Advanced critera: Research

Specialised Research Centres

Name of the specialised research centre/ Centre of excellence	Objectives of the centre	No. of international conferences organised by the centre *(in the last 12 months)*	No. of research reports/ working papers published by the centre *(in the last 12 months)*
Centre of Excellence for Gastroenterology & Minimal Access Surgery	• To provide door step advanced healthcare services • To fill the gap between low socio economic group and multi-speciality care • To provide affordable quality health care services in rural areas • Better learning experience for the students • To promote research in local health issues • Strengthen the collaboration	0	23
Animal House Lab	• To ensure, on behalf of the institution, that all activities relating to the care and use of animals are conducted in compliance with the CCSEA. • Ethically reviewing proposals and animal facility management procedures.	Nil	4
Genetics Lab	The Division of Laboratory Genetics actively contributes to the study of genetic disease and the advancement of genetic testing and related technology, with a goal of enhancing clinical patient care.	0	3
Central Research Lab	Aim : To perform the cutting edge research in therapeutic areas which will make the significant difference in human lives and sufferings. Therapeutic areas as mentioned below: a) Translational research – Infectious diseases, Cancer, Inflammatory diseases b) Drug discovery and Pre-clinical Pharmacology – Anticancer NCE's c) Molecular Pharmacology at miRNA level – Cancer, Inflammation and Infectious diseases.	0	12

Name of the specialised research centre/ Centre of excellence	Objectives of the centre	No. of international conferences organised by the centre *(in the last 12 months)*	No. of research reports/ working papers published by the centre *(in the last 12 months)*
Central Clinical Lab	1) To provide accurate, precise and reliable diagnostic services for prompt patient care. 2) To contribute in patient care for the improvement of patients in OPD & IPD bases by offering a wide range of diagnostic tests for timely diagnosis and treatments of patients. 3) Quality Assurance including proficiency testing, to ensure the reliability of test results, their precision for accurate diagnosis, treatment and prognosis for patient care. 4) Laboratory services are offered for research projects and clinical trials, after fulfillment of all official formalities to support the medical research initiatives. 5) To take efforts for continuous update and improvement in testing methodologies, overcoming the difficulties in different methods of estimations, maintain and update equipments, and staff training to keep laboratory in state of art. 6) To ensure the prompt and accurate lab reports to healthcare providers and patients by minimizing turnaround time (TAT). 7) To adhere to all regulatory and accreditation standards, ensuring patient and staff safety measures and confidentiality with reference to patient.	0	12

Recent Publications per Research Centre

Publications Centre of Excellence

Asian Journal of Case Reports in Surgery

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Adenocarcinoma of Ileum Presenting as Acute Abdomen – A Rare Case Report

Sushil Deshpande[1#], Sangeeta Deshpande[2†], Santosh Kasture[3‡] and Dinesh Kulkarni[4*¥]

[1]Sanket Nursing Home, Aurangabad 431005, Maharashtra, India.
[2]Independent Researcher, Aurangabad, Maharashtra, India.
[3]Kasture Pathology Laboratory, Aurangabad, Maharashtra, India.
[4]Saurabh Histopathology Centre, Aurangabad 431005, Maharashtra, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Case Report

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ABSTRACT

Benign and malignant tumours of small intestine are uncommon. Small bowel malignancies are rare and often present with non-specific symptoms. Because of this, diagnosis of small bowel malignancies is often missed. The primary malignant tumours of small intestine constitute only 1-3% of all primary gastrointestinal malignancies. Adenocarcinoma account for 40-60% of small bowel malignant tumours. Ileum is involved in 13% of patients. Our patient presented with features of acute abdomen. Resected ileal segment revealed thickened wall and histologically it was adenocarcinoma invading upto muscularis, serosa was spared and no nodes were involved. We report this case for uncommon involvement of small intestine with malignant lesion and perforation.

Keywords: Adenocarcinoma; ileum; perforation.

[#]Consultant Surgeon
[†]Consultant Anaestesiologist
[‡]Consultant Pathologist
[¥]Consultant Histopathologist
**Corresponding author: E-mail: drdineshkulkarni@gmail.com;*

1. INTRODUCTION

Although the small bowel represents 90% of the surface area and 75% of the length of the alimentary tract and is located between two organs with high cancer incidence (i.e., stomach and colon), malignant neoplasm of the small bowel fall in the category of rare neoplasms. They account for only 2% of all GI malignancies. Even though, the first collective series of malignant small bowel neoplasm was published by Leichtenstein in 1876, small bowel neoplasms continue to present a challenge to the clinician due to their infrequency, nonspecific symptoms and a delay in diagnosis [1]. Adenocarcinoma of small intestine are seen in elderly persons with median age of 55-67 years and have no sex predilection. U.S. cancer registry for 1993-97 showed highest incidence of small bowel malignancies in U.S and lowest in East Asia [2,3]. In small intestine, these tumours are more common in upper segment with duodenum being involved in 55.2%, jejunum in 17.6% and ileum in 13% patients[3]. Adenocarcinoma of small bowel are described in hereditary nonpolyposis colorectal carcinoma syndrome, Peutz-Jegher's syndrome, bowel duplications, Crohn's disease, at ileostomy sites and ileal segment of defunctionalised ileoplasty [3]. Primary malignant tumors of the small bowel are rare. These are mainly adenocarcinomas followed in decreasing order by carcinoid tumors, non-Hodgkin lymphomas, gastrointestinal stromal tumors, melanomas, and other rare entities. Grossly these tumours can be flat, stenosing, ulcerated, infiltrative or polypoid [4]. The tumour cells show reactivity for mucin and carcinoembyonic antigen (CEA) [5]. Morphologically these are adenocarcinoma but more often papillary in nature. At the time of diagnosis, most of these tumours are deeply invading into intestinal wall and show distant metastasis [6]. Prognosis depends on involvement of mesenteric lymphnodes with 88% deaths in positive nodes and 45% with negative nodes [5].

2. CASE REPORT

Seventy five year old lady presented with sudden onset pain in abdomen since previous night associated with high grade fever. She did not have history of nausea / vomiting. She had tachycardia. Her abdomen was not distended and there was marked tenderness on right side of abdomen. Bowel sounds were absent. She had leukocyte count of 25,220 with 93% Neutrophils. Her other blood investigations were normal. Her ultrasonography revealed edematous bowel loops and mesentery in right iliac fossa? appendicitis. There was no free fluid in peritoneal cavity. She was posted for exploratory laparotomy with high risk consent. On exploration there was pus in peritoneal cavity. There was a gangrenous diverticulum like structure in distal ileum with pus flakes. (Fig. 1) It was thought to be gangrenous Meckel's diverticulum and resection anastomosis was performed. There was no significant lymphadenopathy. The resected specimen was sent for histopatholgy examination. She had uneventful recovery and was discharged from the hospital on seventh post-operative day.

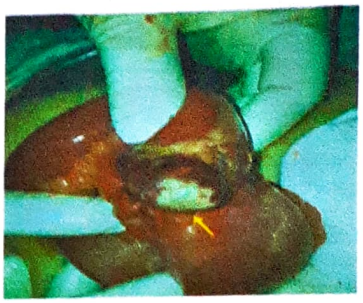

Fig. 1. Intraoperative photograph showing ileal loop with perforation and pus collection (yellow arrow) on serosal surface

A segment of Ileum with attached mesentery, 10 cm in length was received in the lab. External surface showed a perforation of 1 cm on antimesenteric border with whitish flakes on serosal surface. Cut surface showed irregular circumferential ulcerated growth in the centre of segment (Fig. 2), with an area of perforation nearby, having necrotic floor and firm base. The ileal wall was thickened at the site of ulcerated areas. Serosal surface had two grayish soft nodules filled with blood. Resected small bowel ends showed normal mucosa. Two small lymphnodes were palpable in mesentery. Sections from Ulcerated growth showed normal mucosal villi lined by columnar and goblet cells and underneath tumour tissue arranged in glandular and papillary pattern lined by multilayered columnar cells containing hyperchromatic nuclei and clear cytoplasm. The tumour cells had invaded submucosa and muscularis (Fig. 3). Serosa was free from tumour infiltrate. Mesentery along with proximal and distal resected ends of ileum were free from tumour infiltrate. Both lymphnodes in mesentery did not reveal metastasis. Histopathological

Diagnosis was moderately differentiated adenocarcinoma of ileum glands. It involving muscle layer showing stenosis and both mesenteric nodes negative.

Fig. 2. Showing thickened wall of ileum (red arrow) and ulcerated growth on mucosal surface (black arrow)

Fig. 3. Shows normal ileal mucosa (blue arrow) and tumour tissue (red arrows) arranged in glandular pattern consisting of multilayered cells with hyperchromatic nuclei and abundant cytoplasm (H & E 10x X 40x)

3. DISCUSSION

Relative to the length and surface area of small intestine, malignant tumours are rare. Most of the small bowel malignancies are metastatic deposits from tumours arising elsewhere [1]. The clinical presentation in these patients is vague abdominal pain. Sometimes may present with vomittings, weight loss, anaemia, intestinal obstruction or even with signs of perforation peritonitis [3].

The possible reasons for rarity of small bowel cancers are [3]:

1) More liquid content in small bowel
2) Rapid transit time for food
3) Lack of anaerobic bacteria capable of converting bile salts to carcinogen
4) Larger amount of lymphoid tissue like Peyer's patches provide immunity
5) Larger volumes of enzymes detoxify luminal contents and
6) Liquified chyme causes lesser mechanical trauma

Adenocarcinoma of the ileum is a gastrointestinal tumor with a low incidence. Some risk factors are known (e.g., Crohn's disease and celiac disease), but many others have only been postulated and are also associated with colorectal cancer. The clinical presentation is nonspecific, and the first symptoms are usually related to advanced disease. The most common symptoms – abdominal pain, nausea, and vomiting – are frequently present with the majority of intra-abdominal conditions. These factors contribute to delayed diagnosis and treatment, and consequently to a worse prognosis. This rare entity is associated with a nonspecific clinical presentation that contributes to delayed diagnosis and treatment, and consequently to a worse prognosis. Approximately half of the cases are only diagnosed at surgery[7]. The four main histological types of small bowel cancer are adenocarcinomas (30–40%), carcinoid tumours (35–42%), lymphomas (15–20%) and sarcomas (10–15%). The most common location for small bowel adenocarcinoma is in the duodenum (57%), followed by the jejunum (29%) and the ileum (10%)[8]. The adenocarcinoma of the small intestine is a rarely occurring malignant neoplasm. It is mainly located in the duodenum and jejunum, and less frequently located in the ileum. Because of the low frequency of the case, it is very likely that active general surgeons may treat only one or two cases of ileum adenocarcinoma in their surgical life[9].

4. CONCLUSION

This case demonstrates an unusual condition characterized by late and challenging diagnosis. We highlight the importance of an earlier diagnosis and optimal treatment for improved patient outcomes. Because of the vague symptoms, these lesions are diagnosed late. Therefore, on symptoms of partial intestinal obstruction or perforation in an adult, this condition must be suspected.

CONSENT

As per International standard or university standard, patients written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Annals of Clinical Case Reports

Case Series
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Intussusception in Adults: Rare but Grievous Disease

Sushil Deshpande[1]*, Ramesh Satarkar[2] and Dinesh Kulkarni[3]

[1]Department of Surgery, Sanket Nursing Home, India

[2]Department of Gastroenterology, Satarkar Gastroenterology Center, India

[3]Department of Gastroenterology, Saurabh Histopathology and Eye Care Center, India

Abstract

Intussusception is rare in adults. In this age group, symptoms are often misleading, and malignancy stands out as an important etiology, unless proven otherwise. Therefore, surgery remains the treatment of choice. We report two cases of adult intussusception, which presented as sub-acute intestinal obstruction. Both cases had intussusception on CT Abdomen. Both patients underwent surgical intervention. In first case, ileal loop along with mesenteric fat and lymph node was seen in intussusception. In second case colonic mass with lymph node involvement was seen. Hemicolectomy was done in both the patients.

Introduction

Intussusception is common in infants and children. In adults it is rare. Colonic intussusceptions account for 1% of intestinal obstructions in adults [1]. We present our experience of treating two cases of adult colonic intussusception along with review of literature.

Case Series

Case 1

A sixty-year-old female patient presented with abdominal pain, vomiting and fullness of abdomen. She gave history of similar attack one and half month before. She was found to have anemia and leukocytosis with Total Leukocyte Count of 17000/cm. Considering diagnosis of sub-acute intestinal obstruction, she was investigated. On USG there was suspicion of Ileo-colic intussusception, hence a CT abdomen was done, which confirmed the diagnosis of Ileo-colic intussusception leading to sub-acute intestinal obstruction. However, no mass was detected. On exploration Ileo-colic intussusception was extending up to transverse colon (Figure 1). Intussusception was partially reducible, and a large mesenteric node was seen inside the intussusceptum. Right hemicolectomy was performed with end-to-end Ileo-transverse anastomosis. On cutting open the specimen a proliferative lesion was seen in the cecum (Figure 2). Histopathology revealed adenocarcinoma of cecum (Figure 3). Patient made uneventful recovery.

Case 2

Eighty-one-year-old man presented with complaints of abdominal pain, loss of appetite. He was investigated with CECT Abdomen. It revealed a colo-colic intussusception with a mass lesion in ascending colon as a lead point (Figure 4). Enlarged lymph nodes were noted in adjacent mesocolon. Since the lumen was compromised on CT scan, patient was operated upon. On exploration there was colo-colic intussusception with large colonic mass as a lead point. There were

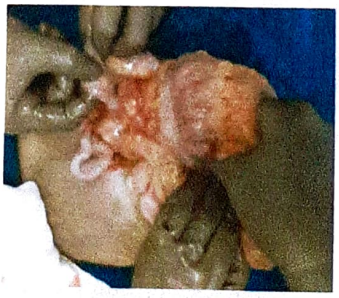

Figure 1: Showing Ileo-colic intussusceptions.

Correspondence:
Sushil Deshpande, Department of Surgery, Sanket Nursing Home, Plot no 5, Balaji Niwas, Deva Nagari, Aurangabad, 431005, India, E-mail: sushildeshpande@hotmail.com

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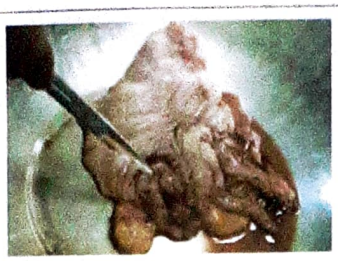

Figure 2: Showing proliferative lesion in cecum.

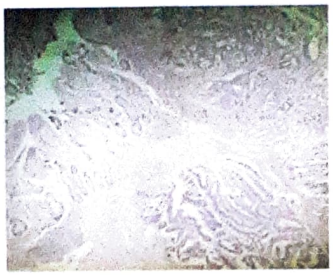

Figure 3: Histopathology H&E, 10x × 10x showing lining mucosa and underneath tumor mass arranged in glandular and papillary pattern, moderately differentiated adenocarcinoma grade II.

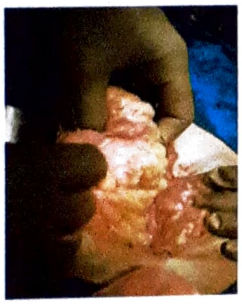

Figure 4: Showing Colo-colic intussusception

large mesenteric lymph nodes. Right hemicolectomy was done with end-to-end Ileo-colic anastomosis. On cutting open, the specimen revealed a proliferative lesion in proximal ascending colon (Figure 5). Histopathology revealed adenocarcinoma of colon grade II. Patient had severe hypoproteinemia post-operatively but responded well to supportive treatment and made uneventful recovery afterwards.

Discussion

Intussusception was first described by Barbette in 1674 [2]. It is defined as the telescoping of a proximal segment of the Gastrointestinal (GI) tract, called intussusceptum, into the lumen of the adjacent distal segment of the GI tract, called intussuscipiens. One of the segments is free and the other is freely moving. The commonest age group for intussusception is from 6 to 18 months of age. In pediatric population its occurrence is usually idiopathic (~90%) and may be treated conservatively by endoscopic or radiological reduction [1]. Intussusception is rare in adults. Less than 5% cases of intussusception occur in adults. In about 80% to 90% adult patients, the underlying cause of intussusception is benign or malignant neoplasm [3-5]. The

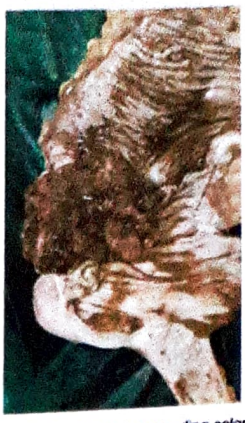

Figure 5: Showing proliferative lesion in ascending colon

classical clinical trial of intussusception is abdominal pain, blood in stool and lump in abdomen. In adults, the patient commonly present with chronic abdominal pain, symptoms of sub-acute intestinal obstruction such as vomiting, constipation and abdominal pain. This makes the clinical diagnosis of intussusception in adults difficult. Various causative factors leading to intussusception in adults are malignant and benign tumors, Meckel's diverticulum, foreign bodies, inflammatory lesions, lymphoid hyperplasia, and sometimes post operative adhesions [6]. In adult colonic intussusception primary adenocarcinoma is the commonest underlying malignant lesion [4,5]. Ten percent of adult cases may present with no demonstrable cause of intussusception and are considered to have idiopathic intussusception [7]. Dean et al. [8] in 1956 classified intussusception in adult as per the location *viz.* Enteric (43%), Colo-colic (22%) and Ileo-cecal (21%). Diagnosis of intussusception in adults is often based on imaging as in majority of patients the clinical findings are inconclusive. Abdominal sonography which has revolutionized the diagnosis of gastrointestinal pathologies is found to be useful in these cases too. Ultrasonography shows typical features like "Target" or "Donut sign" on transverse view while on longitudinal view" Pseudo-kidney" or "Hayfork" sign is seen [8]. However, gas fluid filled intestinal loops and obesity are the major limiting factors for USG [9]. In a meta-analysis of recent studies by Abralena et al. [10] in 2013, abdominal CT was observed to be the most sensitive modality in the diagnosis of intussusception with diagnostic accuracy of 83%. The characteristic features on CT scan include an inhomogeneous "target" or "sausage" shaped soft tissue mass with a layering effect and mesenteric vessels within the bowel lumen [11]. However, possibilities of breathing artifacts in ill patients and exposure to ionizing radiation have been found to be the major drawbacks of CT Abdomen [9]. Considering the high probability of underlying benign or malignant tumor as the etiology in majority of adult patients, surgery is the treatment modality of choice in adults. Azar reported that, for right-sided colonic intussusceptions, resection and primary anastomosis can be performed even in unprepared bowels, while for left-sided or retrosigmoid cases, resection with construction of a colostomy and a Hartmann's pouch with re-anastomosis at a second stage is considered safer, especially in the emergency setting [5]. Both our patients were elderly and had history of abdominal pain for few weeks. They presented with sub-acute obstruction and needed surgery on priority. The typical presentation of intussusception was

absent in both the cases and the diagnosis could be established on imaging modalities. In first case, there was no obvious cause for intussusception on imaging but considering the patient's age and standard treatment protocol, surgery was performed. In second case presentation in acute stage with obstruction, left surgery as the only option even though advanced malignant lesion imaging showed advanced malignant lesion. In both cases, right hemicolectomy was done. Both the patients made good recovery except severe hypoproteinemia in case no 2 which required additional supportive care.

Conclusion

Intussusception is common in pediatric age group and its incidence in adults is rare. The clinical presentation in adult patients is often vague hence diagnosis requires help of imaging modalities like USG and CT Abdomen. In view of the possibility of underlying malignant lesion, surgery is the treatment of choice in adults.

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ORIGINAL ARTICLE

Endoscopic Bile Duct Clearance Followed by Same-Day Cholecystectomy: a Case Series Analysis

Mridul Tantia[1] · **Pravin Suryawanshi**[1] · **Ashish Gandhi**[1]

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Abstract

Early laparoscopic cholecystectomy (LC) after endoscopic retrograde cholangio-pancreaticography (ERCP) is beneficial for patients with gall stone disease (GSD) and common bile duct (CBD) stones. However, there are no clear guidelines for the optimal timing of surgical intervention. This study aimed to assess the feasibility and clinical outcomes of the same-day sequential approach—ERCP followed by LC for management of choledocholithiasis and concomitant cholelithiasis. Between March 2018 and November 2019, 24 patients diagnosed with choledocholithiasis and concomitant cholelithiasis underwent ERCP-guided biliary clearance followed by LC sequentially on the same day. ERCP was done in the endoscopy suite and followed by LC in the operation theatre. Both procedures were performed by the same endoscopist-cum-laparoscopic surgeon proficient in advanced endoscopy and laparoscopy. Success rate, ERCP findings, operative findings, logistic issues, and complications were recorded and analysed. Technical success for ERCP and LC, both were 100%. No major complications like bleeding, perforation, pancreatitis, or mortality were encountered post-ERCP. The rate of conversion to open cholecystectomy was 0%. Post-operative self-resolving minor bile leak was encountered in 1 patient (4.1%) who was managed conservatively. The mean time for ERCP and LC was 33.33 min and 80.4 min, respectively. The mean time interval between the two procedures was 51 min. The mean post-procedure hospital stay was 3.7 days. This study demonstrated a safe, effective, and feasible same-day sequential approach—ERCP-guided biliary clearance followed by LC—in selected patients with choledocholithiasis with concomitant cholelithiasis.

Keywords ERCP · Biliary stenting · Laparoscopic cholecystectomy · Choledocholithiasis · Cholelithiasis

Introduction

Symptomatic gallstone disease is one of the most common gastrointestinal surgical problems faced worldwide, and about 10–18% of patients with gallbladder stones have common bile duct (CBD) stones at the time of cholecystectomy [1, 2]. While various minimally invasive approaches have been described, there is no consensus on the optimum strategy for the management of gallbladder stones with concomitant CBD stones. Minimally invasive surgery offers faster recovery, lesser post-operative pain, and a shorter length of hospital stay. Reducing the length of stay (LOS) in hospitals perhaps remains one of the most cost-effective approaches to reducing the healthcare expenditure of patients. ERCP followed by LC is the most commonly practised method in most hospitals worldwide [3]. Traditionally, these procedures were performed on different days and often with an unnecessary delay. This was in lieu of the anticipated fear arising from post-ERCP-related complications including pancreatitis. However, advanced technology and endoscopic expertise have reduced the rate of such complications. Performing ERCP and LC on the same day is one potential method of decreasing LOS and potentially reducing healthcare expenditures. The benefits of a single-stage approach in the management of cholelithiasis and choledocholithiasis have been studied concerning laparoscopic CBD exploration (LCBDE), or LC with intra-operative ERCP

✉ Mridul Tantia
tantiamridul@gmail.com

Pravin Suryawanshi
drspravin22@gmail.com

Ashish Gandhi
ashishrgandhi@gmail.com

[1] Department of Surgery, Centre of Excellence for Gastroenterology & Minimal Access Surgery, M.G.M. Medical College & Hospital, N-6 Cidco, Maharashtra 431001 Aurangabad, India

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[4–6], but the clinical application of a same-day sequential approach—ERCP followed by LC— has not been adequately analysed. Such a method may provide an alternative approach to managing gallstones and CBD stones, especially in centres where performing laparoscopic CBD exploration or intra-operative ERCP during LC may still not be feasible.

This study aimed to assess the feasibility and clinical outcomes of the same-day sequential approach—ERCP-guided biliary clearance followed by LC in the management of choledocholithiasis with concomitant cholelithiasis. Special emphasis was laid on evaluating the intra-procedural difficulties faced, post-operative complications, and logistical issues encountered in conducting these same-day sequential interventions.

Patients and Methods

This case series analysis was conducted in the Department of Surgery at MGM Medical College and Hospital (Aurangabad, India) between March 2018 and November 2019. Appropriate clearances and approvals were sought from the Institutional Ethics Committee.

Patients presenting to the hospital with choledocholithiasis and concomitant cholelithiasis on an accrual basis were evaluated clinically and with radiological and biochemical investigations. A liver function test was part of the routine biochemical workup. Diagnostic ultrasonography of the abdomen (USG) was performed in all patients, whereas contrast-enhanced computed tomography (CECT), magnetic resonance imaging cholangiopancreatography (MRCP), or endoscopic ultrasound (EUS) were done selectively, for further assessment as clinically warranted.

After obtaining written informed consent, the patients satisfying the inclusion criteria were included in the study. The sole inclusion criterion was the presence of stone in the CBD as evidenced by imaging modalities in patients with symptomatic gallstone disease. Gallbladder wall thickness was measured in millimetres using transabdominal USG preoperatively.

Patients with severe cholangitis, large CBD stones (> 2 cm), complicated cholecystitis, gallstone pancreatitis, and unfit for general anaesthesia (GA) were excluded from the study.

The selected patients underwent ERCP followed by LC on the same day. ERCP was performed in the endoscopy suite, in the left semi-prone position under propofol sedation and CO_2 insufflation. Guidewire-assisted technique was used for selective biliary cannulation. Various parameters including the duration of the procedure (time interval between scope-in and scope-out), number of attempts at guidewire insertion, inadvertent pancreatic duct cannulation, difficulties encountered, and complications were recorded. Once the biliary stone extraction was successful, a CBD stent was placed, and the patient was shifted to the operation theatre for LC. The patient's condition during transport and the time interval between the two procedures were monitored. LC was performed using the standard 4-port technique under general anaesthesia. The total time of surgery was recorded from the time of intubation till the time of extubation. Other parameters like intra-operative difficulties faced, presence of bowel distension or adhesions, use of drains, and conversions to open cholecystectomy or any complications were noted.

A standardised post-operative care protocol was followed for all patients. An oral diet was resumed after 6 h. Serum amylase level was done 4-h post-ERCP to evaluate for pancreatitis. Complications like biliary leaks, haemorrhage, pancreatitis, and bowel injury were recorded if present. Total and post-operative hospital length of stay (LOS) was recorded.

Patients were followed up at 1-week, 4-week, and 28-week intervals. Recurrent biliary events and other complications were noted. CBD stents, if placed, were removed after 4 weeks of surgery, after excluding any residual stones on ultrasonography.

The primary outcome was studied based on parameters like the average time for ERCP and LC, the average time interval between the procedures, ERCP and LC findings, post-ERCP and post-LC complications, perioperative logistical difficulties, total and post-operative LOS (mean no. of days), and follow-up evaluation.

Results

A total of 24 patients (10 men and 14 women) underwent ERCP followed by LC by same-day sequential approach. The average age was 48.88 SD16.29 years, with 25% ($n = 06$) of patients belonging to the elderly age group (age > 65 years), all of whom underwent both procedures safely.

Abdominal pain was the presenting complaint in 100% of patients, whereas jaundice and fever were recorded in 62.5%, and 25% of patients, respectively. The average total bilirubin was 2.65 SD2.24 mg/dL, and the average direct bilirubin was 1.89 SD1.87 mg/dL. The largest CBD stone size in our study was 16 mm, while the average CBD stone size was 8.26 SD3.28 mm, and the average CBD diameter was 10.12 SD3.54 mm. The average GB wall thickness was calculated at 4.90 SD2.24 mm.

During ERCP, selective CBD cannulation and stone extraction were achieved in all the patients (100%). CBD stents were placed in all 24 patients. The average time taken for ERCP was 33.33 SD15.79 min. No major complications (bleeding, perforation, or pancreatitis) were encountered following ERCP. Asymptomatic hyperamylasaemia (Sr.

amylase > 100 U/L) was noted in 5 patients, which settled spontaneously without any further intervention. Inadvertent pancreatic duct cannulation occurred in 2 patients (8.3%) with an uneventful post-ERCP course. We also studied the correlation between the number of attempts at guidewire insertion and post-procedure serum amylase levels and found a positive correlation ($p = 0.024$). This suggested that the more the number of guidewire insertions, the higher the possibility of hyperamylaesemia (Fig. 1). The average post-procedure serum amylase level was 74.29 SD47.93 U/L. No major logistical difficulties were encountered as both procedures were performed in the same centre and by the same surgeon. Additionally, careful patient selection and the use of CO2 insufflation reduced the occurrence of abdominal distension facilitating LC.

The average time interval between the two procedures (ERCP and LC) was 51.25 SD22.47 min. All patients were clinically monitored between the procedures and were stable.

The average time taken for LC was 80.42 SD47.82 min. The rate of conversion to open cholecystectomy was 0%. Bowel distension was also not seen in any patient (0%). Drains were placed in 9 patients (37.5%) based on intra-operative findings. One patient had a complication of a minor bile leak from the cystic duct stump which was self-limiting and managed conservatively.

Additionally, the correlation between the gallbladder wall thickness on preoperative imaging and the time taken for LC was analysed, and a positive correlation was noted ($p = 0.013$), indicating that increased wall thickness could contribute to longer operative times (Fig. 2).

This finding, although positive, needs to be considered in light of various confounding factors of operative time like duration, the number of attacks of cholecystitis, biliary anatomy, condition of the liver, location of stone in the gallbladder, and length of the cystic duct.

The average post-procedure LOS was 3.71 SD1.58 days. A 28-week follow-up of all the patients was recorded. There were no recurrent biliary episodes, and CBD stents were removed at 4 weeks after the procedure.

Discussion

Laparoscopic cholecystectomy for GSD is the most common surgery performed worldwide, and about 10–18% of patients undergoing LC have concomitant CBD stones [1].

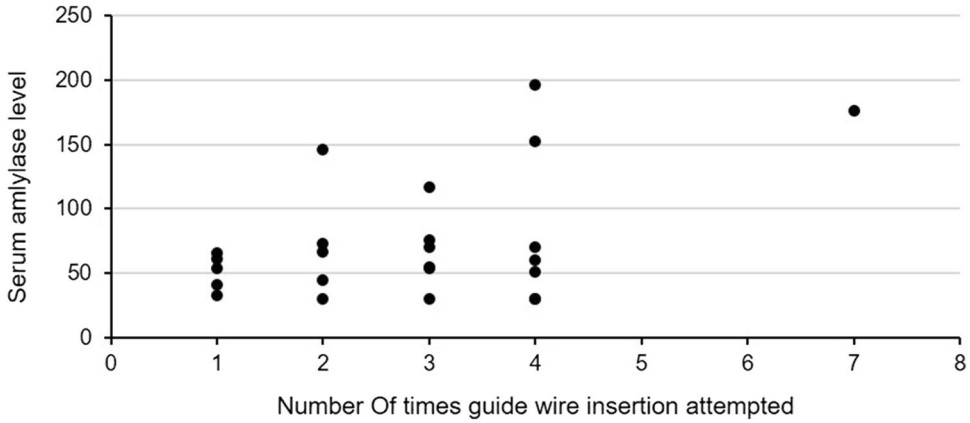

Fig. 1 Plot graph showing the correlation between serum amylase levels with guidewire insertion

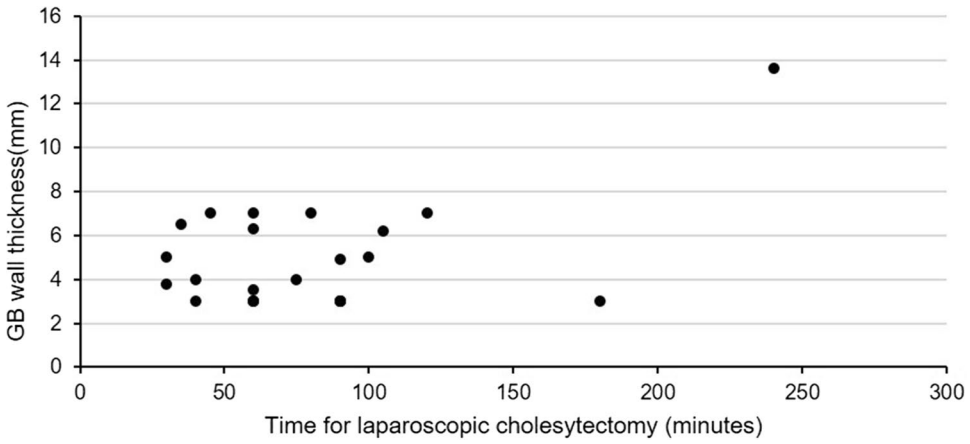

Fig. 2 Plot graph showing the correlation between GB wall thickness and time for laparoscopic cholecystectomy

The appropriate management for these patients involves clearance of the CBD stones, in addition to removal of the gallbladder to prevent recurrent biliary events. The appropriate method of dealing with CBD stones largely rests on the availability of local resources and the technical skills of the surgeon and endoscopist. Therapeutic options range from open CBD exploration to the minimally invasive laparoscopic CBD exploration (LCBDE) and the widely used ERCP. While LCDBE offers a single-stage therapy, it requires considerable surgical skill in laparoscopic suturing and additional resources [7, 8]. ERCP on the other hand is widely practised and is the most common method of dealing with choledocholithiasis worldwide [9]. Studies have shown an early LC following ERCP to be beneficial when the surgery is done before the onset of inflammation in the hepatoduodenal region. Still, no guidelines as to when the surgery should be performed have been established [10, 11]. Although same-day ERCP and LC does not take away the need for CBD exploration, it can offer the benefits of a single-stage procedure, and reduce hospital stay and costs, in patients whose CBD stones can be dealt with endoscopically [12–15].

While some authors have advocated intra-operative ERCP and LC done under the same general anaesthesia as an option, it is worthwhile to note that this method has several logistical drawbacks. It might not always be feasible to have the endoscopy team in the operating theatre, and performing ERCP on a supine patient can be technically challenging even for an experienced endoscopist [16, 17]. To overcome these challenges, we adopted an alternative same-day sequential approach—ERCP preoperatively in the endoscopy suite under propofol sedation, followed by LC in the operation theatre under general anaesthesia. This method offered several advantages—the ERCP could be performed in a comfortable semi-prone position, making cannulation easier, as we reported successful CBD cannulation in all our patients. There were no instances of post-ERCP pancreatitis, although self-resolving asymptomatic hyperamylasemia was seen in 5 patients which was probably related to inadvertent pancreatic duct cannulation and multiple attempts at guidewire insertion, as has been noted in other studies as well [11, 12, 14]. The decision to exclude patients with large CBD stones (> 2 cm) from our study could have contributed to our success in achieving complete CBD clearance in all the patients. The mean total procedure time for ERCP was 33.3 min and was comparable to that of other studies found in the literature [11, 12, 15].

Our study involved immediate shifting of patients from the endoscopy suite to the operating theatre, and the average time interval between the two procedures was only 51 min, which was much less compared to other similar studies [12, 14, 15].

Successful ERCP-guided CBD clearance and the absence of any immediate post-procedure complications enabled us to proceed with LC safely. There was no need for intra-operative cholangiography, as CBD clearance had been documented. Complete CBD clearance must be achieved endoscopically before proceeding with laparoscopic cholecystectomy, as just stenting may add to overconfidence and can lead to CBD injuries. ERCP complications may complicate laparoscopic cholecystectomy. Bowel distension has been reported in the literature to be a problem during LC after ERCP; however, the use of CO_2 for insufflation during ERCP helped us to prevent any bowel distension in our study [11, 13, 14]. Laparoscopic cholecystectomy was completed safely without conversion in all the patients, which reflected results in other studies [11–15]. The mean operating time of 80.4 min was comparable to the literature reviewed [11–15]. Only 1 patient had a complication of a post-operative biliary leak which was self-limiting and managed conservatively. The average post-procedure LOS of 3.7 days was similar to other studies that had performed single-stage procedures [11, 14]. This translates into decreased hospital stay and reduced healthcare expenses as demonstrated in other similar studies [14, 15]. The absence of any mortality or long-term complications further assured us of the feasibility and safety of this novel approach. Another lesson learnt from this case series is that serum amylase may be raised without much implication to the difficulty in cholecystectomy, but if the GB wall thickness is much more, one may wait for 4 to 6 weeks and delay the cholecystectomy.

A few limitations to our study must be kept in mind. The study population was small, it was a single-centre study, and there was no control group. Larger, multi-institutional, randomised controlled trials with a wider range of inclusion criteria are needed before adopting this method as a standard of care. We also feel that further studies need to be done comparing this method to LCBDE or with LC and intra-operative ERCP before this is widely adopted.

Conclusion

In 24 patients with cholelithiasis and choledocholithiasis, endoscopic bile duct clearance followed by same-day laparoscopic cholecystectomy was performed successfully, with minimal complications and no mortality. The success of performing follow on cholecystectomy within a few hours of endo-biliary stone clearance is dependent on the hospital's functionality. While it is preferable that biliary clearance and laparoscopic cholecystectomy are both performed on the same working day, if however, the biliary clearance is achieved during later hours of a working day, and then, cholecystectomy may be performed on the next working day within 24 h. Additionally, other parameters like increased

gallbladder wall thickness, suspicion of malignancy, or lack of expert surgeons may require a delayed cholecystectomy.

Declarations

Ethics Approval The questionnaire and methodology for this study were approved by the Ethics Committee for Research on Human Subjects (ECRHS) of MGM's Medical College Aurangabad (MGM-ECRHS/2018/19).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Conflict of Interest The authors declare no competing interests.

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Kikuchi Fujimoto Disease – A Diagnostic Challenge

Sushil Deshpande[1] and Dinesh Kulkarni[2*]

[1]Sanket Nursing Home, Auranganbad - 431005, Maharashtra, India.
[2]Saurabh Histopathology Centre, Aurangabad - 431005, Maharashtra, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author SD carried out the surgery, searched literature and modified manuscript. Author DK processed the specimen, diagnosed the case, searched literature and prepared the manuscript. Both authors read and approved the final manuscript.

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Case Report

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ABSTRACT

Kikuchi-Fujimoto Disease is an extremely rare entity characterized by subacute necrotizing lymphadenitis and frequently associated with fever. It is known to have a worldwide distribution with higher prevalence among Japanese and other Asiatic young individuals. The clinical, histopathological and immunohistochemical features appear to point to a viral etiology, a hypothesis that still has not been proven and the cause remains uncertain. It is generally diagnosed on the basis of an excisional biopsy of affected lymphnodes. Early diagnosis with excisional lymph node biopsy is crucial as this disease can be mistaken for Systemic Lupus Erythematosus (SLE) or lymphoma and to avoid unnecessary investigations. The treatment is only symptomatic unless complicated, where steroid therapy is considered. Kikuchi's disease has an excellent prognosis with almost no risk. Because of the rarity and difficulty in diagnosis, we thought of publishing the case.

Keywords: Necrotising Lymphadenitis; adults; Asia.

**Corresponding author: E-mail: drdineshkulkarni@gmail.com;*

1. INTRODUCTION

Kikuchi Fujimoto Disease (KFD) was first reported in Japan almost simultaneously by Kikuchi [1] and Fujimoto [2] in 1972. It is a benign and self-limiting disease that classically presents with lymphadenopathy and fever. The etiology is poorly understood. The searched literature did not show incidence rates. Although seen worldwide, it is known to be much more prevalent in Asian populations [3]. Indeed, a comprehensive literature review of KFD cases in 2003 described it as being "scarce in the western world" [4]. KFD is prevalent in younger population [3,4,5]. Histologically, lymphadenitis is characterized by paracortical lymph node expansion with patchy, well-circumscribed areas of necrosis showing abundant karyorrhectic nuclear debris and absence of neutrophils and eosinophils. The histological differential diagnosis of KFD includes reactive lesions as lymphadenitis associated with Tuberculosis, Histoplasmosis, Leprosy, Syphilis, Infectious mononucleosis, SLE or herpes simplex , non-Hodgkin's lymphoma, plasmacytoid T-cell leukemia, myeloid tumor and even metastatic carcinoma [5]. Treatment is symptomatic with analgesics-antipyretics, non-steroidal anti-inflammatory drugs and, rarely, corticosteroids. Spontaneous recovery occurs in 1 to 4 months. Patients with Kikuchi-Fujimoto disease need long term follow up to survey the possibility of the development of SLE.

2. CASE REPORT

A 32-year male was referred for cervical lymphnode biopsy having multiple bilateral neck nodules associated with low grade fever, for last four months. The size of nodules was increasing slowly and didn't respond to a course of antibiotics prescribed by his physician. On examination the nodules were multiple, nonmatted, soft to firm, freely mobile, nontender with largest being of size 2x2 cm. He also had bilateral axillary lymphadenopathy. He didn't have history of allergy. His systemic examination i.e. Cardiovascular, Respiratory, Abdominopelvic and Nervous systems were within normal limits. A possible diagnosis of tuberculosis was considered and the patient was sent for investigations. Complete blood count showed lymphocytosis and elevated E.S.R. (20 mm at the end of one hour by Wintrobe's method). Rest all parameters were within normal limits. His X-ray chest was done and was reported by radiologist as (Rt) hilar lymphadenopathy likely of infective origin, rest of the lung parenchyma and bony cage did not show any abnormality. The Tuberculin test had induration of 3 mm after 48 hours. With multiple lymphadenopathies, he was referred for USG Abdomen and Pelvis, which ruled out hepatosplenomegaly and abdominal lymphadenopathy. (Rt) neck cervical lymphnode biopsy was done, two intact nodes were removed and sent for histopathology examination. On gross examination, two whitish rounded 2x1 and 1.5x1 cm soft tissue masses were seen. Cut surface had capsule and uniform whitish areas. No caseation was seen. Histologically the sections showed fibrous capsule and beneath maintained lymphoid architecture (Fig. 1) with paracortical expansion by necrotic areas showing admixture of histiocytes, plasmacytoid dendritic cells, lymphocytes and karyorrhectic debris.(Fig. 2) No neutrophils or eosinophils were seen. There was no evidence of Reed Sternberg cells, granuloma or metastatic deposits. Ziehl Neelsen (ZN) stain was negative for acid fast bacilli. The diagnosis of Lymphadenitis of Kikuchi Fujimoto type was conveyed. On immunohistochemistry (IHC), CD 20 and CD 3 highlighted "B" and "T" lymphocytes, CD 68 histiocytes, CD 123 plasmacytoid dendritic cells and CD 30 highlighted scattered immunoblasts. Ki 67 was 95% in lymphoid follicles and 15% in interfollicular areas. Thus, IHC ruled out lymphoma and also confirmed our diagnosis.

3. DISCUSSION

Kikuchi Fujimoto Disease was discovered in 1972 in Japan by two separate groups. Its exact incidence is unknown, but it is more prevalent in East Asia. Kikuchi's disease is seen more frequently in young adults, with a mean age between 20–30 years but it can occur in any age group. Even though a female predominance is reported in many previous cases, some studies done in Asia show a male to female ratio of 1:1. [4,5] The definitive diagnosis of KFD can be made only through lymph node biopsy and histological examination. Even with adequate tissue the lymph node appearances can be mistaken for malignant lymphoma; in one study, 30% of lymph node biopsies in KFD were initially misdiagnosed as lymphoma. The histopathological features of KFD have been classified into three stages: (1) proliferative stage, with expression of histiocytes, plasmacytoid monocytes, and lymphoid cells containing karyorrhectic nuclear fragments and eosinophilic apoptotic debris; (2) necrotizing stage, with a degree of coagulative necrosis; and (3) xanthomatous stage, with foamy histiocytes

predominating. A characteristic and useful diagnostic feature is the absence of granulocytes in the "necrotizing stage", which is helpful in distinguishing KFD from SLE and drug induced lymphadenopathy. [4,5].

Fig. 1. shows fibrous capsule (yellow arrow head) and beneath lymphnode architecture maintained and hyperplastic follicles (red arrow) and dilated sinuses. (H & E 5x X 10x)

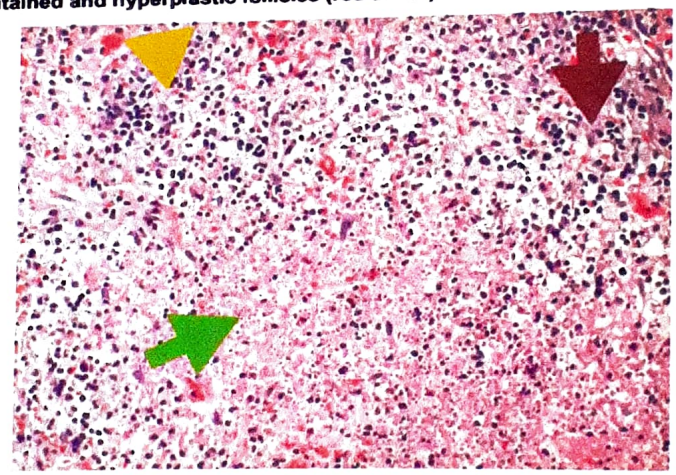

Fig. 2. shows areas of necrosis (green arrow) surrounded by chronic inflammatory cells (yellow arrowhead) and histiocytes (red arrow) (H & E 10x X 40x)

4. CONCLUSION

Kikuchi-Fujimoto disease poses significant diagnostic challenges to pathologists and clinicians as it can easily be mistaken for other benign lymphadenopathies or infectious lymphadenitis as well as lymphomas. Clinicians' and pathologists' awareness of this disorder can help to avoid misdiagnosis of lymphoma and prevent further expensive and invasive investigations, as well as
potentially harmful treatments and psychological stress to the patient. Excisional lymph node biopsy provides the optimal specimen for diagnosis of KFD.

CONSENT

As per international standard or university standard, patient's consent has been collected and preserved by the authors.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Publication in Animal House

Form B (per rule 8(a)* for Submission of Research Protocol (s)

Application for Permission for Animal Experiments

Application to be submitted to the CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC)

Section –I

1.	Name and address of establishment	**Department of Pharmacology, MGM Medical college,N-6, Cidco Aurangabad, Maharashtra.**	
2.	Registration number and date of registration.	Registration Number	**CPCSEA Reg. No. 1777/PO/Re/S/14/CPCSEA**
		Date of registration	**28-08-17**
3.	Name, address and registration nu mber of breeder from which animal s acquired (or to be acquired) for experiments mentioned in parts B & C	Name	**1. National institute of Biosciences**
		Address	**1. Pune, Maharashtra.**
		Registration No.	**Registration No. 1091/GO/Bt/S/07/CPCSEA**
4.	Place where the animals are present ly kept (or proposed to be kept).	**Animal house, Department of pharmacology, MGM medical college Aurangabad.**	
5.	Place where the experiment is to be performed (Please provide CPCSEA Reg. Number)	**Animal house, Department of pharmacology, MGM medical college Aurangabad.(CPCSEA Reg. No. 1777/PO/Re/S/14/CPCSEA)Date:-28-08-17**	
6.	Date and Duration of experiment.	Date	**Feb 2022 to April 22**
		Duration	**6 weeks**
7.	Type of research involved (Basic Research / Educational/ Regulatory / Contract Research)	**Basic research**	

Signatures	
Name of Investigator	**Dr. Deepali Jaybhaye**
Designation of Investigator	**Associate professor**
Signature	
Date	**27/12/2021**
Place	**MGM Medical College & Hospital, Aurangabad.**

Section -II

Protocol form for research proposals to be submitted to the Institutional Animal Ethics Committee/ CPCSEA, for new experiments or extensions of ongoing experiments using animals.

1	**Project / Dissertation / Thesis Title:**	**The study of renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wister rats.**
2	**Principal Investigator / Research Guide / Advisor**	
	Name	**Dr. Deepali Jaybhaye**
	Designation	**Associate professor Department of pharmacology, MGM Medical college Aurangabad**
	Dept / Div/ Lab	**Department of pharmacology, MGM Medical College Aurangabad**
	Telephone No.	**0240 – 6601100 Ext: 1502 , 1501.**
	E-mail Id	**deepalijaybhaye@rediffmail.com**
	Experience in Lab animal Experimentation (Years/Months)	**12 years**
3	**List of all individuals authorized to conduct procedures under this proposal.**	
	Investigator 1	
	a) Name	**Dr. Deepak Bhosle**
	b) Designation	**Professor and HOD**
	c) Dept / Div/ Lab	**Department of pharmacology, MGM Medical College, Aurangabad**
	d) Telephone No.	**0240 – 6601100 Ext : 1502 , 1501.**
	e) E-mail Id	**drdeepakbhosle@gmail.com**
	f) Experience in Lab animal Experimentation (Years/Months)	**17 Years**
4	**Funding Source / Proposed Funding Source with complete address**	**MGM Institute of Health Sciences, Navi Mumbai.**
	(Please attach the proof)	Yes
5	**Duration of the animal experiment.**	
	a) Date of initiation (Proposed)	Jan 22
	b) Date of completion (Proposed)	Jan 2023
6	**Describe details of study plan to justify the use of animals (Enclose Annexure)**	We will use Wistar rats for this study. **Citrus limon and Emblica officinalis has strong antioxidant property. But study of renoprotective activity is not available. So we want to explore this property of above plant.**
7	**Animals required**	
	a. Species	Rats of either sex.
	Strain	Wistar (Rat),

<table>
<tr><td></td><td>b. Age</td><td>8- 20 weeks.</td></tr>
<tr><td></td><td>Weight</td><td>150-250 gms,</td></tr>
<tr><td></td><td>c. Gender</td><td>Both</td></tr>
<tr><td></td><td>d. Number to be used</td><td>Rat-36<table><tr><td></td><td>Available</td><td>Procure</td></tr><tr><td>Rat</td><td>08</td><td>28</td></tr></table></td></tr>
<tr><td></td><td>(Year-wise breakups and total figures needed to be given in tabular form)</td><td><table><tr><th>Year</th><th>Number of Animals</th></tr><tr><td>Feb – 2022 to Feb-2023</td><td>Rat-36</td></tr></table></td></tr>
<tr><td></td><td>e. Number of days each animal will be housed.</td><td>Procurement to life time</td></tr>
<tr><td>8</td><td colspan="2">Rationale for animal usage</td></tr>
<tr><td></td><td>a) Why is animal usage necessary for these studies?</td><td>As animal study is not available. So we have to do it as primary study to see the renoprotective effect. (lemon and Amla having strong antioxidant property and in renal failure the causative agent is oxidative stress but still not a single study is available as renoprotective drug. To explore the renoprotective property we want to do this study.)</td></tr>
<tr><td></td><td>b) Whether similar study has been conducted on <u>in vitro</u> models?</td><td>Not Applicable</td></tr>
<tr><td></td><td>If yes, describe the leading points to justify the requirement of animal experiment.</td><td>Not Applicable</td></tr>
<tr><td></td><td>c) Why are the particular species selected?</td><td>As it is best demonstrated in rats.</td></tr>
<tr><td></td><td>d) Why is the estimated number of animals essential?</td><td>For the statistical significant result.</td></tr>
<tr><td></td><td>e) Are similar experiments conducted in the past <u>in your establishment</u>?</td><td>No</td></tr>
<tr><td></td><td>f) If yes, justify why new experiment is required?</td><td>-</td></tr>
<tr><td></td><td>g) Have similar experiments been conducted by any <u>other organization</u> in same or <u>other in vivo models</u>?</td><td>-</td></tr>
<tr><td></td><td>If yes, enclose the reference.</td><td>-</td></tr>
<tr><td>9</td><td colspan="2">Describe the procedures in detail:</td></tr>
<tr><td></td><td>a) Describe all invasive procedures that animals will be subjected to in the course of the experiments</td><td>Handling of animals and giving standard and test dose of drugs by oral route. (detail Performa attached)</td></tr>
<tr><td></td><td>Describe all potentially stressful non-invasive procedures that animals will be subjected to in</td><td>-</td></tr>
</table>

	the course of the experiments		
	b) Furnish details of injections schedule Substances	Doses	CCl4- intraperitoneal injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil, Acetylcystine 950 mg/kg, Citrus limon 6 ml/kg/oral route for 6 wks, Emblica officinalis 700mg/kg/oral route for six wks.
		Sites	IP and oral
		Volumes	-
	c) Blood withdrawal Details:	Volumes	1 ml
		Sites	retro-orbital plexus
	d) Radiation	Dosage	Not Applicable
		Schedules	Not Applicable
	e) Nature of compound/Broad Classification of drug/NCE:	CCl4 , Acetylcystine, Citrus limon , Emblica officinalis	
10	Does the protocol prohibit use of anesthetic or analgesic for the conduct of painful procedures?	NO	
	If yes, justify.	Not Applicable	
11	Will survival surgery be done?	No	
	If yes, the following to be described.		
	a) List and describe all surgical procedures (including methods of asepsis)	-----	
	b) Personnel involved in surgical procedure	Name 1	-----
		Qualification	----
		Experience in such surgeries	----
		Name 2	----
		Qualification	----
		Experience in such surgeries	----
		Name 3	----
		Qualification	----
		Experience in such surgeries	----
	c) Describe post-operative care	------	
	d) Will major survival surgery is to be performed more than once on a single animal?	NO	
		If Yes, Justify: ------	
12	**Describe post-experimentation procedures.**		
	a) Scope for Reuse	No	

	b) Rehabilitation (if reuse is ti	-	
		-	
	c) Describe method of Euthanasia (If required in the protocol)	After giving CO2 (70 %) animal will be scarified and kidney will be remove for histopathology. (detail of experiment synopsis is attached)	
	d) Method of carcass disposal after euthanasia.	Common biomedical waste facility affiliated to AMC.	
14	**Will extra-institutional transport is envisaged?**	YES	
	If yes, Describe animal transportation methods	Through AC vehicle along with adequate food & water.	
15	**Use of hazardous agents:** (use of recombinant DNA-based agents or potential human pathogens requires documented approval of the Institutional Biosafety Committee (IBC). For each category, the agents and the biosafety level required, appropriate therapeutic measures and the mode of disposal of contaminated food, animal wastes and carcasses must be identified).		
	Does your project involved use of any of the below mentioned agent?		
	(a) Radionucleotides (AERB)	NO	
	(b) Microorganisms / Biological infectious Agents (IBSC)	NO	
	(c) Recombinant DNA (RCGM)	NO	
	(d) Any other Hazardous Chemical / Drugs	NO	
	Have you ticked "Yes" in either of above four hazardous agents? **If so, copy of the approval certificates of the respective agencies:**		
	Certificate attached	Not applicable	

Investigator's declaration.

1. I certify that the research proposal submitted is not unnecessarily duplicative of previously reported research.

2. I certify that, I am qualified and have experience in the experimentation on animals.

3. For procedures listed under item 10, I certify that I have reviewed the pertinent scientific literature and have found no valid alternative to any procedure described herein which may cause less pain or distress.

4. I will obtain approval from the IAEC/ CPCSEA before initiating any changes in this study.

5. I certify that performance of experiment will be initiated only upon review and approval of scientific intent by appropriate expert body (Institutional Scientific Advisory Committee / funding agency / other body).

6. I certify that I will submit appropriate certification of review and concurrence for studies mentioned in point 14.

7. I shall maintain all the records as per format (Form D) and submit to Institutional Animal Ethics Committee (IAEC).

8. I certify that, I will not initiate the study before approval from IAEC/ CPCSEA received in writing. Further, I certify that I will follow the recommendations of IAEC/ CPCSEA.

9. I certify that I will ensure the rehabilitation policies are adopted (wherever required).

Signatures	
Name of Investigator	Dr. Deepali Jaybhaye
Signature	
Date	27/12/2021

CERTIFICATE

This is to certify that,

Project proposal no	002/Pharmac/IAEC/2021
Entitled	---
submitted by Dr./ Mr. / Ms	Dr. Deepak Bhosle and Dr. Deepali Jaybhaye

has been approved/recommended by the **IAEC MGM Medical College & Hospital Aurangabad** in its meeting held on **08-01-2022** (date) and **Rat-36,** have been sanctioned under this.

Authorized by	Name	Signature	Date
Chairman:	Dr. Deepali Jaybhaye		
Member Secretary:	Dr. Sangita Phatale		
Main Nominee of CPCSEA:	Dr. Shrikant Satale		

Annexure: Study Plan / Outline of Reasearch

<table>
<tr><td>Title</td><td colspan="4">The study of renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.</td></tr>
<tr><td>Animal species / strain</td><td colspan="4">Wistar rats</td></tr>
<tr><td>How do this animal relates to human in terms of test item nature</td><td colspan="4">Wistar rats ideal model for human kidney study.</td></tr>
<tr><td rowspan="7">Study design
(In all the six groups renal injury will be induce by CCl4 IP injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil and then give standard drug Acetylcystine and test drugs i.e Citrus limon and Emblica officinalis while group I will serve as control group only distilled water will be given in this)</td><td>Group</td><td>Test item</td><td>Dose</td><td>No of animals</td></tr>
<tr><td>Group I</td><td>CCl4</td><td>1 ml Distilled water /Oral route</td><td>6</td></tr>
<tr><td>Group II</td><td>Acetylcystine</td><td>950 mg/kg/ oral</td><td>6</td></tr>
<tr><td>Group III</td><td>Citrus limon</td><td>6 ml/kg/oral</td><td>6</td></tr>
<tr><td>Group IV</td><td>Emblica officinalis</td><td>700mg/kg/oral</td><td>6</td></tr>
<tr><td>Group V</td><td>Citrus limon + Emblica officinalis</td><td>6 ml/kg + 700 mg /kg/oral</td><td>6</td></tr>
<tr><td>Group VI</td><td>Citrus limon + Emblica officinalis + Acetylcystine</td><td>6 ml/kg + 700 mg /kg + 950 mg /kg/ oral</td><td>6</td></tr>
<tr><td>Rationale for dose selection</td><td colspan="4">Drug dose is selected as per the previous literature.</td></tr>
<tr><td>Duration, dosing schedule, route, other details</td><td colspan="4">Duration of study – 6 weeks
Drugs and doses- CCl4 IP injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil and Acetylcystine 950 mg/kg/ oral , Citrus limon 6 ml/kg/oral route, Emblica officinalis 700 mg/kg/oral route for 6 wks</td></tr>
<tr><td>Parameters to be investigated</td><td colspan="4">BUN and Serum Creatinine levels along with activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) in a homogenized renal tissue will be determined using ELISA kits, on the kit guidelines. This parameters will be taken after inducing the renal injury by CCl4. And after giving standard and test drugs.
Kidney histopathology will be done at the end of study.</td></tr>
<tr><td>Result interpretation criteria</td><td colspan="4">Renoprotective effect of drugs.</td></tr>
<tr><td>How will you correlate/ translate these results to human</td><td colspan="4">Improvement in markers of oxidative stress and BUN and serum Creatinine level.</td></tr>
</table>

What is the use of your obtained results? How it will be taken forward? How it will be used for humans	As Citrus limon i.e lemon and Emblica officinalis i.e Amla is easily available in India having no adverse effects in higher doses. If we got positive result the drug will be available for the treatment of kidney failure in less cost.

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Research Article

Anti-Inflammatory Effect of *Emblica officinalis* in Rodent Models of Acute and Chronic Inflammation: Involvement of Possible Mechanisms

Mahaveer Golechha,[1,2] Vikas Sarangal,[1] Shreesh Ojha,[3] Jagriti Bhatia,[1] and Dharmveer S. Arya[1]

[1] *Department of Pharmacology, All India Institute of Medical Sciences, New Delhi 110029, India*
[2] *Public Health Foundation of India, New Delhi 110070, India*
[3] *Department of Pharmacology and Therapeutics, Faculty of Medicine & Health Sciences, UAE University, P.O. Box 17666, Al Ain, UAE*

Correspondence should be addressed to Dharmveer S. Arya; dsarya16@hotmail.com

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Emblica officinalis, commonly known as amla in Ayurveda, is unarguably the most important medicinal plant for prevention and treatment of various ailments. The present study investigated the anti-inflammatory activity of hydroalcoholic extract of *Emblica officinalis* (HAEEO). Acute inflammation in rats was induced by the subplantar injection of carrageenan, histamine, serotonin, and prostaglandin E_2 and chronic inflammation was induced by the cotton pellet granuloma. Intraperitoneal (i.p.) administration of HAEEO at all the tested doses (300, 500, and 700 mg/kg) significantly ($P < 0.001$) inhibited rat paw edema against all phlogistic agents and also reduced granuloma formation. However, at the dose of 700 mg/kg, HAEEO exhibited maximum anti-inflammatory activity in all experimental models, and the effects were comparable to that of the standard anti-inflammatory drugs. Additionally, in paw tissue the antioxidant activity of HAEEO was also measured and it was found that HAEEO significantly ($P < 0.001$) increased glutathione, superoxide dismutase, and catalase activity and subsequently reduced lipid peroxidation evidenced by reduced malondialdehyde. Taken all together, the results indicated that HAEEO possessed potent anti-inflammatory activity and it may hold therapeutic promise in the management of acute and chronic inflammatory conditions.

1. Introduction

Inflammation plays a major role in rheumatoid arthritis and osteoarthritis [1]. In clinics, the nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for pain relief in arthritic conditions. However, their continual use is associated with serious adverse effects like gastric mucosal damage, occult blood loss and elevation of serum hepatic transaminases, salt and water retention, and also exacerbation of asthma [2]. In order to circumvent these adverse effects associated with conventional NSAIDs, novel selective COX-2 inhibitors are in progress. However, the development of serious adverse reactions, like cardiovascular events with rofecoxib and Stevens-Johnson syndrome with valdecoxib, has compelled their withdrawal from use [3]. Additionally, the clinical uses of the remaining drugs in this class have been prescribed with caution and have consequently decreased [4].

In milieu of these observations patients as well as health care providers prefer to use alternative therapeutic agents as they are considered to be safe and effective in alleviating inflammation associated with arthritis. Several Indian medicinal plants were reported as an important source of new chemical moieties with potential therapeutic effects [5]. The studies on plants with substantiated folkloric use as anti-inflammatory agents are viewed as a productive and logical research strategy in the search for new anti-inflammatory drugs

Emblica officinalis Gaertn. (*Euphorbiaceae*) commonly known as amla grow in the tropical areas of South-East

Asia. The fruit of the plant is one of the most important medicinal ingredients used in Ayurveda, Siddha, Unani, Arabic, Tibetan, and various other folk systems for the management of myriad chronic ailments [6]. Experimental studies have shown potent antioxidant, analgesic, antipyretic, adaptogenic, immunomodulatory, and antiulcerogenic activities of the fruit of *Emblica officinalis* [6–8].

The fruits are reported to contain thermostable vitamin C, minerals, amino acids, tannins, flavonoids, and other important phytochemicals which are believed to possess diverse pharmacological and biological effects [9]. Earlier studies have shown that the leaf extract possesses anti-inflammatory activities in the carrageenan and dextran-induced rat hind paw edema [10]. However, studies on the fruit extract which is the most used part of amla have never been performed. Therefore, the present study was carried out to evaluate the anti-inflammatory activity of the hydroalcoholic extract of the fruit of *Emblica officinalis* (HAEEO) in both acute and chronic models of inflammation in rats. Further, in order to understand the possible underlying mechanism, the effect of extract on the oxidative stress produced by carrageenan was also studied in the rat paw.

2. Methodology

2.1. Plant Extract. The standardized lyophilized hydroalcoholic extract of the fruit of *Emblica officinalis* (HAEEO) was procured from Sanat Products Limited, India (A WHO-GMP and ISO 9001 Accredited Herbal Extract Manufacturer Company). The voucher specimen of lyophilized extract of the fruits of *Emblica officinalis* (number EO 0114) was deposited at Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India. The phytochemical analysis was done by using HPLC (Waters, Milford Massachusetts, USA). The extract obtained was of the highest purity with 28.26% w/w of hydrolysable tannins emblicanin A and emblicanin B on dried weight basis.

2.2. Drugs and Chemicals. Carrageenan, histamine, 5-hydroxytryptamine (serotonin), chlorpheniramine, cyproheptadine, prostaglandin E_2 (PGE_2), and bovine serum albumin were purchased from Sigma Chemicals, St. Louis, MO, USA. Indomethacin was procured from Cipla, India. All other chemicals and reagents were of analytical grade.

2.3. Experimental Animals. All experimental procedures described were reviewed and approved by the Institutional Animal Ethics Committee and care of animals was taken as per guidelines of CPCSEA, Ministry of Environment and Forest, Government of India. Wistar albino rats of either sex weighing 180–200 g were used for the study. The animals were procured from the central animal facility in All India Institute of Medical Sciences, New Delhi. The rats were group-housed in polypropylene cages with no more than four animals per cage. They were maintained under standard laboratory conditions with natural dark-light cycle and were allowed free access to standard pellet diet (Golden Feeds, India) and tap water *ad libitum*. All the experiments were carried out using five groups, each containing 6 animals (Groups I–V) except carrageenan-induced paw edema where Groups I–VI were used.

2.4. Determination of Anti-Inflammatory Activity of HAEEO on Acute Inflammation

2.4.1. Carrageenan-Induced Hind Paw Edema in Rats. Acute inflammation was produced by injecting 0.1 mL of carrageenan (1% in saline) locally into the plantar aponeurosis of the right hind paw of the rats [11, 12]. Group I served as normal control, where no inflammation was induced. This group was used for evaluation of biochemical parameters. Groups II and III received vehicle (saline 1 mL/kg, i.p.) and standard drug indomethacin (10 mg/kg, p.o.), respectively, and served as vehicle and positive controls. HAEEO (300, 500, and 700 mg/kg, i.p.) was administered to Groups IV, V, and VI, respectively. The HAEEO or vehicle was administered 30 min prior to injection of carrageenan and indomethacin was orally administered 1 h prior to the injection of carrageenan. The pedal volume up to the ankle joint was measured using a digital plethysmometer (Ugo Basile, 7140 Comerio, Varese, Italy) at 0 h (just before carrageenan injection) and then at 3 h. The different timing was chosen because of the different route of drug administration. The % inhibition of edema volume between treated and control groups was calculated as follows: % Inhibition = $V_c - V_t \times 100/V_c$, where V_c and V_t represent the mean increase in paw volume in control and treated groups, respectively.

2.4.2. Autacoids-Induced Hind Paw Edema in Rats. This experiment was conducted according to the method described by Singh and Pandey [13]. The autacoids serotonin (1 mg/mL), histamine (1 mg/mL), and prostaglandin E_2 (1 μg/mL) were employed as phlogistic agents. The effect of HAEEO (300, 500, and 700 mg/kg, i.p.) and vehicle was tested individually against each autacoid. The anti-inflammatory effect of HAEEO was compared with that of standard drugs against each autacoid: phenylbutazone (PBZ, 100 mg/kg, p.o.) against prostaglandin E_2, chlorpheniramine (CPM, 3 mg/kg, p.o.) against histamine, and cyproheptadine (CPH, 3 mg/kg, p.o.) against serotonin. Right hind paw edema was induced by the subplantar injection of 0.1 mL of different phlogistic agents in the respective groups. HAEEO was administered i.p. 30 min prior to inflammatory insult and standard reference drugs were administered p.o. 1 h prior to the inflammatory insult. The pedal volume was measured just before (0 h) and then at 3 h after the phlogistic challenge.

2.5. Determination of Anti-Inflammatory Activity of HAEEO on Chronic Inflammation

2.5.1. Cotton Pellet-Induced Granuloma in Rats. The cotton pellet-induced granuloma in rats was studied according to the method of D'Arcy et al. [14]. The animals were divided into five groups with six animals in each group. The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin

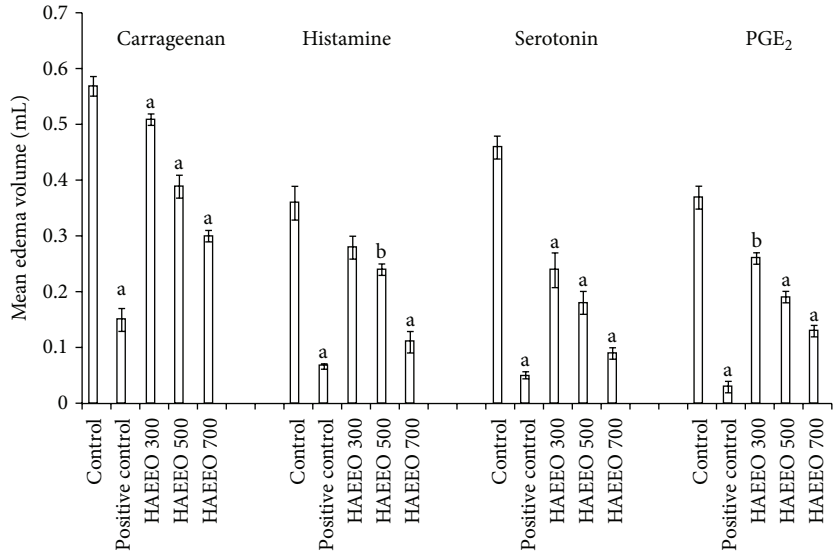

Figure 1: Effect of HAEEO on carrageenan- and autacoids-induced hind paw edema in rats. Each value represents the mean ± S.E.M. (n = 6). [a]$P < 0.001$ and [b]$P < 0.01$ compared to control. Positive control carrageenan (indomethacin 10 mg/kg), histamine (chlorpheniramine 3 mg/kg), serotonin (cyproheptadine 3 mg/kg), and PGE_2 (phenylbutazone 100 mg/kg). HAEEO: hydroalcoholic extract of *Emblica officinalis*.

region of each rat. Group I served as control and received the vehicle. HAEEO in the doses of 300, 500, and 700 mg/kg, i.p. was administered to animals in groups II, III, and IV for seven consecutive days from the day of cotton pellet implantation. Group V received indomethacin (10 mg/kg, p.o.) for the same period. On day 8, the animals were anaesthetized and the pellets together with the attached granuloma tissue were carefully removed and freed from extraneous tissues. The wet pellets were weighed and then dried in an oven at 60°C for 24 h to a constant weight; after that the dried pellets were weighed again. Increment in the dry weight of the pellets was taken as a measure of granuloma formation.

2.6. Determination of Levels of Oxidative Stress Parameters. The biochemical markers of oxidative stress were determined in the carrageenan-induced rat paw edema model. Animals were euthanized 3 h after measurement of paw volume and the inflamed paw tissue was removed and processed for the estimation of oxidative stress. Paw tissue samples were thawed and homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from paw tissue were used to determine the malondialdehyde (MDA) [15] and glutathione [16]. The remaining homogenates were centrifuged at 7000 rpm for 30 min at 4°C temperature and the supernatant was used for estimation of superoxide dismutase (SOD) [17], catalase [18], and protein [19].

2.7. Statistical Analysis. Data were expressed as mean ± S.E.M. Statistical differences between the treatment and the respective control groups were evaluated by one-way ANOVA followed by Tukey-Kramer post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Carrageenan-Induced Hind Paw Edema in Rats. The mean increase in paw edema volume was 1.0 ± 0.02 mL in the vehicle-treated control rats. All the three doses of HAEEO (300, 500, and 700 mg/kg, i.p.) produced a dose-dependent significant ($P < 0.001$) reduction in the mean paw edema volume (Figure 1). The percentage inhibition in paw edema volume as compared to the vehicle treated group was 48.9, 60.2, and 70.0% for HAEEO, respectively. The standard drug, indomethacin (10 mg/kg, p.o.), exhibited maximum anti-inflammatory activity with 84.27% inhibition.

3.2. Effect of HAEEO on Changes in Tissue Levels of MDA, GSH, SOD, and Catalase. Carrageenan injection into the subplantar tissue of the rat paw decreased the tissue GSH, catalase, and SOD levels (Table 1). Both HAEEO and indomethacin produced a significant increase in the endogenous antioxidants in a dose dependent manner to maintain oxidative homeostasis. Carrageenan injection produced significant lipid peroxidation, as evidenced by a marked increase in the levels of MDA. Both HAEEO and indomethacin produced a significant decrease in the levels of MDA. HAEEO at 700 mg/kg dose most effectively stabilized the oxidative stress parameters.

3.3. Autacoid-Induced Hind Paw Edema in Rats. A dose-dependent effect of HAEEO on hind paw edema was observed. The 700 mg/kg dose of HAEEO was the most effective (Figure 1). It significantly ($P < 0.001$) inhibited hind paw edema induced by histamine (68.47%), serotonin (79.26%), and PGE_2 (64.00%). Phenylbutazone (100 mg/kg, p.o.), chlorpheniramine (3 mg/kg, p.o.), and cyproheptadine

Table 1: Effect of HAEEO on oxidative stress parameters in carrageenan-induced paw edema in rats.

Treatment	GSH (μg g^{-1} tissue)	MDA (nmol g^{-1} tissue)	SOD (U mg^{-1} protein)	Catalase (U mg^{-1} protein)
Normal control	32.91 ± 2.13	27.14 ± 2.96	40.54 ± 2.23	57.19 ± 2.48
Carrageenan control (vehicle treated)	13.33 ± 1.39[a]	88.45 ± 4.79[a]	15.19 ± 1.21[a]	14.48 ± 0.75[a]
Indomethacin (10 mg kg^{-1})	26.66 ± 1.66[b]	28.54 ± 6.85[b]	31.96 ± 1.08[b]	49.30 ± 1.86[b]
HAEEO (300 mg kg^{-1})	19.16 ± 1.53	63.18 ± 4.51[d]	21.18 ± 1.80	27.44 ± 1.66[b]
HAEEO (500 mg kg^{-1})	22.29 ± 2.80[d]	49.14 ± 5.83[b]	24.87 ± 0.98[d]	36.1 ± 0.83[b]
HAEEO (700 mg kg^{-1})	26.25 ± 2.18[c]	35.10 ± 2.78[b]	29 ± 1.66[b]	41.82 ± 1.41[b]

Values given are mean ± S.E.M. ($n = 6$). [a]$P < 0.001$ compared to normal control and [b]$P < 0.001$, [c]$P < 0.01$, and [d]$P < 0.05$ compared to carrageenan control. HAEEO: hydroalcoholic extract of *Emblica officinalis*; GSH: glutathione; MDA: malondialdehyde; SOD: superoxide dismutase.

Table 2: Effect of HAEEO on cotton pellet-induced granuloma in rats.

Group	Weight of cotton pellet granuloma (mg)	Protection percentage
Control (vehicle treated)	53.81 ± 1.94	—
Positive control (indomethacin 10 mg kg^{-1})	18.96 ± 2.18[a]	64.76
HAEEO (300 mg kg^{-1})	35.23 ± 1.48[a]	34.52
HAEEO (500 mg kg^{-1})	30.30 ± 0.94[a]	43.69
HAEEO (700 mg kg^{-1})	25.63 ± 1.29[a]	52.36

Each value represents the mean ± S.E.M. ($n = 6$). [a]$P < 0.001$ compared to control. HAEEO: hydroalcoholic extract of *Emblica officinalis*.

(3 mg/kg, p.o.) also significantly ($P < 0.001$) inhibited hind paw edema induced by PGE_2 (92.00%), histamine (82.06%), and serotonin (89.56%), respectively (Figure 1).

3.4. Cotton Pellet-Induced Granuloma. The study of HAEEO on proliferative phase of inflammation indicated that HAEEO (300, 500, and 700 mg/kg, i.p.) significantly ($P < 0.001$) and dose-dependently reduced the granuloma formation (Table 2). Indomethacin (10 mg/kg, p.o.) exhibited significant ($P < 0.001$) and maximum inhibition on granuloma formation.

4. Discussion and Conclusion

In the present study, it was observed that *Emblica officinalis* possessed potent anti-inflammatory activity both in acute and chronic rat models of inflammation. Inflammation is part of the host defense system and is triggered by a variety of noxious stimuli. It involves a complex interplay between cell-cell, cell-mediator, and tissue interactions [20]. Carrageenan-induced rat paw edema model is a well-established model for evaluating anti-inflammatory drugs [21]. The edema and inflammation induced by carrageenan are a biphasic event. In the initial 1 h after carrageenan administration, the edema and inflammation are mediated by histamine and serotonin. Later, the increased vascular permeability is maintained by the release of kinins up to about 2.30 h. Thereafter from 2.30 h to 6 h, inflammation is mediated by prostaglandins and is also associated with migration of leucocytes into the inflamed site [22].

Carrageenan-induced paw edema model in rats is known to be sensitive to cyclooxygenase (COX) inhibitors and has been used to investigate the effect of nonsteroidal anti-inflammatory agents [23]. The result of the present study indicated that HAEEO afforded protection against the carrageenan-induced acute inflammation in dose dependent manner. HAEEO at a dose of 700 mg/kg exhibited significant anti-inflammatory activity with 70.0% inhibition of paw edema and was comparable to the indomethacin group. In autacoid-induced models of inflammations (against serotonin, histamine, and PGE_2), HAEEO produced significant inhibitory activity. The present study exhibited HAEEO's anti-inflammatory action by means of inhibiting the synthesis, release, or action of inflammatory mediators like histamine, serotonin, and prostaglandins that are involved in inflammation. In earlier study on the anti-inflammatory activity of leaf extracts of *Emblica officinalis* in carrageenan- and dextran-induced rat paw edema models, it was reported that the extracts did not inhibit the synthesis of the lipid mediators LTB_4, TXB_2, or PAF [24]. Therefore, it is quite possible that a composite effect may have been responsible for the observed protection against autacoids-induced inflammation.

The role of excess generation of nitric oxide (NO) in inflammatory response is well studied. Inflammation or tissue damage leads to induction of iNOS (inducible nitric oxide synthase); consequently large amounts of NO are generated at the site of inflammation [25]. NO reacts with superoxide anion to form peroxynitrite, an oxidizing molecule capable of eliciting lipid peroxidation. In lipid peroxidation there is oxidative deterioration of polyunsaturated lipids to form radical intermediates that causes cellular damage [26]. MDA is a major end product of this reaction and an index of lipid peroxidation that is measurable by estimating as thiobarbituric acid reactive substance (TBARS) [27]. The present study showed that both HAEEO (500 and 700 mg/kg) and indomethacin (10 mg/kg) decreased the levels of MDA.

The infiltrating inflammatory cells also generate reactive oxygen species (ROS) and free radicals. The most common

ROS include the superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide. The enzyme superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. The activity of SOD reduces during severe inflammation as well as in the presence of oxidative stress [28]. The large quantities of hydrogen peroxide generated are then taken care of by catalase and glutathione peroxidase (GPx) to water. Excessive production of lipid hydroperoxide may also lead to reduced activity of GPx in inflammatory conditions [29]. Besides the enzymatic antioxidants, the level of glutathione, a nonenzymatic reducing agent that traps free radicals and prevents oxidative damage, is also diminished in inflammatory conditions [30]. Both HAEEO (700 mg/kg) and indomethacin (10 mg/kg) maintained the oxidative homeostasis, and the levels of reduced glutathione and activities of catalase and SOD were comparable to the control animals.

Experimental studies have shown the potent antioxidant property of the fruit of *Emblica officinalis* [31]. Various phytochemical constituents of the plant such as emblicanins A and B, gallic acid, and ellagic acids have been identified as powerful free radical scavengers [9]. Moreover, other phytochemicals with NO scavenging properties like Geraniin, Corilagin, and Furosin have been reported in the *Emblica officinalis* fruit extract [32]. Recently, it has also been reported that the superoxide scavenging properties of *Emblica officinalis* extract approximate those of L-ascorbic acid, a well-established antioxidant [33].

In order to assess the efficacy of HAEEO against chronic inflammation, the cotton pellet granuloma model in rats was employed. HAEEO at all doses tested significantly ($P < 0.001$) reduced the granuloma formation. The maximum effect was observed at the dose of 700 mg/kg with 52.36% inhibition in granuloma formation as compared to the control group. Although the exact mechanism of anti-inflammatory activity of HAEEO on proliferative phase of inflammation in this model is not known, it may be hypothesized that both the antioxidant and the immunomodulatory properties of the plant may have been responsible for the protective action of the extract. *Emblica officinalis* extract has been reported to inhibit NF-κB activation, a key transcription factor involved in chronic inflammatory response and ageing [34]. The inhibition of NF-κB leads to reduction in the iNOS and COX-2 enzyme levels.

The main adverse effect of nonsteroidal anti-inflammatory drugs is their ability to produce gastric lesions [35]. Furthermore, Sairam et al. [36] demonstrated the ulcer protective potential of *Emblica officinalis* in different acute gastric ulcer models in rats induced by aspirin, ethanol, cold restraint stress, and pyloric ligation and healing effect in chronic gastric ulcers induced by acetic acid in rats. The antiulcerogenic activity of *Emblica officinalis* is definitely complementary to the good anti-inflammatory and antioxidant activity observed in the present study. Further, it has been shown that *Emblica officinalis* was well tolerated in mice even at the dose of 2.5 g/kg [37].

In conclusion, the present study clearly demonstrated that HAEEO possessed potent anti-inflammatory activity and also scientifically validated the traditional use of this plant for treating inflammatory disorders in the folk medicine. The advantages of HAEEO, namely, better and safer anti-inflammatory profile with potent antiulcerogenic activity, deserve further studies to establish the therapeutic value and elucidate the mechanism of action in the treatment of different inflammatory diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Citric Acid Effects on Brain and Liver Oxidative Stress in Lipopolysaccharide-Treated Mice

Omar M.E. Abdel-Salam,[1] Eman R. Youness,[2] Nadia A. Mohammed,[2] Safaa M. Youssef Morsy,[2] Enayat A. Omara,[3] and Amany A. Sleem[4]

Departments of [1]Toxicology and Narcotics, [2]Medical Biochemistry, [3]Pathology, and [4]Pharmacology, National Research Center, Cairo, Egypt

ABSTRACT Citric acid is a weak organic acid found in the greatest amounts in citrus fruits. This study examined the effect of citric acid on endotoxin-induced oxidative stress of the brain and liver. Mice were challenged with a single intraperitoneal dose of lipopolysaccharide (LPS; 200 μg/kg). Citric acid was given orally at 1, 2, or 4 g/kg at time of endotoxin injection and mice were euthanized 4 h later. LPS induced oxidative stress in the brain and liver tissue, resulting in marked increase in lipid peroxidation (malondialdehyde [MDA]) and nitrite, while significantly decreasing reduced glutathione, glutathione peroxidase (GPx), and paraoxonase 1 (PON1) activity. Tumor necrosis factor-alpha (TNF-α) showed a pronounced increase in brain tissue after endotoxin injection. The administration of citric acid (1–2 g/kg) attenuated LPS-induced elevations in brain MDA, nitrite, TNF-α, GPx, and PON1 activity. In the liver, nitrite was decreased by 1 g/kg citric acid. GPx activity was increased, while PON1 activity was decreased by citric acid. The LPS-induced liver injury, DNA fragmentation, serum transaminase elevations, caspase-3, and inducible nitric oxide synthase expression were attenuated by 1–2 g/kg citric acid. DNA fragmentation, however, increased after 4 g/kg citric acid. Thus in this model of systemic inflammation, citric acid (1–2 g/kg) decreased brain lipid peroxidation and inflammation, liver damage, and DNA fragmentation.

KEY WORDS: • ***antioxidant activity*** • ***citric acid*** • ***cytokines*** • ***dietary supplementation*** • ***peripheral infection*** • ***systemic inflammation***

INTRODUCTION

Oxidative stress is the term used to indicate the imbalance between reactive oxygen species and antioxidant defense mechanisms. Under physiological conditions, reactive oxygen species play integral roles in intracellular signaling, physiological immunological responses, and gene expression. Reactive oxygen metabolites can be generated in excess from many sources. The most important source is the leakage of electrons from the mitochondrial electron transport chain to generate superoxide radical ($O_2^{\bullet -}$). Other sources are xanthine oxidase, NADPH oxidases, activated phagocytes, and nitric oxide synthases (NOSs). When excessively produced, however, these species could result in potential cellular and tissue damage. Being highly unstable molecules with unpaired electrons, reactive oxygen metabolites, such as superoxide radical and hydroxyl radical, react with the cellular membrane polyunsaturated fatty acids to form lipid peroxides, oxidize and cross-link proteins including enzymes, or oxidize DNA, with the potential to produce a harmful or even lethal event.[1–3] Cellular defenses against free radicals and reactive oxygen species include enzymes, such as catalase, glutathione peroxidase (GPx), and superoxide dismutase, as well as nonenzymatic antioxidant mechanisms, for example, glutathione (GSH), ascorbic acid, carotenoids, and vitamin E.[2,4] Oxidative stress occurs when redox homeostasis is tipped toward an overbalance of free radicals, due to either their overproduction or deficiencies in antioxidant defense.[5] Oxidative stress has been implicated in the pathogenesis of numerous diseases, such as diabetes mellitus, cardiovascular disease, and neurodegenerative and psychiatric disorders.[6,7] The brain is considered particularly vulnerable to oxidative damage because of its high oxygen utilization and hence generation of free radical byproducts, the high content of polyunsaturated lipids, the biomacromolecules most susceptible to oxidation, its modest antioxidant defenses, and the presence of redox-catalytic metals, such as iron and copper.[7,8]

Citric acid (2-hydroxy-1,2,3-propane-tricarboxylic acid) is a weak organic acid found in the greatest amounts in citrus fruits, such as lemon, grapefruit, tangerine, and orange. Lemon and lime juices are rich sources.[9] It is used as a natural preservative and also to add an acidic (sour) taste to foods and soft drinks.[10] Being a component of the tricarboxylic acid or Krebs cycle, citric acid is found in all animal

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Address correspondence to: Omar M.E. Abdel-Salam, MD, PhD, Department of Toxicology and Narcotics, National Research Centre, Tahrir Street, Dokki, Cairo 12311, Egypt, E-mail: omasalam@hotmail.com

tissues as an intermediary substance in oxidative metabolism. Studies indicated that citrate decreases lipid peroxidation and downregulates inflammation by reducing polymorphonuclear cell degranulation and attenuating the release of myeloperoxidase, elastase, interleukin (IL)-1β, and platelet factor 4.[11–13] *In vitro*, citrate improved endothelial function by reducing the inflammatory markers and decreasing neutrophil diapedesis in hyperglycemia.[14] Moreover, citric acid has been shown to reduce hepatocellular injury evoked in rats by carbon tetrachloride.[15] Citric acid might thus prove of value in decreasing oxidative stress.

Thus, in view of the antioxidant and anti-inflammatory effects for citrate reported just now and since citrate anticoagulation has been employed in the critically ill patients, it looked pertinent to study the effect of citric acid administration on oxidative stress and tissue injury in a model of systemic inflammatory illness caused by intraperitoneal (i.p.) lipopolysaccharide (LPS) administration in mice. LPS is a constituent of the cell walls of gram-negative bacteria. When given systemically, LPS potently stimulates the immune cells in the periphery (through plasma membrane proteins, *e.g.*, the toll like receptor 4 [TLR4] and CD14) to release proinflammatory cytokines, such as necrosis factor-alpha (TNF-α), IL-1β, and IL-6 in the periphery and brain. This results in the development of systemic and neuroinflammation.[16–19] LPS-induced endotoxemia is a well-established model for infection with gram-negative bacteria and is widely used to study endotoxin effects on peripheral tissue/organs and the influence of systemic inflammation on the brain.

MATERIALS AND METHODS

Animals

Swiss male albino mice that weigh 22–25 g (age 5–6 weeks) were used. Mice were obtained from animal house colony of the National Research Centre. Standard laboratory food and water were provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Drugs and chemicals

A purified, lyophilized *Escherichia coli* endotoxin (Serotype 055:B5; Sigma) was used; it was dissolved in sterile physiological saline, aliquoted, and frozen at −20°C. The same stock solutions were used for all experiments. Citric acid and all other chemicals were of analytical grade and were obtained from Sigma. The dose of LPS (200 μg/kg) and the time for tissue sampling were based on previous studies.[20]

Study design

Mice were randomly divided into five equal groups (six mice each). Mice were treated with either 0.2mL of: sterile physiological saline (group 1) or citric acid at doses of 1, 2, and 4 g/kg, orally (groups 2–4). Treatments were given just prior to endotoxin administration (LPS: 200 lg/kg, injected intraperitoneally, 0.1 mL). The fifth group received just the vehicle, no LPS (negative control). Mice were euthanized after 4 h of LPS or vehicle injection by decapitation under ether anesthesia, where the brain and liver of each mouse were then removed, washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), weighed, and stored at −80°C until the biochemical analyses. The tissues were homogenized with 0.1 M PBS at pH 7.4, to give a final concentration of 0.1g/mL for the biochemical assays. Reduced GSH, malondialdehyde (MDA), nitric oxide (nitrite), GPx, and paraoxonase 1 (PON1) activity was determined in brain and liver tissues. TNF-α was measured in brain tissue. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and DNA fragmentation were measured in liver tissue.

Determination of lipid peroxidation, reduced GSH, and nitrite levels

Lipid peroxidation was assayed by measuring the level of MDA in brain tissue using the method of Ruiz-Larrea *et al.*[21] Reduced GSH was determined in tissue by Ellman's method.[22] Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage *et al.*[23]

Determination of GPx activity

GPx activity in supernatants was determined spectrophotometrically at 340 nm by the analysis of NADPH oxidation using glutathione peroxidase kit (Biodiagnostics).[24] One unit of GPx activity is defined as the amount of protein that oxidized 1 mM NADPH per minute. The activity of GPx is expressed as mU/mL.

Determination of paraoxonase activity

Arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenyl acetate as a substrate.[25,26] In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol is measured by monitoring the increase in absorbance at 270 nm at 25°C. The working reagent consisted of 20 mM Tris/HCl buffer (pH 8.0) containing 1 mM calcium chloride and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer are added and the change in absorbance is recorded following a 20 s lag time. Absorbance at 270 nm was taken every 15 s for 120 s using a UV-Vis Recording Spectrophotometer (Shimadzu Corporation). One unit of arylesterase activity is equal to 1 μM of phenol formed per minute. The activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 M/cm at 270 nm, pH 8.0, and 25°C. Blank samples containing water are used to correct for the spontaneous hydrolysis of phenyl acetate.

Determination of TNF-α, DNA fragmentation, and liver enzymes

Tissue TNF-α was determined in brain tissue according to Chen *et al.*[27] by enzyme-linked immunosorbent assay using TNF-α kits (Biosource International) and microtiter plate reader (Fisher Biotech). Quantitation of DNA fragmentation in liver tissue was done according to the method described by Gercel-Taylor.[28] ALT and AST activities in liver were measured using commercially available kits (BioMérieux).[29,30]

Histological assessment of liver injury

The liver from each mouse was rapidly removed and fixed in freshly prepared 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Sections of 5-μm thick were cut and stained by hematoxylin and eosin (H&E) for histopathological examination. All sections were investigated by the light microscope.

Immunohistochemistry for caspase-3 and inducible nitric oxide synthase

Paraffin-embedded liver sections were deparaffinized, and hydrated. Immunohistochemistry was performed with a mouse monoclonal caspase-3 and inducible nitric oxide synthase (iNOS) for detection of the caspase cleavage and iNOS activity. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with either anti-caspase or iNOS antibodies (1:50 dilution) overnight at 4°C. After washing with PBS, followed by incubation with biotinylated goat-anti-rabbit-immunoglobulin G secondary antibodies (1:200 dilution; Dako Corp.) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) for 30 min at room temperature, the binding sites of antibody were visualized with DAB (Sigma). After washing with PBS, the samples were counterstained with H&E for 2–3 min, and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylen at room temperature for 5 min, mounted, examined, and evaluated by high-power light microscope.[31]

Statistical analysis

Data are expressed as mean ± standard error. Data were analyzed by one-way analysis of variance, followed by Duncan's multiple-range test for *post hoc* comparison of group means. Effects with a probability of $P < .05$ were considered to be significant.

RESULTS

Effect of citric acid on LPS-induced oxidative stress

Lipid peroxidation. The administration of LPS resulted in a significant increase in the level of MDA in brain and liver tissues by 140.3% (23.1 ± 1.0 vs. 55.5 ± 2.7 nmol/g tissue) and 62.9% (66.8 ± 3.8 vs. 41.0 ± 2.2 nmol/g tissue), respectively, compared with the saline control group (Fig. 1A, B).

Brain MDA was significantly decreased by 40.4% and 58% after treatment with 1 and 2 g/kg citric acid, respectively, compared with the LPS control group (33.1 ± 1.9 and 23.3 ± 1.4 vs. 55.5 ± 2.7 nmol/g tissue). The higher dose of citric acid (4 g/kg) resulted in 33.3% inhibition of brain MDA (Fig. 1A).

In contrast, no significant effect on liver MDA has been observed after treatment with citric acid (1–4 g/kg; Fig. 1B).

Reduced GSH. Following LPS challenge, the level of GSH decreased in brain and liver tissues by 72.1% (1.21 ± 0.07 vs. 4.1 ± 0.28 μmol/g tissue) and 46.9% (4.16 ± 0.29 vs. 7.83 ± 0.36 μmol/g tissue), respectively.

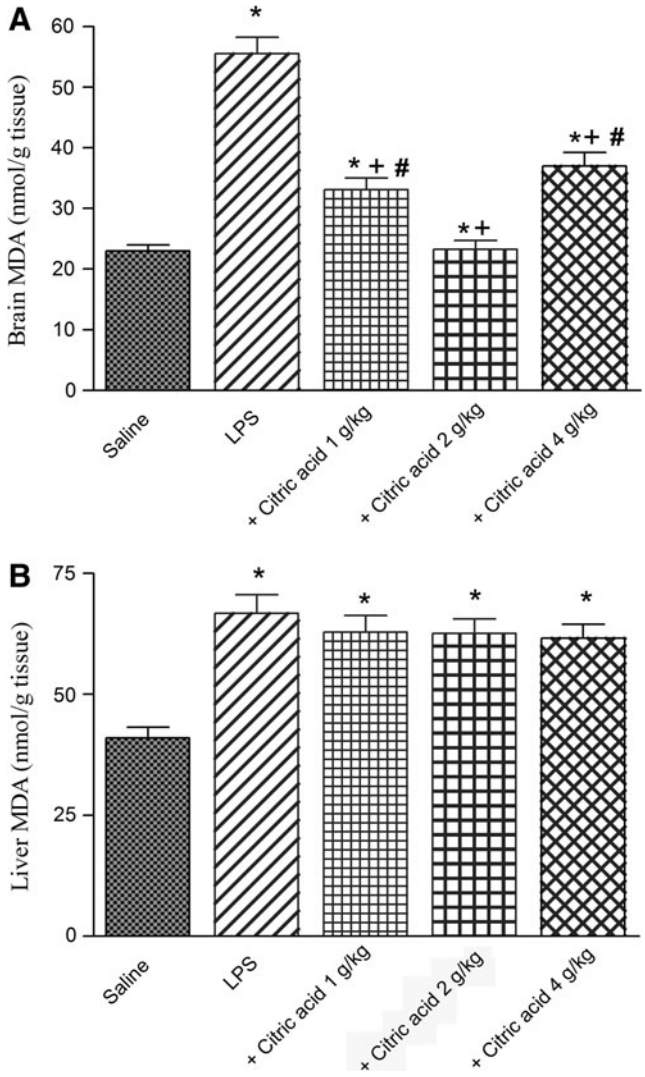

FIG. 1. **(A)** Brain and **(B)** liver tissue concentrations of malondialdehyde (MDA: nmol/g tissue) in mice given lipopolysaccharide (LPS) or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 2 g/kg of citric acid. p.o., per os.

Treatment with citric acid (1–4 g/kg) had no significant effect on brain or liver GSH (Fig. 2A, B).

Nitrite. Marked and significant increase in brain nitrite was observed after treatment with LPS compared with the vehicle-treated group (93.0 ± 4.6 vs. 31.0 ± 1.8 μmol/g tissue; Fig. 3A). Similarly, the level of liver nitrite was significantly increased by 86.3% after LPS administration compared with vehicle-treated group (123.7 ± 8.6 vs. 66.4 ± 4.1 μmol/g tissue; Fig. 3B).

In LPS-treated mice, the level of nitrite in brain tissue was markedly inhibited by 74.6% and 82.8% by citric acid at 1–2 g/kg (23.6 ± 1.2 and 16.0 ± 0.63 vs. 93.0 ± 4.6 μmol/g tissue). Nitric oxide decreased by 48.1% after citric acid at 4 g/kg, compared with the LPS-only group (Fig. 3A).

In the liver, nitrite decreased significantly by 17% by citric acid given at 1 g/kg compared with the LPS control group. The higher doses of citric acid, however, failed to significantly alter nitrite in liver tissue (Fig. 3B).

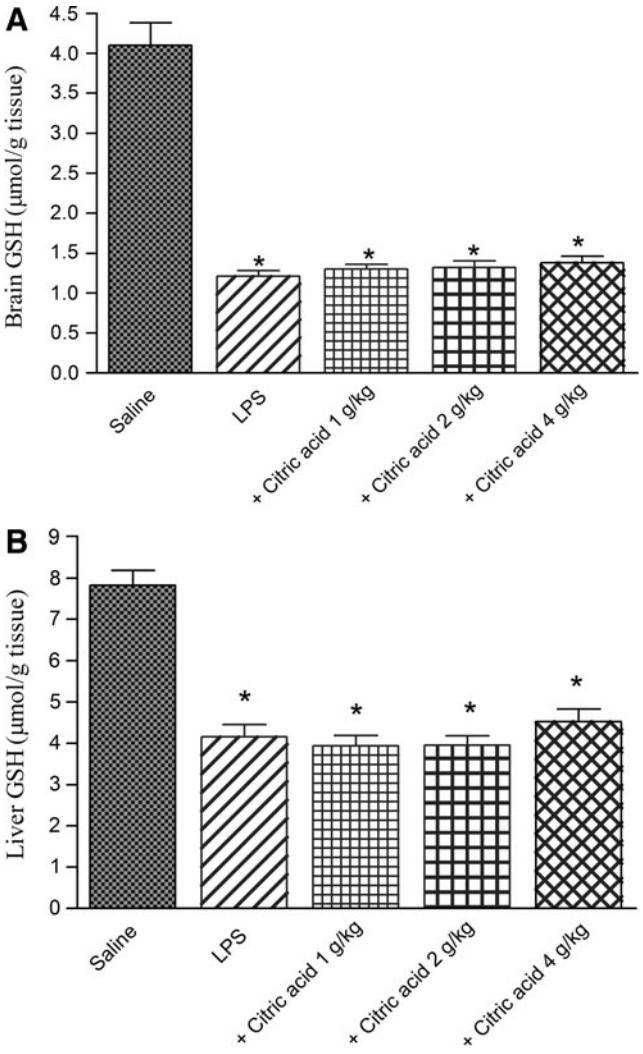

FIG. 2. **(A)** Brain and **(B)** liver tissue concentrations of reduced glutathione (GSH: μmol/g tissue) in mice given LPS or LPS + citric acid (1–4 g/kg, p.o.). $*P < .05$ versus saline control.

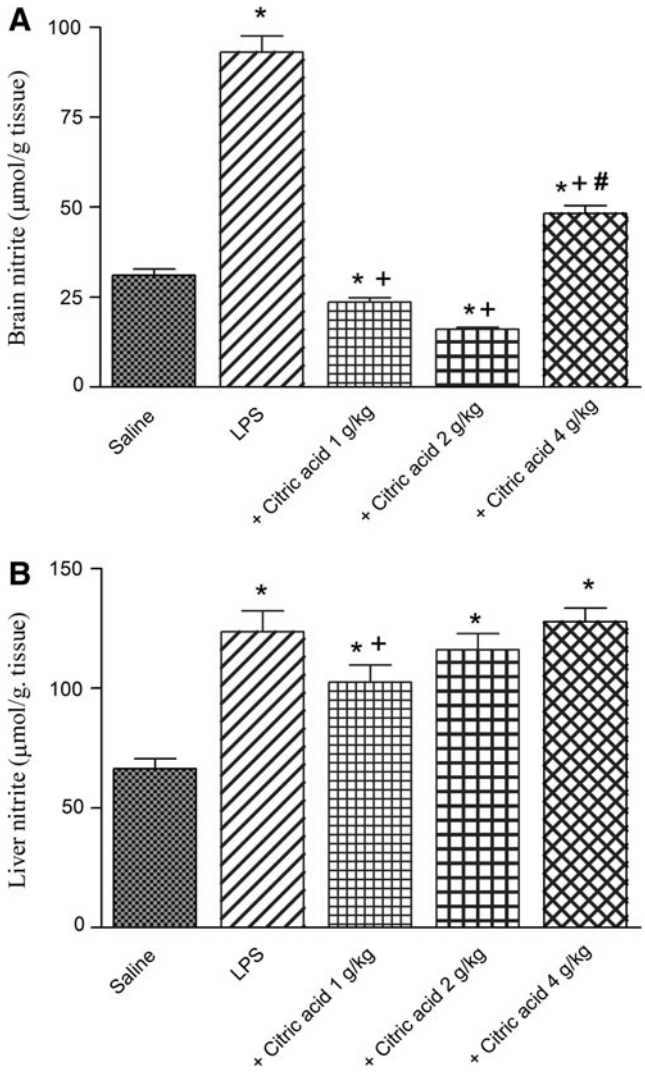

FIG. 3. **(A)** Brain and **(B)** liver tissue concentrations of nitrite (μmol/g tissue) in mice after treatment with LPS or LPS + citric acid (1–4 g/kg, p.o.). $*P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 2 g/kg of citric acid.

GPx activity. GPx activity showed a significant decrease in brain (by 81.6%) and liver tissues (by 47.3%) after LPS challenge compared with the vehicle-treated group. Brain GPx activity increased by 82.6% after treatment with 1 g/kg citric acid ($P < .05$) compared with the LPS control group (0.80 ± 0.052 vs. 1.015 ± 0.061 U/g tissue). No significant effect was observed in brain GPx activity after treatment with citric acid at 2 or 4 g/kg (Fig. 4A). On the other hand, liver GPx activity significantly increased by 29.7%, 79.6%, and 56.5% after treatment with 1, 2, and 4 g/kg of citric acid, respectively (Fig. 4B).

Paraoxonase activity. Paraoxonase activity significantly decreased in brain and liver tissues by 54.2% (11.3 ± 0.7 vs.

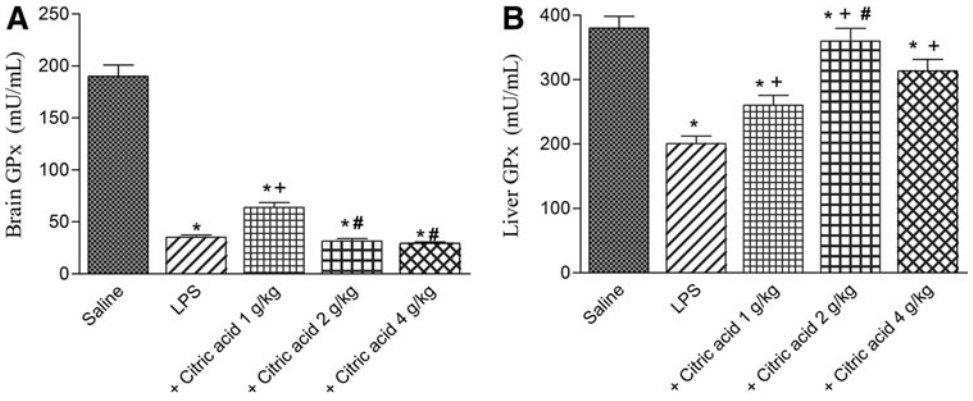

FIG. 4. Glutathione peroxidase (GPx) activity in **(A)** brain and **(B)** liver of mice after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 1 g/kg of citric acid.

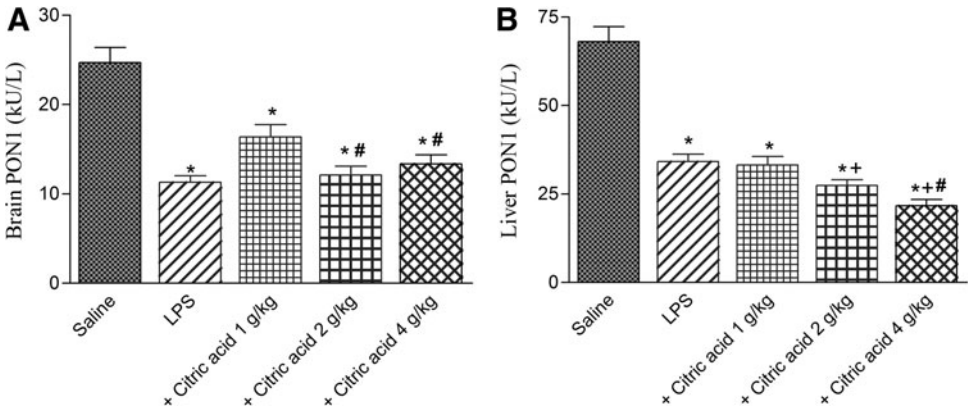

FIG. 5. Paraoxonase 1 (PON1) activity in mice **(A)** brain and **(B)** liver after treatment with LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 1 g/kg of citric acid.

24.7 ± 1.8 kU/L) and 49.8% (34.2 ± 2.1 vs. 68.1 ± 4.2 kU/L), respectively, after LPS challenge (Fig. 5A, B). Brain PON1 activity increased by 44.9% following treatment with citric acid at 1 g/kg. Higher doses, however, failed to significantly alter PON1 activity (Fig. 5A). On the other hand, liver PON1 activity significantly decreased by 19.7% and 36.6% after treatment with citric acid at 2 and 4 g/kg, respectively, compared with the LPS control group (Fig. 5B).

TNF-α in brain tissue. A pronounced increase in TNF-α in mice brain was observed following i.p. injection of LPS (324.9% increase: 182.7 ± 6.2 vs. saline control value of 43.0 ± 2.7 pg/g tissue). TNF-α showed a significant decrease by 48.4% and 28.8% after treatment with citric acid at 1 and 2 g/kg (93.3 ± 3.8 and 130.0 ± 4.3 vs. LPS control value of 182.7 ± 6.2 pg/g tissue). The administration of citric acid at 4 g/kg failed to decrease the level of TNF-α (Fig. 6).

DNA fragmentation in the liver. DNA fragmentation in the liver was significantly and markedly increased by 633.5% after LPS injection compared with the vehicle-treated mice. It showed a 49.4% and 82.6% decrease after treatment with citric acid at 1 and 2 g/kg, respectively, compared with the LPS control value. However, a 92.5% increment in DNA fragmentation was observed after the highest dose of citric acid (4 g/kg; Fig. 7).

Liver transaminases. In LPS-treated mice liver, ALT and AST significantly increased by 145.4% and 204.8% compared with the saline-treated group. ALT significantly decreased by 22.5% after treatment with 1 g/kg of citric acid. The higher doses of citric acid, however, failed to

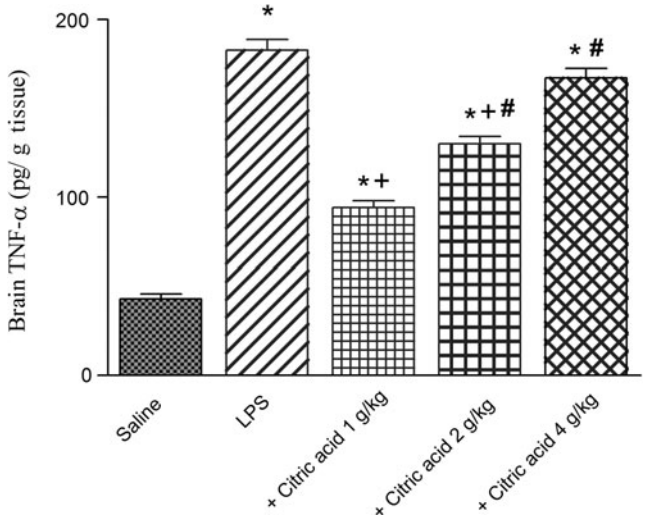

FIG. 6. Brain tissue tumor necrosis factor-alpha (TNF-α; pg/g tissue) in mice given LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus the saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus the LPS + 1 g/kg of citric acid.

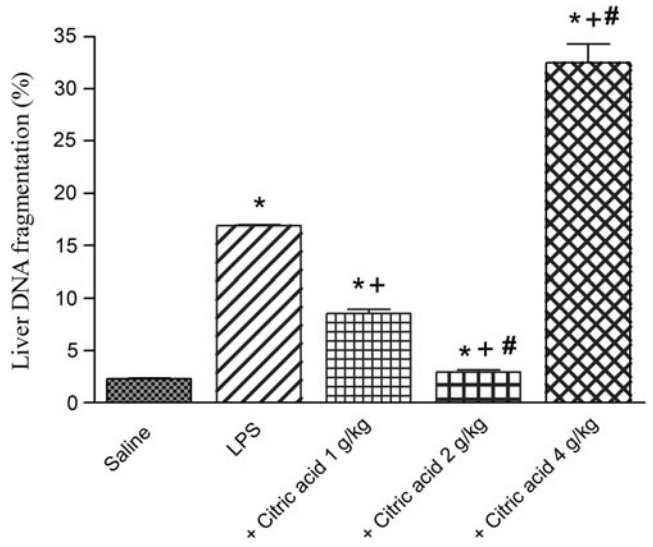

FIG. 7. DNA fragmentation (%) in mice liver after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 1 g/kg of citric acid.

significantly alter ALT in the liver of LPS-treated mice. Meanwhile, AST significantly decreased by 26.5% and 30.4% after treatment with 1 and 2 g/kg citric acid, respectively. The highest dose of citric acid, however, had no significant effect on liver AST in LPS-treated mice (Fig. 8A, B).

Histological results

The control livers showed normal hepatic architecture with distinct hepatic cells, sinusoidal spaces, and a central vein (Fig. 9A).

Histological examination of the liver from LPS-treated mice revealed mononuclear cell infiltrations, bile duct proliferation in the periportal areas, and minimal enlargement in the periportal areas. In the LPS group we also observed dilatation and congestion of the central vein and blood sinusoids that showed numerous Kupffer cells. Hepatocytes exhibited necrotic changes in the form of small pyknotic nuclei with condensed or marginated chromatin, lack of nucleoli, and strongly acidophilic cytoplasm (Fig. 9B, C).

On the other hand, histological examination of liver sections from mice treated with LPS + citric acid at 1 g/kg showed nearly normal hepatic architecture. The hepatic lobules appeared with prominent central vein with less sinusoidal dilatation and decreased number of Kupffer cells compared with the LPS-only-treated group (Fig. 9D).

The improvement in histological appearance was more pronounced after treatment with citric acid at 2 g/kg, evidenced in normal appearance of liver lobules with strains of hepatocytes compared with section of LPS groups (Fig. 9E).

In contrast, sections from mice treated with LPS and 4 g/kg of citric acid showed mild improvement with dilated portal areas. The hepatocytes exhibited some degree of histological regeneration with less sinusoidal dilatation and with decreased number of Kupffer cells and less necrotic cells (Fig. 9F).

Caspase-3 and iNOS immunoreactivity

Activated caspase-3 labeling was specific in delineating morphologically apoptotic cells. Caspase-3 and iNOS expression was localized in the cytoplasm of hepatocytes. There was negligible caspase-3 (Fig. 10A-i) and iNOS (Fig. 10A-ii) immunopositivity in the livers of vehicle-treated mice. After treatment with LPS strong expression of caspase-3 (Fig. 10B-i) and iNOS (Fig. 10B-ii) was observed compared with the vehicle control group. In these sections, caspase-3 and iNOS immunoreactivity was observed mainly around central vein.

Caspase-3 and iNOS immunopositivity decreased in the livers of LPS-intoxicated mice treated with 1 g/kg of citric acid (Fig. 10C) and 2 g/kg of citric acid (Fig. 10D), respectively. In contrast, citric acid in the high dose of 4 g/kg was not effective in reducing caspase-3 (Fig. 10E-i) and iNOS expression (Fig. 10E-ii).

DISCUSSION

In the present model of mild systemic inflammation caused by a subseptic dose of LPS endotoxin and associated with increased oxidative stress in brain and liver tissues,

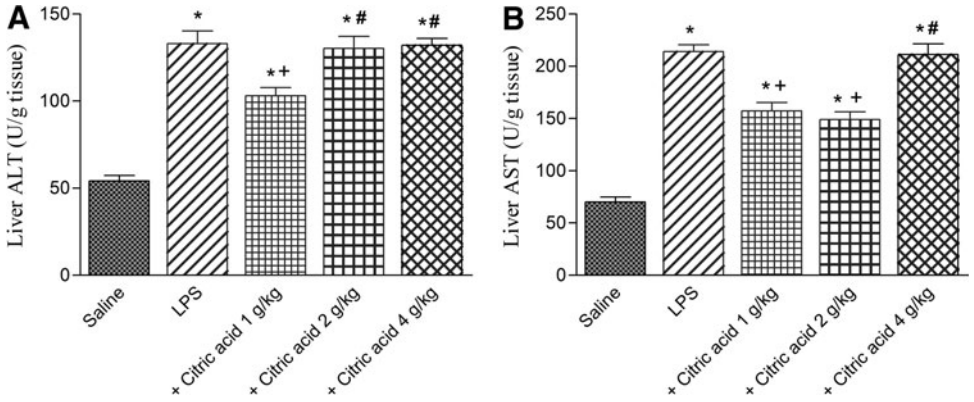

FIG. 8. **(A)** Alanine aminotransferase (ALT) and **(B)** aspartate aminotransferase (AST) activities in mice liver after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P < .05$ versus saline control. $^{+}P < .05$ versus LPS control group. $^{\#}P < .05$ versus LPS + 1 g/kg of citric acid.

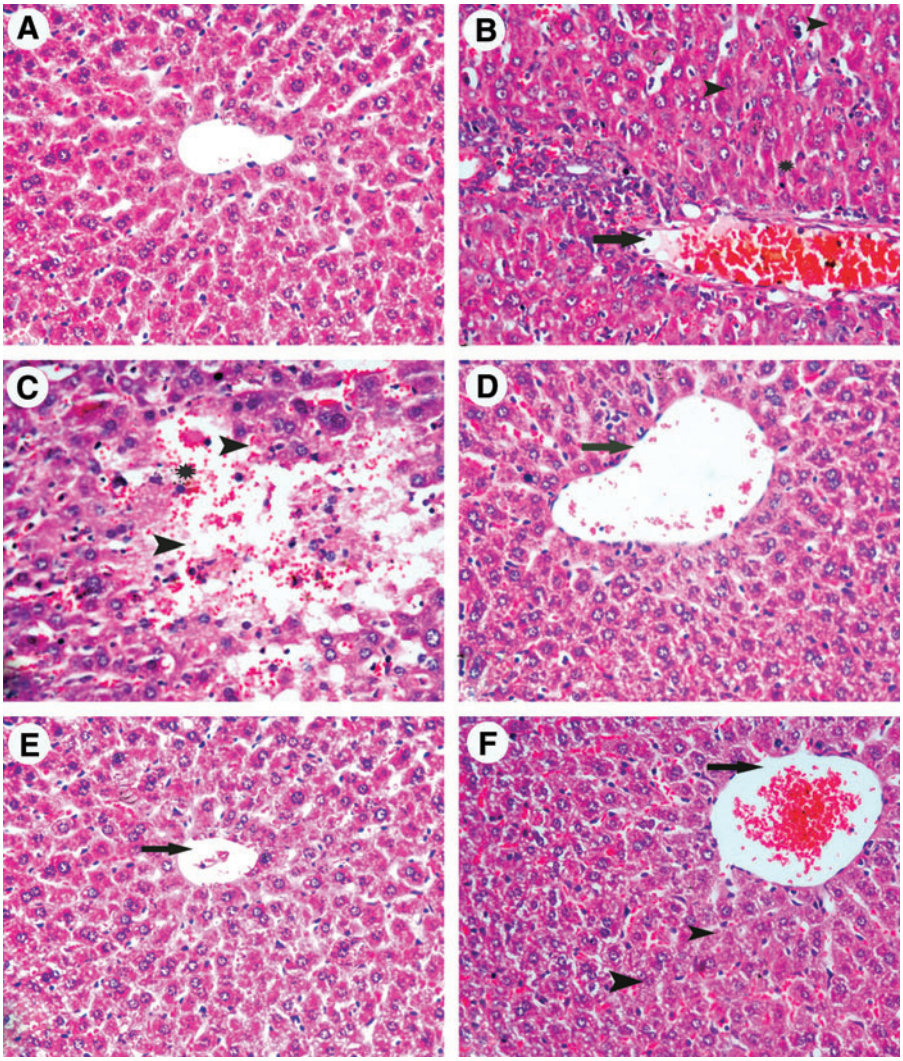

FIG. 9. Hematoxylin and eosin (H&E)–stained liver sections from mice treated with **(A)** saline (control). **(B)** LPS: inflammatory leukocytic cell infiltration around portal area (long arrow), necrosis (arrow head), dilated and congested blood sinusoids, and marinated nuclear chromatin (star). **(C)** LPS: focal necrotic area (arrow head), activated Kupffer cells, dilated and congested blood sinusoids, and pyknotic nuclei (star). **(D)** LPS + citric acid 1 g/kg: congestion of central vein (long arrow), dilated blood sinusoids, and few necrotic cells (arrowhead). **(E)** LPS + citric acid 2 g/kg: normal central vein (long arrow), minimally dilated blood sinusoids, and few Kupffer cells. **(F)** LPS + 4 g/kg of citric acid: congestion of central vein (long arrow), signs of degeneration of hepatocytes, dilated congested blood sinusoids, and few Kupffer cells (H&E × 400). Color images available online at www.liebertpub.com/jmf

citric acid exerted important pharmacological effects. A significant and marked decrease in lipid peroxidation (measured as MDA) was observed in brain tissue after treatment with citric acid, thereby suggesting decreased free radical attack on polyunsaturated fatty acids. In contrast, no significant effect on liver MDA has been observed after treatment citric acid. In both the brain and liver, however, citric acid displayed marked inhibitory effect on nitric oxide. Under physiological conditions, this free radical gas synthesized from the amino acid L-arginine by the enzyme NOS is important in neurotransmission, maintaining vascular tone, immune regulation, synaptic plasticity, and many other functions.[32,33] Increased levels of nitric oxide generated by glial cells, including astrocytes and microglia, due to action of inducible NOS, however, contributes to neuronal cell death in inflammatory, infectious, ischemic, and neurodegenerative diseases.[34] This is due to the ability of nitric oxide to react with other free radicals, especially with the oxygen radical superoxide (O_2^-), to form peroxynitrite ($ONOO^-$), decomposing to form the powerful and cytotoxic oxidants hydroxyl radical and nitrogen dioxide.[35,36]

In face of increased free radicals and reactive oxygen species, cells are equipped with a number of antioxidant mechanisms, such as catalases, GPxs, glutathione transferase, superoxide dismutase, and GSH.[3] The administration of LPS was associated with an increase in lipid peroxidation and a drop in GSH level and GPx activity in brain and liver tissues, which indicates increased generation of free radicals. In LPS-treated mice, brain and liver GSH were not altered by citric acid. Meanwhile, treatment with citric acid at 1 g/kg was associated with increased GPx activities in brain and liver tissues, possibly due to an antioxidant effect of citric acid. In the current study, decreased brain and liver PON1 activity was observed after the administration of LPS. PON1 enzyme that plays an important role in the metabolism of many xenobiotic compounds has recently drawn attention, for a possible role in protecting cellular membranes against lipid peroxidation.[25,26] In brain tissue, PON1 activity was improved by citric acid given at 1 g/kg. PON1 activity in liver tissue, however, decreased following higher doses of citric acid, possibly reflecting consumption or inactivation of the enzyme by increased free radicals with high concentration of citric acid.

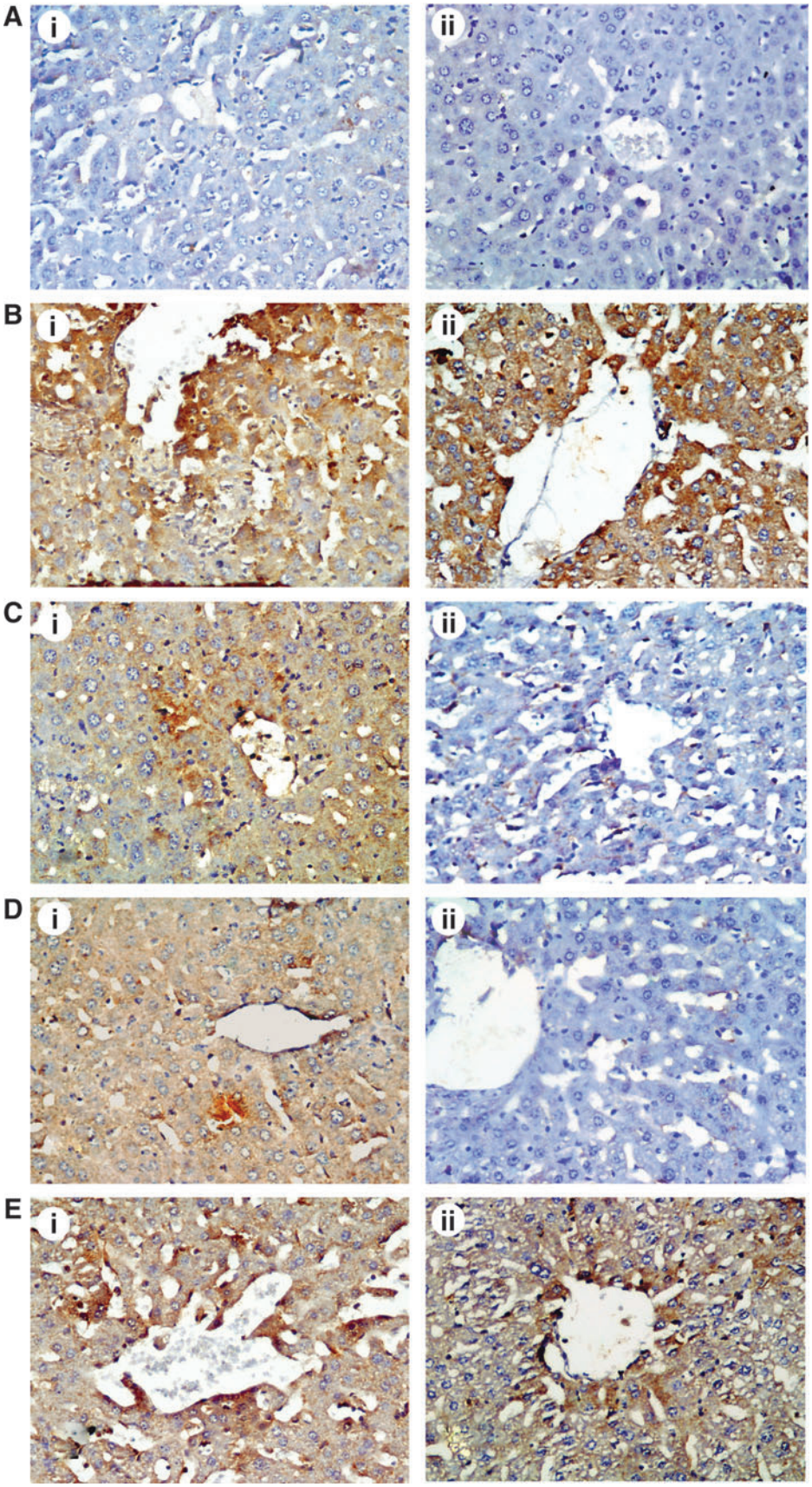

FIG. 10. The effect of LPS and citric acid treatment on hepatic caspase-3 (**i**) and inducible nitric oxide synthase (iNOS) (**ii**) immunostaining: **(A)** control liver; **(B)** LPS; **(C)** LPS + citric acid 1 g/kg; **(D)** LPS + citric acid 2 g/kg; **(E)** citric acid 4 g/kg (caspase-3 and iNOS immunohistochemistry, hematoxylin counterstain ×400). Color images available online at www.liebertpub.com/jmf

One potent proinflammatory cytokine is TNF-α, which is produced in the brain by glial cells in response to various stimuli and induces astrocytes and microglial cells to secrete several inflammatory mediators, such as chemokines, lipid mediators, nitric oxide, and other free radicals. TNF-α has been demonstrated to play an important role in central nervous system neuroinflammation-mediated cell death in various neurodegenerative conditions.[37,38] In the present study, the cytokine was markedly increased in brain tissue after LPS administration. Here we demonstrate that citric acid treatment was associated with marked inhibitory effect on TNF-α production within brain tissue after LPS challenge. This ability of citric acid to decrease pathological TNF-α production in the brain might be of value in relevance to neurodegenerative diseases. TNF-α expression appears to be upregulated in several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, and pharmacological manipulation of TNF-α within the brain has been proposed as one potential target in the treatment of these conditions and may represent a valuable target for intervention.[39–41]

The present data indicate that citric acid can act directly on brain cells to inhibit their production of TNF-α and nitrite. LPS acts on TLR4 receptors on macrophages, dendritic cells, and other immune cells to release proinflammatory cytokines, such as TNF-α and IL-1β, which might gain direct access to the brain via the blood–brain barrier or signal to the brain via the vagus nerve, the so-called gut–brain immune communication.[42,43] Thus it is also possible that the effects of citric acid on brain are accounted for by modulating the release of inflammatory mediators from leucocytes in the periphery.

Differences in results for the same markers in liver and brain tissues were observed. Thus, in contrast to the effects of citric acid in decreasing lipid peroxidation in the brain, no significant effect on liver MDA was observed. Moreover, PON1 activity in liver tissue decreased following citric acid at high doses. This might be due to the particular metabolic pathway interrelationships within each so different organ. Nevertheless, DNA fragmentation, serum transaminase elevations, caspase-3 and iNOS expression, and histological damage were all attenuated by 1–2 g/kg of citric acid. These data clearly indicated a protective effect for citric acid administration within this dose range on hepatic damage during endotoxemia. Citric acid intake, therefore, is likely to have a beneficial effect on the liver under toxic and inflammatory conditions. Citric acid might prevent liver injury through (1) reducing polymorphonuclear cell degranulation and attenuating the release of myeloperoxidase, elastase, IL-1β, and platelet factor; (2) stimulation of glycolysis and the tricarboxylic acid cycle; (3) increased production of bicarbonate with improvement of tissue acidosis in inflammatory conditions and therefore maintains tissue and cellular integrity; and (4) stimulation of vagal sensory afferents involved in signaling hepatic protection.[11–13,15,44,45]

It should be noted, however, that some of the beneficial effects observed for citric acid in brain and liver tissues were only in the dose range of 1–2 g/kg. This protective effect is lost when the dose is increased to 4 g/kg; for example, GPX and PON1 activities were increased only with 1 g/kg and TNF was decreased only by 1–2 g/kg of citric acid; the doses that were most effective in inhibiting brain nitrite. The protective effects on the liver were also lost with the higher dose of 4 g/kg, which also increased liver DNA fragmentation. Since citric acid is found in all animal tissues as an intermediate in the Krebs cycle, no limit has been set on the acceptable daily intake for humans for either the acid or salt.[46] It is possible, however, that at higher concentrations, citric acid acts as a pro-oxidant. Several antioxidants show pro-oxidant effects at higher doses/concentrations, for example, carotenoids,[47] vitamin E, and vitamin C.[48,49] Natural compounds also display double-edged effects on inflammatory reactions, depending potentially on their concentrations: physiologic doses leading to beneficial effects whereas high doses may result in harmful effects.[50]

In summary, the present data suggest an antioxidant and anti-inflammatory effect for orally given citric acid at 1–2 g/kg in brain tissue. Citric acid also demonstrated a beneficial hepatic protective effect at this dose range. Given that both increased brain oxidative stress and chronic inflammation have been linked to the development of neurodegenerative diseases, citric acid might thus prove of clinical benefit in such conditions. The present study suggests that citric acid might find utility in treatment of toxic and inflammatory conditions of the brain and liver tissues. This can take the form of supplementation as nutraceutical citric acid. Meanwhile, citric acid is naturally concentrated in citrus fruits with lemon juice and lime juice being rich sources of citric acid and intake of these has been suggested as an effective means of treating oxalate stones.[1,51,52] These studies have addressed the utility of dietary intervention with fruits and fruit juices with high citrate content (orange juice and lemonade) as an alternative to potassium citrate in increasing urinary pH and citrate, but the combination of citrate supplementation and fruit juices was not evaluated. This latter approach might prove a useful one combining the advantages of both classes of food additives. The presence of flavonoids and vitamin C in citrus fruits and juices makes the latter option an attractive one.

AUTHOR DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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Lemon juice has protective activity in a rat urolithiasis model

Mohammed Touhami*[1,4], Amine Laroubi[1], Khadija Elhabazi[1], Farouk Loubna[1], Ibtissam Zrara[2], Younes Eljahiri[3], Abdelkhalek Oussama[4], Félix Grases[5] and Abderrahman Chait[1]

Address: [1]Laboratory of Animal Physiology. Ecophysiology Unit. Department of Biology, Faculty of Sciences Semlalia, University Cadi Ayyad, PO Box 2390, Marrakech, 40000, Morocco, [2]Laboratory of Anatomopathology, Avicenne Military Hospital, Marrakech, 40000, Morocco, [3]Laboratory of Biochemistry, Avicenne Military Hospital, Marrakech, 40000, Morocco, [4]Laboratory of Applied and Environmental Chemical Spectroscopy, Urolithiasis Unit, Faculty of Science and Techniques, University Cadi Ayyad, PO Box 523, Beni-Mellal, 23000, Morocco and [5]Laboratorio de Investigación en Litiasis Renal. Edificio Mateu Orfila Universitat de les Illes Balears, Ctra. de Valldemossa km 7.5, E-07071, Palma de Mallorca, Spain

Email: Mohammed Touhami* - mohammed.touhami@gmail.com; Amine Laroubi - lar_amine@hotmail.com; Khadija Elhabazi - kelhabazi@hotmail.com; Farouk Loubna - loubna_fa@hotmail.com; Ibtissam Zrara - ibtissam.zrara@yahoo.fr; Younes Eljahiri - younes.eljahiri@yahoo.fr; Abdelkhalek Oussama - oussamaabdelkhalek@yahoo.fr; Félix Grases - fgrases@uib.es; Abderrahman Chait - chait@ucam.ac.ma

* Corresponding author

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Abstract

Background: The use of herbal medicines (medicinal plants or phytotherapy) has recently gained popularity in Europe and the United States. Nevertheless the exact mechanism of the preventive effects of these products is still far to be clearly established, being its knowledge necessary to successfully apply these therapies to avoid stone formation.

Methods: The effect of oral lemon juice administration on calcium oxalate urolithiasis was studied in male Wistar rats. Rats were rendered nephrolithic by providing drinking water containing 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC) for 10 days. In addition to EG/AC treatment, three groups of rats were also gavage-administered solutions containing 100%, 75% or 50% lemon juice [v/v] (6 μl solution/g body weight). Positive control rats were treated with EG/AC but not lemon juice. Negative control rats were provided with normal drinking water, and were administered normal water by gavage. Each group contained 6 rats. After 10 days, serum samples were collected for analysis, the left kidney was removed and assessed for calcium levels using flame spectroscopy, and the right kidney was sectioned for histopathological analysis using light microscopy.

Results: Analysis showed that the rats treated with EG/AC alone had higher amounts of calcium in the kidneys compared to negative control rats. This EG/AC-induced increase in kidney calcium levels was inhibited by the administration of lemon juice. Histology showed that rats treated with EG/AC alone had large deposits of calcium oxalate crystals in all parts of the kidney, and that such deposits were not present in rats also treated with either 100% or 75% lemon juice.

Conclusion: These data suggest that lemon juice has a protective activity against urolithiasis.

Background

Kidney stone formation or urolithiasis is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidneys [1]. The recurrence of urolithiasis represents a serious problem as patients who have formed one stone are more likely to form another. Not all standard pharmaceutical drugs used to prevent urolithiasis are effective in all patients, and many have adverse effects that compromise their long-term use [2].

Renal calculi can be broadly classified in two large groups: tissue attached and unattached [3]. Attached calculi are mainly integrated by calcium oxalate monohydrate (COM) renal calculi, with a detectable attachment site to the renal papilla and basically consisting of a core located near to the attachment site (concave zone) and radially striated concentrically laminated peripheral layers. Unattached calculi, with no detectable site of attachment to papilla, are developed in renal cavities of low or reduced urodynamic efficacy and can exhibit diverse composition and structures. Several reports have been published since Randall's first description of papillary calcifications and their possible active role in the genesis of COM papillary calculi [4-6]. At present, it seems clear that renal epithelial cell injuries play a decisive role in such a type of renal calculi development [7,8], and in fact the lithogenic effect caused by ethylene glycol (EG) must be mainly attributed to the oxidative damage caused by the high level of oxalate generated by EC. Thus, although EC rat model can be questioned as a general model to study renal stone formation, it must be considered as an interesting model to evaluate renal papillary stone development, at least for those stones which genesis is linked to oxidative cell damage. Thus, the first studies on experimental EC renal lithiasis appeared in the 60' decade [9,10] but the importance of the oxidative damage caused by hyperoxaluria was not clearly proposed until the end of the century [11]. From this last period it appeared several prophylaxis proposals on EC induced nephrolithiasis using herbal extracts and antioxidants [12-19]. In all these papers the effects of these compounds did not seem to be mediated by diuretic or other urinary biochemical changes and positive effects on calcium oxalate lithiasis are most likely due to antioxidative effects.

To further investigate the potential of lemon juice as a therapy for lithiasis, the present study examined the effect of lemon juice on experimentally EG-induced calcium oxalate (CaOx) nephrolithiasis in rats.

Methods

Animals

Thirty male Wistar rats weighing approximately 280 g were acclimated for 3 days in cages before experiments commenced. Experiments were conducted in accordance with internationally accepted standard guidelines for the use of animals. Rats had *ad libitum* access to standard chow and tap water, and were kept under a controlled 12 h light/dark cycle at 22 ± 2°C.

Ethylene glycol-induced urolithiasis

The thirty rats were divided into five groups comprising six animals per group. Each group underwent a different treatment protocol for 10 days. Group 1: negative control, *ad libitum* access to regular food and drinking water, and administered 6 μl distilled water per 1 g of body weight by gavage (intra-gastric administration). Groups 2, 3, 4 and 5: *ad libitum* access to regular food, and *ad libitum* access to drinking water containing 0.75% [v/v] ethylene glycol (EG) and 2% [w/v] ammonium chloride (AC) in order to promote hyperoxaluria and CaOx deposition in the kidneys. Groups 2, 3 and 4 were also administered 6 μl lemon juice solution/g body weight by gavage at the following concentrations: Group 2, 100% lemon juice; Group 3, 75% [v/v] and Group 4, 50%. Group 5 rats were administered 6 μl distilled water/g body weight by gavage (positive control). All rats were weighed daily.

Assessment of antiurolithic activity

Kidney and serum analysis

After the 10-day experimental period, rats were anaesthetized and blood was collected from the retro-orbital region, centrifuged at 10,000 × g for 10 min [20], and the serum collected and analyzed for calcium, phosphorus, urea and creatinine using an automated system (Cobas Integra 400 plus). The rats were then sacrificed by cervical dislocation, the abdomen opened and both kidneys removed. The left kidney was dried in an oven at 100°C for 24 h, after which the kidney was weighed and then minced in a beaker containing 7 ml 0.5 N nitric acid. The mixture was then heated until the liquid became transparent. After calibration using a standard calcium solution, the calcium content of the mixture was determined using flame spectroscopy. The amount of calcium is expressed as μg/g dry kidney. The right kidney was fixed in bouin liquid [21,22], soaked in paraffin, cut at 3–4 μm intervals, and the slices stained using hematoxylin and eosin [21]. Tissue slices were photographed using optical microscopy under polarized light (Olympus BX41).

Statistical analysis

Results are presented as mean ± standard error (S.E.). A one-way ANOVA was used to determine the significance of differences among groups. Student's *t*-test was used to assess differences between means. Conventional Windows software was used for statistical computations. A P value < 0.05 was considered to indicate a significant difference.

Results

Serum analysis

Serum analysis showed that urea and creatinine levels were higher in Groups 2, 3, 4 and 5 compared to Group 1 (Fig. 1). These data indicate marked renal damage in the EG/AC-treated rats. The data also showed that urea, creatinine, calcium and phosphorus levels were lower in rats treated with lemon juice (Groups 2, 3 and 4) compared to rats treated with EG/AC alone (Group 5, positive control).

Body weight

EG/AC-treated rats (Groups 2, 3, 4 and 5) weighed less than the negative control rats (Group 1) at the completion of the experiment (Fig. 2).

Calcium levels in the kidneys

The left kidneys were assessed for calcium levels. EG/AC treatment alone (Group 5) resulted in increased kidney calcium levels compared to the negative control rats, while the administration of 100% lemon juice reduced this calcium accumulation (Group 2) (Fig. 3).

Histological examination

Examination of kidney paraffin sections showed that Group 5 rats (EG/AC alone, positive control) had the greatest amount of CaOx deposition, and this was present in all parts of all three major areas of the kidney. Intratubular and interstitial crystals were observed on the cortex (Figs. 4d and 4e). There was greater calcification on surface of the renal parenchyma (Fig. 5) and the papillary tip (Fig. 6) in Group 5 rats compared to the Groups 2, 3 and 4 rats (EG/AC and lemon juice). Longitudinal sections showed the papillary tips were encrusted with CaOx crystals (Figs. 6d and 6e). Analysis of portions of these crystalline deposits removed from the papillary tip showed they were composed of CaOx monohydrate and CaOx dihydrate. No papillary encrustations were seen in tissue from the negative control rats (Group 1) (Fig. 6a) or rats treated with EG/AC and 100% lemon juice (Group 2) (Fig. 6b). Major calcium deposits were observed on the surface of the papillary tips in 33% of the positive control rats (Group 5) and 17% of the rats treated with EG/AC and 75% lemon juice (Group 3). All positive control rats (Group 5) had major calcium deposits on the surface of the cortex and medulla, while no such deposits were

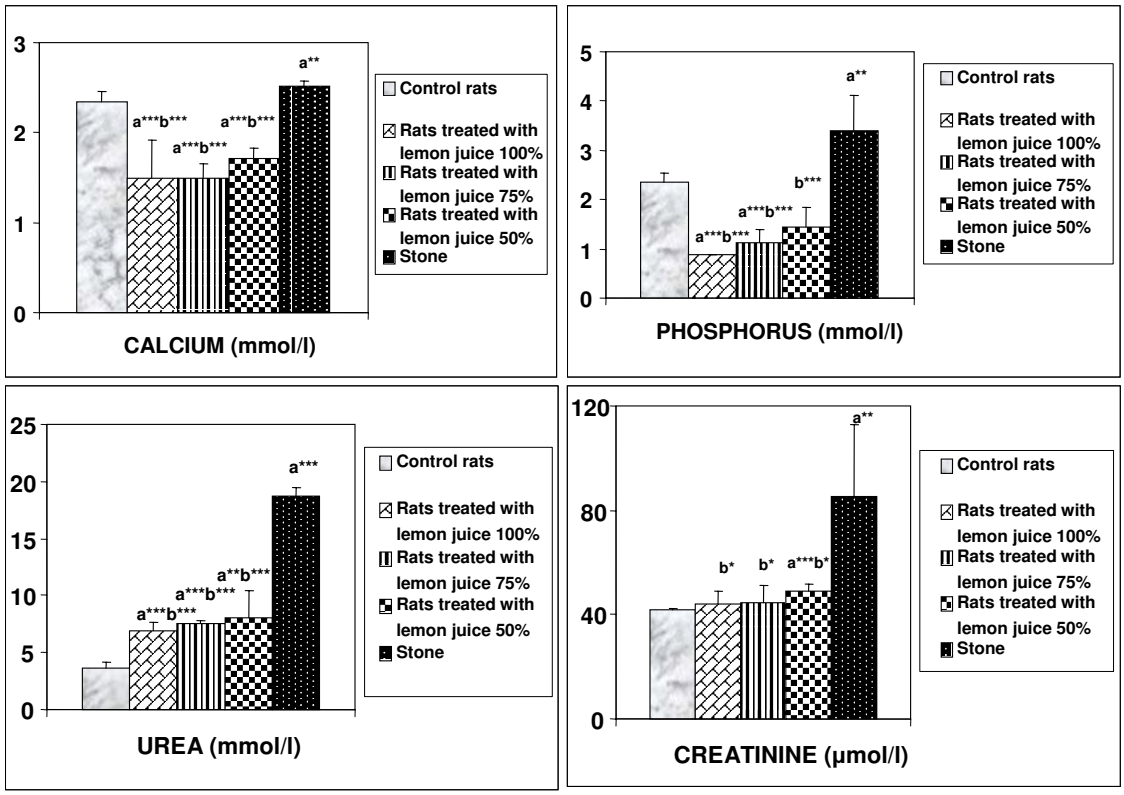

Figure 1

Serum biochemical data. Values represent mean ± SD for six animals in each group. [a] Values are significantly different from the negative control group: *p < 0.05, **p < 0.01, ***p < 0.001. [b] Values are significantly different from the positive control group: * p < 0.05, **p < 0.01, ***p < 0.001.

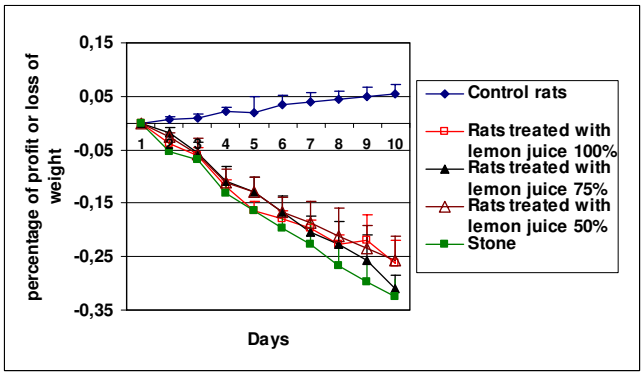

Figure 2
Changes in body weight in the various rat groups over the ten days of the experiment.

observed in the negative control rats (Group 1) (Tables 1 and 2). These morphological findings were consistent with the left kidney calcium level data.

Discussion

Urinary lithiasis is generally the result of an imbalance between inhibitors and promoters in the kidneys. Human kidney stones are usually composed of CaOx [1], and several studies have examined the effect of the citrus juices on calcium salt crystallization [23-27]. However, the conclusions from those studies were not consistent.

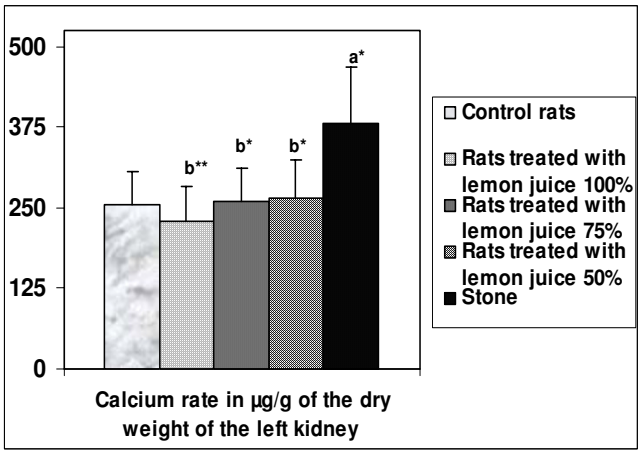

Figure 3
Amount of calcium in the left kidney. Values represent mean ± SD (µg/g) for six animals in each group. [a] Values are significantly different from the negative control group: *p < 0.05, **p < 0.01, ***p < 0.001. [b] Values are significantly different from the positive control group: * p < 0.05, **p < 0.01, ***p < 0.001.

Many in vivo models have been developed to investigate the mechanisms involved in the formation of urinary stones, and to ascertain the effect of various therapeutic agents on the development and progression of the disease [28-33]. Rats are the most frequently used animals in models of CaOx deposition in the kidneys, a process that mimics the etiology of kidney stone formation in humans [28]. Rat models of CaOx urolithiasis induced by either EG alone or in combination with other drugs such as AC, are often used to study the pathogenesis of kidney crystal deposition [30]. Using the accelerated model [32], in the present study rats were treated with 0.75% EG and 2% AC for 10 days. All positive control rats (Group 5) developed CaOx depositions during that time.

The present study examined the effect of various lemon juice concentrations on the deposition of CaOx crystals within the rat kidney. Previous studies concluded that medicinal plants had little effect on the urinary chemistry of urolithiasis [34,35]. The current study analyzed body weight, kidney calcium level, serum concentrations of calcium, phosphorus, urea and creatinine, and the histopathology of the kidney. We found that Group 1 rats (negative controls) remained active and gained weight, while Group 2, 3, 4 and 5 rats lost weight over the 10 days of treatment. Microscopic examination using polarized light of kidney sections derived from nephrolithiasic rats showed intratubular and interstitial crystal deposits, consistent with the findings of others [36]. These crystals were intensely birefringent, polycrystalline, and arranged in a rosette characteristic of CaOx crystals. The presence of such deposits is evidence of adhesion and retention of particles within the renal tubules. These crystal deposits were observed in the kidneys of all Group 5 rats. Moreover, 33% of these rats showed major calcifications on the papillary tip. In contrast, no rats treated with lemon juice showed such papillary crystalline deposits. Rats treated with 100% or 75% lemon juice had far less kidney calcification and lower renal tissue calcium levels than the positive control rats (Group 5) (Table 1 and 2). No papillary encrustations were seen in 100%, 83% and 50% of rats treated with 100%, 75% and 50% lemon juice, respectively. Furthermore calcic parenchymatous deposits were not observed in 83% of rats treated with 100% and 75% lemon juice. These results clearly demonstrate the ability of the lemon juice to prevent the development of papillary and renal parenchymatous calcifications on the kidney, consequently preventing the development of papillary and parenchymatous calculi. All rats treated with 50% lemon juice showed fewer calcium deposits on the kidney surface than positive control rats (Group 5). While treatment with 100% and 75% lemon juice appeared to be more beneficial that treatment with 50% juice, this difference was not found to be statistically significant.

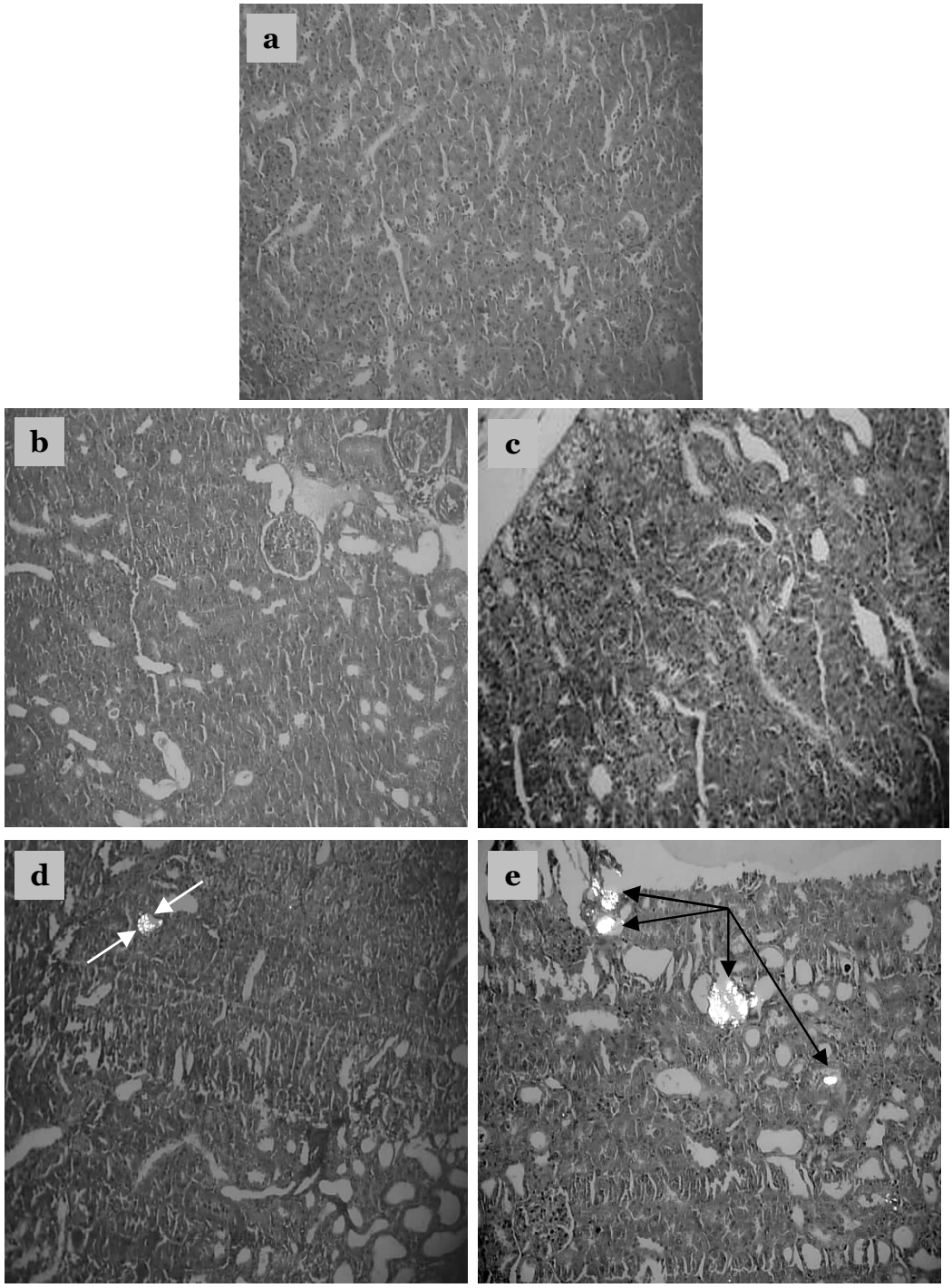

Figure 4
Crystalline formations in the rat kidney cortex. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal cortex are indicated by arrows. Magnification ×100.

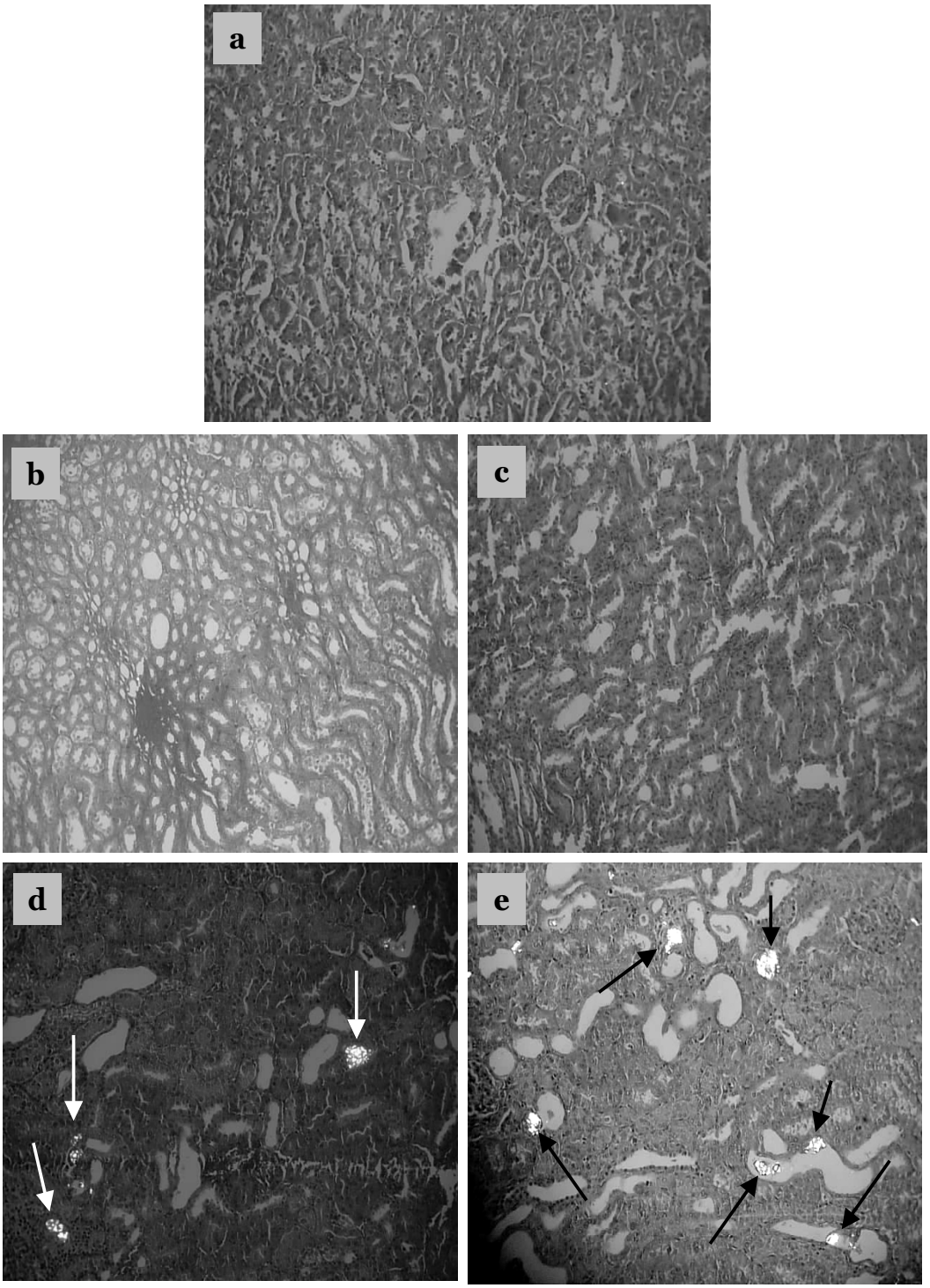

Figure 5
Crystalline formation in the renal parenchyma. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal parenchyma are indicated by arrows. Magnification ×100.

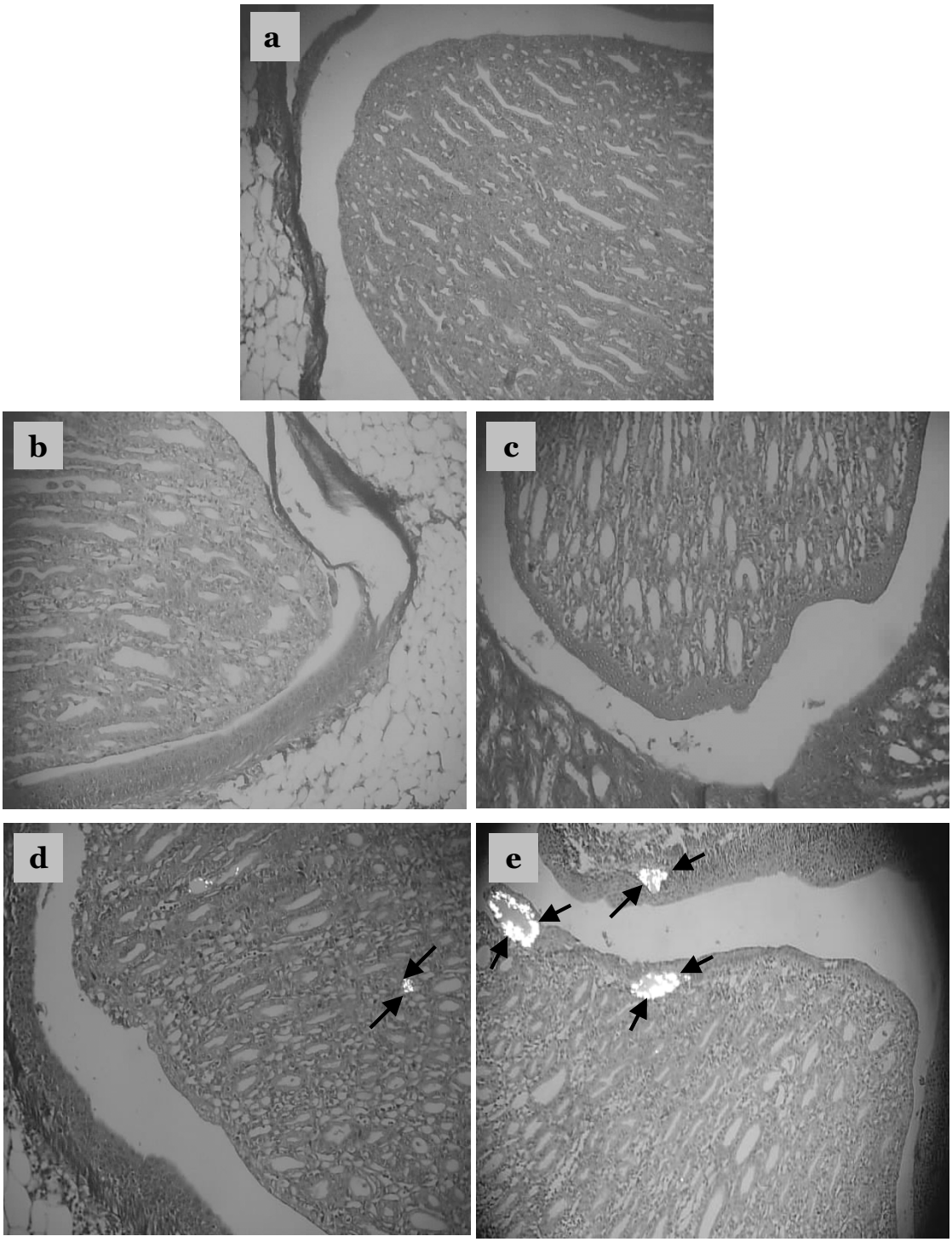

Figure 6
Crystalline formations in the renal papilla. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal papilla are indicated by arrows. Magnification ×100.

Table 1: Number and type of calcifications observed

Groups	Percentage of rats with major calcifications on the papillary tip (> 90% of the papillary tip calcified)	Percentage of rats with some area of the papillary tip calcified	Percentage of rats with some calcified points on the papillary tip	Percentage of rats without calcifications on papillary tip
1. Negative controls	-	-	-	100
2. EG, AC and 100% lemon juice	-	-	-	100
3. EG, AC and 75% lemon juice	17	-	-	83
4. EG, AC and 50% lemon juice	-	-	50	50
5. EG and AC (positive controls)	33	33	17	17

The association of crystals with renal tubular cells is considered a potential factor in the process of renal stone formation. Indeed, calculations considering the rate of crystal growth even at its maximum speed and tubular fluid flow suggest that a single crystal would not become large enough to be retained and occlude the lumen during its normal transit through the nephron [28]. Furthermore, it is established that crystals, especially calcium oxalate monohydrate crystals, can be retained by attachment to the surface of renal epithelial cells and be internalized [28].

Lemon juice has a high antioxidant capacity due to the presence of citrate, vitamin C, vitamin E and flavonoids such as eriocitrin, hesperetin [37,38] and limonoids [39]. Vitamin E may prevent calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface (lipid peroxidation) [40,41], which in turn can prevent calcium oxalate crystal attachment and subsequent development of kidney stones [41,42].

In urolithiasis, the glomerular filtration rate (GFR) decreases due to stones in the urinary system obstructing urine outflow. This leads to the accumulation of waste products in the blood, particularly nitrogenous substances such as urea, creatinine and uric acid. In addition, increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi-producing diet [20]. In this context, oxalate has been reported to induce lipid peroxidation and to cause renal tissue damage by reacting with polyunsaturated fatty acids in cell membranes [20]. In the present study, the positive control calculi-induced rats (Group 5) were found to have marked renal damage, consistent with the elevated serum levels of creatinine and urea. The administration of lemon juice inhibited these changes that would otherwise promote new stone formation in the urinary system. In rats treated with lemon juice, we attribute the lower serum creatinine and urea levels to an enhanced GFR and the anti-lipid peroxidative property of lemon juice [20]. As commended, the lithogenic effects of EG must be mainly attributed to the oxidative damage caused by the high level of oxalate generated by this substance. For this reason, the presented studies were focused to evaluate the effects on renal papillary tissue through histological studies and the protective effects caused by the consumption of lemon juice. Previous studies evaluated the effects of citrate on renal lithiasis induced by EG [43,44]. Nevertheless, to attain an increase in citrate excretion it is necessary to induce metabolic acidosis in rats and to achieve this condition it is necessary to increase the doses of EG to 2%. In such case, urinary pH of EG treated rats was clearly inferior to urinary pH of control group, the treatment with high doses of potassium citrate significantly increased the urinary pH and, as a consequence, the urinary citrate excretion notably rose. Nevertheless, EG doses of 0.75% practically did not change the urinary pH value when compared with control group [36,44] and consequently the administration of citrate did not cause important changes in urinary citrate excretion [45].

Table 2: Cortex and medullar tissue data (see text for description of various groups)

Crystal deposits	Group 1 n = 6	Group 2 n = 6	Group 3 n = 6	Group 4 n = 6	Group 5 n = 6
None	6	5	5	-	-
Crystals: +	-	1	1	2	-
Crystals ++	-	-	-	4	-
Crystals +++	-	-	-	-	6

Conclusion

The present study found that the administration of lemon juice effectively prevented the development of urolithiasis in rats. These findings support the use of lemon juice as an alternative medicine to prevent urolithiasis. Further research is necessary to clarify the mechanism underlying this preventative effect of lemon juice.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MT participated in this study by gavage of rats, measurement of body weight and analysis of kidney calcium levels. AL performed the statistical analysis. KE participated in the animal experiments. FL participated in laboratory management. IZ examined the histological samples. YE participated in analytical determinations. AO performed image processing. FG participated in the evaluation and discussion of the obtained results. AC participated in coordination. All authors read and approved the final manuscript.

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To study the renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.

Introduction-

Reactive oxygen species (ROS) are various forms of activated oxygen. A disproportionately large amount of ROS and the absence of their scavenging systems in cells lead to oxidative stress and increase the risk of several human diseases, including hepatic injury, carcinogenesis, and inflammation [1]. The liver plays a central role in the maintenance of systemic lipid homeostasis and is especially susceptible to ROS-induced damage. Carbon tetrachloride (CCl4) is widely used to develop experimental animal models of liver failure (caused by free radical production) that mimic human hepatic toxicity. Although the main target organ of CCl4 is the liver, toxic effects of CCl4 are also observed in other organs, including the kidneys, testis, and brain [2–5], and the nephrotoxic effect of CCl4 is also associate with free radical production [2].

To prevent the damage caused by ROS, living organisms have developed an antioxidant system, which includes nonenzymatic antioxidants and enzymes, such as catalase, superoxide dismutase, and peroxidase [6]. In addition to these natural antioxidants, other synthetic or natural ROS scavengers may reduce the incidence of free radical-mediated diseases. The use of antioxidants in the prevention and cure of various diseases is intensifying, and there is considerable interest in the study of antioxidant activities of molecules, such as Citrus limon and Emblica officinalis [6–8]. Antioxidants appear to act against disease processes by increasing the levels of endogenous antioxidant enzymes and decreasing lipid peroxidation [9, 10]

Citrus lemon (Citrus limon Burm.F) is a source of vitamin C, flavonoids and carotenoids [11]. Eriocitrin and hesperidin are the main flavonoids in lemon. The antioxidant activity of eriocitrin is more potent than other citrus flavonoids [12] , so we use the lemon as a rich source of antioxidant in present study.

Emblica officinalis Gaertn. (Euphorbiaceae) commonly known as amla. Experimental studies have shown potent antioxidant, analgesic, antipyretic, adaptogenic, immunomodulatory, and antiulcerogenic activities of the fruit of Emblica officinalis [13,14] As Amala has antioxidant property we included it in our study to see the nephroprotective activity.

Aim and objective-

Aim-

To study the renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.

Objectives-

1) To evaluate the renoprotective activity of Citrus limon in comparison with standard drug and in control group in wistar rats.

2) To evaluate the renoprotective activity of Emblica officinalis in comparison with standard drug and in control group in wistar rats.

3) To evaluate the mixture of Citrus limon and Emblica officinalis with standard drug.

Materials and Methods-

Citrus limon-

The fresh Citrus limon will properly identified and purchase. The Citrus limon will be authenticated from the botanist in Aurangabad. Juice will be collected and will be stored in jar.

Emblica officinalis -

Emblica officinalis will be purchase from market and will be authenticated from the botanist in Aurangabad.

Preparation of extract Emblica officinalis

The Emblica officinalis will be dried and powdered by using mixer . Emblica officinalis powdered (5.0 g) will be extracted with mixture of distilled water 25 mL and 75 ml of ethanol i.e hydroalcoholic extract will be prepared by using percolater. The extract will be dried in fan air and stored in cool and dry place.

Chemicals-

Acetylcistein (granules)

Carbon tetrachloride-

Animals –

Albino wistar rats of either sex of weight (150-250 gm) will be use for the study. Animal will housed in ventilated animal rooms having free supply of standard laboratory diet ad libitum and allowed free access to drinking water. The animals will also kept in 12:12 hour light/dark cycle. The experimental rats will be handled in strict compliance.

Experimental induction of CCl4 nephrotoxicity

CCl4-induced acute renal injury will be initiated by intraperitoneal injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil as described by Lu et al. (2002).[15] CCl4 will injected intraperitoneally in wistar rats to produce nephrotoxicity. Blood will be collect by retro-orbital plexus and sent for estimation of BUN and Serum Creatinine levels and Oxidative Stress Parameters - activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) in a homogenized renal tissue will be determined using ELISA kits, on the kit guidelines. The above test will be done before giving standard and test drug and after giving standard and test drugs.

.The animals will then randomly divided into six experimental groups as shown in below.

Groups	Drugs	Drug dose
Group I	CCl4	1 ml Distill water /Oral route
Group II	Acetylcystine	950 mg/kg
Group III	Citrus limon	6 ml/kg/oral route for six wks.
Group IV	Emblica officinalis	700mg/kg/oral route for six wks
Group V	Citrus limon + Emblica officinalis	6 ml/kg + 700 mg /kg/oral route for six wks.
Group VI	Citrus limon + Emblica officinalis + Acetylcystine	6 ml/kg + 700 mg /kg + 950 mg /kg/ oral route for six wks.

Only single dose of CCl4 will given in animals for induction of renal injury.

The dose of Citrus limon, Emblica officinalis , and acetyl cystine is selected as per it were use in previous literature. [16-18]

Histological Evaluation of kidney.- The animals will be sacrificed by giving CO2 and kidney will be removed for histological assessment, kidney tissue samples will be will be fixed in 10% formalin solution for one week. After embedding in paraffin, the tissues will cut into 3-4 μm sections. The sections will be mounted on the glass slides, stain with hematoxylin-eosin (H&E) reagent, and finally survey by a pathologist in a blinded way.

Statistical Analysis-

Results will be done by ANOVA test using SPSS.

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The association of mucormycosis co-infection in patients with COVID-19 pneumonia: experience at tertiary care hospital in India

L.B. Dagdia[1], S.U. Joshi[1], P.S. Joshi[2], A.R. Patil[1], A.D. Kulkarni[1]

[1]Department of Orthopedics, [2]Department of Anaesthesiology, MGM Medical College and Hospital, Aurangabad, India

ABSTRACT:

Objective: We performed this study to explore the impact of multiple co-morbidities, different treatment strategies and vaccination in patients diagnosed with mucormycosis co-infection during the ongoing COVID-19 pandemic.

Patients and methods: This is an observational study of 60 patients out of 3000 admitted from March 2021 to May 2021 for treatment of COVID-19 pneumonia, with confirmed diagnosis of opportunistic fungal infection. Characteristics like age, antibiotic usage, steroid usage, and associated co-morbidities, need of oxygen or ventilator support and status of vaccination were studied.

Results: Out of 60 patients studied, maximum 37 (61.6%) belonged to 40 to 60 years age group and 38 (63.3%) were male. Fifty-two (86.6%) patients had one or other co-morbidities, while 56 (93.3%) of these patients received steroids in oral or intravenous form. Fifty-one (85%) patients received one or more than one higher grade antibiotics during treatment in hospital. Forty-two (70%) patients required Intensive Care Unit (ICU) admission out of which 4 (6.7%) required ventilator support, 10 (16.6%) required Non-Invasive Ventilation (NIV) while 28 (46.6%) were managed with high flow oxygen.

Conclusions: Our observations suggest for judicious use of steroids and higher antibiotics during treatment of COVID-19 pneumonia as it is associated with increased risk of opportunistic fungal infections. Strict control of blood glucose levels, multidisciplinary approach to reduce the impact of opportunistic fungal infection on patient morbidity and widespread vaccination especially among patients with co-morbidities will help in mitigating the impact of opportunistic fungal infections in patients with COVID-19 pneumonia.

Keywords: COVID-19, Mucormycosis, Fungal osteomyelitis prevention, Treatment.

INTRODUCTION

Around the end of year 2019, a number of patients with symptoms of pneumonia of unknown cause were detected in Wuhan, China. A novel coronavirus was identified as the causative pathogen, provisionally named as 2019 novel coronavirus (2019-nCoV) by the World Health Organization (WHO)[1,2]. Within two years this virus has spread from China to the whole world affecting more than 150 countries across all continents and causing morbidity and mortality across all age groups. This human-to-human transmitted disease, coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has been an emerging global public health event.

SARS-CoV-2 is mainly a lower respiratory tract infection causing Acute Respiratory Distress Syndromes (ARDS)[3]. In addition to widespread alveolar damage

Corresponding Author: Laxmikant Babulal Dagdia, MD; e-mail: dr.ldagdia@gmail.com

and inflammatory exudation, COVID-19 patients also develop immunosuppression due to a reduction in CD4 T and CD8 T cells. Such patients turn critical rapidly and require intensive care unit (ICU) admission along with mechanical ventilation[4]. These patients stand a very high risk of developing fungal co-infections. Many studies[5-7] have demonstrated multiple fungal co-infections like *Aspergillus flavus*, *A. fumigates*, *Candida albicans*, *C. Grabrata* in COVID-19 patients.

Mucormycosis is a type of opportunistic fungal infection caused by micro-organisms belonging to the phylum glomeromycota. Once a rare fungal infection, it is now seen as emerging threat in the wake of increased incidence of opportunistic fungal infections in COVID-19 patients[8]. Mucormycoses are life-threatening fungal infections mostly affecting diabetic, patients on immunosuppressant and solid organ recipients. Mucormycosis infection is characterised by infarction and necrosis of host tissues that results from invasion of vasculature by hyphae. Mucormycosis is most commonly present as rhino-orbito-cerebral and pulmonary infection[8,9]. In this short period of time, no studies have been conducted that determine the incidence of mucormycosis infections in COVID-19 patients and also the causative factors leading to a sudden increase in incidence[10,11]. Hence, we performed a study in the Indian population where the caseload of COVID-19 infections is extremely high.Our aim was to calculate the incidence of mucormycosis co-infection in patients suffering from COVID-19 pneumonia by finding the risk factors associated with increased incidence of mucormycosis co-infection in COVID-19 pneumonia, to determine the effectiveness of current treatment protocol of mucormycosis co-infection and to determine whether COVID-19 vaccination is effective in preventing fungal co-infections.

PATIENTS AND METHODS

This observational descriptive type of study was carried out over a period of three months from March 2021 to May 2021 and the patients admitted in Mahatma Gandhi Mission Hospital and Medical College, Aurangabad, Maharashtra for treatment of COVID-19 pneumonia were included in our study.

The inclusion criteria for our study were patients with confirmed diagnosis of COVID-19 pneumonia by RT-PCR test with testing device INSTA Q 9 (Equipment Number: ML01 – manufactured by Himeda Serial Number HN550988). Patients with proven diagnosis of fungal co-infection on laboratory tests (potassium hydro-oxide KOH mount of scrapping from infected tissue).

Exclusion criteria for our study were patients with history of fungal infection in the past and patients with fungal infection but not associated with COVID-19 infection.

A total of 3000 patients with confirmed diagnosis of COVID-19 pneumonia were admitted from March 2021 to May 2021. Amongst these patients those developing clinical symptoms of fungal infection and proven as mucormycosis infection on direct examination in 10% potassium hydro-oxide (KOH) of sample from scrapping of infected tissue, histopathology and culture reports were studied. A total of 60 patients were diagnosed with mucormycosis co-infection over a period of 3 months and these patients were followed up regularly throughout their course of disease.

When the patient first arrived in the fever clinic of our hospital (during COVID-19 pandemic special fever clinic and emergency section were established in our hospital campus to segregate patients with acute onset high grade fever with/without breathing difficulty from other emergency patients) an exhaustive history was taken regarding the type, severity and duration of symptoms. Specific information was obtained regarding the presence of co-morbid conditions, its duration and the type of treatment that is being carried out. A thorough general and system specific examination was then carried out with special attention towards the respiratory system for severity of symptoms. As soon as the patient was admitted a nasal swab was sent for RT-PCR which detects the spike gene and the N gene on viral RNA and is considered gold standard for diagnosing the presence of COVID-19 pneumonia[12]. Apart from this a battery of laboratory and radiological investigations comprising of Complete blood count, Renal Function Test, Liver Function Test, Serum Electrolytes, CReactive Protein, Serum ferritin, Arterial blood analysis, Erythrocyte sedimentation rate,X-ray chest, High resolution computed tomography of chest were done to assess severity of the disease and plan an appropriate course of action for the same. Patients who developed symptoms of fungal co-infection in addition to above investigations also underwent tests like direct microscopy of KOH mounted samples taken from specific sites of suspected infection, fungal cultures for detection of causative organism and magnetic resonance imaging of the brain, orbit or paranasal sinuses to evaluate of extent of disease.

Patients developing mucormycosis after admission for COVID-19 pneumonia had symptoms of lid edema and soft tissue swellings along the para nasal sinuses. Severe cases present with orbital cellulitis, para nasal sinusitis with osteomyelitic changes or neurological symptoms if the infection spreads to the brain. Mucormycosis was detected on nasal and conjunctival swabs subjected to direct microscopy and fungal cultures. MRI of the brain as well as orbit and para nasal sinuses gave an idea about the extent of spread of infection.

The treatment protocols for COVID-19 pneumonia are not well documented but the basic regime followed in our hospital included supportive treatment including intravenous fluids and oxygen support. According to the severity of the symptoms patients were started on oral or intravenous steroids, as well as antiviral drugs like Remdesevir with dosage – Day 1: Inj. Remdesivir 200 mg in 100 ml NS IV OD, Day 2 to 5: Inj. Remdesivir 100 mg in 100 ml NS IV OD. As a cover to protect the patients from secondary bacterial infections broad spectrum antibiotics and higher antibiotics like Meropenem (Inj. Meromac 500 mg IV in 100 cc NS IV BD), Tigecycline (Inj. Teganex 100 mg IV od followed by Inj.

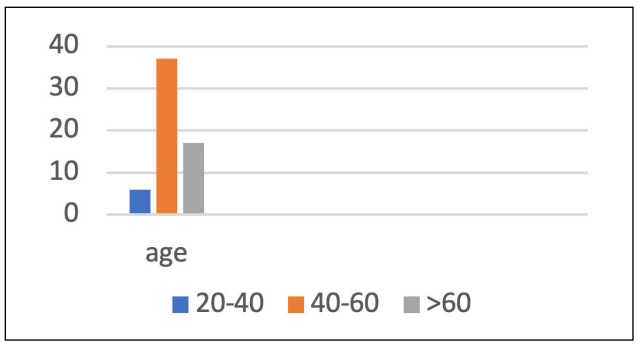

Figure 1. Age wise incidence of fungal osteomyelitis.

Teganex 50 mg BD) and Piperacillin tazobactum (In. Piptaz 4.5 gm iv TDS) were given. Enoxaparin (Inj. Clexane 0.4 cc or 0.6 SC HS) and other anti-thrombotic agents (Tab. Ecosprin 75 mg or 150 mg HS) were given to the patients to prevent life-threatening thrombotic events. In cases of fungal co-infections patients were started on antifungal like Amphoterecin B-Inj. Liposomal Amphotericin 5 amp 250 mg in 250 ml D5 IV OD for 21 days or Inj. Amphotrate (1 amp) 150 mg in 250 ml D5 OD for 21 days under all photosensitivity precautions and Posaconazole-Tab. Posaconazole 300 mg OD for 3 months. Surgery for the infected paranasal sinuses and orbital cellulitis was reserved for cases not responding to medical treatment or as a salvage procedure.

RESULTS

There were a total of 3000 patients admitted in our hospital for COVID-19 pneumonia out of which 60 patients suffered from Mucormycosis within a time period of 3 months with an incidence of 2%. Among these 60 patients there were 6 (10%) patients in the age group of 20-40, 37 (61.6%) patients belonged to the age group of 40-60 and17 (28.3%) patients above the age of 60 years who suffered from mucormycosis (Figure 1). Total 38 (63.3%) patients were male and 22 (36.7%) were female with a male to female ratio of 1.7:1 (Figure 2).

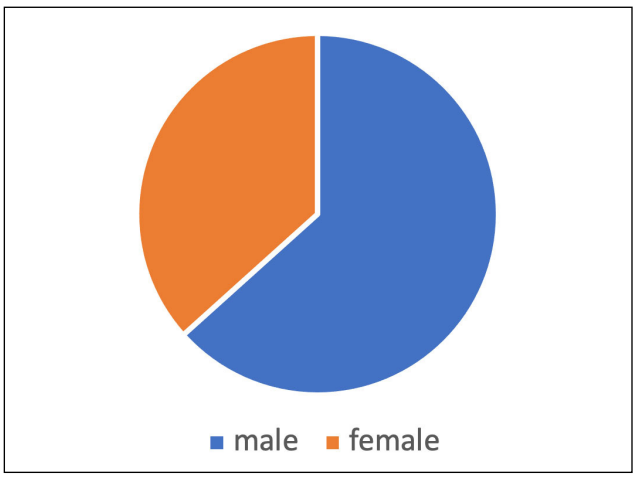

Figure 2. Sex wise incidence of fungal osteomyelitis.

From a total of 60 patients who suffered from fungal infections, 52 (86.6%) patients had presence of co-morbidities. Amongst these, diabetes mellitus was the most common co-morbidity seen in 34 (65.3%) patients with mucormycosis infections, followed by hypertension seen in 19 (36.5%) patients (Figure 3). There were also 14 (26.9%) patients who suffered from a combination of co-morbidities.

Steroids were one of the first line drugs used to counter the inflammatory response of the body to COVID-19 pneumonia and were administered either orally or intravenously in 56 (93.3%) of the 60 patients suffering from mucormycosis (Figure 4). Most of these patients received steroids for more than 5 days amongst which 8 patients consumed oral dexamethasone while 48 patients were administered IV methyl prednisolone.

The viral pneumonia affecting the lungs increased the susceptibility of patients to various super added bacterial infections. These infections were treated using both broad spectrum and higher antibiotics. Amongst the 60 patients maximum, 25 (41.66%) were treated with high end antibiotics like Inj piperacillin tazobactum, Inj meropenem in 9 (15%) patients and Inj tigecycline in 2 (3.33%) patients (Figure 5). Broad spectrum antibiotics like ceftriaxone, doxycycline or azithromycin were

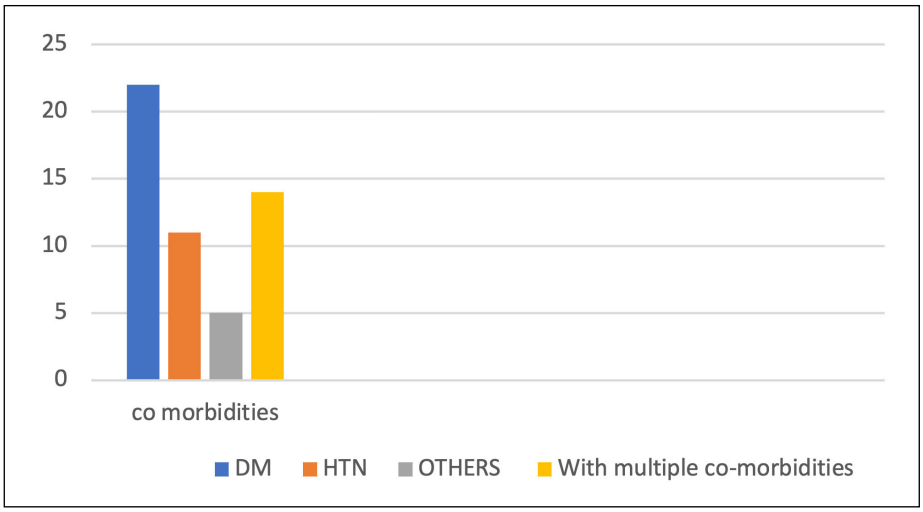

Figure 3. Incidence of co-morbidities in patients with mucormycosis.

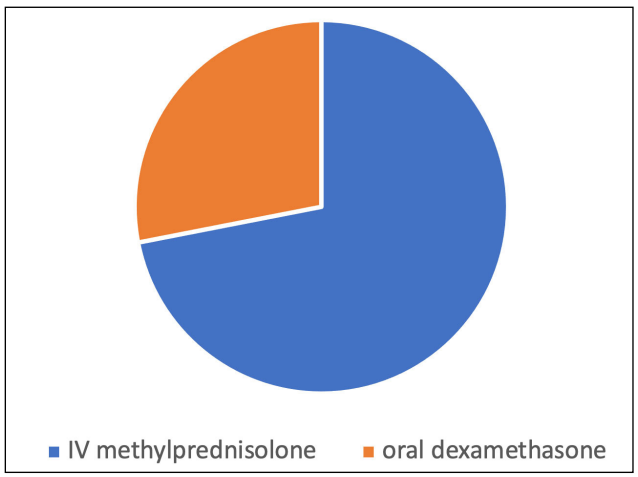

Figure 4. Steroid use.

used in 17 (28.33%) patients. Seven (11.66%) patients received a combination of above antibiotics.

In our study amongst 60 patients who suffered from COVID-19 pneumonia, 42 (70%) patients required ICU admission at some point in their course of disease. Four (6.7%) patients had to be put on ventilator support, 10 (16.6%) patients required non-invasive ventilation and 28 (46.6%) patients needed high flow oxygen with canula or reservoir bag (Figure 6). The rest 18 (30%) patients were treated in ward with intermittent need for O_2 support.

The role of vaccines in preventing COVID-19 infections has not yet been proven but studies suggest that previously vaccinated individuals are more likely to suffer from a mild illness without any serious complications. An observation was made that from the 60 patients who suffered from Mucormycosis only 9 patients had taken at least one dose of COVID-19 vaccine before suffering from the disease and amongst these only 3 patients required intensive care with others being managed in the ward on intermittent oxygen support.

Out of 60 patients in our study 9 (15%) patients died during course of follow-up, 4 (6.66%) patients required re-exploration surgery for residual infection, while 47 (78.33%) patients had an uneventful recovery at 3 months follow-up.

Mucormycosis occurring as a result of COVID-19 infection mainly affected the face with the nasal sinuses being the most common site of fungal infection seen in 36(60%) patients followed by orbit in 9 patients (15%) and brain in 6 (10%) patients (Figure 7). Nine (15%) patients presented with fungal infections in more than one site, the orbit and para nasal sinuses being the most common sites.

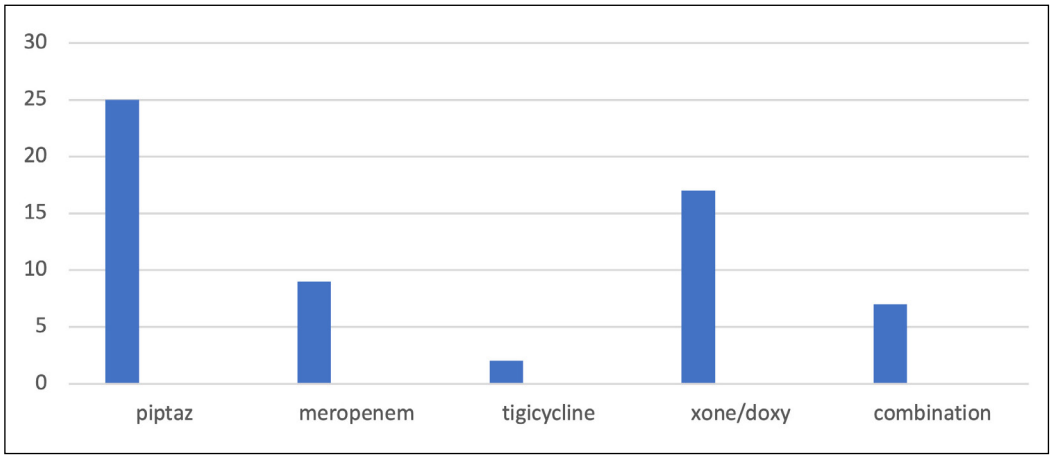

Figure 5. Antibiotics used.

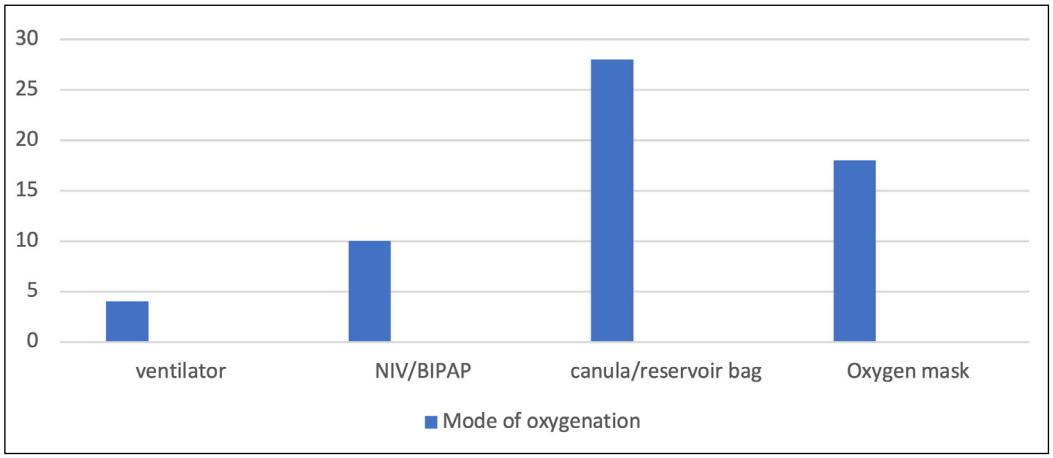

Figure 6. Oxygen support.

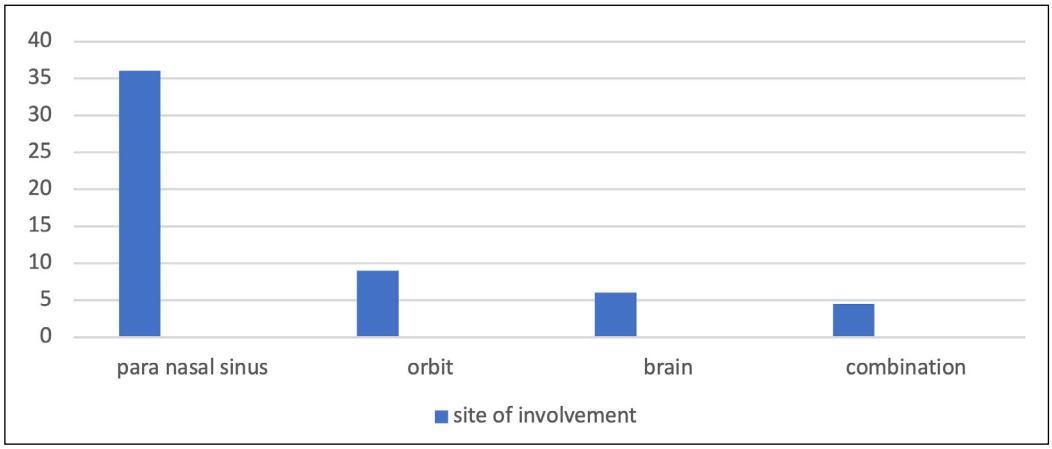

Figure 7. Site of occurrence of mucormycosis.

DISCUSSION

Mucormycosis is a deadly opportunistic fungal infection caused by fungus originating from mucorales order and includes Mucor, *Rhiopus*, *Rhizomucor*, *Abdidia*, *Apophysomyces* and *Cunninghumella*. Fungal spores enter *via* inhalation and then reach up to paranasal sinuses. Spores may also be acquired by contaminated food ingestion. Affected individuals usually present with acute sinusitis, fever, nasal congestion, purulent nasal discharge and headache[16]. If not treated early, contiguous spread to adjacent structures may occur, resulting in various clinical symptoms[16]. The orbital cavity is accessible through the ethmoid bone *via* the lamina papyracea, infratemporal fossa, inferior orbital fissure or orbital apex. Contiguous intracranial extension can occur through the ethmoid cribriform plate, supraorbital fissure and perineural routes[17]. Cavernous sinus or sagittal sinus thrombosis, carotid occlusion, cerebral infarction, intracranial aneurysm, intracranial haemorrhage and cerebral abscesses are potential *sequelae*[17-24].

In our study conducted over a period of 3 months there were a total of 3000 patients admitted in our hospital from which 60 patients developed Mucormycosis as a complication with an incidence of 2%. Jeong et al[14] in their study found an incidence a rate of 0.005-1.7 per million population globally. Alanio et al[25] screened 135 adults with COVID-19 infection and reported an incidence of invasive fungal infections of 26.7%. Patients with invasive fungal diseases had higher mortality (53% with *vs.* 31% without), which was significantly reduced by appropriate therapy. Corticosteroid therapy and a past history of chronic pulmonary disease were associated with a higher risk of invasive fungal disease[25]. Similarly, high incidences have been observed in Pakistan (23/147, 15.6%) and Italy (30/108, 27.7%), and with the authors suggesting that the development of invasive fungal infections alters the natural history of the disease[26,27].

In our study the para nasal sinuses were the most common site of affection for the fungal spores followed by the orbit and then brain. Similar results were observed in studies conducted by Selarka et al[28] where the most common site was rhino-cerebro-orbital (44%-49%), followed by cutaneous (10%-19%), pulmonary (10%-11%), disseminated (6%-11%) and gastrointestinal (2%-11%).

Mucormycosis is known to affect immunocompromised patients especially those with diabetes mellitus, prolonged corticosteroid use, solid organ transplant recipients, neutropenia and haematological malignancies[29-31]. The overall immunity of the patient suffering from COVID-19 infection has been observed to decline due to a decrease in CD4 and CD8 counts which is further aggravated by medical co-morbidities such as diabetes mellitus, hypertension and bronchial asthma. Diabetes mellitus is known to cause microangiopathy reducing tissue perfusion[13-15]. So, the deadly triad of diabetes mellitus, rampant use of steroids in the background of COVID-19 infection appears to increase risk of mucormycosis. All efforts should be made to maintain optimum glucose levels along with judicious use of steroids in COVID-19 treatment. In our study 52 patients were suffering from one or more co-morbidities with diabetes mellitus being the most common, 34 (56.66%) patients playing a major role in the severity of infection. In a cohort study presented by Erener et al[32] amongst patients diagnosed with COVID-19 pneumonia and mucormycosis, about three-quarters had a pre-existing history of diabetes mellitus along with a poor glycaemia control at presentation. The excessive use of broad spectrum antibiotics and immunosuppressive agents such as steroids and Remdesevir has also adversely affected the immunity of the individual. In our study, almost 93% of the patients suffering from Mucormycosis had received steroids for more than 5 days and almost all the patients had received complete courses of higher end antibiotics and Remdesivir to tackle the COVID-19 infection, all laying foundation for opportunistic infections like Mucormycosis. In addition, COVID-19 patients were more prone to develop secondary infections if they had decompensated pulmonary functions or required invasive mechanical ventilation. Our study showed that 42 patients required ICU admission with half of them requiring either ventilatory or non-ventilatory support of oxygen which was similar to studies conducted by Sharma et al[17] showing 82% of their study population required large amounts of oxygen through ventilator support.

The role of vaccination in preventing COVID-19 infection is still debatable but observations from our study show that Mucormycosis was fairly more common in individuals who had not received any previous dose of vaccinations and the severity of infection was comparatively lesser in those patients that had been vaccinated previously.

LIMITATIONS

Some limitations in our study were that the data represented the experience of loading in a single tertiary care centre, which often treat most of the sick patients with severe complications. Thus, the data may not be generalisable. Second, we could not perform blood investigations in all study participants due to lack of affordability by the patients, as well as limited availability of test kits among rapidly rising cases of COVID-19 patients. Third, a case series of 60 patients might be considered a small sample size and various associations could not be evaluated. However, given the rarity of the disease, it still accounts for a large case series. In fact, according to the published literature, 101 cases of mucormycosis in patients with COVID-19 have been reported so far, of which 82 cases belong to India[30]. Lastly, being an observational study, there is no control group to evaluate reliable differences and association.

CONCLUSIONS

The incidence of mucormycosis in the COVID-19 pandemic is likely to increase and can result in significant morbidity and mortality. While treating COVID-19 patients, we should have a high index of suspicion of mucormycosis especially when corticosteroids are used during the course of disease. Optimised glycaemic control should be achieved to control mucormycosis. Comprehensive monitoring of blood sugar levels on daily basis should be encouraged. Use of antifungal therapy with surgical debridement of affected tissue together should be undertaken and it remains the mainstay of treatment. Precautions need to be practised with regard to the widespread usage of corticosteroids and broad-spectrum antibiotics, with an emphasis to administer corticosteroids only in severe COVID-19 pneumonia and to reduce super-infections. Excessive use of corticosteroids should be restricted. A multidisciplinary approach involving an intensivist, diabetologist, otolaryngologist, ophthalmologist, infectious diseases specialist, neurologist and/or neurosurgeon is needed for the management of mucormycosis. An accelerated COVID-19 vaccination programme should be the highest priority in a country with high prevalence of diabetes and relatively poor resources to avoid massive outbreaks, morbidity and mortality during the current pandemic.

Conflicts of Interest:
The authors declare that they have no conflict of interests.

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Data available upon request from hospital records section.

ORCID ID:
Dr. LB Dagdia – 0000-0003-4990-6180
Dr. AR Patil – 0000-0001-9748-4029
Dr. AD Kulkarni – 0000-0002-2860-5103
Dr. SU Joshi – 0000-0001-6761-6684

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Analysis of Risk Factors for Mucormycosis in COVID-19 Patients Admitted in Tertiary Care Hospital Aurangabad

M. Naik [a,bφ], Shamisha Khade [a,b#*], Vinayak Bangi [a,b†] and Nilofer Banoisa Patel [a,b†]

[a] Department of Medicine, MGM Medical College and Hospital, Aurangabad, (MS), India.
[b] Constituent Unit of MGM Institute of Health Sciences, Navi Mumbai, (MH), India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Mucormycosis manifests as various syndromes in human beings, particularly in immunocompromised patients and those with diabetes mellitus. Recently, several cases of mucormycosis in people with COVID19 have been increasingly reported worldwide, especially from India. This prompted us to conduct a study in mucormycosis patients with COVID-19, to know its clinical profile of the COVID-19 patients with mucormycosis and identify of various risk factors in mucormycosis patients with COVID-19 infection.
Materials and Methods: This Cross sectional Retrospective Qualitative Descriptive study was conducted in Department of Medicine, MGM Medical College and Hospital, Aurangabad [Maharashtra]. A total of 100 patients admitted from April 2021 to August 2021 were enrolled as study participants. All COVID-19 patients admitted in MGM who are diagnosed with mucormycosis by microbiologically (KOH mount) or radiologically (CT/MRI) or by histopathology.
Observations and Results: The mean age of patients was 59.72±12.47 years. The male 73 (73.0%) predomianance than female 27(27.0%). 88(88.0%) of patients were having Diabetic Mellitus and 31(31.0%) of patients were having hypertension. All the patients were given Antibiotic

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[φ] Professor and Head;
[#] Chief Resident;
[†] Senior Resident;
**Corresponding author: E-mail: shamishakhade@gmail.com;*

& steroids during treatment of COVID-19 at hospitalisation. 15(15.0%) of patients were admitted in ICU during treatment of COVID-19. 76(76.0%) patients were required Oxygen, 03(3.0%) were on NIV/Ventilator and 08 (8.0%) patients were on HFOT during treatment of COVID-19. Overall (97.0%) of patients were recovered.

Conclusion: Diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients. Also use of steroid, duration of use of steroid, and oxygen therapy during the treatment of COVID-19 were risk factors observed in the patients with mucormycosis.

Keywords: Mucormycosis; steroid in COVID-19; post-COVID-19.

1. INTRODUCTION

Mucormycosis is manifested by a variety of different syndromes in humans, particularly in immunocompromised patients and those with diabetes mellitus. Devastating rhino-orbital-cerebral and pulmonary infections are the most common syndromes caused by these fungi.

"Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory distress syndrome. It has been associated with a wide range of opportunistic bacterial and fungal infection" [1]. "Both Aspergillosis and candida have been reported as the main fungal infections in patients with COVID-19" [2]. Recently, several cases of mucormycosis in people with COVID19 have been increasingly reported worldwide, especially from India. The main reason that appears to be facilitating mucorales spores to germinate in patients with COVID-19 is favourable conditions of low oxygen (hypoxia), high glucose(diabetes, new onset hyperglycemia, steroid induced hyperglycemia), acidic medium (metabolic acidosis, diabetic ketoacidosis [DKA]) ,high iron levels(increased ferritins) and decreased phagocytic activity of WBC due to immune suppression along with several other risk factors like prolonged hospitalization with or without mechanical ventilators.

"The genera in the order mucorales are responsible for causing Mucormycosis in humans. These organisms are universal in nature and can be found on decaying vegetations and in the soil. These fungi grow rapidly and release large numbers of spores which are air borne. Because the agents of mucormycosis are common in the environment hence they are most common contaminants in the clinical microbiology laboratory; all humans have plenty of exposure to these fungi during day to day activities. The fact that mucormycosis is a rare human infection reflects the effectiveness of the intact human immune system. This is further supported by the finding that almost all human infections due to the agents of mucormycosis occur in the presence of some underlying compromising condition" [2].

"The genera most commonly found in human infections are *Rhizopus*, *Mucor* and *Rhizomucor*; *Cunninghamella*, *Absidia Saksenaea*, and *Apophysomyces*are genera that are less commonly implicated in infection. *Rhizopus oryzae* is most common type and responsible for nearly 60% of mucormycosis cases in humans and also accounts for 90% of Rhino-orbital –cerebral (ROCM) form" [3].

"Globally, the prevalence of mucormycosis varied from 0.005-1.7 per million population, while its prevalence is nearly 80 times higher (0.14 per 1000) in India compared to developed countries in a recent estimate of year 2019-20" [4,5,6] "in other words, India has highest cases of mucormycosis in the world. Notwithstanding, India is already having second largest population with diabetes mellitus (DM) and was the diabetes capital of the world, until recently. Importantly, DM has been the most common risk factor linked with mucormycosis in India, although haematological malignancies and organ transplant takes the lead in Europe and USA" [7].

While long term use of corticosteroids has often been associated with several opportunistic fungal infections including aspergillosis and mucormycosis [8], even a short course of corticosteroids has recently been reported to link with mucormycosis especially in people with DM. A total prednisolone dose of more than 600mg or total methyl prednisolone dose of 2-7gm given during the month before, puts immunocompromised patients at risk of mucormycosis [9]. The case reports of mucormycosis resulting from even a short course

(5-14 days) of steroid therapy in patients of DM are rare [10]. “Surprisingly 46% of the patients had received corticosteroids within the month before the diagnosis of mucormycosis in the European Confederation of Medical Mycology study” [11].

“These findings need to be considered with respect to COVID-19 pandemic where corticosteroids are commonly used. There has been a rampant rise in case reports/series of mucormycosis patients with COVID-19 infection especially in India. Similarly, several case reports are being reported from various continents. These findings are unprecedented and are of great public health importance especially because there is high fatality rate with mucormycosis. Intracranial involvement of mucormycosis increases the fatality rate to as high as 90%” [12].

Moreover, rapidity of dissemination of mucormycosis is an extraordinary phenomenon and even a delay of 12 hours in the diagnosis could be fatal, the reason 50% of cases of mucormycosis have been historically diagnosed only in the post-mortem autopsy series. This prompted us to conduct a study in mucormycosis patients with COVID-19, to know its clinical profile of the COVID-19 patients with mucormycosis and identify of various risk factors in mucormycosis patients with COVID-19 infection.

1.1 Aim and Objectives

- To study the clinical profile of the COVID-19 patients with mucormycosis
- To identify the various risk factors in mucormycosis patients with COVID-19 infection.
- To study the outcome of mucormycosis patients in COVID-19 infection.

2. MATERIALS AND METHODS

2.1 Study Design

Cross sectional Retrospective Qualitative Descriptive study.

2.2 Study Area

Department of Medicine, MGM Medical College and Hospital, Aurangabad [Maharashtra], India.

2.3 Sample Size

100 mucormycosis patients

2.4 Study Duration

A total of 100 patients admitted from April 2021 to August 2021 were enrolled as study participants.

2.5 Inclusion Criteria

All COVID-19 patients admitted in MGM who are diagnosed with mucormycosis by microbiologically (KOH mount) or radiologically (CT/MRI)or by histopathology.

2.6 Exclusion Criteria

- COVID-19 patients with mucormycosis who were not willing to participate in study.

2.7 Elimination Criteria

All COVID-19 patients who are microbiologically and radiological diagnosed to have Mucormycosis but have failed to follow up.

2.8 Methodology

After getting ethical permission from ethics committee of MGM Medical College & Hospital, Aurangabad [MH], India, data was collected from COVID-19 with mucormycosis patients who satisfying inclusion and exclusion criterion of study. The purpose of the study was explained to the study participants. Only after their written consent patients were enrolled in the study. Confidentiality of the information was ensured. For the purpose of data collection a detailed proforma was prepared. The proforma was included demographic profile (Name, age, sex and BMI), Personal history, comorbidity and detailed history of COVID-19, treatment during COVID-19.

Also diagnosis method of mucormycosis patients, patients according to involvement, anti-fungals received surgical intervention and outcome of mucormycosis patients.

2.9 Statistical Analysis

The collected data was entered in Microsoft excel and analysed using SPSS version 24th. Mean and SD was calculated for quantitative variables and proportions were calculated for categorical variables.

3. RESULTS

In present study out of 100 patients, maximum patients i.e. 49 (49.0%) were from age more than 60 years, 40(40.0%) were age-group of 45-60 years and only 11(11.0%) of patients were from age-group 15-45 years. The mean age of patients was 59.72±12.47 years. The male 73 (73.0%) predominance than female 27(27.0%).

In present study, 88(88.0%) of patients were having Diabetic Mellitus, 31(31.0%) of patients were having hypertension and 12(12.0%) of patients were having CHD/IHD, one patient was having Hypothyroidism and Asthma.

In present study, all 100(100%) of patients were diagnosed on Imaging and 55(55.0%) were positive on KOH and 36(36.0%) patients were positive on histopathology.

All 100 patients were reported Sinuses involvement, 70(70.0%) patients were having ocular, 02(2.0%) Pulmonary and 03(3.0%) patients were having cerebral involvement.

All the patients were given Antibiotic & steroids during treatment of COVID-19 at hospitalisation. 15(15.0%) of patients were admitted in ICU during treatment of COVID-19. 76(76.0%) patients were required Oxygen, 03(3.0%) were on NIV/Ventilator and 08 (8.0%) patients were on HFOT during treatment of COVID-19. 67 (67.0%) patients were used steroids for COVID-19 treatment for 6–10 days, 28(28.0%) study participants used steroids for 11-15 days. Where as 5(5.0%) patients were used steroids more than 15 days.

All the 100 patients were given Posaconazole & Amphotericin, 17(17%) & 16(16.0%) patients were given Liposomal Amphotericin and Lyophilized Amphotericin respectively. 67(67.0%) of patients were given Lipid Emulsion Amphotericin.

All the patients required Functional endoscopic sinus surgery (FESS), 78 (78.0%) of patients were done Endoscopic Debridement where as 16(16.0%) patients done Maxillectomy.

Out of 100 patients 97(97.0%) of patients were recovered and 03(3.0%) were died during treatment of mucormycosis.

4. DISCUSSION

In present study out of 100 patients, maximum patients i.e. 49 (49.0%) were from age more than 60 years, 40(40.0%) were age-group of 45-60 years and only 11(11.0%) of patients were from age-group 15-45 years. The mean age of patients was 59.72±12.47 years. Similar findings was reported by Bhagyashri Jadhav et al. [13] “the mean age was 54.46±13.13, years ranging from 28 to 77 years”. Also Ganesh Lokhande et al [14] observed “mean age of the patient was 52.47 ±12.84 years with a minimum age of 26 and maximum age of 83 years”. Study conducted by Sen et al. [15] observed that “the mean age of the study participants was 51.9”. A study conducted by Gupta [16] revealed that “the mean age of the study participants was 50 years. Maximum study reported mean age of mucormycosis patients were above 50 years”.

Table 1. Distribution of patients according to demographic profile of patients

		No. of patients	Percentage
Age-group in years	15-45	11	11.0
	45-60	40	40.0
	>60	49	49.0
	Total	100	100%
	Mean±SD	59.72±12.47 years	
Gender	Male	73	73.0
	Female	27	27.0

Table 2. Distribution of patients according to co-morbidities

Comorbidities	No. of patients (n=100)	Percentage
Hypertension	31	31.0
Diabetic Mellitus	88	88.0
IHD/CHD	12	12.0
Hypothyroidism	01	01.0
Asthama	01	01.0

Table 3. Distribution of patients according to diagnosis method

Diagnosis method	No. of patients (n=100)	Percentage
KOH	55	55.0
Hisopathology	36	36.0
Imaging	100	100.0

Table 4. Distribution of patients according to involvement

Involvement	No. of patients (n=100)	Percentage
Sinuses	100	100.0%
Occular	70	70.0%
Pulmonary	02	02.0%
Cerebral	03	03.0%

In present study the male 73 (73.0%) predominance than female 27(27.0%). Similar male predominance was observed by Patel et al [17] 69.5% of participants affected by mucormycosis were men. Sen et al. [15] observed 71% of the male. Bhagyashri Jadhav et al [13] Observed 75% of male patients. , Lokhande GS et al [14] also reported 61.34% were males.

In present study, 88.0% of mucormycosis patients were having Diabetic Mellitus. John et al. [18] observed that 94% of the patients with mucormycosis were diabetic. In 73.5% of cases with mucormycosis, diabetes was observed as a risk factor in India [19]. Sen et al. [15] observed that 78% of the patients with mucormycosis were having diabetes. 77% found by Priya et al. [20] In contrast to the Findings in this study, Lokhande GS et al [14] reported (57%) were diabetic. COVID-19 cases with a history of diabetes are at increased risk of developing the severe disease and these patients are also at higher risk of fungal infections. Globally diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients [21].

In present study 76.0% patients were required Oxygen, 3.0% were on NIV/Ventilator and 8.0% patients were on HFOT during treatment of COVID-19. Similarly Sen et al. [15] observed that 79% of the patients with mucormycosis received O2 therapy for the treatment of COVID-19. Whereas Afroze SN et al [22] reported 80.22%. Whereas Bhagyashri Jadhav et al. [13] reported 18.75% patients gave the history of receiving oxygen or mechanical ventilation during the treatment of COVID-19.

In present study, all 100 patients were given steroids during treatment of COVID-19 at hospitalisation. Lokhande, et al [13] found that more than 90% of patients had a history of steroid use for the treatment of COVID-19. Also Sen et al. [15] revealed a history of use of steroids in 87% of patients admitted with mucormycosis. Use of corticosteroids was observed in 88% of the study participants with mucormycosis in the study conducted by John et al. [18]. In present study (67.0%) patients were used steroids for COVID-19 treatment for 6–10 days, (28.0%) study participants used steroids for 11-15 days. Where as (5.0%) patients were used steroids more than 15 days. Lokhande, et al [14] reported "(77.11%) study participants used steroids for COVID-19 treatment for 7–14 days, whereas (20.48%) study participants used steroids for less than 7 days. The National Institute of Health recommends the use of dexamethasone (6 mg per day for a maximum of 10 days) in patients who are ventilated or require supplemental oxygen but not in milder cases. 17 The guidelines specifically mention the risk of developing a secondary infection".

In present study, all (100%) of patients were diagnosed on Imaging and (55.0%) were positive on KOH and (36.0%) patients were positive on histopathology. Lokhande, et al. [14] reported 57.14% of patients found positive on KOH.

In this study, All 100 patients were reported Sinuses involvement, (70.0%) patients were having ocular, (2.0%) Pulmonary and (3.0%) patients were having cerebral involvement. Singh et al. [22] found that 88.9% of Sinuses involvement, ocular (1.0%), Pulmonary (7.9%) and Cerebral (22.2%).

Table 5. Distribution of patients according to treatment during COVID-19

		No. of patients(n=100)	Percentage
Antibiotic		100	100.0%
Steroid		100	100.0%
ICU admission		15	15.0%
Oxygen requirement		76	76.0%
NIV/ventilator		03	3.0%
HFOT		08	8.0%
No. of days steroid given in COVID-19	0-5 Days	00	00
	6-10 Days	67	67.0%
	11-15 Days	28	28.0%
	>15 Days	5	5.0%

Table 6. Distribution of patients according to anti-fungals received

Antifungals received	No. of patients (n=100)	Percentage
Posaconazole	100	100.0
Amphotericin	100	100.0
Liposomal amphotericin	17	17.0
Lipid emulsion amphotericin	67	67.0
Lyophilized amphotericin	16	16.0

Table 7. Distribution of patients according to surgical intervention

Surgical intervention	No. of patients (n=100)	Percentage
Functional Endoscopic Sinus Surgery (FESS)	100	100.0%
Endoscopic debridement	78	78.0%
Maxillectomy	16	16.0%

Table 8. Distribution of patients according to Outcome

Surgical intervention	No. of patients (n=100)	Percentage
Recovered	97	97.0%
Death	03	3.0%
Total	**100**	**100.0%**

In present study All the patients required Functional endoscopic sinus surgery (FESS), 78 (78.0%) of patients were done Endoscopic Debridement where as 16(16.0%) patients done Maxillectomy. Whereas contrast finding was reported by Bhagyashri Jadhav et al [13] that in "(25%) patients only medical line of treatment was sufficient whereas (62.5%) patients required surgical debridement during the treatment. In our study, (97.0%) of patients were recovered and (3.0%) were died during treatment of mucromycosis". Bhagyashri Jadhav et al. [13] reported Overall survival was 90.62%.

5. CONCLUSION

In Post COVID-19 patients, Mucormycosis is one of the complications observed in the later stage of the disease. Diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients. Also use of steroid, duration of use of steroid, and oxygen therapy during the treatment of COVID-19 were risk factors observed in the patients with mucormycosis. A high clinical suspicion and early and accurate diagnosis of AIFR in COVID-19 patients are essential for better prognosis.

6. LIMITATIONS OF STUDY

The study doesn't do justice in the aspect that it doesn't include all the cases of Mucormycosis on a single based criteria, i.e some are included on the basis of histopathology, while some on the basis of microbiology and the rest on basis of radiological diagnosis.

CONSENT

As per international standard or university standard, patient (s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The study is subjected for approval to "Ethical Committee" of MGM Medical College & Hospital Aurangabad [MH], India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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ORIGINAL ARTICLE

Impact of COVID-19-related Stress on Glycaemic Control in Hospitalized Patients with Type 2 Diabetes Mellitus

Rohini Patil[1,2], PhD candidate;
Kamal Shah[3], PhD;
Deepak Bhosle[4], MD

[1]Department of Information Technology, Thakur college of Engineering and Technology, Mumbai, Maharashtra, India
[2]Department of Computer Engineering, Terna Engineering College, Navi Mumbai, Maharashtra, India
[3]Department of Information Technology, Thakur College of Engineering and Technology, Mumbai, Maharashtra, India
[4]Department of Pharmacology, MGM Medical College, Aurangabad, Maharashtra, India

Correspondence:
Rohini Patil, PhD candidate;
Department of Information Technology, Thakur college of Engineering and Technology, Mumbai, Maharashtra, India
Tel: +91 98 20167350
Email: rohinipatil@ternaengg.ac.in
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Abstract

Background: Evaluation of the impact of stress on glycaemic control in hospitalized type-2 diabetes (T2DM) patients with coronavirus disease (COVID-19).

Methods: In this retrospective study conducted at a single centre in Maharashtra from May to July 2020 on hospitalized COVID-19 patients with T2DM who reported having stress of pandemic; they were selected using purposive sampling. DASS-12 stress sub-scale was used to estimate the severity of their stress. Fasting blood glucose (FBG) and post-prandial blood glucose (PPBG) before admission and at the time of discharge were compared.

Results: One hundred and ninety-nine patients (mean age 54 years; 61.30% females) were included. Mean±SD FBG before admission was 168.4±30.6 mg/dl which increased to 195.9±28.8 mg/dl at the time of discharge (P<0.001). Also, Mean±SD PPBG before admission was 312±62.3 mg/dl which increased to 351.6±61.9 mg/dl (P<0.001). A total of 73 (36.7%) participants had perceived stress. Moderate and severe/extremely severe stress was found in 44 (27.1%) and 19 (9.6%) patients, respectively. A significant difference was observed in the mean FBG before and during discharge in patients who had no stress and those with moderate stress (P<0.001). There was no difference in FBG in patients with severe/extremely severe stress (P=0.43). Similar observations were seen for PPBG (no stress P<0.001; moderate stress P<0.001; severe/extremely severe stress P=0.06).

Conclusion: There was a rise in the glucose level in T2DM patients discharged after COVID-19 treatment. The increase was significant in T2DM without stress and those with moderate stress. In addition to traditional treatment, measures for psychological stress control should also be taken for such patients.

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Introduction

Diabetes mellitus is a challenging and prevalent chronic metabolic disorder from psychosocial and behavioural perspective. Untreated diabetes can result in serious short-term or long-term complications resulting in significant morbidity and mortality. According to global report of International Diabetes Federation (IDF 2017), there are 463 million people with diabetes.[1] In 2019, the global prevalence of diabetes was 9.3%. It is expected to rise to 10.2% and 10.9% by 2030 and 2045, respectively.[2] Overall type 2 diabetes (T2DM) prevalence in India is 8.9%.[1] Uncontrolled T2DM can result in several acute and chronic complications. Although traditional risk factors contribute to the high risk of T2DM development, its incidence continues to increase despite strategies to

control traditional risk factors.[3]

Stress contributes to many pathological conditions and hormonal imbalance; during stress, situations can adversely affect the normal glycaemic control in people with diabetes.[4] Perceived stress can contribute to the risk of T2DM development.[3] A 12- year longitudinal study on women showed three years later stress levels were associated with a higher risk of diabetes.[3] Apart from traditional stress factors, during the last year corona virus disease (COVID-19) emerged and caused a significant stress on people, families and community. Diabetes is expected to have poor outcomes after COVID-19 infection.[5] Patients with diabetes are reported to have COVID-19-specific concerns regarding their disease.[6] A cross-sectional study from South India reported unhealthy dietary habits, mental stress, and sleep disturbances during COVID-19 lockdown period. However, the same study reported no major difference in overall glycaemic control among patients with T2DM during lockdown.[7] Therefore, we aimed to study the effect of stress on T2DM patients. The objective was to evaluate the impact of stress on glycaemic control in T2DM patients hospitalized for COVID-19 treatment.

Methods

A retrospective single-centre questionnaire based on a study in Maharashtra, with patients of both gender with T2DM who received anti-diabetes medications with a history of hospitalization for the treatment of COVID-19 from May to July 2020 and those whose readings for glycaemic parameters, i.e. fasting blood glucose (FBG) level and post-prandial blood glucose (PPBG) level were available were included in the study. Type 1 diabetes patients, newly diagnosed cases of T2DM who did not receive any anti-diabetic medication, patients with T2DM with no history of hospitalization for COVID-19 treatment, and those with gestational diabetes were excluded. Demographic details [gender, age, weight, body mass index (BMI)] and duration of T2DM were noted. DASS-12 stress sub-scale was used to estimate the severity of stress.[8] There are four items in the stress sub-scale which are rated as "Never (0), Sometimes (1), Often (2), and Almost Always (3)". Based on the stress scores, the patients were classified as (0-4) normal, (5) moderate, (6) severe and (≥7) extremely severe.[8] Face validity and content validity of the questionnaire were checked with an expert.

Difference in glycaemic parameters (FBG and PPBG) before and at the time of discharge was estimated. Gender-wise and age-wise (<34 years, 35-49 years and >50 years) comparison was done for estimating the difference in the severity of stress.

The data were entered into MS-EXCEL sheet. Number and percentages are provided for categorical data whereas Mean±SD are provided for continuous data. With the use of paired t-test, the difference in glycaemic parameters before and after COVID-19-related admission was compared. Unpaired t-test was used to estimate the statistical difference in glycaemic parameters between different groups. Chi-square test was used for comparing the categorical variables among the two groups. Results were found statistically significant (P<0.05).

Results

A total of 199 patients with a Mean±SD age of 54±12.8 years were included, of whom 122 (61.30%) were female and 77 (38.7%) were male. The Mean±SD weight and BMI of patients were 78.1±14.6 kg and 30.9±7.5 kg/m^2. A total of 116 (58.3%) patients were from urban areas, whereas 83 (41.7%) were from rural areas (Table 1). A total of 114 (57.3%) patients were housewives.

Table 1: Demographics characteristics of the study participants

Parameter	Result
Mean±SD age	54±12.8 years
Age range (minimum, maximum)	30-92 years
Gender n (%)	
Male	77 (38.7%)
Female	122 (61.3%)
Mean±SD weight	78.1±14.6 kg
Range of weight	30-114 kg
Mean±SD BMI	30.9±7.5 Kg/m^2
Range of BMI	10.6-51.5 Kg/m^2
Residence n (%)	
Rural	83 (41.7%)
Urban	116 (58.3%)
Profession n (%)	
Business	7 (3.5%)
Service	10 (5.03%)
Housewife	114 (57.3%)
Other	68 (34.2%)
Mean±SD duration of diabetes	5±3.6 years
Range of duration of diabetes (minimum, maximum)	0.08-25 years

Mean±SD FBG before admission was 168.4±30.6 mg/dl which increased to 195.9±28.8 mg/dl after discharge (P<0.001). Similarly, Mean±SD PPBG before admission was 312±62.3 mg/dl which increased to 351.6±61.9 mg/dl (P<0.001), as shown in Table 2.

Overall, out of 199 participants, 54 (27.1%) patients had moderate stress. Severe or extremely severe stress was observed in 19 (9.6%) patients (Figure 1). Thus, out of 199 participants, 73 (36.7%) had perceived stress.

In the group of moderate stress, 23 out of 54 patients (42.6%) were females and 31 (57.4%) were male. In the group of patients with severe/extremely severe stress, 11 out of 19 patients (57.9%) were females and 08 (42.1%) were male. Gender-wise as well as age-wise comparison showed a significant difference in the severity of stress (P<0.05).

Comparison of glycemic parameters, weight, age, and BMI between the groups is shown in Table 3.

Table 2: Glycaemic parameters before and at the time of hospital discharge after COVID-19 treatment

	Before hospital admission	After discharge	P value
Mean±SD fasting blood glucose mg/dl	168.4±30.6	195.9±28.8	<0.001
Mean±SD post prandial blood glucose mg/dl	312±62.3	351.6±61.9	<0.001

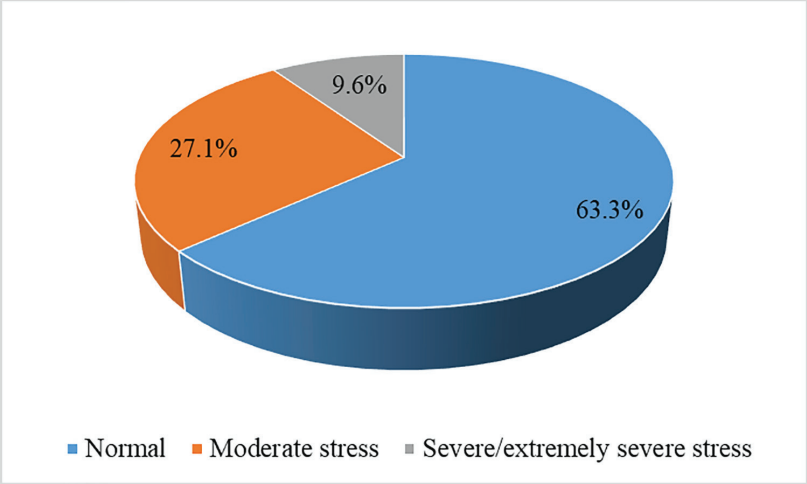

Figure 1: Distribution of patients based on the severity of stress as assessed by DASS-12 score (n=199)

Table 3: Comparison of glycemic parameters based on the severity of stress

	No stress (n=126)	Moderate stress (n=54)	Severe/Extremely severe stress (n=19)
Mean±SD age in years	52.6±13	57.3±12.5	54.2±10.6
Mean±SD weight in Kg	80±13.9	74±15.1	77.2±16.5
Mean±SD BMI in kg/m^2	32.6±7.3	27.8±6.7	28.3±7.5
Gender n (%)			
Male	38 (30.16%)	23 (42.59%)	8 (42.11%)
Female	88 (69.84%)	31 (57.41%)	11 (57.89%)
Mean±SD fasting blood glucose (mg/dl)			
Before admission	164.7±30	171.3±24.5	185±43.2
After admission	195.1±27.8	198.3±30.8	194.2±30.8
P value	<0.001	<0.001	0.43
Mean±SD post-prandial blood glucose (mg/dl)			
Before admission	309.2±62.3	315.8±59	319.7±72.8
After admission	347.3±64.7	359±52.6	358.7±68.1
P value	<0.001	<0.001	0.06

Table 4: Comparison of glycemic parameters in three groups with different levels of stress

	Without stress versus moderate stress	Moderate versus severe/ extremely severe stress	Without stress versus severe/ extremely severe stress
Fasting blood glucose before admission	0.15	0.09	0.01
Fasting blood glucose at the time of discharge	0.497	0.68	0.98
Post-prandial blood glucose before admission	0.51	0.82	0.50
Post-prandial blood glucose at the time of discharge	0.24	0.99	0.48

There was a significant difference in the mean FBG before and after admission in patients without stress and those with moderate stress. There was no significant difference in the mean FBG in patients with severe/extremely severe stress (P=0.43). Similar observations were seen for PPBG (without stress P<0.001; moderate stress P<0.001; severe/extremely severe stress P=0.06) (Table 3).

There was no difference in the FBG or PPBG in patients without stress versus moderate stress, moderate versus severe/extremely severe stress, no stress versus severe/extremely severe stress before admission or after discharge (Table 4).

In patients with stress, there was significant difference in the FBG and PPBG based on their residence, i.e. urban versus rural population (P<0.001).

Discussion

COVID-19 pandemic has impacted every individual's life, resulting in significant changes in their lifestyle. A study on 435 patients has reported increase in perceived

stress among patients with diabetes.[9] In the current study, we investigated the impact of perceived stress on glycaemic control in 199 T2DM patients discharged after their COVID-19 treatment. Generally, male patients are at higher risk of complications related to COVID-19 disease as compared to female. Similarly, male predominance is observed among hospitalized diabetic patients with COVID-19.[10] However, in our study there was female predominance. Our observations are in accordance with those of Yoshida et al. who conducted a study to evaluate the gender differences in clinical presentations and outcomes in hospitalized patients for COVID-19. In their study, there were 61.4% females, and women had a significantly higher prevalence of diabetes as compared to males (38.2% vs. 31.8%).[11] We focused only on patients with diabetes.

In our study, out of 73 patients with stress, 46.6% patients were female. A study from urban slums of Bangalore reported higher prevalence of diabetes in females as compared to males.[12] In our study, the prevalence of severe stress was more common in females.

Negative effect of stress on glycaemic control in patients with diabetes is known. Direct as well as indirect effects both contribute to the glycaemic impairment in patients with diabetes. Direct effects are related to the stress hormones, and indirect ones are due to changes in the lifestyle and behaviour.[4] Chronic stress can lead to neuroendocrine changes and dysregulation of physiological systems.[13]

A study from South India reported no major change in the overall glycaemic control among patients with T2DM due to lockdown after COVID-19 pandemic.[7] We observed a significant increase in the mean FBG and PPBG in T2DM patients at the time of discharge as compared to before admission for COVID-19 treatment. Suboptimal glycaemic control during infectious diseases is known.[14] However, we analysed the data at the time of discharge from the hospital.

Depending on the duration of exposure to stressors, patients with diabetes may be exposed to acute or chronic stress. Acute stress, because of its short duration, may not affect HbA1c which indicates glucose control over several weeks.[13] Considering this, we did not focus on changes in HbA1c. However, a retrospective study from Japan reported a significant rise in HbA1c levels after the outbreak of COVID-19 as compared to before the pandemic. There have been changes in the physical and psychological health of patients during this period. Behavioural changes have been suggested to affect the level of HbA1c in these patients.[15] Although not specifically examined, stress contributing to glycaemic derangement cannot be ruled out. In the same study, when compared by age, a significant increase in HbA1c was observed in patients with age more than 65 years. Also, there was a significant increase in HbA1c in patients with BMI more than 25 kg/m^2, but not in those with lower BMI. We focused on T2DM patients hospitalized for the treatment of COVID-19, unlike outpatients in a study by Tanji et al.[15]

Faulenbach et al. evaluated the effect of acute stress on glycaemic control in 30 patients with T2DM with a mean age of 60 years. In this study, experience of stress after the meals resulted in a significant increase in the post-prandial blood glucose level.[16] Another cross-sectional study from Chennai, India, has reported a positive correlation between both FBS and PPBG levels and the stress levels.[17] Another study has reported the association of increased stress with difficulty in glycaemic control.[9]

Perceived intensity of stress can also vary between different individuals. To categorise the patients into different levels of stress, we used DASS-12 stress scale. In our study, 36.7% had stress, of whom 74% had moderate stress and 26% had severe or extremely severe stress. In our study, a significant difference was observed in the mean FBG before admission and at the time of discharge in patients with moderate stress. However, there was no significant difference in the mean FBG in patients with severe/extremely severe stress. Similar observations were seen for PPBG (moderate stress P<0.001; severe/extremely severe stress P=0.06). We could not findany study on the effects of severity of stress on glycaemic control in T2DM patients. Furthermore, deterioration in HbA1c values has been reported, in particular among women, patients aged more than 65 years, those with body mass index of more than 25 kg/m^2, and those that were not using insulin.[15] Further studies on evaluation of the effect of stress on glycaemic parameters in these subgroups are recommended.

Intergroup analysis showed no difference in the FBG or PPBG in patients without stress versus moderate stress, moderate versus severe/extremely severe stress, no stress versus severe/extremely severe stress before admission or after discharge.

Diabetes is a known risk factor for hospitalization and mortality due to infections.[18] It has also been reported as a risk factor for severity and mortality in patients with COVID-19.[19, 20] Patients with diabetes may get frustrated with experience of hyperglycemia despite the lifestyle modifications.[21] Stress may further add to the impairment of glycaemic control. Thus, it is essential to address psychological issues of vulnerable groups during the COVID-19 pandemic.[22] Considering the adverse impact on glycaemic control, patients with diabetes should be counselled effectively to control stress.

This was a retrospective study; hence, a definite cause and effect relationship between stress and glycaemic parameters cannot be ascertained. The single centre study with limited sample size is

another limitation. COVID-19 may contribute to the development of hyperglycaemia.[23] Moreover, steroids used in the treatment of COVID-19 can also contribute to the hyperglycaemia. Because of lack of pharmacotherapy details in these patients, we could not conduct separate analysis of patients who received steroids versus those who did not. Larger prospective studies are recommended to be conducted to confirm our observations.

Conclusion

Overall, the study population showed a rise in fasting and postprandial glucose level in T2DM patients discharged after COVID-19 treatment. The rise was significant in T2DM without stress and those with moderate stress. Studies with larger sample size on T2DM patients with stress may be needed to provide more insights regarding the difference between those without stress and moderate to severe/extremely severe stress. In addition to traditional treatment of diabetes, measures for control of psychological stress should also be taken in patients with COVID-19.

Conflicts of interest: None declared.

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Review

Advances in the colon-targeted chitosan based multiunit drug delivery systems for the treatment of inflammatory bowel disease

Nilesh Kulkarni [a,*], Priti Jain [b], Amol Shindikar [c,d,e], Pravin Suryawanshi [f], Nanasaheb Thorat [d,g,**]

[a] Department of Pharmaceutics, R. C. Patel Institute of Pharmaceutical Education and Research, Karwand Naka, Shirpur, 425405, Dist. Dhule, Maharashtra, India
[b] Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Delhi Pharmaceutical Sciences and Research University (DPSRU-Govt. of NCT Delhi), Mehrauli-Badarpur Road, Pushp Vihar Sector 3, New Delhi, 110017, India
[c] In-charge, Central Research Laboratory, MGM Medical College and Hospital, MGM Campus, N-6, CIDCO, Aurangabad, 431003 (A constituent college of MGMIHS, Navi Mumbai), Maharashtra, India
[d] Cluster of Drug Discovery, Development and Translational Research, Center of Multidisciplinary Research (CMR), MGM University, Aurangabad, 431003, Maharashtra, India
[e] Department of Pharmaceutical Sciences, Birla Institute of Technology and Science (BITS), Pilani, 333031, Rajasthan, India
[f] Center of Excellence for Endoscopy and Advanced Minimally Invasive Surgery, Department of Surgery and Gastroenterology, MGM Medical Center and Research Institute, Aurangabad, 431003, Maharashtra, India
[g] Nuffield Department of Women's and Reproductive Health, John Radcliffe Hospital, Medical Sciences Division, University of Oxford, Oxford OX3 9DU, United Kingdom

ARTICLE INFO

Chemical compounds studied in this article:
Chitosan (7057928)
Sodium alginate (5102882)
Starch (24836924)
Xanthan gum (131750926)
Pectin (5381226)
Carboxymethyl cellulose sodium (23706213)
Dextran sulphate (4125253)
5-Aminosalicylic acid (4075)
Eudragit S-100 (65358)
Sodium tripolyphosphate (24455)

Keywords:
Chitosan

ABSTRACT

Chitosan is the polymer of choice for delivery of the active moieties to the colon due to its cationic nature that enables strong mucosal attachment. Chitosan is explored for formulations such as pellets, beads, microspheres, nanoparticles and drug-polymer conjugates for colon targeting of various therapeutic agents in inflammatory bowel disease (IBD). The major challenge in the colonic delivery of drugs in IBD is altered physiological pH, which can be addressed *via* chitosan containing multiparticulate drug delivery systems owing to their biodegradability in the colon. Its ionic interaction with anionic polymers forms gastro-resistant multi-unit systems that ensures safe delivery of payloads to the colon. In contrast to commercial grade gastro-resistant polymers, chitosan has GRAS (generally regarded as safe) status that ensures safety for long-term therapy in case of chronic diseases such as IBD. Here, we review in detail essential properties of chitosan and chitosan based multiunit formulations for treatment/mitigation of IBD.

Abbreviations: APIs, active pharmaceutical ingredients; GRAS, generally regarded as safe; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; 5-ASA, 5-amino salicylic acid, mesalamine; LD_{50}, lethal dose; GIT, gastrointestinal tract; EC, ethylcellulose; ROS, reactive oxygen species; TLRs, Toll-like receptors 4; NODs, Nod-like receptors; LPS, lipopolysaccharides; NF-κB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; JNK, C-JUN-N-terminal kinase; DCs, dendritic cells; COS, chitosan oligosaccharides; TNF-α, tumor necrosis factor-α; IL, interleukins; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; ($PG-E_2$), prostaglandin E_2; NO, nitric oxide; PF, platelet factor; MCC, microcrystalline cellulose; SIF, simulated intestinal fluid; EDTA, ethylenediaminetetracetic acid; CHI-g-AAm, acrylamide grafted chitosan; MPO, myeloperoxidase; TNBS, 2,4,6-trinitrobenzenesulfonic acid; M, mannuronic acid; G, guluronic acid; CAB, cellulose acetate butyrate; DSS, dextran sulphate; PCR, polymerase chain reaction; MyD 88, myeloid differentiation primary response 88; GMPs, glucan mannan particles; HNT, halloysite nanotubes; LMWH, low molecular weight heparin; TMC, trimethyl chitosan; KPV, Lys-Pro-Val; Map4k4, mitogen-activated protein kinase kinase kinase kinase 4; MGL, macrophage galactose-type lectin; PLGA, poly lactic-co-glycolic acid; US-FDA, US-Food and Drug Administration; CMC, carboxymethyl chitosan; 6-MP, 6-mercaptopurine; GSH, glutathione; CH-EDTA, chitosan-ethylenediaminetetracetic acid; I.V., intravenous; S.C., subcutaneous; aPTT, ATPP, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; CRC, colorectal cancer; BBE, Brush border enterocytes; mRNA, messenger RNA; IRF, interferon regulatory factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; VEGF, vascular endothelial growth factor.

* Corresponding author.
** Correspondence to: N. Thorat, Cluster of Drug Discovery, Development and Translational Research, Center of Multidisciplinary Research (CMR), MGM University, Aurangabad, India.
E-mail addresses: nilesh.kulkarni9@gmail.com (N. Kulkarni), pritijain@dpsru.edu.in (P. Jain), crliic@mgmmcha.org (A. Shindikar), thoratnd@gmail.com (N. Thorat).

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Inflammatory bowel disease
Colon targeting
Sodium alginate
Biopolymers
Multiparticulate drug delivery

1. Introduction

1.1. Inflammatory bowel disease and its current status worldwide

The term IBD represents two clinically defined conditions known as Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by chronic inflammation of the gastrointestinal tract, collectively affecting 6.8 million population worldwide in the year 2017 (Alatab et al., 2020; Shah, Palakurthi, Khare, Khare, & Palakurthi, 2020). The mortality rate of IBD is 40% as compared to those without evidence of the disease (Oz & Ebersole, 2008). Despite the large population suffering from IBD, the exact etiology of the disease is not yet completely understood, however, extrinsic factors such as microorganisms and chemical compounds or autoimmune disease are known contributors. Other factors reported to have role in the causation of the disease are genetics, unhygienic conditions, immune dysfunction, environmental factors such as diet, stress, pollution, cigarette smoking, pathogenic infections and microbiome imbalance (Hua, Marks, Schneider, & Keely, 2015a; Zhang, Langer, & Traverso, 2017). As shown in Fig. 1, CD usually affects terminal ileum and the colon in the discontinuous pattern of mucosal inflammation, on the other hand UC limits itself to the large intestine and may extend to rectum or entire colon with continuous pattern of mucosal inflammation. IBD manifests clinically in the form of abdominal pain, weight loss, vomiting, fever, or bloody diarrhea. IBD have been associated with extra-intestinal manifestations in the form of arthritis, sacroiliitis, and ankylosing spondylitis (Langhorst, 2009). There are a number of factors associated with the disease that affect a patient's quality of life significantly, *viz.* early onset, no permanent cure, and frequent flare-ups; all these factors lead to severe morbidity among the patients. Previously, the disease was considered to be confined to the western/developed world, but in the last decade or two there has been significant rise in the number of cases reported in Asian countries, which can also be attributed to the availability of advanced detection techniques, such as endoscopy and colonoscopy, which were not available earlier, especially during 80's. The rise in the incidence of the disease is also correlated to industrialization which has started in the western world and is now growing significantly in the Asian countries (Das et al., 2009; Kedia & Ahuja, 2017).

As there is no permanent cure of the disease, therapy options are mainly aimed at maintenance of remission and prevention of flare-ups of inflammation. If untreated, IBD exacerbate itself in the form of clinical or paraclinical symptoms such as rectal bleeding, anemia, gastrointestinal (GI) spasm, nausea, fever, fatigue, loss of weight and loss of appetite. These symptoms form a base for classification of the disease into three stages as mild, moderate or severe. Treatment options for IBD includes 5-amino salicylic acid (5-ASA) (an anti-inflammatory agent (for mild IBD), corticosteroids such as prednisolone, budesonide, beclomethasone dipropionate, *etc.* (for moderate stage), immunosuppressive agents, like methotrexate, azathioprine, 6-mercaptopurine (6-MP), cyclosporin-A, *etc.* and anti-TNF-α-antibodies, like infliximab, adalimumab, certolizaumab, *etc.* for the severe stage of the disease (Lautenschläger, Schmidt, Fischer, & Stallmach, 2014; Rawla, Sunkara, & Raj, 2018; Talaei, Atyabi, Azhdarzadeh, Dinarvand, & Saadatzadeh, 2013; Zhang & Merlin, 2018).

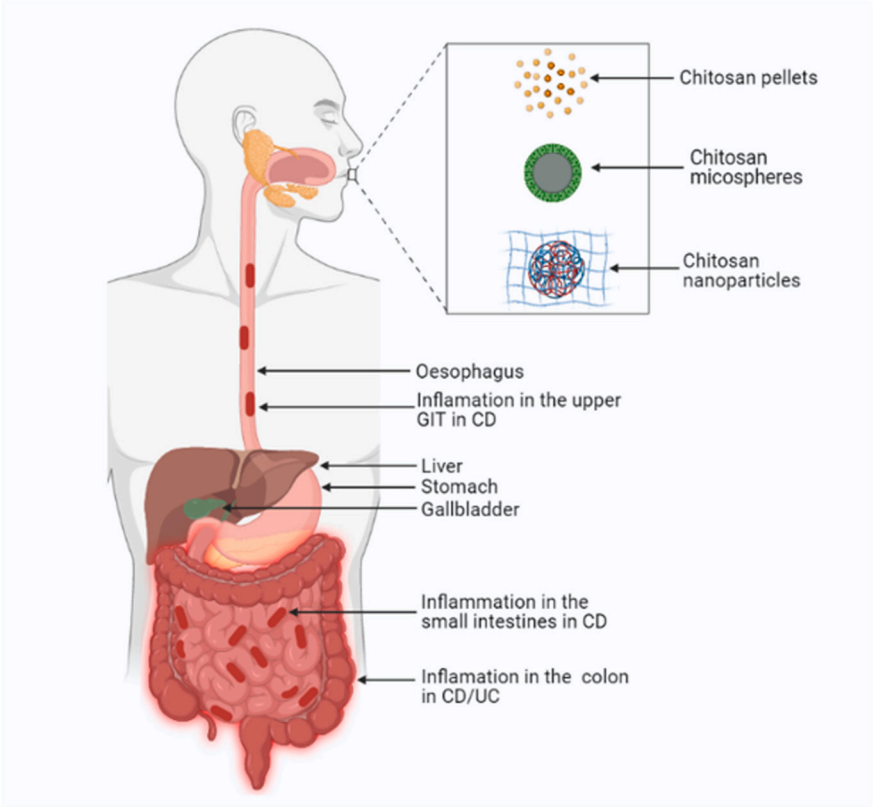

Fig. 1. Term IBD encompasses both the conditions, Crohn's disease (CD) and ulcerative colitis (UC), while UC and CD share many clinical features, UC remains confined to terminal ileum and the colon, on the other hand CD can affect any region of the GIT. The other differentiating point is depth of inflammation. UC could affect up to the innermost mucosal layer, and CD penetrates the deeper portions instead of being confined to the mucosal layers. Chitosan based drug delivery systems have the potential of targeting the payloads to the colon.

Table 1
Comparison of normal and altered physiology of the gastrointestinal tract in IBD.

Gastrointestinal organs	Normal luminal pH	pH in active IBD	Normal GI transit time (h)	GI Transit time in IBD (h)	References
Stomach	1.2–2 (fasted) 2–6 (fed)	~2	2–3	N/A	(Hua, Marks, Schneider, & Keely, 2015b; Nugent, Kumar, Rampton, & Evans, 2001; Zeeshan, Ali, Khan, Khan, & Weigmann, 2019)
Proximal small intestine	5.5–7.0	6.1–7.3	4–6	Prolonged	
Distal small intestine	6.5–7.5	7.2–8.3			
Caecum/right colon	5.5–7.5	2.3–7.2	41.1–62.3	9.5–39.1	
Left colon/ rectum	6.5–7.5	5.3–7.5			

The oral route for drug administration is the preferred one because of ease of administration, patient compliance, cost effectiveness, and ease to cut-off absorption of a drug at any time point. However, due to physiology of the GI tract, a dosage form exposes to different environmental conditions at different gastrointestinal sites, such as acidic pH in the stomach, slightly acidic to neutral in the small intestine and slightly basic in the large intestine. Besides, there is also a large variation in the gastrointestinal transit time post meals. Alteration in the gastrointestinal physiology in IBD patients is described in Table 1. All the above-mentioned factors collectively pose a serious challenge for the formulation scientists to target a drug to the colonic site *via* oral administration.

The commonly used formulation strategies for drug targeting to the colon include time-dependent release, pH responsive polymeric coating, pro-drug approach, colonic microbiota initiated drug delivery, conjugation of a drug with a polymer/biopolymer, bioadhesive drug delivery and osmotically controlled drug delivery (Cesar et al., 2018; Chourasia & Jain, 2003; Kotla et al., 2019; Sinha & Kumria, 2003; Vass et al., 2019).

2. General properties of chitosan in view of the colon targeting

2.1. Chemistry of chitosan

Chitosan, synthesized from chitin, is a polysaccharide with exceptional physical and biological properties. Chitin is generally present in the shells of crustaceans and shrimps and fungal cell walls. Chitin was introduced to the world in the year 1884, which is the second most abundant polysaccharide in nature after cellulose (Zeeshan et al., 2019). Chitosan is obtained from chitin by deacetylation, which is only naturally occurring cationic polymer. It consists of β-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose (Fig. 2). Chemically, chitosan is a linear amino-polysaccharide chain comprising of randomly linked-β (1→4) linked D-glucosamine and *N*-acetyl D-glucosamine units arranged in a random fashion. Elemental analysis demonstrated that chitosan possesses greater than 7% nitrogen content and less than 0.04% degree of acetylation. Normally, the commercial chitosan has 60–100% degree of deacetylation and its molecular weight ranges between 20 and 1200 kDa. Synthesis of chitosan from chitin requires harsh conditions needed for removal of acetyl groups using concentrated sodium hydroxide solutions; this is an issue warrants economic as well as ecological problems. Therefore, techniques have been sought to design synthetic methods that would employ less amount of sodium hydroxide solution (Nugent et al., 2001).

The presence of deactylated primary amine group in chitosan is important to elicit the ability to undergo desired modifications for the site-specific drug delivery. Chitosan is a weak base with p*Ka* values ranging from 6.2 to 7.0, therefore, it is insoluble at neutral and alkaline pH. In acidic conditions, the amine groups of the polymer undergoes protonation followed by solubilization, resulting in the positively charged polysaccharide with high charge density. This positively charged polymer has ability to interact with the GI mucosa, which is essential for long residence time at the site, desirable in case of IBD, as diarrhea is very common symptom of the disease. Apparently, chitosan is degraded by hydrolysis in humans primarily by enzyme lysozyme and bacterial enzymes in the colon such as Chitinases secreted by the Bacteroids. Looking at the physiology of the GIT, due to the acidic pH in the stomach, concentration of bacteria is very low, that gradually increases along small intestine and there is significant rise in their number in the colon probably due to favorable pH. Therefore, chances of chitosan being metabolized in the upper GIT are very rare. Other factor that controls rate and extent of degradation of chitosan is its degree of deacetylation, higher it is less is its degradation. Thus, degradation by colonic microflora makes chitosan a potential polymer for colon specific drug delivery (Hejazi & Amiji, 2003a, b; Kalantari, Afifi, Jahangirian, & Webster, 2019b; Kean & Thanou, 2010; Ray, 2019). All these properties possessed by chitosan makes it very special, displaying advanced physiochemical properties that are explored for biomedical applications. Polymer chain length and varying acetyl group distribution are important governing factors for the biodegradation kinetics and sustained release of the drugs which may prove important for management of IBD (Kalantari et al., 2019a, b).

2.2. Biocompatibility and biodegradability

Owing to the semi-crystalline nature, chitosan is insoluble in aqueous solutions above pH 7; however, it is freely soluble below ~pH 5 due to protonation of the amino group present on it. Chitin is also semi-crystalline, with intermediate level of degree of deacetylation that imparts minimum crystallinity (Chourasia & Jain, 2003; Vass et al., 2019). Chitosan metabolizes within the human body *via* hydrolytic cleavage of the glycosidic bond joining polysaccharide units in the polymer. This degradation converts the polymer into glucosamine and saccharide units. Apparently, degradation kinetics is dependent upon the degree of crystallinity and degree of acetylation of the polymer, as the latter regulates the former, greater the acetylation the more crystalline the polymer (Chourasia & Jain, 2003; Francis Suh & Matthew, 2000;

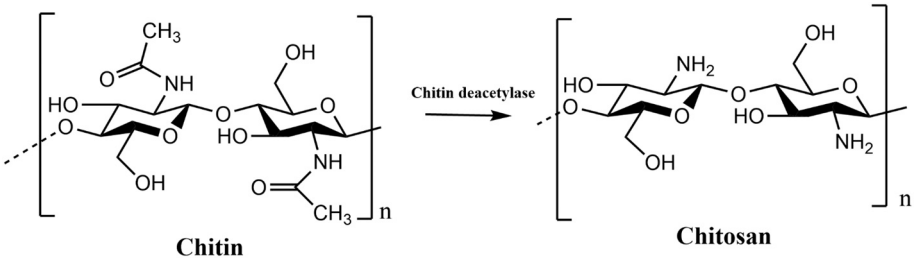

Fig. 2. Synthesis of chitosan from chitin.

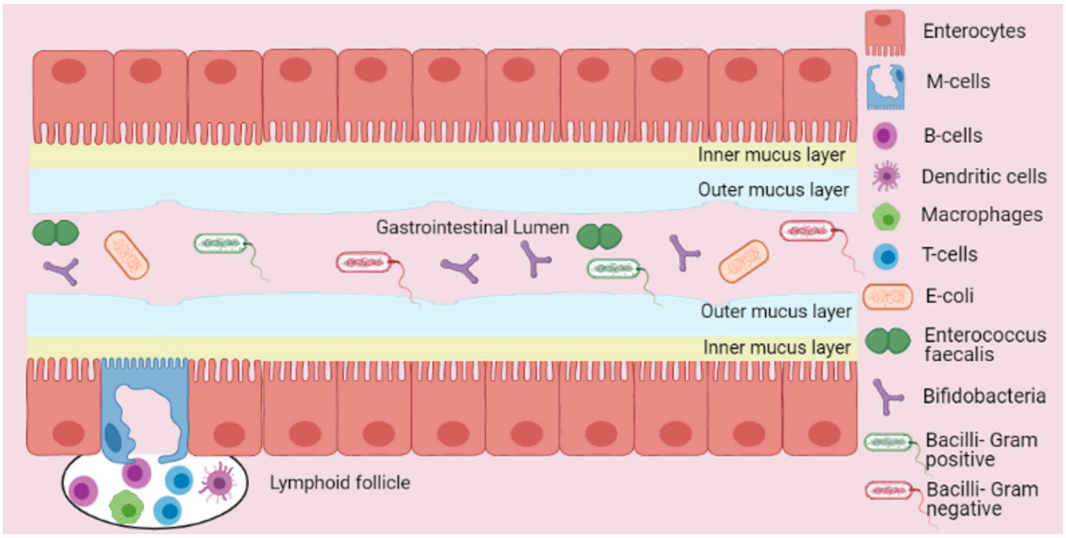

Fig. 3. Healthy colonic mucosa has closely packed enterocytes over which inner and outer layers of mucus are present; these two features jointly prevent entry of foreign materials and luminal contents, but absorption of nutrients. Here, commensal microbiota and immune system are in harmony with each other. Out of the number of components responsible for IBD, imbalance of microbiota (dysbiosis) is one. M-cells lying in lymphoid tissues initiate immune response. Various cells responsible for the immune responses in gastrointestinal lumen reside in the lymphoid follicles.

Kalantari, Afifi, Jahangirian, & Webster, 2019a; Lorenzo-Lamosa, Remuñán-López, Vila-Jato, & Alonso, 1998; Vass et al., 2019). Another distinctive attribute of chitosan which contributes towards its biocompatibility is its low LD_{50} value, which is reported to be greater than 16 g/kg in mice (Dodane & Vilivalam, 1998). In addition to the above mentioned features another advantage of chitosan is its degradation by colonic microflora which enables its exploration for colon specific drug delivery in IBD (Hejazi and Amiji, 2003a, b). Mcconnell et al. tried to answer the fundamental question, does chitosan metabolize by the enzymes present in the colon? They used human faecal material and porcine pancreatic enzymes in the study. Authors concluded that colonic microflora metabolizes chitosan which is a function of its cross-linking. Non cross-linked films of chitosan were metabolized within 4 h, however when cross-linked by using glutaraldehyde and sodium tripolyphosphate, it resisted digestion over 4 h. Further, sodium tripolyphosphate cross-linked films resisted the metabolism by pancreatic enzymes for up to 18 h (Mcconnell, Murdan, & Basit, 2008). Tokazi et al. reported chitosan capsules for colonic delivery of insulin. Authors coated the capsules by an enteric polymer to protect it from the harsh acidic environment in the stomach; outcomes of the study revealed chitosan

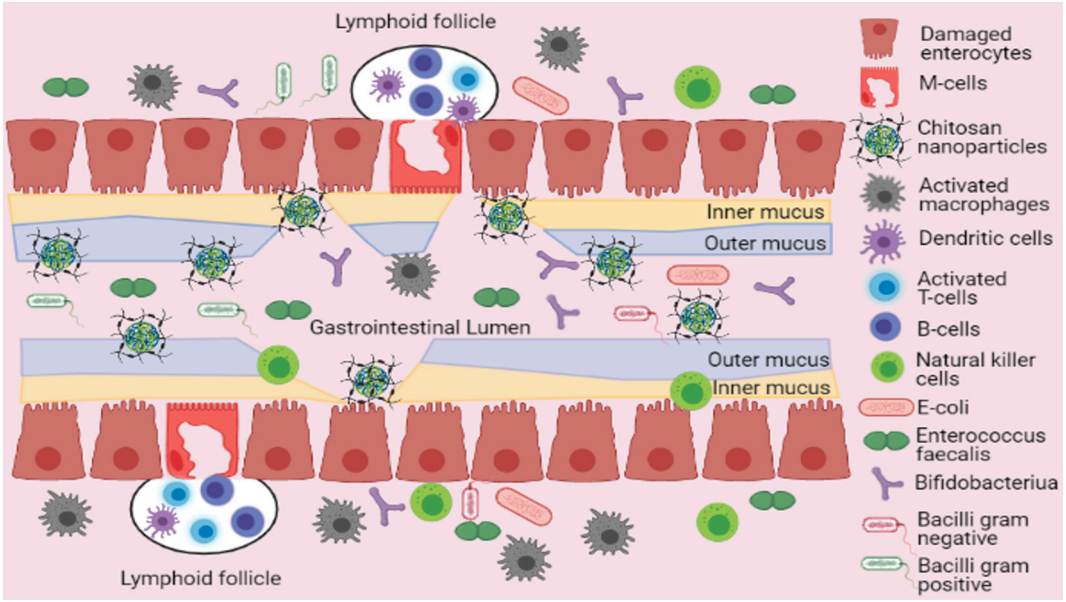

Fig. 4. Inflamed colonic mucosa has distorted morphology of enterocytes, due to erosion of the microstructures present on their surface there is loss of selective permeability of the cells. There is also disruption of the inner and outer layers of mucus. External stimuli triggers response initiated by the M-cells, cells involved in innate immunity-macrophages, dendritic cells, and natural killer cells and cells involved in adaptive immunity-lymphocytes such as B-cells and T-cells called at the site of stimuli. Chemokines such as IL-8, platelet factor-4 (PF-4) produced by activated lymphocytes play an important role as chemoattractants. Balanced microbiota not only facilitates differentiation of naïve gut DCs into tolerogenic DCs but also generate regulatory T cells thereby establishing immune homeostasis. The harmony between intestinal mucosa and microbiota is lost in severe inflammation that leads to increased infiltration across epithelial barriers by the intestinal bacteria. Chitosan nanoparticles interact with the anionic sialic acid groups of the mucus that improves their retention time in the inflamed colon, where of the several clinical manifestations of IBD, diarrhea is predominant.

capsules were degraded by proteolytic enzymes present in rat cecal content (Tozaki et al., 1997).

2.3. Delayed and controlled/sustained release of drugs

Delayed release has significance in terms of site-specific drug release in the gastrointestinal tract (GIT), which offers number of advantages, such as, 1. No unwanted distribution of the drugs in the body, 2. Possible reduction of the dose, and 3. Achieves maximum concentration of the drugs at the desired site. Delayed release of an API can be achieved through various formulation approaches using chitosan that are discussed in subsequent sections in detail. Sustained/Controlled release of the drugs ensures prolonged action at the site, which is essential for mitigation of chronic inflammation in IBD. Multiparticulate dosage forms such as nano- or microparticles are especially important in achieving sustained as well as site-specific drug release. Because of their small size, they are taken-up easily by the cells involved, and if made-up of chitosan, its mucoadhesive properties ensures prolonged stay at the site, which is otherwise difficult using available/marketed polymers in case of inflammatory bowel disease due to severe diarrhea. In addition to that, chitosan can reversibly open tight junctions between the epithelial cells and promotes paracellular transport of the encapsulated drugs (Du, Liu, Yang, & Zhai, 2015).

In case of chitosan its crystallinity and molecular weight is important to regulate its dissolution at the acidic pH of the stomach. Crystallinity can be controlled by degree of deacetylation, lesser is the degree of deacetylation more crystalline is chitosan. A new dimension in the controlled/sustained release of payloads has been introduced since the biodegradable (natural and synthetic) polymers are employed for the purpose. Encapsulated drug materials show slow and controlled diffusion through these polymeric membranes/matrices. In another mechanism of controlling drug release using chitosan, drugs are covalently attached to the polymer or they are dispersed into its matrix, its biodegradation/erosion would facilitate the release. Chitosan also has gel-forming ability at low pH that may provide rate-controlling barrier (Ravi Kumar, 2000). Multilayer coatings using natural polymers by exploring opposite charge present on their surface is also a suggested approach for controlled release applications (N. Mengatto, Helbling, & Luna, 2012).

Chitosan acetate, a derivative of chitosan along with ethylcellulose (EC) was explored for pH-, time-, and enzyme- controlled release of the model drug 5-ASA in the compressed coated tablet formulation (Nunthanid et al., 2009). Besides, various research groups has reported controlled/sustained release applications of chitosan in the form of multi-particulate formulations (Bharathala, Singh, & Sharma, 2020; Murali et al., 2020).

2.4. Anti-inflammatory effect of chitosan in IBD

Inflammations are the protective biological reactions that intend to protect a human body from the harmful stimuli. These stimuli can be triggered by infectious agents such as virus or bacteria or their components, physical agents, reactive oxygen species (ROS), hypoxia, to name a few (Chovatiya & Medzhitov, 2014). In certain situations, inflammatory reactions may go dysregulated and cause acute or chronic inflammation that leads to tissue or organ damage. Heathy *vs* inflamed mucosa is depicted in Figs. 3 and 4, respectively. Microbial components are one of the contributory factors to the etiopathology of IBD. In a series of events that lead to inflammation in IBD, firstly, the pattern recognition proteins of toll-like receptors 4 (TLRs) and nod-like receptors (NODs) recognize pathogen-associated molecular patterns associated with bacterial components such as lipopolysaccharides (LPS) which then initiates innate immune response by degradation of IκB which allows translocation of nuclear factor kappa B (NF-κB) into the nucleus of macrophages. NF-κB accounts for the regulation of pro-inflammatory mediators responsible for the inflammation. Another pathway that initiates immune responses is mitogen-activated protein kinase (MAPK)-dependent pathways (Muanprasat & Chatsudthipong, 2017; Ngo et al., 2015; Tu, Xu, Xu, Ling, & Cai, 2016). MAPK has three distinct downstream mediators: extracellular-signal-regulated kinase (ERK), P 38 MAPK, and C-JUN-N-Terminal kinase (JNK).

Innate immune response acts as a frontline defense system against stimuli and in case of IBD it is microbial and environmentally borne antigens. This immune response is non-specific and does not grant long-lasting immunity. It is mediated by a variety of cells that includes typical immune cells such as neutrophils, monocytes, dendritic cells (DCs), macrophages and non-immune cells such as intestinal epithelial cells, endothelial cells, and myofibroblasts (Fig. 4). Adaptive immune response is a result of inability of acute immune response in clearing antigenic materials due to defective autophagy and recognition of microbial and inflammatory debris. Adaptive immune response is highly specific, and provides long lasting immunity. T-cells are the important mediators of the adaptive immune response. Dysregulated innate and adaptive immune pathways contribute towards intestinal inflammation in IBD (Dave, Papadakis, & Faubion, 2014; de Souza & Fiocchi, 2015; Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014).

Chitosan oligosaccharides (COS) exhibit anti-inflammatory activity by inhibiting responses initiated by macrophages that are induced by microbial debris. Large amount of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E_2 (PG-E_2), and nitric oxide (NO) produced by macrophages contribute significantly to the pathogenesis, onset and progression of UC; COS has been shown to inhibit expression and release of these pro-inflammatory mediators. Mechanisms involve downregulation of JNK ½, prevention of phosphorylation of p38 MAPK, and IκB degranulation (Etzerodt et al., 2012; Muanprasat & Chatsudthipong, 2017; Song et al., 2016). Intestinal barrier dysfunction is another clinical manifestation of IBD. Very viscous inner layer and less viscous outer layer of mucus forms a formidable intestinal barrier that prevents translocation of pathogens across the epithelial layer. Partial or complete erosion of the mucus layers and disruption of the tight junctions between epithelial cells allow entry of harmful pathogens. COS has been reported to improve integrity of the intestinal epithelial barrier by promoting tight junction assembly (Chovatiya & Medzhitov, 2014; Muanprasat et al., 2015; Yousef, Pichyangkura, Soodvilai, Chatsudthipong, & Muanprasat, 2012). In the first of its kind of study, Wang and research group has reported intestinal mucus modulating activity of COS on human colonic mucus secreting HT-29 cells (Wang, Wen, et al., 2021). In a histological event infiltration of innate immune cells (macrophages, neutrophils, dendritic cells, and natural killer cells), and adaptive immune cells (lymphocytes such as T and B cells) takes place into the lamina. Neutrophils, basophils are the granulocytes; they actively participate in inflammation by secreting pro-inflammatory cytokines. Neutrophils, earlier thought to be brave warriors fighting against bacterial infiltration, over the time emerged as major damage causing cells that worsen inflammation by releasing ROS, proteinases, and cationic peptides (Zhang, Jiang, et al., 2020). COS obstruct activation of basophils, neutrophils and lymphocytes. Moreover, COS prevents histamine release and thereby production of pro-inflammatory cytokines such as IL-1β, IL-4, IL-6, IL-8 and IL-13, in basophils, by suppressing the calcium-induced activation of MAPK signaling pathways that include ERK1/2, and p38 (Muanprasat & Chatsudthipong, 2017). Oxidative stress mediated *via* ROS such as superoxide radicals, hydroxyl radicals, peroxyl, alcoxyl, and hydroperoxyl has significant role in worsening of IBD in term of damaging mucosal lining and bacterial invasion (Tian, Wang, & Zhang, 2017). COS is reported to attenuate oxidative stress induced apoptosis in human colonic epithelial cells (T84 cells) (Yousef et al., 2012).

Controlling inflammation and achieving mucosal healing are the main goals of UC treatment (Iacucci, De Silva, & Ghosh, 2010; Pineton De Chambrun, Peyrin-Biroulet, Lémann, & Colombel, 2010), multiple drug combination therapy has been proposed as a potential strategy

(Lee, Gangireddy, Khurana, & Rao, 2014; Ni et al., 2016). For restoration of intestinal homeostasis, there have been numerous studies reported benefits of a pro-healing cytokine, Interleukin-22 (IL-22) as: (1). It facilitates the proliferation, survival and reconstitution of epithelial cells, avoiding microbiota from further penetrating into the colonic tissues (Ouyang, 2010; Zindl et al., 2013), (2). It enhances production of mucus-associated proteins and induces regeneration of goblet cells, that lead to the formation of essential static external barrier which separates intestinal flora from intestinal epithelial cells (Sugimoto et al., 2008), and (3). It stimulates secretion of a large amount of antimicrobial peptides through expression of intestinal epithelial cells and Paneth cells, which kill invading or sequester pathogens (Sugimoto et al., 2008; Zenewicz et al., 2008). It is reported in some preclinical studies based on wild-type mice or IL-22-deficient mice subjected to dextran sulphate sodium (DSS)-induced UC, treatment with a IL-22-neutralizing antibody augmented damage in the colonic epithelial layer, induced severe weight loss and increased inflammation in the colon (Neufert et al., 2010; Pickert et al., 2009). In the research work reported by Sugimoto et al., it is concluded that, IL-22 is a vital and important therapeutic molecule to enhance intestinal healing in patients with UC based on the observations in T cell receptor-alpha-deficient mice, wherein, IL-22 is over expressed, which ultimately reduced the disease score and colonic thickness in DSS-induced UC animal model (Sugimoto et al., 2008).

3. Multiparticulate formulation approaches for the colon targeting of the drugs using chitosan

Many drugs used in the treatment of IBD are associated with adverse effects, *viz.* Cushing's syndrome, glaucoma, osteoporosis, hepato- and nephrotoxicity, peptic ulcers, pruritus, diarrhea, pancreatitis, and malignancies. Optimal therapeutic efficacy and reduction of adverse effects of the drugs is the key for successful treatment of the disease and there is great probability of achieving this through multi-particulate drug delivery systems designed for the release of the drugs at the inflamed sites (Lautenschläger, Schmidt, Lehr, Fischer, & Stallmach, 2013; Rogler, 2010).

3.1. Chitosan containing pellets

Pellets have several advantages as a multiparticulate dosage form — i. Rapid transit through the upper GI tract in the presence of food if particle size is less than 2–3 mm, ii. Lesser chances of burst release, iii. No or minimal gastric irritation because of the distribution of an API in the stomach, and iv. Incompatible drugs can be encapsulated separately and mixed later, to name a few. Steckel H. et al. has reported development of chitosan pellets using extrusion-spheronization technique and explored possibilities of using chitosan as a major component of pellet formulation. Water and different concentrations of acetic acid was used as an extrusion liquid. Results suggested that 1:1 concentration of chitosan and microcrystalline cellulose (MCC) can be extruded successfully and with ease using water as granulating liquid; however, extrusion of 100% chitosan is possible only when higher acetic acid with normality of 0.2 N is used as granulating liquid (Steckel & Mindermann-Nogly, 2004). Recently, chitosan was reported to be used as a major component for preparation of pellets by extrusion spheronization technique for the colonic release of metronidazole, effect on the drug release was evaluated on uncoated and enteric coated pellets. It was concluded that, pellets containing chitosan showed extended release of the drug after enteric coating in gastric fluid as compared to the pellets devoid of chitosan. In contrast, there was no significant difference in the drug release when the pH of the dissolution medium was shifted to 6.8 (Ferrari et al., 2012). Chitosan was also employed as a pore former in the enteric coated core beads for the colonic delivery of 5-ASA in combination with ethylcellulose as a coating material for core beads. The criterion for taking the beads for further level of evaluation was set as not more than 10% drug released in simulated intestinal fluid (SIF) in 6 h. It was observed that as the level of chitosan goes up in the coating mixture, drug release was quicker. The reason cited was the hydrophilicity of chitosan, which swells in the presence of SIF while on the other hand ethylcellulose in sufficient quantity prevents wetting and swelling of chitosan and further resists entry of dissolution medium into dosage forms and slows down the drug release (Omwancha, Mallipeddi, Valle, & Neau, 2013).

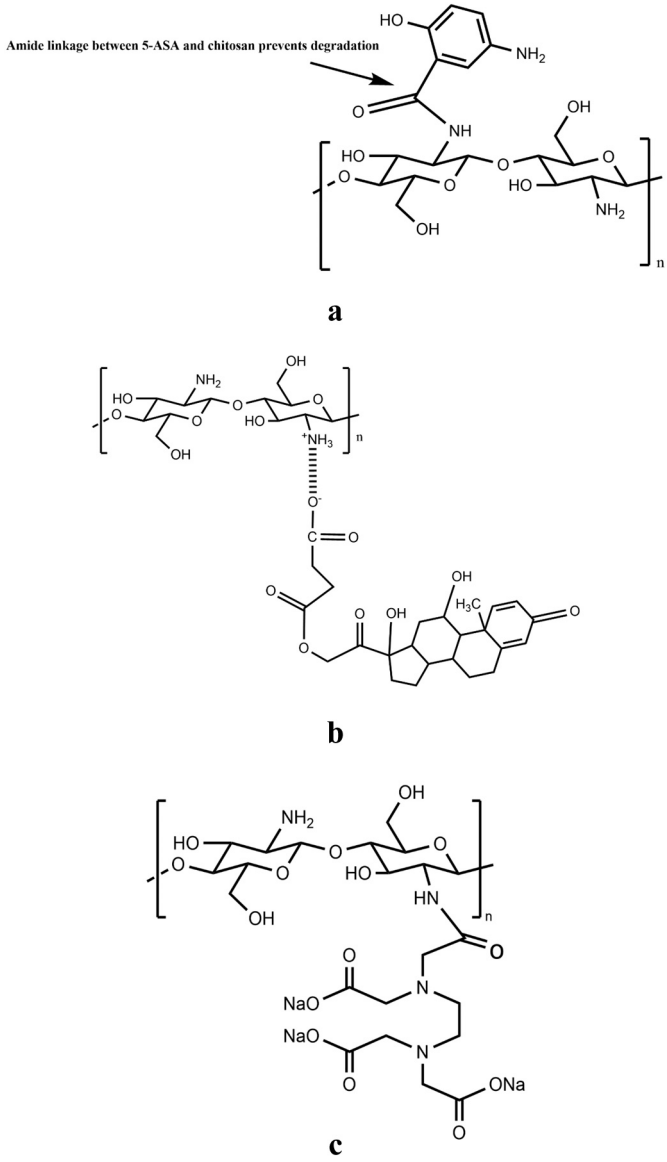

Fig. 5. a. Chemical structure representing the amide linkage between chitosan and 5-ASA. The amide linkage did not allow drug release because of amide bond stability hence the chitosan-5-ASA-azo linked compounds have been synthesized, b. Chitosan-Prednisolone succinate complex, and c. Chitosan-EDTA complex.

Controlled/sustained release of drugs after oral administration is important in prolonging action, reducing dosing frequency, and for the patient's convenience. Apart from sustained release applications, chitosan has been employed for delayed release of APIs in the pellet formulations. In one of the research reports, chitosan and pectin were used for the preparation of core pellets using extrusion-spheronization technique for controlling release of model drugs theophylline, dimenhydramine and ibuprofen; effect of porosity of pellets on the drug release was reported. Chitosan and pectin containing pellets have shown highest porosity against MCC containing pellets and this led to faster drug

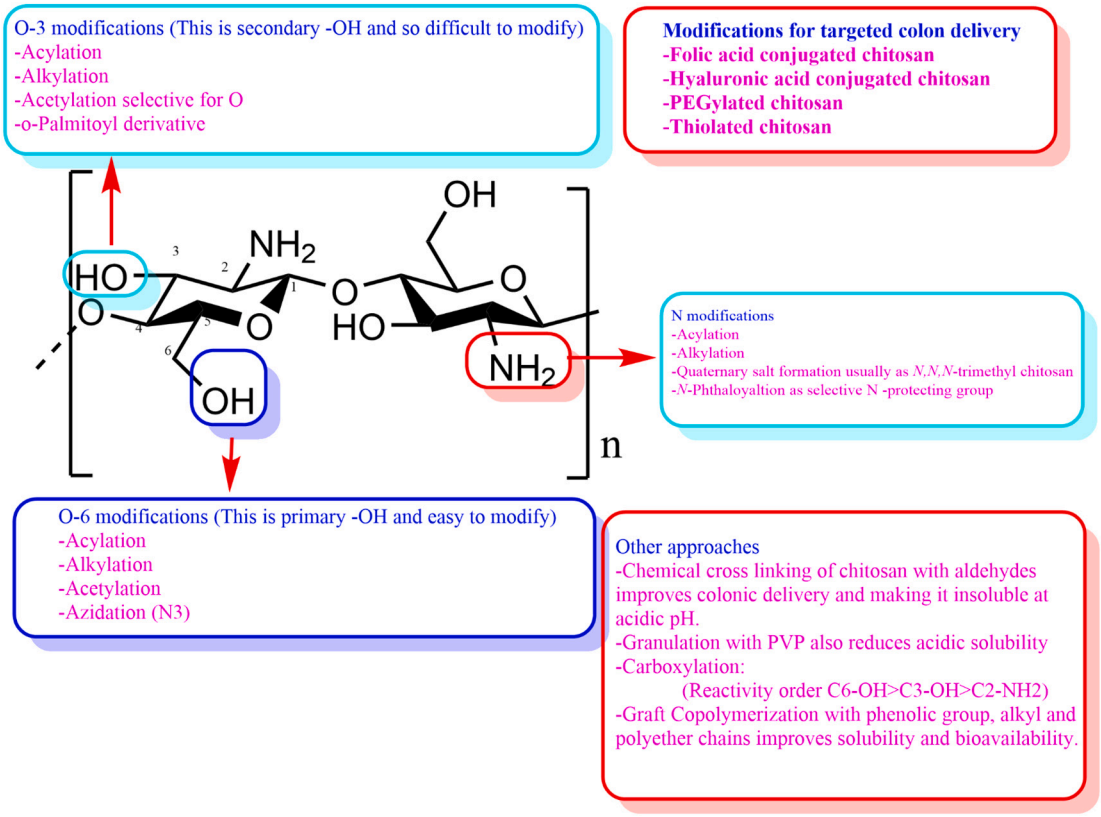

Fig. 6. Various approaches for modification in chitosan: Chitosan shows limited applications due to poor solubility in aqueous solutions. Hence, chemical modifications need to be done to chitosan so as to enhance solubility at physiological pH. Fig. 6 displays that chitosan can be chemically modified at O-3, O-6 and at N. The reactions at O-6 are easier to perform as this is primary hydroxyl group while the –OH at third position is secondary and difficult to modify. The figure represents the most common modifications that are performed to these three functional groups. Quaternization of NH_2 is important modification as it not only increases solubility but also improves absorption across biological membranes. Besides these common modifications, the figure also represents other approaches adopted for colon-targeted delivery.

release from the former as compared to the latter (Nejati et al., 2018). In another research work, surface coating by chitosan was evaluated for sustaining the drug release of an anti-diabetic drug Metformin hydrochloride, which was adsorbed onto the mesoporous silica nanoparticles. Chitosan paste was mixed with these mesoporous silica nanoparticles for the preparation of quasi-spherical pellets using molding technique and in the second stage five layers of chitosan coating was applied over these pellets to prevent release of the drug in the acidic pH. Surface coating led to prolongation of drug release in neutral pH, however, burst release was observed in pH 1.2, that resulted in 100% of the drug release at the end of 17 h of dissolution study as against merely 26% drug released in pH 7.0 in the same time (Patiño-Herrera et al., 2019). There are primarily four parameters, which govern the effect of chitosan on dissolution; these are degree of deacetylation, percentage content in a formulation, viscosity grade and solubility of an API. For an acidic drug piroxicam, chitosan was reported to have a solubility enhancing effect in acidic pH, this effect has shown to be increased exponentially with the grade of chitosan used. At the optimized ratio of chitosan and piroxicam, chitosan facilitated complete release in an extended manner over 8 h. (Partheniadis, Gkogkou, Kantiranis, & Nikolakakis, 2019). Chemical structure of Chitosan linked with 5-ASA, predisolone and Ethylenediaminetetracetic acid (EDTA) are shown in Fig. 5. Fig. 5b represents an electrostatic interaction between quaternary amine and carboxylate ion. This interaction takes place due to protonation of amine in aqueous acidic media that leads to reaction of chitosan bearing amine moiety with prednisolone succinate.

3.2. Chitosan microspheres

Amino group present on the chitosan undergoes protonation in the acidic environment due to which chitosan shows good solubility at acidic pH (Park, Saravanakumar, Kim, & Kwon, 2010). As mentioned in Table 1, there are marked changes observed in the pH gradient of the GIT in IBD patients and solubility of chitosan at acidic pH becomes an important property for colon specific release considering shift in the colonic pH towards acidic side. Table 2 lists the techniques used in the preparation of chitosan microspheres for encapsulation of various drug molecules having different aqueous solubility, targeted to the colon for alleviating symptoms of IBD.

3.3. Nanoparticulate drug delivery systems comprising of chitosan

Nanoparticles have been explored as a very effective tool for targeting the drugs to the inflamed sites. Owing to their small size they can accumulate in large quantities in the specific tissues. Drug targeting to the colon using nanocarrier approach can be achieved by different mechanisms either alone or in combination, these are time-, pH-, pressure- or gut microflora responsive. There were incidences wherein therapy has failed due to insufficient drug deposition or drugs have shown adverse effects because of the lack of release at the desired site, these untoward incidences can be prevented *via* nanoparticulate drug delivery. Over the years multiparticulate drug delivery systems especially micro- and nano- sized particles have proven ability of drug targeting to the specific sites in the GIT and this ability is further amplified because of the mucoadhesive characteristics of the polymers such as chitosan. Commonly used techniques for the preparation of chitosan

Table 2
Some examples of colon targeting of drugs achieved through chitosan comprising microspheres and summary of the results obtained.

Technique/s used for the preparation of microspheres	Polymers/material used alongside chitosan in the study	Drug/s encapsulated	Significant outcomes of the study	Reference
Spray-drying	–	5-ASA	■ Enhancement in the solubility of the drug, and improvement in intrinsic dissolution was observed. ■ Microspheres did not show cytotoxicity and reduced messenger RNA (mRNA) levels responsible for the release of IL-1β and IL-8.	(Aguzzi et al., 2011)
Spray-drying	–	5-ASA	■ N-succinyl-chitosan was explored for colon specific release because of its stability in acidic pH, biocompatibility, low toxicity and mucoadhesive property.	(Mura et al., 2012)
Spray-drying	–	Mesalazine	■ Chitosan microspheres were designed for the rectal administration of mesalazine to bypass the variable physiological environment in the GIT after oral administration. ■ In an *in-vitro* study chitosan microspheres showed certain degree of cytotoxicity at polymer concentration greater than 200 μg/ml. ■ Half of the dose of the drug given through microparticles as compared to marketed formulation Asamax® produced the same effects in alleviation of the disease.	(Palma et al., 2019)
Spray-drying followed by ionotropic gelation/ polyelectrolyte complexation	Sodium alginate	5-ASA	■ Prolong release of the drug was expected based on the physicochemical properties of the polymers *i.e.* mucoadhesiveness and pH sensitive solubility. ■ Physicochemical properties of the drug such as its p*Ka* (2.3 and 5.4) and log *P* (1.4) were also taken in to account to predict the release. ■ Microspheres prepared by using high viscosity alginate richer in mannuronic acid (M) content, exhibited faster release *i.e.* between 40 and 50% in the first 02 h of the study carried out in acidic conditions where drug has good solubility as well (8.65 mg/ml). ■ Slower release of the drug in phosphate buffer pH 6.8 was also attributed to its lesser solubility (3.94 mg/ml) in pH 6.8. ■ When alginate richer in guluronic acid (G) was used, slower release of the drug was observed as compared to alginate richer in M, in the first 08 h of the dissolution study. ■ This is due to, formation of high porosity, low shrinking ability and no swelling after drying (rigid) of gels having higher content of G. In contrast gels formed by the alginate having higher content of M are elastic, softer, shrink/swell more. ■ Microspheres exhibited dominant localization of 5-ASA in the colon and low systemic bioavailability in the bio-distribution studies.	(Mladenovska et al., 2007)
Ionic cross-linking and ionotropic gelation	β-Glucan and sodium alginate	Tylophorine malate (NK007)	■ A beta-glucan, glucan mannan particles (GMPs) were separated from the commercially available yeast cells (cell wall). ■ These GMPs were labeled with Rhodamine and then incubated with NK007 in chitosan solution for 2 h, to allow GMPs to swell and engulf NK007. Chitosan was cross-linked with mixture of solution of tripolyphosphate and sodium alginate. ■ Beta-glucans show specificity and selectivity for macrophages, when GMP containing microspheres were assayed for cellular uptake in RAW 264.7 cells, exhibited efficient internalization. ■ Intestinal uptake studies were carried out using mice model. These studies revealed efficient uptake of microspheres by epithelial cells into the intestinal mucosal layer. ■ Disease activity index (weight loss, stool consistency, and faecal bleeding) was measured in the DSS induced colitis model. GMP-NK007 microspheres and plain NK007 treatments were compared. It was observed that disease activity was controlled by both the treatments, but there was no significant difference between them. ■ The disease activity was controlled efficiently because of suppression of pro-inflammatory cytokine TNF-α.	(Chen, Wang, et al., 2015)
Crosslinking by TPP, polyelectrolyte complexation	Sodium alginate, pectin, Eudragit S 100	Ketoprofen, and ascorbic acid	■ Waxy materials and hydrophilic polymers were used for encapsulation. ■ Hydrophilic polymers showed good entrapment efficiency for both the APIs as compared to waxy materials. ■ Hydrophilic polymers despite cross-linking and combined use of two polymers (Chitosan-alginate) could not prevent	(Maestrelli, Zerrouk, Cirri, & Mura, 2015)

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Table 2 (*continued*)

Technique/s used for the preparation of microspheres	Polymers/material used alongside chitosan in the study	Drug/s encapsulated	Significant outcomes of the study	Reference
			release of ascorbic acid, more hydrophilic drug than ketoprofen, in gastric pH. ■ Microspheres made up of the hydrophilic polymers need to be enteric coated to prevent early drug release.	
Emulsification and gelation	Sodium alginate	5-ASA, and zinc	■ Microspheres exhibited pH dependent release of 5-ASA and Zn^{2+}, with higher percent release of the drugs at pH 7.4. ■ Increase in cross-linking by Zn^{2+} ions decrease release of 5-ASA. ■ Sharp decline in the clinical scores in colitis induced rat model after treatment with 5-ASA and Zn^{2+} containing microspheres was observed.	(Duan et al., 2017)
Emulsification (w/o) followed by crosslinking with glutaraldehyde	Eudragit S100	Aceclofenac	■ Eudragit S100 coating prevents early release of the drug at gastric pH. ■ Biodistribution studies revealed efficient targeting to the colonic site by the Eudragit S100 coated chitosan microspheres as compared to uncoated chitosan microspheres.	(Umadevi, Thiruganesh, Suresh, & Reddy, 2010)
Emulsification followed by cross-linking	Eudragit S100	5-ASA, camylofine dihydrochloride	■ There was no significant difference observed in the drug release when evaluated in gastric fluid and phosphate buffer saline pH 7.4. ■ Significant increase in the drug release was reported when 3% rat caecal contents were used in the dissolution medium. ■ Enhanced release of both the drugs was observed after colonic enzymes were induced in the rat by oral administration of chitosan. ■ Microspheres released the drugs after a lag time of 9 h; hence possesses drug targeting potential to the colon.	(Dubey, Dubey, Omrey, Vyas, & Jain, 2010)
Emulsion cross-linking	Eudragit S-100	Curcumin	■ pH sensitive microspheres of curcumin were prepared for colon specific drug release. ■ Microsphere showed high entrapment efficiency over the range of 74–83% across all formulation batches. ■ Chitosan microspheres displayed burst release in the first four hours of dissolution study, to minimize this quick release, was later coated with Eudragit S100. ■ Curcumin containing microspheres exhibited better control over disease activity in acetic acid induced colitis model.	(Sareen, Jain, Rajkumari, & Dhar, 2016)
Emulsification internal gelation	Sodium alginate	Icariin	■ A flavonoid, Icariin, which has poor solubility and low bioavailability, was encapsulated successfully in the microspheres. ■ Fluorescence labeled microspheres indicated high retention in the colon for more than 12 h. ■ Microspheres effectively reduced colon mucosa damage index and also reduced production as well as gene expression of inflammatory mediators and cytokines in 2,4,6-trinitrobenzenesulfonic acid (TNBS)/ethanol induced colonic inflammation.	(Wang, Wang, Zhou, Gao, & Cui, 2016)
Water-in-oil (w/o) emulsification and cross-linking with glutaraldehyde	Acrylamide grafted chitosan polymer	5-ASA	■ Acrylamide grafted chitosan (CHI-g-AAm) polymer was synthesized for colon specific drug delivery. ■ Drug release was significantly higher in the simulated colonic fluid containing caecal and colonic content against the simulated stomach and small intestinal fluid. ■ Microspheres were evaluated for their healing capacity against 2,4,6-trinitrobenzene sulfonic acid sodium salt induced colitis in rats. ■ Colonic inflammation was assessed by measuring Myeloperoxidase activity, colon/body weight ratio and damage score. ■ CHI-g-AAm microspheres showed better activity against all the mentioned parameters as compared to the drug solution administered orally.	(Jain et al., 2008)
Emulsification cross-linking and emulsion solvent evaporation	Eudragit	Sinomenine	■ Newly developed microspheres were evaluated in DSS induced mice model. ■ The disease activity index was measured on the basis of combined scores of weight loss, stool consistency, and bleeding, which was found to be insignificantly lower in sinomenine microspheres group than in plain sinomenine group. ■ Immunohistochemistry and real-time polymerase chain reaction (PCR) studies for the expression of TLR4, Myeloid differentiation primary response 88 (MyD88) and NF-κBp65 revealed lowering in the expression in the animal groups treated with sinomenine-chitosan and sinomenine enteric microspheres treated groups in comparison to plain sinomenine and salicylazosulfapyridine treated groups.	(Xiong et al., 2017)

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Table 2 (*continued*)

Technique/s used for the preparation of microspheres	Polymers/material used alongside chitosan in the study	Drug/s encapsulated	Significant outcomes of the study	Reference
Emulsification cross-linking and emulsion-solvent evaporation	Eudragit S 100 and halloysite nanotubes	Paeoniflorin	■ The halloysite nanotubes (HNT) contain hydroxyl and siloxyl groups on its surface which assists in adsorption of various chemicals and drugs. ■ Chitosan, being cationic, spontaneously binds to the negatively charged surface of HNT. ■ Paeoniflorin containing HNT/Chitosan microspheres were prepared by W/O emulsification technique and chitosan was cross-linked using glutaraldehyde. ■ Secondary coating of Eudragit S100 was applied over the microspheres using emulsion solvent evaporation technique. ■ Presence of chitosan facilitated permeation of microspheres in the colonic tissue, evaluated through everted gut sac technique.	(H. Li et al., 2021)
Emulsification and solvent evaporation	Eudragit L 100 and Eudragit S 100	Prednisolone, and prednisolone 21-hemisuccinate sodium salt	■ Prednisolone 21-hemisuccinate was conjugated with chitosan and chitosan microspheres of the conjugate were prepared which were then coated with the enteric polymer. ■ No significant difference in the drug release was observed at pH 1.2 between plain chitosan and enteric polymer coated chitosan microspheres. This is due to slow hydrolysis of the ester bond present in the conjugate. ■ Drug release was quicker in plain chitosan microspheres than enteric polymer coated microspheres at pH 6.8 due to quick hydration of the former.	(Oosegi, Onishi, & Machida, 2008)
Emulsification and solvent evaporation	Eudragit L 100	Prednisolone 21-hemisuccinate sodium salt	■ In continuation to the previous work, Eudragit L 100 coated microspheres were prepared and evaluated in TNBS induced colitis model. ■ On the basis of measurement of different inflammation indices such as myeloperoxidase (MPO) activity and ratio of proximal colon weight to body weight and distal colon weight to body weight, it was concluded that enteric coated microspheres has better efficacy and lesser toxicity than plain chitosan microspheres.	(Onishi, Oosegi, & Machida, 2008)
Emulsion solvent evaporation technique based on multiple emulsion (w/o/w)	Cellulose acetate butyrate (CAB)	5-ASA	■ Here, chitosan was selected as a drug carrier material based on its ability to undergo biodegradation by the enzyme lysozyme present abundantly in the colon, secreted by colonic microflora. ■ Core chitosan microspheres were coated with Cellulose acetate butyrate (CAB) for preventing the drug release in the acidic environment. ■ Chitosan molecular weight and core/coat ratio of the polymers has significant effect on the % bioadhesion of the microspheres, which was performed using averted sac technique. ■ Rat caecal content has positive effect on the release of the drug from microspheres; this is due to biodegradation of chitosan, and reduction of the pH by the products of bacterial fermentation.	(Varshosaz, Jaffarian Dehkordi, & Golafshan, 2006)
Emulsification solvent evaporation (o/o)	Eudragit S 100 and Ethylcellulose	5-ASA	■ Blend of ethylcellulose and Eudragit S 100 was used in a few formulation batches for the preparation of microspheres. ■ Production yield obtained for all the batches was very high ranging from 84 to 99%. ■ Reduction in concentration of Eudragit S 100 in the internal phase of an emulsion led to significant decrease in the particle size distribution due to increase in shearing action as a result of decreased viscosity of the internal phase. ■ A combination of time dependent approach and pH dependent approach using ethylcellulose and eudragit S 100 respectively was explored to prolong the drug release after reaching the colon. ■ Optimized batch of formulation released minimum amount of 5-ASA in first 4 h of dissolution studies, and drug release was prolonged over 12 h after reaching the colon.	(El-Bary, Aboelwafa, & Al Sharabi, 2012)
Emulsion polymerization	Wheat germ agglutinin	Reduced brominated derivative of noscapine	■ Chitosan microspheres were prepared by emulsion polymerization method and later coated with wheat germ agglutinin for enhancement of bioadhesive properties. ■ Microspheres exhibited affinity towards colonic mucin secreting cells in simulated colonic fluid of ~pH 7.2. ■ Microspheres showed pH sensitive release of the drug in simulated colonic fluid with colonic milieu (pH ~ 4.7).	(Kaur et al., 2015)
Single-step electrospraying	Sodium alginate	IL-1 Ra (Recombinant IL-1 receptor antagonist)	■ Microcapsules were prepared by single-step electrospraying technique and sodium alginate coating was hardened with Ca^{2+} ions in the presence of chitosan.	(Cao et al., 2019)

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Table 2 (*continued*)

Technique/s used for the preparation of microspheres	Polymers/material used alongside chitosan in the study	Drug/s encapsulated	Significant outcomes of the study	Reference
			■ Microspheres showed pH dependent drug release, with slow and smaller amount was released in simulated gastric fluid. In simulated intestinal fluid drug release shot up to 86% within 2 h of the study. ■ Treated mice with microspheres showed decrease in the disease activity estimated on the basis of disease activity index which was evaluated in dextran sulphate (DSS) sodium induced colitis model. ■ IL-1 Ra containing microspheres exhibited improvement in the damaged colonic tissue evaluated by histological studies. ■ Serum concentration of different cytokines such as TNF-α, and IL-1β was evaluated in animal model, post treatment concentration of the cytokines was found to decrease significantly.	

nanoparticles include ionic gelation, spray-drying, emulsion followed by cross-linking and complex coacervation (Saboktakin, Tabatabaie, Maharramov, & Ramazanov, 2011).

Scalability of nanoparticles has always been challenging for formulation scientists across the world and manufacture must be supplemented with narrow particle size distribution. To address the issue, Huanbutta et al. had developed chitosan nanoparticles coated with poly (methyl acrylates) using spinning disc processing technique. High entrapment efficiency of 88% was reported with over 90% drug release in the simulated colonic fluid within 8 h. With this novel approach, researchers achieved selective targeting to the colon with minimum potential of drug release in the upper GIT (Huanbutta et al., 2013).

3.3.1. *Colonic delivery of small molecules via chitosan-based nanoparticles*

Mucus is indigenous to the intestine majorly made-up of mucin, a glycosylated glycoproteins possessing negative charge. Specialized cells known as Goblet cells secrete the mucus. Alteration in the number of goblet cells and reduction in the thickness of the mucus is observed in the IBD (Brown, Whitehead, & Mitragotri, 2020; Michielan and D'Incà, 2015). Mucus varies in thickness and level of adherence, it has thickness of 13–167 μm of firmly attached layer and 97–823 μm of loosely bound layer on the surface (Hunter, Elsom, Wibroe, & Moghimi, 2012). Chitosan is a cationic polymer, nano-carriers made-up of it interact with the mucus by electrostatic interaction and exhibit prolonged residence time at the desired site, which is advantageous considering rapid bowel movements in IBD, over and above this property also enables higher drug permeation across the epithelium (Grenha, 2012; Hua et al., 2015a). Production of mucus is reported to be high in active UC, and as mentioned, chitosan containing nanoparticles show adherence to the mucus, owing to their small size nanoparticles are taken up well by the macrophages in an inflamed area. Mongia et al. had developed mucoadhesive chitosan nanoparticles of curcumin for the treatment of UC. Nanoparticles were prepared by ionic gelation technique using tripolyphosphate as a cross-linker. The only limitation of chitosan for colon targeting when administered *via* oral route its protonation in the acidic environment of the stomach that leads to its solubilization, here, this problem is addressed by coating of nanoparticles by Eudragit FS 30 D. Because of the coating nanoparticles showed good accumulation in the colonic area as revealed in the biodistribution studies carried out using gamma scintigraphy (Raj, Raj, Kaul, Mishra, & Ram, 2018). On the similar lines, chondroitin sulphate functionalized nanoparticles of curcumin were developed for targeting colonic macrophages, for their protection in harsh conditions of upper GIT, nanoparticles were encapsulated in chitosan-alginate hydrogel (Zhang et al., 2019). Natural polymers for the site-specific drug delivery to the colon have grabbed more attention over the past few years due to low toxicity as compared to the synthetic polymers. In conditions like colon cancer and Crohn's disease, increased secretion of the mucus suits well for mucoadhesion and this ultimately leads to increased flux of the drugs across colonic mucosa. In an attempt to increase propensity of mucoadhesion, Sabra et al. have reported modified pectinate-chitosan nanoparticles prepared by ionic gelation technique. Mucin, from the porcine stomach at four different concentrations 10, 50, 100 and 150 μg/ml was used to evaluate the mucoadhesion propensity. Change in the zeta potential of the nanoparticles was evaluated after incubating them in simulated pH media for stomach and colon *i.e.* pH 1.2 and 7.0, respectively. Authors reported that zeta potential reduces to +18.2 mV from +35.7 mV in acidic conditions and to +1.22 mV in pH 7.0. From the above readings, nanoparticles were inferred to be stable in acidic conditions; however, at neutral pH drastic reduction in the zeta potential is due to Van der Waals inter-particle attraction and this aggregation affects mode of cellular uptake and biological response. Further, drop in zeta potential can be attributed to electrostatic interaction between positive charge of chitosan and negative charge of sialic acid moieties of the mucin. Therefore, significant drop in the zeta potential indicates extent of mucoadhesion. It is concluded that pectinate-chitosan nanoparticles are more mucoadhesive at neutral pH than acidic (Sabra, Roberts, & Billa, 2019). In another research work, chitosan-dextran sulfate nanoparticles were evaluated for the colonic delivery of a model drug 5-ASA. Sustained drug release over the period of 10 h. was reported, and there was no significant effect of chitosan-dextran sulfate observed to be on the drug release (Saboktakin, Tabatabaie, et al., 2010). On the similar line, same research group has reported successful delivery of a model drug 5-ASA to the colon *via* carboxymethyl starch-chitosan nanoparticles (Saboktakin, Maharramov, & Ramazanov, 2010).

Chemical modification of chitosan is reported in various studies for improvement of the physicochemical properties of the natural polymer, especially to improve its solubility over a wide pH range. In one such study, for the colon targeting of low molecular weight heparin (LMWH) which has strong therapeutic activity in UC because of its anti-inflammatory, anticoagulant and mucosal healing effects, authors developed trimethyl chitosan (TMC) and sodium alginate coated-TMC (SA-TMC) nanoparticles containing LMWH. There are several issues associated with oral administration of heparin- low bioavailability due to poor absorption in the GIT, high anionic charge density, first pass metabolism, and enzymatic degradation. Therefore, colon targeted nanoparticulate delivery offers an advantage in terms of passive targeting to the inflamed colon owing to enhanced permeability and retention effect. As an outcome, *in-vitro* mucosal permeation study revealed significantly enhanced passage of the drug across rat intestine by 3.45 fold through TMC nanoparticles and 2.67 fold through SA-TMC nanoparticles when compared against free LMWH. This enhancement is attributed to reversible opening of tight junctions of epithelial cells mediated by chitosan-based nanoparticles (Wang et al., 2017; Wang &

Kong, 2017; Yeh et al., 2011). Furthermore, anticoagulant effect of LMWH was determined *in-vitro* in human plasma; nanoparticles exhibited significant extension of the prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) as compared to normal saline. In continuation, for determination of effect of nanoparticles on oral absorption of LMWH in rats, *in-vivo* studies were performed by activated partial thromboplastin time (APTT) assay. In the study, TMC nanoparticles exhibited highest anticoagulant activity, maximum APTT significantly prolonged to 2 h, which is 1.60 fold rise as compared to free LMWH, this observation highlights strong anti-coagulant activity and better oral absorption. On the other hand, SA-TMC nanoparticles showed 1.31 fold increase in APTT value. This significant rise in ATPP through TMC nanoparticles was due to the cationic charge that facilitated penetration of LMWH *via* paracellular transport thus improving oral absorption and anti-coagulant activity. In the pharmacodynamics, ameliorative effects of nanoparticles was evaluated in TNBS induced colitis mice model. Here, nanoparticles treatment group exhibited significantly positive effect on some key indicators of IBD as in better control over weight loss, reduced disease activity score, restoration of the colon length and finally, decline in MPO activity as compared to free LMWH (Mittal et al., 2018; Yan Yan et al., 2020).

Chitosan and its derivatives are widely used for coating liposomes in achieving site specific gastrointestinal delivery because of their mucoadhesive/bio-adhesive property (Fatouh, Elshafeey, & Abdelbary, 2020; Huang, Wang, Chu, & Xia, 2020; Tai et al., 2020), however, chitosan alone cannot protect the lipid vesicles in the acidic environment of the stomach. In a novel approach, Castingia et al. reported colonic delivery of quercetin, a potent anti-inflammatory and antioxidant drug, achieved through its encapsulation into phospholipid vesicles (nanoparticles) coated with chitosan/nutriose. Phospholipid vesicles are prone to acidic pH and enzymatic degradation that can be protected by employing coating of suitable polymers. Herein, chitosan and nutriose was used for coating of nanovesicles. These nanosystems exhibited better control of the disease activity (weight loss, and rectal bleeding) over uncoated ones as revealed through a pre-clinical study using rat model in which IBD was induced with the help of TNBS (Castangia et al., 2015).

In a recent study, researchers have proposed novel chitosan coated c-SLN (Solid-lipid nanoparticles) delivery system. This research group have designed an effective hybrid system for chemoprevention, which is the combination of SLNs and chitosan-based drug delivery systems. c-SLNs have wide range of pharmaceutical applications and employed as a promising vehicles for oral drug delivery systems because of their characteristics such as permeation enhancer, bioadhesion and biodegradation. In addition, cationic nature of chitosan leads to prolonged residence time at the negatively charged epithelia in the small intestine that shows significantly enhanced drug concentration at the site of absorption. In addition, chitosan can facilitate reversible opening of the tight junctions between neighboring epithelial cells, promoting drug molecule's paracellular transport, thereby enhancing bioavailability of the encapsulated drugs. In conclusion, chitosan as a drug delivery vehicle, and SLNs combine the advantages of both the carrier materials (Fonte et al., 2012; Thakkar, Chenreddy, Wang, & Prabhu, 2015).

3.3.2. *Chitosan nanoparticles in the colonic delivery of macromolecules*

Therapy of IBD with macromolecules has a unique advantage in addressing the underlying cause of the inflammation as compared to conventional treatments using small molecules. Macromolecule therapy options include immunosuppressants such as cyclosporine-A, anti-TNF-α agents *e.g.* infliximab, adalimumab, certolizumab pegol, natalizumab, interleukin-12/23 antibodies, interleukin-6-receptor antibodies, vaso-active intestinal peptide, bioactive natural peptides *e.g.* Lys-Pro-Val (KPV), and RNA based therapeutics. Presence of gastric acid and proteolytic enzymes in the GIT that would degrade macromolecules administered orally without any protection, in addition to that colonic microbiota-mediated metabolism, mucus layer, intestinal epithelium and basement membrane limits oral administration of macromolecules. Aforementioned advantage of macromolecules suffers a major blow due to no alternative route other than injectable is available for their systemic administration. Altered structural intactness of the apical side of the gastrointestinal mucosa in IBD provides an opportunity for oral delivery and localization of macromolecules (Stallmach, Hagel, & Bruns, 2010; Zhang, Thanou, & Vllasaliu, 2020). Selective drug targeting to the desired tissue/site is a key in the treatment of the IBD. Conventional drug delivery systems are unable to release the required amount of the drug in the colon; apart from that, drug absorbed in the upper part of the GIT produces adverse reactions and side effects considering long-term medications in the IBD patients. Therefore, a new drug delivery system that can release the drugs in the colon is the need of the hour. Polysaccharide polymers are ideal for colon-targeting of the payloads because of their degradation by the enzymes produced by the microflora and structural modifications of these polymers is also an formidable alternative (Chen et al., 2020; Deng et al., 2019; X. Li, Lu, Yang, Yu, & Rao, 2020). In an attempt to target anti-inflammatory tripeptide KPV to the colonic region Laroui et al. have developed biodegradable nanoparticles which were further encapsulated into polysaccharide hydrogel made-up of chitosan and alginate. The polysaccharides specifically selected because of their ability to protect the payloads from the harsh acidic environment of the stomach and ability to degrade by the colonic microflora, which makes site-specific delivery of macromolecules possible. *In-vitro* interaction between the nanoparticles and Caco 2-BBE (brush border enterocytes) showed drug release in the vicinity and inside the cell membrane (intracellular space). Similarly, LPS induced inflammatory response in Caco 2-BBE was controlled by downregulation of inflammatory cytokines in the dose dependent manner by the drug-loaded nanoparticles. *In-vivo* studies in DDS-induced colitis mice model revealed improved disease activity index, and reduced intestinal inflammation, which was further confirmed by determination of pro-inflammatory cytokines (downregulated) in the colonic tissue. Dose of KPV reduced by 12,000 times post encapsulation into nanoparticles, probably due to endocytosis of the nanoparticles by epithelial cells and drug release in the extra- and intra-cellular space. KPV nanoparticles encapsulated in hydrogel matrix prevented early drug release in the upper GIT hence facilitated drug deposition at the inflamed site. Further, high level mucus secretion is reported during intestinal inflammation, here, chitosan played its role in enhancing anti-inflammatory response of the drug by interacting with the mucus owing its cationic charge hence maintaining the drug molecules at the inflammation site for longer duration (Laroui et al., 2010). In another research work, Rivera et al. have reported sequentially assembled hollow nanocapsules made-up of chitosan-alginate developed by using layer-by-layer deposition technique by exploring opposite charges on the polymers. The technique is inexpensive, adaptable and easy to perform; in the study glycomacropeptide and 5-ASA was used as model drugs. This study focuses on finding release mechanisms of both the drugs from the nanoscale particles; concludes that release of the drugs followed i. Fickian diffusion, and ii. Polymeric relaxation, which is due to hydrophilic nature of the polymers. At pH 7.0, the anionic alginate from the complex may have been displaced by hydroxyl ions and chitosan losing its positive charge facilitating quick release of encapsulated drugs (Rivera, Pinheiro, Bourbon, Cerqueira, & Vicente, 2015). Chitosan offers several advantages for the delivery of small interfering RNA (siRNA), such as bioadhesion, biodegradability, and strong affinity for nucleic acid. The modification of primary amino groups of chitosan with glycidyltrimethylammonium chloride gives quaternary chitosan. This quaternization has shown enhanced nucleic acid binding capacity and improves cellular uptake by facilitating electrostatic affinity between chitosan and cell membrane (Xiao et al., 2017). However, its solubility at acidic pH and insolubility at neutral and alkaline pH limits its utility for the colon specific drug delivery. Trimethylation of chitosan enhances its solubility over a wide pH range and incorporation of thiol group further improves its bioadhesion through covalent bonding with mucin

glycoproteins thereby facilitates cellular uptake and gene transfection efficiency by solubilizing at extra- and intra-cellular pH. TNF-α, a pro-inflammatory cytokine plays central role in progression of IBD. Monoclonal antibody treatment is worthy, but also has high cost and side effects. Knockdown of functional proteins at mRNA level mediated by siRNA provides another approach for the treatment of various inflammatory diseases due to its high specificity and efficacy. For targeting mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) a siRNA, which is known as key upstream mediator of TNF-α action, to the macrophages at the inflamed colon Zhang et al. have reported galactosylated TMC nanoparticles for oral delivery. Owing to the high affinity between galactose residue and macrophage galactose-type lectin (MGL) receptors expressed on the surface of macrophages, nanoparticles exhibited good binding affinity towards them when evaluated in *in-vitro* cell culture of Raw 264.7 cells, similarly, significant knockdown of TNF-α secretion was observed in LPS-stimulated Raw 264.7 cells. These nanoparticles significantly suppress TNF-α production in DSS-induced colitis mice tissue, improved disease activity in the animals, and inhibited MPO activity (Zhang, Tang, & Yin, 2013). Further, in a similar approach Huang et al. reported TNF-α siRNA containing nanoparticles encapsulated within the poly lactic-*co*-glycolic acid (PLGA) matrix by employing a coating of galactosylated chitosan to target at MGL (Huang, Guo, & Gui, 2018). There are two best characterized phenotypes of macrophages, M1 and M2 believed to have a vital role in the inflammation. M1 macrophages are responsible for secretion of pro-inflammatory cytokines and subsequently worsening of the situation, whereas M2 macrophages are involved in tissue repair. M1 macrophages are dominant in the early stage of inflammation later on they are replaced by M2 phenotype. MiR146b is up-regulated in human monocytes and acts as an anti-inflammatory agent by inhibiting TLR4 signaling pathway, it also targets interferon regulatory factor (IRF5) and thereby inhibit activation of M1 macrophages. Considering this, mannose-modified TMC nanoparticles (MTC), which are taken up by macrophages *via* mannose receptor-mediated endocytosis, are conjugated with miR-146b mimic. Herein, Deng et al. explored molecularly targeted immunotherapeutic strategy using MTC for inhibition of M1 macrophage activation and subsequent pro-inflammatory cytokine release; in an attempt to promote mucosal healing and suppress the development of colitis-associated carcinoma. As compared to negative control MTC, MTC- miR-146b mimic nanoparticles significantly inhibited inflammation and promoted epithelial regeneration in DSS-induced colitis mice model (Deng et al., 2019; He et al., 2016). Nearly 25% of the patients treated with monoclonal antibody, infliximab, suffered from at least one of the serious adverse effects, namely pneumonia, cancer or acute inflammation, may probably be due to lack of drug targeting and hence taking over dose of the drug. As mentioned, TNFα plays crucial role in the progression of inflammation, Laroui et al. had reported TNFα siRNA loaded biodegradable nanoparticles made up of poly (lactic acid) poly (ethylene glycol) block copolymer for macrophage targeting by covalently attaching Fab' portion of F4/80 antibody on to the surface. These nanoparticles were further coated with chitosan-alginate as this hydrogel collapses at pH 5 or 6 and ensures colon specific drug release. Phagocytosis of Fab′ portion bearing nanoparticles by macrophages took place very quickly when evaluated *in-vitro* on RAW 264.7 cells, similarly these nanoparticles reduced TNF-α expression in inflamed macrophages. Hydrogel-encapsulated Fab'-bearing TNF-α siRNA-loaded nanoparticles improved disease activity index and attenuated inflammation in DSS induced colitis mice model. TNF-α is a major upstream regulator of the NF-κB pathway; authors evaluated concentration of IKβα protein, an inhibitor of NF-κB, in the colon of DSS induced colitis mice model. Concentration of IKβα protein found to be higher in mice administered with Fab'-bearing siRNA-loaded nanoparticles as compared to Fab'-bearing scrambled siRNA-loaded nanoparticles, indicating that chitosan-alginate coating enabled cell specific accumulation of the siRNA thereby allowing disease attenuation by targeting an activity at the molecular level (Laroui et al., 2014). In another study, Wu et al. developed PLGA nanoparticles containing cyclosporine-A functionalized with KPV, a tripeptide targeted at oligopeptide transporter receptors (PepT1) that are overexpressed only in inflammatory condition on colonic epithelial cells and macrophages. Nanocarriers further coated with montmorillonite/chitosan for preventing early drug release at the acidic pH of the stomach. Montmorillonite chosen as a coating material because of its ability to interact with mucin by transforming into viscous gel; similarly, chitosan is well established as a coating material due its mucoadhesion ability and anti-inflammatory effect. It was hypothesized that these coated nanoparticles would increase retention time *via* time-, pressure-, pH-, or bacteria responsive mechanism and hence would target the colonic site. Fluorescent dye tagged nanocarriers exhibited 23-fold higher concentration in the inflamed colon than that in the healthy colonic tissue. Chitosan/montmorillonite coating contributed in the gathering of the nanocarriers at the inflamed sites by interacting with glycoprotein and mucin that made oral site-specific drug delivery possible thereby avoiding systemic distribution of the drug. In addition to that, PLGA-KPV could bind to macrophages and colonic epithelial cells and transported into the inflammatory colon cells *via* PepT1 as proven through confocal microscopic studies. In acute DSS induced colitis mice model, mRNA levels of TNF-α and IL-1β evaluated using RT-PCR, found to be significantly lowered in the nanoparticle treatment group when compared with marketed preparations of cyclosporine-A (Sandimmune). Interestingly, not just cyclosporine-A loaded nanoparticles exhibited anti-inflammatory response but, blank nanoparticles also exhibited the same response, probably due to synergistic action of KPV, MMT and chitosan. Of note, chitosan is reported to have anti-inflammatory response comparable to prednisolone (Wu et al., 2019). Higher than usual intestinal expression of NF-κB considered as important factor for progression of IBD, which is associated with altered intestinal barrier function and activation of pro-inflammatory signaling. On the other hand complete ablation of intestinal p65 (a subunit of NF-κB) expression in mice led to deregulation of response to injury and inflammation. Therefore, drug molecules balancing both sides *i.e.* inhibition of NF-κB but not to the extent of its abolishment would be an optimal therapeutic option. Prohibitin, a protein responsible for various cellular processes such as protein folding, proliferation control, suppression of oncogenesis, mitochondrial functions, and regulation of transcription processes, its expression is downregulated in UC and CD. Prohibitin has antioxidant and anti-inflammatory activity, its sustained expression in intestinal epithelial cells decreases TNF-α-stimulated NF-κB activation *in-vivo*. In the research work reported by Theiss et.al, prohibitin was deliver to the colon through adenovirus-directed administration *via* enema and orally through PLGA nanoparticles coated with chitosan-alginate hydrogels. Both the methods of administration of prohibitin proved beneficial *in-vivo*. Increased level of prohibitin was observed in the colonic tissue leading to reduced severity of DSS-induced colitis in mice model revealed through improved disease activity index, reduced MPO activity, reduced pro-inflammatory cytokine expression, improved histological score and reduced oxidative stress. pH- and time-dependent collapse of alginate-chitosan hydrogel enabled oral site-specific drug delivery of prohibitin thereby avoided its non-specific uptake that would have happened had it been administered *via* intravenous route (Theiss et al., 2011).

CD98, a type II transmembrane glycoprotein transporter over expresses on the surface of colonic epithelial cells and on intestinal macrophages in inflammation, it plays a vital role in the activation of the latter, thereby in the progression of IBD. The cytoplasmic domain of CD98 can interact with β_1- integrin and regulates cell homeostasis, epithelial adhesion and immune responses. Targeted drug delivery that can block CD98 could be a potential therapeutic target to ameliorate the disease progression. An effective tool to curb inflammation in this case is RNA interference (RNAi) *via* siRNA, which brings about post-transcriptional gene silencing (genes related to the disease) and inhibits CD98 expression on the macrophages. To exhibit its potential effects, siRNA has to enter cytoplasm by crossing cell barrier where it

can cause sequence-specific deterioration of mRNA. For entering in to the cytoplasm, a siRNA has to avoid degradation mediated *via* acidic endosomes/lysosomes. This degradation can be avoided by proton bearing buffering constituents, such as imidazole group containing compounds, polyethylenimine and chloroquine by disrupting endosomal/lysosomal membranes. Mucus is another barrier that resist localized drug delivery to the colonic mucosal surface when taken by oral route. Chitosan is biocompatible, biodegradable and positively charged that can form complex with siRNA by polyelectrolyte complexation which can be transformed into nanoparticles, apart from this chitosan has high transfection efficiency (Xiao et al., 2014; Yan, Yutao, Vasudevan, Nguyen, & Merlin, 2008). Considering this, Xiao et al. have reported uronic acid modified chitosan nanoparticles containing siCD98 as payload using complex coacervation technique. Single-chain CD98 antibody was conjugated to the surface of the nanoparticles using polyethylene glycol to enhance interaction between functionalized nanoparticles and CD98 protein. Nanoparticles were evaluated for uptake by colon-26 and RAW 264.7 cell lines, found to be taken-up very rapidly through active targeting; similarly, significant knockdown of CD98 expression was reported in the same cell lines as compared to scrambled siRNA loaded nanoparticles. A T-cell transfer and DSS-induced colitis mice model was explored for *in-vivo* studies. Antibody functionalized nanoparticles embedded in chitosan-alginate hydrogel after oral administration reduced weigh loss in the treatment group of mice in both type of colitis mice model. There was also significant reduction in MPO activity, and of CD98 expression in the treatment group (Xiao, Ma, Viennois, & Merlin, 2016). Further, in an attempt to treat UC through a combination therapy, Xiao et al. reported PLGA nanoparticles containing siTNF-α (a siRNA) and IL-22, a pro-healing cytokine, embedded in chitosan-alginate hydrogel for the colon targeting. Nanoparticles encapsulated in the hydrogel exhibited very strong inhibition of pro-inflammatory factors and promoted mucosal healing *in-vivo* in DSS induced colitis mice model (Xiao et al., 2018).

Eggshell membrane, formed as a one of the byproducts during egg processing, has demonstrated inhibitory effect on production of TNF-α and other pro-inflammatory cytokines such as IL-1β, MCP-1 (monocyte chemoattractant protein), MIP-1α and β (Macrophage inflammatory protein), RANTES (regulated upon activation, normal T cell expressed and presumably secreted), and VEGF (vascular endothelial growth factor). For its targeted delivery to the colon, Chen et al. reported novel chitosan-fucoidan nanoparticles. Fucoidan, an anionic polysaccharide extracted from brown seaweeds, used for the cross-linking of chitosan. Nanoparticles exhibited delayed release in the dissolution conditions mimicking GI pH, and strong antioxidant, immunomodulatory activity evaluated *in-vitro* (Lee & Huang, 2019).

US- food and drug administration (US-FDA) has approved several biosimilars that are used in the treatment of IBD; they are listed in Table 1 in the supplementary material. There are some research articles available in the literature on the colon specific drug delivery *via* oral administration of Infliximab using various available polymers (Foong, Patel, Forbes, & Day, 2010; Gareb et al., 2021; Maurer et al., 2016; Pabari et al., 2019). However, as they are out of the purview of this review hence not discussed here.

4. Chitosan-drug conjugates for the colon targeting

Of the several approaches employed for the colon targeting, drug-polymer conjugation *via* covalent linking for the production of the prodrugs is explored in the several marketed technologies. This approach is advantageous considering that it clubs physicochemical properties of drugs, polymers and physiological conditions of the GIT (Shahdadi Sardo et al., 2019).

To explore benefits of biodegradability, biocompatibility and GRAS status associated with chitosan, Nalinbenjapun et al. reported chitosan-5-ASA azoconjugate for colon specific drug release. Previously, Zou and co-workers had reported the same conjugate, but the conjugation was carried out through an amide bond between 5-ASA and chitosan. This conjugate failed to release the drug in simulated gastrointestinal fluid containing rat caecal or colonic content. The failure was attributed to a very stable amide bond, which did not break during *in-vitro* dissolution studies. However, the newly developed azo-conjugate was stable in *in-vitro* stability studies carried out using simulated gastric, intestinal and colonic fluids indicating it would avoid premature drug release in the upper GIT. *In-vitro* release studies demonstrated that there was no drug release from the conjugate in simulated gastric conditions over 24 h and in simulated intestinal conditions over 6 h, 15% of drug released over 6–24 h. In simulated colonic fluid, 10% of drug released over 6 h and maximum drug release was 25% over 24 h. Insufficient drug release in comparison to sulfasalazine (around 70%) is due to protection of the azo bond from enzymatic attack by steric hindrance brought about by the large molecule (Nalinbenjapun & Ovatlarnporn, 2020; Zou et al., 2005).

Amphiphilic polymers have attracted the attention of drug delivery scientists over the last few years. Especially amphiphile made-up of natural polysaccharides are of interest due to non-toxicity and biodegradability. These polymers have hydrophilic tail and hydrophobic core for effectively encapsulation of hydrophobic drugs (Hsu et al., 2020; Liu, Du, & Zhai, 2015). BCS class II drugs poses a significant challenge for effective absorption in the therapeutic concentration. When these drugs are used in the treatment of cancer, parenteral administration is preferred; however, it does not go well with the patients. Biocompatibility and chemical modifiability of chitosan is advantageous for enhancing absorption of these drugs especially in the form of amphiphilic structure. In a research work, two compounds belonging to BCS class II, curcumin, an anti-inflammatory agent and 7-ethyl-10-hydroxycamptothecin, a cytotoxic agent, were individually conjugated with chitosan to form chitosan-drug amphiphile for enhancing GI absorption of the drugs for the treatment of colitis associated colorectal cancer (CRC). These novel formulations pre-clinically evaluated using CRC mouse model. Anti-inflammatory effect of curcumin nanoparticles was evaluated by using Raw 264.7 and murine bone marrow-derived cells. Pre-incubated cells with curcumin nanovesicles showed decreased secretion of pro-inflammatory cytokines, on the other hand, in case of CRC induced mice model; these nanoparticles reversed upregulation of ROS, which is involved in signal transduction and genomic instability, as compared to non-incubated cells. On the other hand, 7-ethyl-10-hydroxycamptothecin nanovesicles and free drug exhibited comparable anti-proliferation when evaluated in human colorectal DLD1 and HCT-116 cell lines, suggesting conjugation does not deter anti-proliferative activity of the drug. *Ex-vivo* near infrared imaging and confocal microscopy studies revealed accumulation of nanovesicles in the colon, as hypothesized by the authors. In DSS induced colitis mice model, authors reported that, administration of curcumin nanovesicles substantially reduced mice mortality, and typical colitis symptoms significantly. Similarly, these nanoparticles effectively targeted the tumor site, as revealed by tumor growth inhibition. Finally, low molecular weight chitosan platform made oral delivery of poorly aqueous soluble drugs possible and played vital role in drug targeting owing to its mucoadhesive potential, and negligible cytotoxicity (Han et al., 2019). Conventional therapies in the treatment of IBD (or other colonic conditions) lacks specificity, has poor bioavailability and retention and severe side effects upon long-term therapy. Administration by injectable route to overcome some of the above-mentioned lacunae, however, has poor patient compliance. Considering these factors oral nanotherapeutics is the emerging strategy. Chitosan-drug conjugates in amphiphilic form seems to bypass absorption from upper GIT and accumulates at the inflammatory/tumor sites, presumably due to reduced solubility of chitosan at acidic pH, increased molecular weight after conjugation and enhanced permeability and retention effect at the inflammation site.

Hydrophobic modification of chitosan by acetylation, or alkylation allows encapsulation of hydrophobic drugs, these drug molecules get physically embedded in the self-assembled nanocarriers and

Table 3
Examples of drug delivery systems developed by exploring interaction of chitosan with other natural polymers for the intestinal delivery of probiotics.

Polymer combined with chitosan for encapsulation	Probiotic encapsulated	Delivery system	Preparation technique	Reference
Sodium alginate	*Bifidobacterium breve*	Chitosan coated alginate microcapsules	External/ionic gelation, immersion coating	(Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011)
	Lactobacillus plantarum	Chitosan coated alginate microcapsules	Electrospray	(Phuong Ta, Bujna, Kun, Charalampopoulos, & Khutoryanskiy, 2021)
	Lactobacillus casei 01	Cross-linked beads	Ionic gelation, polyelectrolyte complexation	(Ta et al., 2021)
	Bifidobacterium longum	Chitosan coated alginate microcapsules	Emulsification, internal gelation and immersion coating	(Ji et al., 2019)
	Lactobacillus plantarum 25	Chitosan-alginate microcapsules	Extrusion and cross-linking	(Jiang et al., 2013)
	Five strains of *Bifidobacterium* and *Lactobacillus*	Chitosan coated microcapsules prepared by	Emulsification, cross-linking and immersion coating	(Lohrasbi et al., 2020)
	Lactobacillus rhamnosus GG	Chitosan coated sodium alginate hydrogel particles	Extrusion and cross-linking	(Qi, Simsek, Ohm, Chen, & Rao, 2020)
	Bifidobacterium pseudocatenulatum G4	Chitosan coated microcapsules	Emulsification, internal gelation and immersion coating	(Kamalian, Mirhosseini, Mustafa, & Manap, 2014)
	Bacillus licheniformis	Chitosan hydrochloride-alginate micro-carriers	Polyelectrolyte complexation *via* orifice-polymerization	(Wu, Xu, Xie, Tong, & Chen, 2016)
	Escherichia coli strain Nissle 1917	Alginate coated chitosan microparticles	Layer-by-layer deposition by ionic gelation/polyelectrolyte complexation	(Luo et al., 2020)
	Lactobacillus gasseri and *Bifidobacterium bifidum*	Cross-linked beads	Ionic gelation/polyelectrolyte complexation	(Chávarri et al., 2010)
	Lactobacillus plantarum TN8	Chitosan coated alginate beads	Ionic gelation and immersion coating	(Trabelsi et al., 2013)
	Lactobacillus plantarum and *Bifidobacterium lactis*	Chitosan coated alginate microcapsules	Electro-hydrodynamic atomization, ionic gelation and immersion coating	(Zaeim, Sarabi-Jamab, Ghorani, & Kadkhodaee, 2019)
	Lactobacillus casei 01	Chitosan coated alginate microparticles	Spray-drying, ionic gelation, and polyelectrolyte complexation	(Ivanovska et al., 2017)
	Lactobacillus salivarius	Chitosan coated alginate beads	Emulsification, ionic gelation and immersion coating	(Youssef et al., 2021)
	Lactobacillus casei	Chitosan and carboxymethyl-chitosan coated beads	Ionic gelation, and immersion coating	(Li, Chen, Sun, Park, & Cha, 2011)
	Ligilactobacillus salivarius Li01	Carboxymethyl-chitosan and alginate microparticles	Layer-by-layer deposition, ionic gelation	(Yao et al., 2021)
	Bifidobacterium longum	Chitosan coated alginate microcapsules	Injection-gelation, immersion coating	(Yeung, Üçok, Tiani, McClements, & Sela, 2016)
	Bacillus coagulans	Chitosan-alginate microcapsules	Layer-by-layer deposition by polyelectrolyte complexation	(Anselmo, McHugh, Webster, Langer, & Jaklenec, 2016)
	Lactobacillus reuteri	Chitosan, thiolated chitosan coated alginate microcapsules	Emulsification ionic gelation, immersion coating	(Song Chen, Cao, Ferguson, Shu, & Garg, 2013)
	Bifidobacterium animalis	Microparticles	Atomization, ionic gelation, polyelectrolyte complexation	(Liserre, Ré, & Franco, 2007)
Alginate-starch	*Lactobacillus casei* and *Bifidobacterium bifidum*	Chitosan coated calcium alginate-gelatinized starch microcapsules	Emulsification ionic gelation, immersion coating	(Khosravi Zanjani, Tarzi, Sharifan, & Mohammadi, 2014)
Alginate-xanthan gum	*Lactobacillus plantarum*	Chitosan coated alginate-xanthan gum beads	Ionic gelation, and immersion coating	(Fareez, Lim, Mishra, & Ramasamy, 2015)
Starch	*Lactobacillus rhamnosus*	Chitosan- carboxymethyl high amylose starch tablets coated double-faced with carboxymethyl high amylose starch	Direct compression	(Calinescu & Mateescu, 2008)
Agar-gelatin	*Lactobacillus plantarum*	Chitosan coated agar-gelatin particles	Immersion coating	(Albadran, Monteagudo-Mera, Khutoryanskiy, & Charalampopoulos, 2020)
Pectin	*Lactobacillus casei*	Chitosan coated pectin microcapsules	Ionic gelation, immersion coating	(Bepeyeva et al., 2017)
Sodium alginate-pectin	*Lactobacillus acidophilus*	Chitosan coated pectin-alginate microbeads	Emulsification ionic gelation, immersion coating	(Odun-Ayo, Mellem, & Reddy, 2017)
Carboxymethyl cellulose	*Lactobacillus acidophilus*	Microcapsules	Layer-by-layer deposition by immersion	(Priya, Vijayalakshmi, & Raichur, 2011)
Dextran sulphate	*Lactobacillus acidophilus*	Hydrogels and beads	Polyelectrolyte complexation, cross-linking	(Yucel Falco, Falkman, Risbo, Cárdenas, & Medronho, 2017)
Xanthan gum	*Lactobacillus acidophilus*	Hydrogels and beads	Polyelectrolyte complexation	(Chen, Song, et al., 2015)

instantaneously dissolve when contacted with the GI fluids (Almeida et al., 2020; Kumar et al., 2020). Carboxymethyl chitosan (CMC), a water soluble derivative of chitosan has reported to increase activity of the drugs, therefore it has been explored extensively in the drug delivery systems to the colon (Vaghani, Patel, & Satish, 2012; Zhang et al., 2021). In the research work reported by Zheng H. and co-workers, water insoluble molecule 6-MP which is also one of the drugs used in the treatment of IBD is conjugated with CMC through disulphide bond for the hydrophobic modification of the polymer. Self-assembled carriers of this conjugate were anticipated to release the drug in the controlled

manner in the target cells as disulphide bond would reduce to free sulfhydryl group in response to the higher levels of glutathione present in the cytoplasm. Due to the lower pH in the intracellular environment (5.0–6.5), polymers can get shrunken and does not release the drug, even pH dependent polymers are not an exception to this, in contrast 6-MP-CMC conjugate released the drug at 10 mM concentration of Glutathione (GSH) at pH 5 as evident in the *in-vitro* dissolution studies (Zheng et al., 2011). Effective targeting to the colon is still a sought after drug delivery issue. Novel carrier mediated approaches seem to have prospects to address this issue due to their drug localization potential (Teruel et al., 2018; Turanlı & Acartürk, 2021; Wang, Han, et al., 2021). In monolithic dosage forms, combination of pH and time dependent release approach has been explored, but, with limited success (Patel, Shah, Amin, & Shah, 2009). Mesalamine, which is the first-line treatment for the IBD, rapidly gets absorbed from the small intestine as compared to the colon. In intestinal epithelial cells mesalamine undergoes rapid and extensive metabolism by the enzyme *N*-acetyltransferase to its *N*-acetyl-mesalamine derivative. This derivative is rapidly absorbed from the small intestine and may cause systemic toxicity and also reduces availability of mesalamine to the colon. Therefore, in one such research work, covalent interaction between EDTA and chitosan-EDTA (CH-EDTA) which resulted into a clear solution has been used as a polymer for coating of mesalamine tablet in a view to protect the drug release in the upper GIT. These coated tablets showed significant control over the release of the drug in *in-vitro* dissolution conditions mimicking stomach and small intestine as compared to eudragit coated marketed formulation of mesalamine. Due to permeation enhancing properties of chitosan, CH-EDTA coated tablets showed enhancement in bioavailability of mesalamine against uncoated ones and thereby achieved effective targeting to the colonic region/cells (Singh, Suri, Tiwary, & Rana, 2013). In another research work by Onishi and co-workers, succinyl-prednisolone was conjugated with chitosan as chitosan matrix alone was not able to control the release of the drug, later this conjugate was formulated into microspheres that were then coated with pH responsive polymer eudragit L-100. Due to small size below 10 μm micro- and nanoparticles retain better at the colitis sites than solid unit dosage forms. A combination of both pH and time controlled release was employed successfully to ameliorate 2,4,6-trinitrobenzenesulfonic acid-induced colitis in rat model (Onishi et al., 2008). Similarly, colon specific delivery of 5-ASA was achieved using N-succinyl chitosan matrices, which showed controlled release of the drug at acidic and alkaline pH against burst release observed at acidic pH through plain chitosan matrices (Mura et al., 2011).

5. Chitosan containing matrices in the delivery of probiotics to the colon

One of the causes of IBD is considered to be an imbalance between useful and harmful colonic bacteria and host-activated immune response against them (Lee & Chang, 2021; Prudhviraj et al., 2015). Probiotics are live microorganisms known to have several health benefits in humans when administered in adequate amounts. Apparently probiotics seems to be effective in the treatment of IBD due to their ability to stimulate anti-inflammatory cytokines, inhibition of pro-inflammatory cytokines, strengthening of the intestinal barrier function, and antagonistic action against pathogens (Asgari, Pourjavadi, Licht, Boisen, & Ajallouеian, 2020; Guandalini & Sansotta, 2019; Laroui et al., 2010). The major hurdle in the delivery of probiotics to the colon is the harsh acidic environment of the stomach where the bacteria may get killed if exposed (Dodoo, Wang, Basit, Stapleton, & Gaisford, 2017). Chitosan is an important polymer for the delivery of probiotics to the colon due to its ability to delay entry of acids and bile salts into the capsules *via* ion-exchange reactions due to formation of a thicker less porous membrane (Vaghani et al., 2012; Zhang et al., 2021; Zheng et al., 2011). Cross-linking of chitosan by anionic polymers and various chitosan derivatives are frequently used in the delivery of probiotics, related reports are summarized in Table 3.

6. Conclusion

There are five basic properties of chitosan, which are, 1. Biocompatibility, 2. Biodegradability especially by colonic microflora, 3. Cationic nature thereby enhanced mucoadhesion, and residence time at the inflamed colonic site, 4. Safety for long term administration due to GRAS status, and 5. Easy to modify chemical structure, underscores its utility for the colon targeted drug delivery. Multiparticulate dosage forms made up of chitosan and other natural polymers by cross-linking has shown pH dependent release of the payloads, hence they can protect sensitive molecules in the harsh acidic environment of the stomach. Structurally modified chitosan also has a potential to avert its dissolution at acidic pH. Therapy with proteins and peptides is the future of the treatment of the IBD, however suffers due to inappropriate route of administration, *i.e.* parenteral route and results into severe adverse effects; oral route certainly offers potential advantages. Delivery of proteins and peptides through chitosan based multiparticulate formulations achieve site specific drug release as well as localization into inflamed tissue. Targeting specific cells, such as macrophages and epithelial cells in the colon is a key therapeutic target, structurally modified chitosan based drug delivery systems offers formidable carrier for drug delivery to these targets. Chitosan based pellets using extrusion-spheronization can be formulated with ease due to good elastic properties of the polymer, which can be further coated with enteric polymers for the colon targeting. Due to sustained release properties of chitosan, better management of the IBD is possible. Chitosan itself has exhibited anti-inflammatory and immunomodulatory properties, it would be interesting if the polymer could produce synergistic/additive effect alongside therapeutic moieties in IBD. Finally, there is certainly a potential in this polymer for colon specific drug delivery, however, further studies are needed to prove commercial utility.

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ORIGINAL ARTICLE

Mupirocin resistance in methicillin-resistant *Staphylococcus aureus* isolates from anterior nares of healthcare workers of a tertiary care hospital

Manjushree V. Mulay[1], Smita S. Kulkarni[1], Vaishnavi V. Mulay[1]*

[1]Department of Microbiology, MGM Medical College and Hospital, Aurangabad-431003 (Maharashtra) India

Abstract

Background: Nasal colonization of *Staphylococcus aureus* is very common among health care workers, as part of a comprehensive Methicillin Resistant *Staphylococcus aureus* (MRSA) decolonization strategy, Mupirocin (Pseudomonic acid) is a topical antibiotic largely used to eradicate staphylococcal nasal carriage. Increased mupirocin use predisposes to mupirocin resistance, which is significantly associated with persistent MRSA carriage. This resistance is both low level as well as high level among the isolated strains. *Aim and Objectives:* To estimate the nasal carriage of MRSA in Healthcare Workers (HCWs) and to detect level of Mupirocin resistance in isolated MRSA strains. *Material and Methods*: A total 670 nasal swabs of HCWs (doctors, nursing staff and housekeeping staff) from various high risk areas were tested. High level and low level Mupirocin resistance among the isolated MRSA strains was detected by Kirby Bauer disc diffusion method. Minimum Inhibitory Concentration (MIC) of Mupirocin resistance was determined by E test. *Results:* Among 670 nasal swabs, 280 (41.79 %) showed growth of *Staphylococcus aureus* and 353 (52.68%) were Coagulase Negative Staphylococci (CONS). Of 280 *Staphylococcus aureus* strains, 61 (21.78%) strains were methicillin-resistant (MRSA). Mupirocin resistance both low level and high level was observed in 1 (1.63%) MRSA carrier only. Conclusion: The present study showed a high incidence of nasal carriage of MRSA among health care workers. Therefore we suggest MRSA screening of HCWs as a routine practice and insist on Mupirocin resistance detection so that in case if resistance detected alternative treatment can be given.

Keywords: Antibiotic resistance, Methicillin Resistant *Staphylococcus aureus*, Low-level Mupirocin Resistance, High-level Mupirocin Resistance

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is an increasingly common pathogen associated with both nosocomial and community-acquired infections [1]. Colonization of the anterior nares with Staphylococcus aureus is common and reported in various studies [2-3]. Mupirocin is an antimicrobial agent that inhibits the synthesis of bacterial proteins by competitive inhibition of bacterial Isoleucyl-tRNA Synthetase (IRS) enzyme [4]. Mupirocin resistance in clinical isolates of MRSA is reported worldwide [5-6]. S. aureus in the nose is a risk factor for endogenous staphylococcal infection. Intranasal application of mupirocin is used widely to eliminate S. aureus colonization. With the increased use of mupirocin, both low and high level resistance has been reported during treatment with nasal mupirocin [7]. Low-level resistance [Minimal Inhibitory Concentration (MIC) 8-256 μg/ml] is usually associated with point mutations in the chromosomally encoded ileS

gene whereas high-level resistance (MICs, ⩾ 512 µg/ml) is generally due to a plasmid-mediated gene, MupA (also referred to as ileS2), which encodes an additional modified IRS and is typically located on mobile genetic elements, which likely facilitates the dissemination of this resistance mechanism. The MupA gene is typically plasmid mediated, and some of these plasmids are conjugative. MupB is a new high level mupirocin resistance mechanism in S. aureus [8]. Detection and differentiation of low-level and high-level resistance has important clinical application. The presence of high-level Mupirocin resistance (MuH) excludes its clinical use as has been associated with decolonization failure and increased use leads to increased resistance rate. Low-level Mupirocin resistance (MuL) can be overcome by using higher than usual dosage [8-10]. It is therefore essential not only to differentiate between susceptible and resistant strains but also to determine the level of resistance.

The extent of mupirocin-resistance and level of resistance among Healthcare Workers (HCWs) in our area is unknown. So, with this background this study was undertaken to detect mupirocin resistance and also to determine the level of resistance in MRSA isolates from healthcare workers in our setup. This study was designed with following objectives to find the prevalence of nasal carriage of MRSA from the nasal swabs of HCWs, to find the prevalence of mupirocin resistance amongst the nasal isolates of MRSA from the healthcare workers of a tertiary care hospital, to determine the rates of MuH and MuL in nasal isolates of MRSA by disc diffusion.

Material and Methods

A prospective observational study was conducted for the period of two years, from February 2018 to January 2020 in the Microbiology department of MGM Medical College and Hospital, Aurangabad, Maharashtra. This study was started after obtaining the institutional ethical committee's approval (Letter no –MGM-ECRHS/2017, Date-07/04/2017).

A total of 670 nasal swabs were collected from HCWs including doctors, nursing staff and housekeeping staff after obtaining their informed consent. The age, sex, designation and other relevant information was obtained. Healthcare workers from various intensive care units and operation theatres were included in this study. HCWs having rhinitis, pharyngitis, upper respiratory tract infection, and who were on oral antibiotics were excluded from the study.

Nasal swabs were collected using a sterile cotton swab with transport tube. The sample was collected by rotating the sterile, normal saline moistened swab five to six times in the anterior nares of both nostrils. The swabs were immediately reinserted in transport tubes, labeled properly and transported to the Microbiology laboratory for further processing.

All nasal swabs were inoculated on 5% sheep blood agar and incubated at 37°C for 24 hours. After incubation, identification of *Staphylococcus aureus* was done on the basis of colony morphology, Gram stain and standard biochemical reactions [11].

Detection of MRSA

All the confirmed *S. aureus* strains were subsequently tested for methicillin resistance by Kirby-Bauer disk diffusion method using 30 µg

cefoxitin disc (Himedia ltd, Mumbai) as per Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. The isolates were considered methicillin-resistant if the zone of inhibition was 21 mm or less.

Mupirocin susceptibility testing

The methicillin-resistant *S. aureus* isolates were then tested for mupirocin resistance. Mupirocin resistance was detected by Kirby-Bauer disk diffusion method using 5 µg and 200 µg mupirocin discs (Himedia ltd, Mumbai) which differentiate between low and high level resistance respectively. Criteria of zone diameter breakpoints for susceptible and resistant isolates were set at > 14 mm and < 13 mm respectively [12-13]. The isolate is mupirocin sensitive when a zone diameter of ⩾ 14 mm is obtained for both 5 µg and 200 µg discs.

Isolates that showed zone diameters < 14 mm in the 5 µg disc but ⩾ 14 mm in the 200 µg disc were considered MuL.

Isolates with zone diameter <14 mm for both 5 µg and 200 µg disc were considered MuH. MIC of mupirocin was also determined by Epsilometer-strip (E-Strip) for the isolate of MRSA showing resistance to mupirocin by disc diffusion test.

All HCWs who were MRSA carriers were advised to apply mupirocin ointment locally twice daily for seven days along with relocation from duty. Nasal swab was repeated after seven days for detection of carrier stage persistence. In those with persistence of carrier stage even after application of mupirocin for seven days, we further advised them to extent application for seven more days. Repeat swab was collected after total 14 days of application of mupirocin. Data were entered in Microsoft excel and analyzed using SPSS version 24.0th mean and SD was calculated for quantitative variables and proportions was calculated for categorical variables, chi-square test was applied to check significance association between attributes: Value of p was checked at 5% level of significance.

Results

Among 670 nasal swabs, *S. aureus* was isolated in 280 (41.79%) and Coagulase negative *Staphylococci* (CONS) in 353 (52.68%) swabs. Other organisms were isolated in 23 (3.43%) nasal swabs and there was no any growth in 14 (2.08%) swabs (Table 1). Of 280 *S. aureus* strains, 61(21.78%) strains were MRSA. Over all, MRSA nasal carriage rate was 9.10 % in our study.

Table 1: Nature of organisms isolated from 670 nasal swabs

Isolates	**Number (%)**
Staphylococcus aureus	280 (41.79%)
Coagulase negative Staphylococcus (CONS)	353 (52.68%)
Others	23
No growth	14
Total	**670**

In female HCWs the prevalence of the nasal *S. aureus* colonization rate was 214(76.42%) and MRSA carriage rate was 38(17.75%). In male HCWs the prevalence of the nasal *S. aureus* colonization rate was 66(23.57%) and MRSA carriage rate was 23(34.84%)) and that is statistically significant (Table 2).

Critical area wise total number of samples examined and the rate of colonization of *S. aureus*, MRSA carriage along with mupirocin resistance has been shown in Table 3 and that is statistically significant. The highest prevalence of *S. aureus* colonization was observed in SICU 39 (68.42%) followed by CCU 33 (60%) and PICU16 (59.2%) whereas the MRSA carriage rate was highest in MICU 14 (70%) followed by endoscopy 04 (57.1%) and COT 02 (33.3%).

In relation to the professional category, housekeeping staff 54 (80.59%) have presented the highest rate of colonization followed by Doctors 52 (44.06%) and the lowest rate of colonization was found in Nursing staff 174 (35.87%) which is statistically significant (Table 4). The MRSA carrier rate was highest among doctors16 (30.76%) followed by housekeeping staff 11 (20.37%) and lowest in nursing staff i.e. 34 (19.54 %) (Table 4) which is statistically not significant.

In our study among the 61 MRSA isolates, only one (1.63%) isolate from nursing staff of MICU showed both low level and high level Mupirocin resistance by disk diffusion method. We have confirmed the result with Etest and this test also showed the same results (Table 4).

We have noted that decolonization was achieved in 47 (77%) HCWs within seven days of application of Mupirocin twice daily regularly. In 13 (21.31 %) HCWs colonization persists even after seven days application of Mupirocin and decolonized thereafter by extending application for seven more days. One HCW who showed Mupirocin resistance there was no decolonization even fourteen days application of Mupirocin, advised to take alternative method of eradication.

Table 2: Gender wise rate of *S. aureus* colonization, MRSA carrier and Mupirocin resistance (MuH and MuL)

Gender	Total Number	*S. aureus* n=280 (41.79%)		MRSA n=61 (21.78%)		MuH and MuL resistance
Male	157	66 (23.57%)	Chi-square =8.52 p=0.04	23 (34.84%)	Chi-square =8.65 p=0.03	00
Female	513	214 (76.42%)		38 (17.75%)		01 (2.12%)
Total	670	280 (41.79%)		61		

Methicillin resistant Staphylococcus aureus: MRSA, Mupirocin high level resistance: MuH, Mupirocin low level resistance: MuL

Table 3: Critical area wise rate of *S. aureus*, MRSA and Mupirocin resistance (MuH and MuL)

Area	Total Number of samples tested	Prevalence of *S. aureus*	p	Prevalence of MRSA	p	MuH and MuL resistance
EICU	50	27 (54%)	Chi-square =19.4 p=0.013	04 (14.3%)	Chi-square =17.1 p=0.029	
RGY ICU	23	11 (47.82%)		02 (18.1%)		
CCU	55	**33 (60%)**		02 (6.0%)		
PICU	27	**16 (59.2%)**		03 (18.7%)		
NICU	58	32 (55.17%)		03 (9.3%)		
SICU	57	**39 (68.42%)**		08 (20.5%)		
OBGY ICU and LR	91	36 (39.56%)		11 (30.5%)		
KT ICU and Dialysis	42	25 (59.52%)		03 (12%)		
Endoscopy	22	07 (31.81%)		**04 (57.1%)**		
OT General	117	28 (23.93%)		05 (17.8%)		
COT	59	06 (10.16%)		**02 (33.3%)**		
MICU	69	20 (28.98%)		**14 (70%)**		**0191.63%)**
Total	**670**	**280**		**61**		**01**

Emergency ICU: EICU), Rajiv Gandhi Yojna ICU: RGY ICU, Cardiac Care Unit: CCU, Paediatric ICU: PICU, Neonate ICU: NICU, Surgical ICU: SICU, Obstretic & Gynec ICU: OBGY ICU, Labour Room: LR), Kidney Transplant ICU: KT ICU, Operation theatre: OT, Cardiac OT: COT, Medical ICU: MICU

Table 3: Category wise rate of *S. aureus*, MRSA carriage status and high, low level Mupirocin resistance (MuH and MuL)

Category	Total No of Nasal Swabs	*S. aureus* Grown		MRSA positive		MuH and MuL resistance
Doctor	118	52 (44.06%)	Chi-square =48.7 p<0.0001	16 (30.76%)	Chi-square =3.04 p=0.219	
Nursing staff	485	174 (35.87%)		34 (19.54%)		01
Housekeeping	67	54 (80.59%)		11 (20.37%)		
Total	670	280 (41.7%)		61 (21.78%)		01 (1.63%)

Methicillin resistant Staphylococcus aureus: MRSA, Mupirocin high level resistance: MuH, Mupirocin low level resistance: MuL

Figure 1: Demonstrating resistance of *S. aureus* to Cifoxitin 30 µg Mupirocin 5 µg and 200 µg

Figure 2: Demonstrating low and high levels of Mupirocin resistance in MRSA

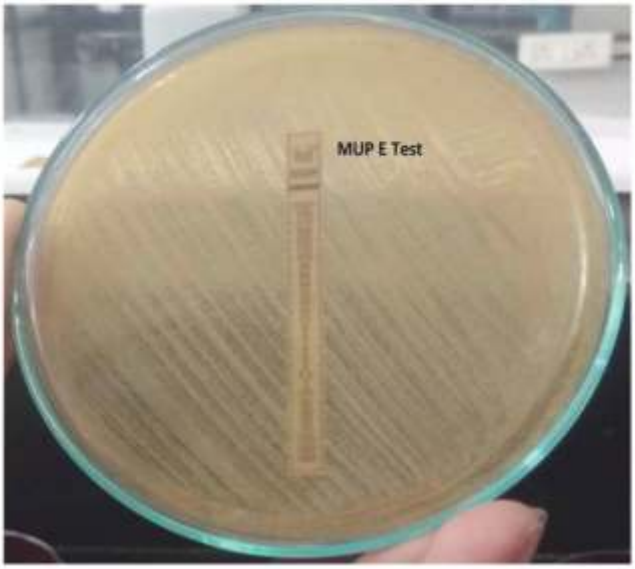

Figure 3: Mupirocin E test showing resistance to Mupirocin

Discussion

Methicillin-resistant *S. aureus* is one of the most common nosocomial pathogen. The main sources of MRSA in the hospital environment are the colonized patients and HCWs who are asymptomatic and they may serve as reservoir and disseminator of MRSA in hospitals. MRSA infections lead to prolong hospital stay, and increase in treatment cost.

In present study the rate of nasal carriage of *S. aureus* in HCWs was 280 (41.79%) which was higher than 14.44% in the studies conducted by Moghadam *et al*. [9], 14.28% by Kaur and Pandey [10], 17.5% in Radhakrishna *et al*. [3], and has been reported as 27.5 % and 28.91% by various authors [14-15]. The results of present study are comparable with the studies 48% and 47.5% conducted by Agrawal *et al*. [2] and AlAbdli and Baiu [14] respectively. Sing *et al*. have detected colonization at two different sites ,in nasal swab only it was 28.91%, in hand swab only it was 9.63% and both nasal swab and hand swab 7.22% total colonization rate was 45.7% [15].

Among 280 *S. aureus* methicillin resistance were seen in 61 isolates. Thus 21.78% of the HCWs were harboring the MRSA. Our results are in accordance with the study conducted by AlAbdli and Baiu (21.4%) [14]. Little lower rates were reported by the studies conducted in different hospital setting worldwide which has been reported in the range of 9.7% to 14.28% [2, 10, 14-15]. Higher percentage of MRSA carriage has been reported by Moghadam *et al*. 43.58% [9]. This difference in rate of nasal carriage of *S. aureus* and MRSA in various hospitals may be due to difference in effectiveness of hospital infection control measures.

In the present study, in relation to the professional category, housekeeping staff (80.59%) have presented the highest rate of nasal carriage of *S. aureus* followed by doctors (44.06 %) and the lowest rate of colonization was found in nursing staff (35.87%). Radhakrishna *et al*. [3] reported MRSA carriage rate 13.3% in housekeeping staff and 2.7% in nursing staff. In majority of studies the colonization rate was higher amongst the nursing staff, followed by housekeeping staff and then in doctors [2, 3, 10].

MRSA carriage rate was particularly high among the doctors 16 (30.76%) which was similar with the findings of Al Abdli and Baiu (30.6%) [14]. In our study, only one case (1.63%) of Mupirocin resistance (MuH and MuL) was reported. Kaur and Pandey [10] reported 1.43 % and Agarwal *et al*. [2] reported 2% Mupirocin resistance. Solmaz *et al*. [9] reported 1.85% Mupirocin resistance with one MRSA strain showed high level mupirocin resistance.

Conclusion

The present study showed a high prevalence of nasal carriage of MRSA among health care workers, these HCWs can be the source of MRSA infections. Therefore we suggest MRSA screening of HCWs as a routine practice. We also insist on mupirocin resistance detection so that in case if resistance detected alternative treatment can be given.

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Author for Correspondence:
Dr. Smita S. Kulkarni, Department of Microbiology, MGM Medical College and Hospital, Aurangabad-431003, Maharashtra, India
Email: gsmita.85@gmail.com, Cell: 9272548907

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The association of mucormycosis co-infection in patients with COVID-19 pneumonia: experience at tertiary care hospital in India

L.B. Dagdia[1], S.U. Joshi[1], P.S. Joshi[2], A.R. Patil[1], A.D. Kulkarni[1]

[1]Department of Orthopedics, [2]Department of Anaesthesiology, MGM Medical College and Hospital, Aurangabad, India

ABSTRACT:

Objective: We performed this study to explore the impact of multiple co-morbidities, different treatment strategies and vaccination in patients diagnosed with mucormycosis co-infection during the ongoing COVID-19 pandemic.

Patients and methods: This is an observational study of 60 patients out of 3000 admitted from March 2021 to May 2021 for treatment of COVID-19 pneumonia, with confirmed diagnosis of opportunistic fungal infection. Characteristics like age, antibiotic usage, steroid usage, and associated co-morbidities, need of oxygen or ventilator support and status of vaccination were studied.

Results: Out of 60 patients studied, maximum 37 (61.6%) belonged to 40 to 60 years age group and 38 (63.3%) were male. Fifty-two (86.6%) patients had one or other co-morbidities, while 56 (93.3%) of these patients received steroids in oral or intravenous form. Fifty-one (85%) patients received one or more than one higher grade antibiotics during treatment in hospital. Forty-two (70%) patients required Intensive Care Unit (ICU) admission out of which 4 (6.7%) required ventilator support, 10 (16.6%) required Non-Invasive Ventilation (NIV) while 28 (46.6%) were managed with high flow oxygen.

Conclusions: Our observations suggest for judicious use of steroids and higher antibiotics during treatment of COVID-19 pneumonia as it is associated with increased risk of opportunistic fungal infections. Strict control of blood glucose levels, multidisciplinary approach to reduce the impact of opportunistic fungal infection on patient morbidity and widespread vaccination especially among patients with co-morbidities will help in mitigating the impact of opportunistic fungal infections in patients with COVID-19 pneumonia.

Keywords: COVID-19, Mucormycosis, Fungal osteomyelitis prevention, Treatment.

INTRODUCTION

Around the end of year 2019, a number of patients with symptoms of pneumonia of unknown cause were detected in Wuhan, China. A novel coronavirus was identified as the causative pathogen, provisionally named as 2019 novel coronavirus (2019-nCoV) by the World Health Organization (WHO)[1,2]. Within two years this virus has spread from China to the whole world affecting more than 150 countries across all continents and causing morbidity and mortality across all age groups. This human-to-human transmitted disease, coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has been an emerging global public health event.

SARS-CoV-2 is mainly a lower respiratory tract infection causing Acute Respiratory Distress Syndromes (ARDS)[3]. In addition to widespread alveolar damage

Corresponding Author: Laxmikant Babulal Dagdia, MD; e-mail: dr.ldagdia@gmail.com

and inflammatory exudation, COVID-19 patients also develop immunosuppression due to a reduction in CD4 T and CD8 T cells. Such patients turn critical rapidly and require intensive care unit (ICU) admission along with mechanical ventilation[4]. These patients stand a very high risk of developing fungal co-infections. Many studies[5-7] have demonstrated multiple fungal co-infections like *Aspergillus flavus*, *A. fumigates*, *Candida albicans*, *C. Grabrata* in COVID-19 patients.

Mucormycosis is a type of opportunistic fungal infection caused by micro-organisms belonging to the phylum glomeromycota. Once a rare fungal infection, it is now seen as emerging threat in the wake of increased incidence of opportunistic fungal infections in COVID-19 patients[8]. Mucormycoses are life-threatening fungal infections mostly affecting diabetic, patients on immunosuppressant and solid organ recipients. Mucormycosis infection is characterised by infarction and necrosis of host tissues that results from invasion of vasculature by hyphae. Mucormycosis is most commonly present as rhino-orbito-cerebral and pulmonary infection[8,9]. In this short period of time, no studies have been conducted that determine the incidence of mucormycosis infections in COVID-19 patients and also the causative factors leading to a sudden increase in incidence[10,11]. Hence, we performed a study in the Indian population where the caseload of COVID-19 infections is extremely high.Our aim was to calculate the incidence of mucormycosis co-infection in patients suffering from COVID-19 pneumonia by finding the risk factors associated with increased incidence of mucormycosis co-infection in COVID-19 pneumonia, to determine the effectiveness of current treatment protocol of mucormycosis co-infection and to determine whether COVID-19 vaccination is effective in preventing fungal co-infections.

PATIENTS AND METHODS

This observational descriptive type of study was carried out over a period of three months from March 2021 to May 2021 and the patients admitted in Mahatma Gandhi Mission Hospital and Medical College, Aurangabad, Maharashtra for treatment of COVID-19 pneumonia were included in our study.

The inclusion criteria for our study were patients with confirmed diagnosis of COVID-19 pneumonia by RT-PCR test with testing device INSTA Q 9 (Equipment Number: ML01 – manufactured by Himeda Serial Number HN550988). Patients with proven diagnosis of fungal co-infection on laboratory tests (potassium hydro-oxide KOH mount of scrapping from infected tissue).

Exclusion criteria for our study were patients with history of fungal infection in the past and patients with fungal infection but not associated with COVID-19 infection.

A total of 3000 patients with confirmed diagnosis of COVID-19 pneumonia were admitted from March 2021 to May 2021. Amongst these patients those developing clinical symptoms of fungal infection and proven as mucormycosis infection on direct examination in 10% potassium hydro-oxide (KOH) of sample from scrapping of infected tissue, histopathology and culture reports were studied. A total of 60 patients were diagnosed with mucormycosis co-infection over a period of 3 months and these patients were followed up regularly throughout their course of disease.

When the patient first arrived in the fever clinic of our hospital (during COVID-19 pandemic special fever clinic and emergency section were established in our hospital campus to segregate patients with acute onset high grade fever with/without breathing difficulty from other emergency patients) an exhaustive history was taken regarding the type, severity and duration of symptoms. Specific information was obtained regarding the presence of co-morbid conditions, its duration and the type of treatment that is being carried out. A thorough general and system specific examination was then carried out with special attention towards the respiratory system for severity of symptoms. As soon as the patient was admitted a nasal swab was sent for RT-PCR which detects the spike gene and the N gene on viral RNA and is considered gold standard for diagnosing the presence of COVID-19 pneumonia[12]. Apart from this a battery of laboratory and radiological investigations comprising of Complete blood count, Renal Function Test, Liver Function Test, Serum Electrolytes, CReactive Protein, Serum ferritin, Arterial blood analysis, Erythrocyte sedimentation rate,X-ray chest, High resolution computed tomography of chest were done to assess severity of the disease and plan an appropriate course of action for the same. Patients who developed symptoms of fungal co-infection in addition to above investigations also underwent tests like direct microscopy of KOH mounted samples taken from specific sites of suspected infection, fungal cultures for detection of causative organism and magnetic resonance imaging of the brain, orbit or paranasal sinuses to evaluate of extent of disease.

Patients developing mucormycosis after admission for COVID-19 pneumonia had symptoms of lid edema and soft tissue swellings along the para nasal sinuses. Severe cases present with orbital cellulitis, para nasal sinusitis with osteomyelitic changes or neurological symptoms if the infection spreads to the brain. Mucormycosis was detected on nasal and conjunctival swabs subjected to direct microscopy and fungal cultures. MRI of the brain as well as orbit and para nasal sinuses gave an idea about the extent of spread of infection.

The treatment protocols for COVID-19 pneumonia are not well documented but the basic regime followed in our hospital included supportive treatment including intravenous fluids and oxygen support. According to the severity of the symptoms patients were started on oral or intravenous steroids, as well as antiviral drugs like Remdesevir with dosage – Day 1: Inj. Remdesivir 200 mg in 100 ml NS IV OD, Day 2 to 5: Inj. Remdesivir 100 mg in 100 ml NS IV OD. As a cover to protect the patients from secondary bacterial infections broad spectrum antibiotics and higher antibiotics like Meropenem (Inj. Meromac 500 mg IV in 100 cc NS IV BD), Tigecycline (Inj. Teganex 100 mg IV od followed by Inj.

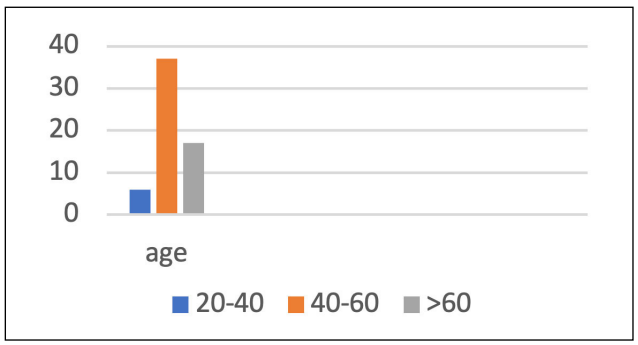

Figure 1. Age wise incidence of fungal osteomyelitis.

Teganex 50 mg BD) and Piperacillin tazobactum (In. Piptaz 4.5 gm iv TDS) were given. Enoxaparin (Inj. Clexane 0.4 cc or 0.6 SC HS) and other anti-thrombotic agents (Tab. Ecosprin 75 mg or 150 mg HS) were given to the patients to prevent life-threatening thrombotic events. In cases of fungal co-infections patients were started on antifungal like Amphoterecin B-Inj. Liposomal Amphotericin 5 amp 250 mg in 250 ml D5 IV OD for 21 days or Inj. Amphotrate (1 amp) 150 mg in 250 ml D5 OD for 21 days under all photosensitivity precautions and Posaconazole-Tab. Posaconazole 300 mg OD for 3 months. Surgery for the infected paranasal sinuses and orbital cellulitis was reserved for cases not responding to medical treatment or as a salvage procedure.

RESULTS

There were a total of 3000 patients admitted in our hospital for COVID-19 pneumonia out of which 60 patients suffered from Mucormycosis within a time period of 3 months with an incidence of 2%. Among these 60 patients there were 6 (10%) patients in the age group of 20-40, 37 (61.6%) patients belonged to the age group of 40-60 and17 (28.3%) patients above the age of 60 years who suffered from mucormycosis (Figure 1). Total 38 (63.3%) patients were male and 22 (36.7%) were female with a male to female ratio of 1.7:1 (Figure 2).

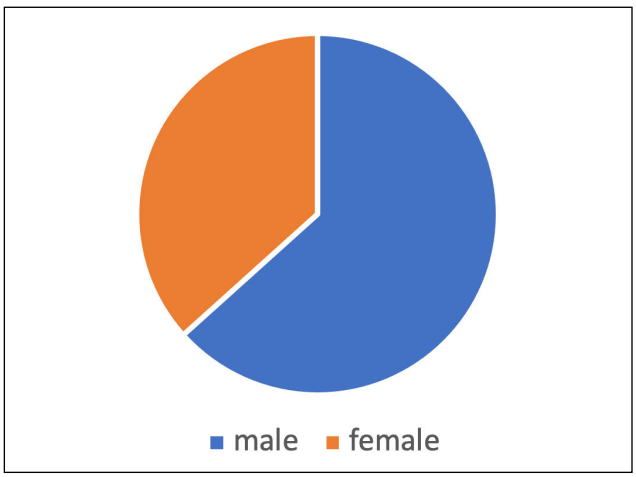

Figure 2. Sex wise incidence of fungal osteomyelitis.

From a total of 60 patients who suffered from fungal infections, 52 (86.6%) patients had presence of co-morbidities. Amongst these, diabetes mellitus was the most common co-morbidity seen in 34 (65.3%) patients with mucormycosis infections, followed by hypertension seen in 19 (36.5%) patients (Figure 3). There were also 14 (26.9%) patients who suffered from a combination of co-morbidities.

Steroids were one of the first line drugs used to counter the inflammatory response of the body to COVID-19 pneumonia and were administered either orally or intravenously in 56 (93.3%) of the 60 patients suffering from mucormycosis (Figure 4). Most of these patients received steroids for more than 5 days amongst which 8 patients consumed oral dexamethasone while 48 patients were administered IV methyl prednisolone.

The viral pneumonia affecting the lungs increased the susceptibility of patients to various super added bacterial infections. These infections were treated using both broad spectrum and higher antibiotics. Amongst the 60 patients maximum, 25 (41.66%) were treated with high end antibiotics like Inj piperacillin tazobactum, Inj meropenem in 9 (15%) patients and Inj tigecycline in 2 (3.33%) patients (Figure 5). Broad spectrum antibiotics like ceftriaxone, doxycycline or azithromycin were

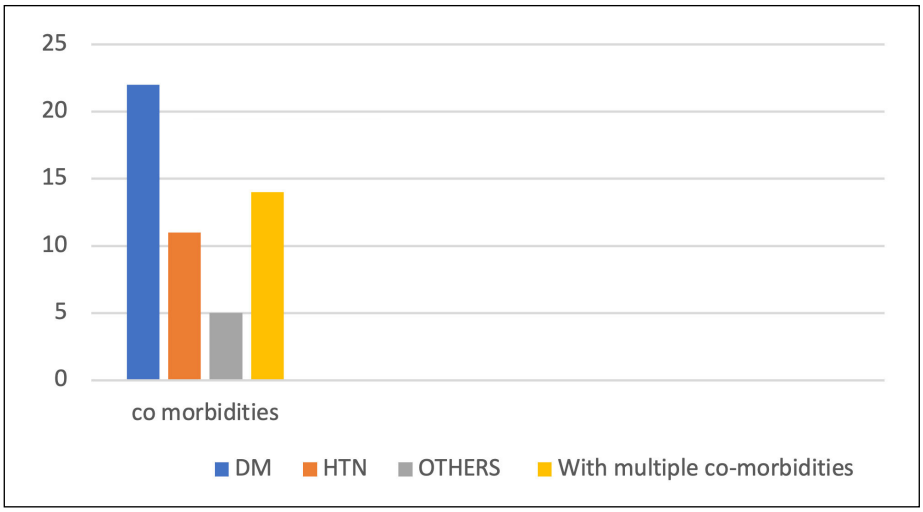

Figure 3. Incidence of co-morbidities in patients with mucormycosis.

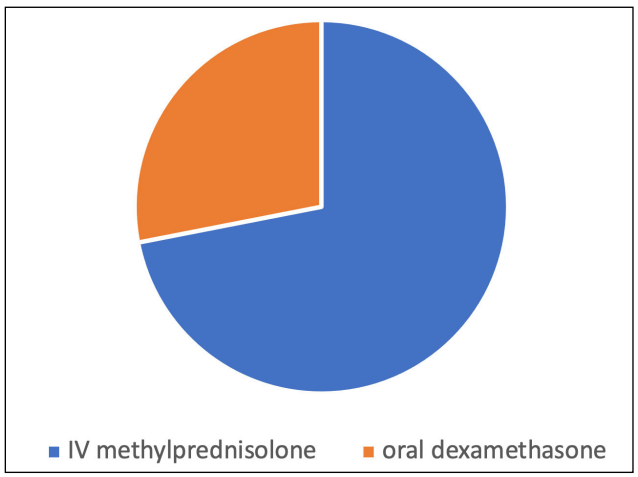

Figure 4. Steroid use.

used in 17 (28.33%) patients. Seven (11.66%) patients received a combination of above antibiotics.

In our study amongst 60 patients who suffered from COVID-19 pneumonia, 42 (70%) patients required ICU admission at some point in their course of disease. Four (6.7%) patients had to be put on ventilator support, 10 (16.6%) patients required non-invasive ventilation and 28 (46.6%) patients needed high flow oxygen with canula or reservoir bag (Figure 6). The rest 18 (30%) patients were treated in ward with intermittent need for O_2 support.

The role of vaccines in preventing COVID-19 infections has not yet been proven but studies suggest that previously vaccinated individuals are more likely to suffer from a mild illness without any serious complications. An observation was made that from the 60 patients who suffered from Mucormycosis only 9 patients had taken at least one dose of COVID-19 vaccine before suffering from the disease and amongst these only 3 patients required intensive care with others being managed in the ward on intermittent oxygen support.

Out of 60 patients in our study 9 (15%) patients died during course of follow-up, 4 (6.66%) patients required re-exploration surgery for residual infection, while 47 (78.33%) patients had an uneventful recovery at 3 months follow-up.

Mucormycosis occurring as a result of COVID-19 infection mainly affected the face with the nasal sinuses being the most common site of fungal infection seen in 36(60%) patients followed by orbit in 9 patients (15%) and brain in 6 (10%) patients (Figure 7). Nine (15%) patients presented with fungal infections in more than one site, the orbit and para nasal sinuses being the most common sites.

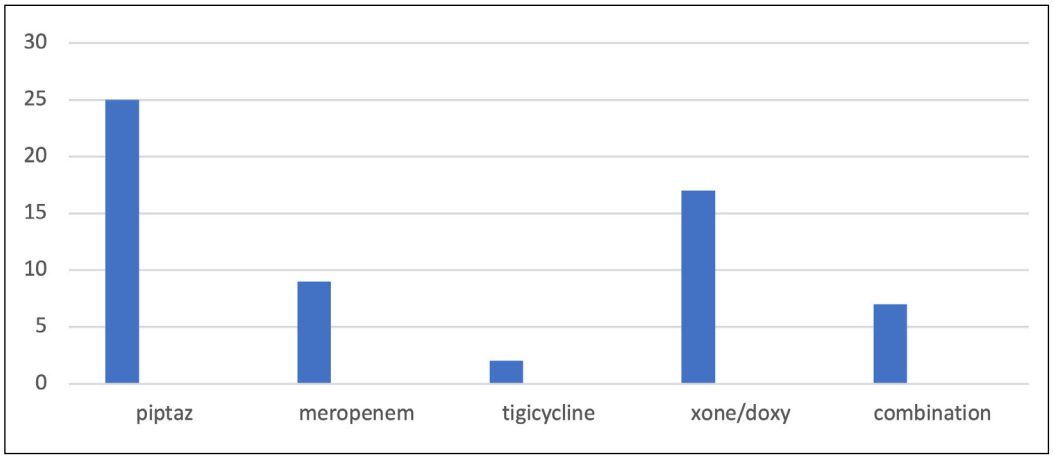

Figure 5. Antibiotics used.

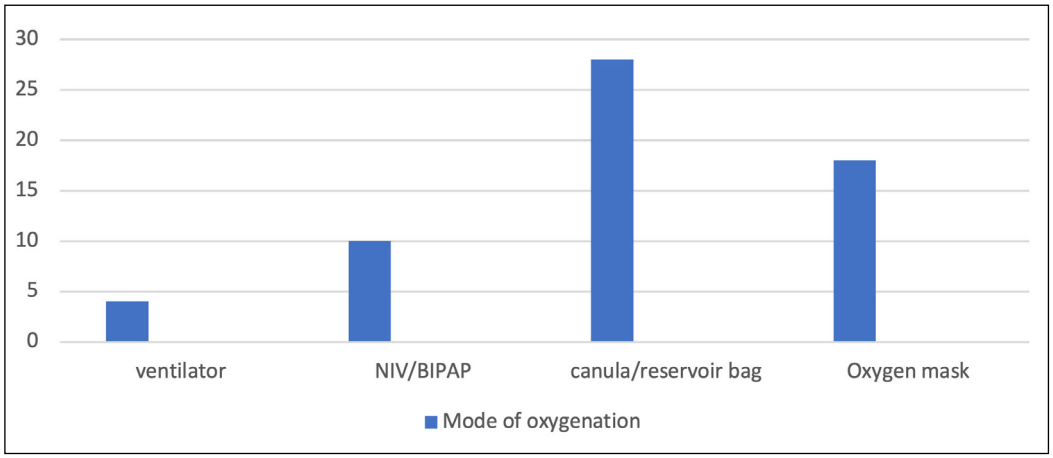

Figure 6. Oxygen support.

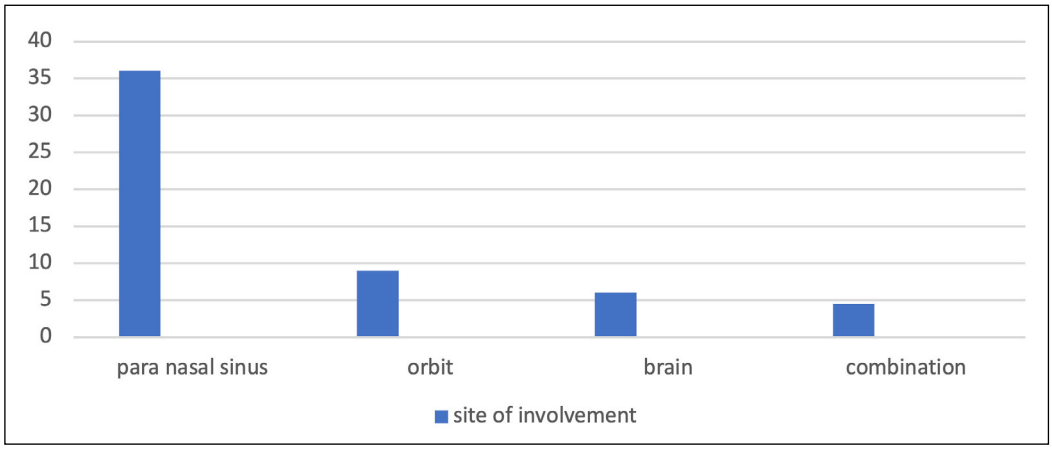

Figure 7. Site of occurrence of mucormycosis.

DISCUSSION

Mucormycosis is a deadly opportunistic fungal infection caused by fungus originating from mucorales order and includes Mucor, *Rhiopus*, *Rhizomucor*, *Abdidia*, *Apophysomyces* and *Cunninghumella*. Fungal spores enter *via* inhalation and then reach up to paranasal sinuses. Spores may also be acquired by contaminated food ingestion. Affected individuals usually present with acute sinusitis, fever, nasal congestion, purulent nasal discharge and headache[16]. If not treated early, contiguous spread to adjacent structures may occur, resulting in various clinical symptoms[16]. The orbital cavity is accessible through the ethmoid bone *via* the lamina papyracea, infratemporal fossa, inferior orbital fissure or orbital apex. Contiguous intracranial extension can occur through the ethmoid cribriform plate, supraorbital fissure and perineural routes[17]. Cavernous sinus or sagittal sinus thrombosis, carotid occlusion, cerebral infarction, intracranial aneurysm, intracranial haemorrhage and cerebral abscesses are potential *sequelae*[17-24].

In our study conducted over a period of 3 months there were a total of 3000 patients admitted in our hospital from which 60 patients developed Mucormycosis as a complication with an incidence of 2%. Jeong et al[14] in their study found an incidence a rate of 0.005-1.7 per million population globally. Alanio et al[25] screened 135 adults with COVID-19 infection and reported an incidence of invasive fungal infections of 26.7%. Patients with invasive fungal diseases had higher mortality (53% with *vs.* 31% without), which was significantly reduced by appropriate therapy. Corticosteroid therapy and a past history of chronic pulmonary disease were associated with a higher risk of invasive fungal disease[25]. Similarly, high incidences have been observed in Pakistan (23/147, 15.6%) and Italy (30/108, 27.7%), and with the authors suggesting that the development of invasive fungal infections alters the natural history of the disease[26,27].

In our study the para nasal sinuses were the most common site of affection for the fungal spores followed by the orbit and then brain. Similar results were observed in studies conducted by Selarka et al[28] where the most common site was rhino-cerebro-orbital (44%-49%), followed by cutaneous (10%-19%), pulmonary (10%-11%), disseminated (6%-11%) and gastrointestinal (2%-11%).

Mucormycosis is known to affect immunocompromised patients especially those with diabetes mellitus, prolonged corticosteroid use, solid organ transplant recipients, neutropenia and haematological malignancies[29-31]. The overall immunity of the patient suffering from COVID-19 infection has been observed to decline due to a decrease in CD4 and CD8 counts which is further aggravated by medical co-morbidities such as diabetes mellitus, hypertension and bronchial asthma. Diabetes mellitus is known to cause microangiopathy reducing tissue perfusion[13-15]. So, the deadly triad of diabetes mellitus, rampant use of steroids in the background of COVID-19 infection appears to increase risk of mucormycosis. All efforts should be made to maintain optimum glucose levels along with judicious use of steroids in COVID-19 treatment. In our study 52 patients were suffering from one or more co-morbidities with diabetes mellitus being the most common, 34 (56.66%) patients playing a major role in the severity of infection. In a cohort study presented by Erener et al[32] amongst patients diagnosed with COVID-19 pneumonia and mucormycosis, about three-quarters had a pre-existing history of diabetes mellitus along with a poor glycaemia control at presentation. The excessive use of broad spectrum antibiotics and immunosuppressive agents such as steroids and Remdesevir has also adversely affected the immunity of the individual. In our study, almost 93% of the patients suffering from Mucormycosis had received steroids for more than 5 days and almost all the patients had received complete courses of higher end antibiotics and Remdesivir to tackle the COVID-19 infection, all laying foundation for opportunistic infections like Mucormycosis. In addition, COVID-19 patients were more prone to develop secondary infections if they had decompensated pulmonary functions or required invasive mechanical ventilation. Our study showed that 42 patients required ICU admission with half of them requiring either ventilatory or non-ventilatory support of oxygen which was similar to studies conducted by Sharma et al[17] showing 82% of their study population required large amounts of oxygen through ventilator support.

The role of vaccination in preventing COVID-19 infection is still debatable but observations from our study show that Mucormycosis was fairly more common in individuals who had not received any previous dose of vaccinations and the severity of infection was comparatively lesser in those patients that had been vaccinated previously.

LIMITATIONS

Some limitations in our study were that the data represented the experience of loading in a single tertiary care centre, which often treat most of the sick patients with severe complications. Thus, the data may not be generalisable. Second, we could not perform blood investigations in all study participants due to lack of affordability by the patients, as well as limited availability of test kits among rapidly rising cases of COVID-19 patients. Third, a case series of 60 patients might be considered a small sample size and various associations could not be evaluated. However, given the rarity of the disease, it still accounts for a large case series. In fact, according to the published literature, 101 cases of mucormycosis in patients with COVID-19 have been reported so far, of which 82 cases belong to India[30]. Lastly, being an observational study, there is no control group to evaluate reliable differences and association.

CONCLUSIONS

The incidence of mucormycosis in the COVID-19 pandemic is likely to increase and can result in significant morbidity and mortality. While treating COVID-19 patients, we should have a high index of suspicion of mucormycosis especially when corticosteroids are used during the course of disease. Optimised glycaemic control should be achieved to control mucormycosis. Comprehensive monitoring of blood sugar levels on daily basis should be encouraged. Use of antifungal therapy with surgical debridement of affected tissue together should be undertaken and it remains the mainstay of treatment. Precautions need to be practised with regard to the widespread usage of corticosteroids and broad-spectrum antibiotics, with an emphasis to administer corticosteroids only in severe COVID-19 pneumonia and to reduce super-infections. Excessive use of corticosteroids should be restricted. A multidisciplinary approach involving an intensivist, diabetologist, otolaryngologist, ophthalmologist, infectious diseases specialist, neurologist and/or neurosurgeon is needed for the management of mucormycosis. An accelerated COVID-19 vaccination programme should be the highest priority in a country with high prevalence of diabetes and relatively poor resources to avoid massive outbreaks, morbidity and mortality during the current pandemic.

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The authors declare that they have no conflict of interests.

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ORCID ID:
Dr. LB Dagdia – 0000-0003-4990-6180
Dr. AR Patil – 0000-0001-9748-4029
Dr. AD Kulkarni – 0000-0002-2860-5103
Dr. SU Joshi – 0000-0001-6761-6684

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Case Report

Peripartum Sepsis Induced Thrombotic Microangiopathic Hemolytic Anemia: A case report with clinical dissection

Talib SH[1]*, Amjad SA[2], Sachin P[2], Pranita B[2]

[1]Department of Medicine, MGM Medical College, Aurangabad, India, [2]Department of Medicine, Resident in Medicine, Aurangabad, India

Corresponding author:
Dr. S.H. Talib,
Professor Emeritus,
Department of Medicine,
MGM Medical College,
Aurangabad, India,
E-mail: sftalib@gmail.com

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Abstract

A case of microangiopathic hemolytic anemia with peripartum TTP in a 24 yr old female patient who developed E.coli sepsis presented with unusual features of leukemoid reaction, anemia and thrombocytopenia. TTP was suspected on finding 3-4/hpf of schistocytes on peripheral blood smear. The importance of peripheral examination for schistocytes in peripartum period for evidence of Microangiopathic Hemolytic Anemia (MAHA) is discussed and described.

Keywords: MAHA; TTP; Schistocytes; Peripartum; *E. coli*; Sepsis

Introduction

A 24 yr old girl presented to the hospital for full term vaginal delivery. She denied history of hypertension, diabetes and tobacco chewing, alcohol or use of any illicit drugs or any medical illness. She had visited the hospital couple of times for ANC checkups and for bilateral pedal edema. The systemic examination was unrevealing. Neurological examination was unremarkable. She was hospitalized as a case of pre-eclampsia with blood pressure was 140/80 mm Hg with mild to moderate pedal edema and puffiness of face. Documents revealed hemoglobin 10.5 g %, TLC 11450/cumm, platelets 107000/cumm, normal kidney function, HbA1c 5.2%, urine albumin 1+, serum sodium and potassium within normal range.

Normal vaginal delivery with episiotomy undertaken. A baby girl of 2.7 kg was delivered. Her condition was described healthy. The patient was discharged on 6th day of hospitalization with advice to take supplemental medications. At the time of discharge, her Hb was 8 g %, platelets counts was 91000/cumm, electrolytes and kidney function were normal within limits. Details of investigation are shown in Table 1.

Two days later post discharged, she was rehospitalized with high grade fever with chills. The patient was toxic, drowsy, but responsive and arousable to deep pain stimulation. Glassgow coma scale was 8. Her pulse rate was 112/min regular low volume, respiration rate was 40/min, SPO2 70% on room air, BP was 90/60 mm Hg, temperature was 101 F. Patient had no meningeal signs, petechial hemorrhages or any rashes over the body.

On examination, the patient had generalized edema with moderate pedal edema along with signs of failure (Pro BNP of >35000 pg/dl). She had signs of dehydration with decreased urine output. X-ray chest was normal. Her PV examination revealed a large abcess at incisional site of episiotomy, incised and 200 ml of pus drained. Blood culture and pus culture were sent. The patient was intubated on emergency basis and was put on ionotropic support and Piperacillin-Tazobactam provided initially and later meropenem as per sensitivity.

Laboratory examination at this stage revealed urine albumin-3+ (300 mg/dl), urea-313 mg/dl, creatinine-9.1 mg/dl, Na+-124 meq/dl, K+-7.3meq/dl, Hb-7.1 g %, TLC-34460/cumm, Platelet-106000/cumm, serum bilirubin (total) -1.6 mg/dl, direct-1.5 mg/dl, SGOT-61U/L, SGPT-47U/L, ALP-110U/L, lactate-9 mmol/L, procalcitonin 22.3. Urine output was nil suggestive of acute kidney injury with sepsis.

Pus culture and blood culture showed the presence of E.coli. The peripheral blood smear showed presence of schistocytes 3-4/hpf (Figure 1). Other laboratory parameters revealed depleted complement levels of C3, C4, decreased haptoglobin levels,

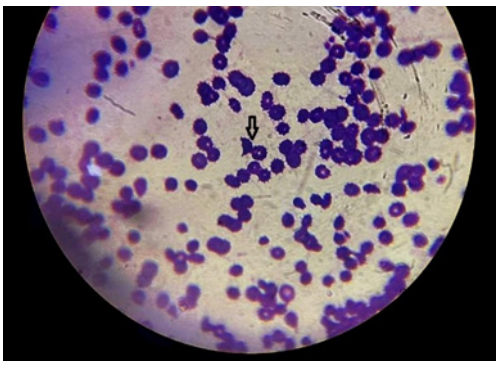

Figure 1: Legends to figure I-Peripheral smear showing presence of Schistocytes (black arrow).

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Table 1: Pre and Post labor investigations undertaken during hospitalization.

Sr. No.	Investigation	02-09-2021 Pre Labour (Before Delivery)	16-09-2021 At Delivery	19-09-2021 Post-delivery on Discharge	25-09-2021 Peripartum Re-hospitalization	28-09-2021 to 13-10-2021	15-10-2021 Investigation done a day prior to death
1	Hb g% (N=11-13g%)	10.4	7.08	8	7	07-Apr	6.3
2	TLC /cumm (N=3500-9,000)	11450	12560	11180	34460	47870 -11610	8590
3	Platelet /cumm (N=165000-415000)	107000	85000	91000	1,06,000	11,9000 -35000	65000
4	PS (SCHISTOCYTES)	Not Seen	Not Reported	Not Done	2-3 /hpf	2-3/hpf	3-4/hpf
5	Blood urea (N=7-20 mg/dl)	17	17	18	303	270-125	169
6	Serum Creatinine (N=0.5-1.2 mg%)	0.9	0.5	6	9.1	7-2.6	4.8
7	Serum .Na^+ (136-146meq/L)	139	139	140	124	127-134	135
8	Serum .K^+ (3.5-5.5meq/L)	3.6	4	5.2	7.3	5.9-3.5	3
9	LDH (140-280 mg/dl)	201	293	220		894-450	
10	PT/INR (N 10 tO 13.5)	10.20/0.85	10.5 /0.9	106 /1.0	10.6/088	10.8 / 0.9	10.9 / 1.12
11	Serum Fibrinogen (N=200-400mg/dl)	Not Done				280mg/dl	
12	Urine albumin	Nil	+	+	+++	++	
13	Urine sugar	Nil	NIL	NIL	Nil	Nil	
14	HbA1C (5.7%-6.2%)	5.2	5.6		5.7	6	
15	Serum Bilirubin Direct (0.6-1.2 mg/dl)	0.4	1.5		1.9	0.9	
17	LFT enzymes	WNL	WNL		WNL	WNL	
19	Total protein (6.7-8.8 mg/dl)	6.1	5.5	5.8	5.8	5.0 -6.4	
20	Serum Albumin (3.5-5.5 mg/dl)	3.8	2.5	2.7	2.7	1.9 – 2.6	
22	Blood Culture				E.coli		
23	Pus Culture (episiotomy site)				E.coli		
23	C3 (N=80-178 mg/dl)				65		
24	C4 (N=12-42 mg/dl)				10		
25	Coombs Test Direct And Indirect				Negative		
26	Haptoglobulin (N=41-165 mg/dl)				20		
27	Lactate (N=0.5-1.6 mmol/L)				9	7 to 0.8	
28	Procalcitonin (N=<0.1 ng/dl)				22.3		
29	Pro BNP (age adjusted) (N<125 pg/dl)				>35000		

increased LDH, negative direct and indirect Coomb's test.

Emergency hemodialysis was initiated. She received multiple cycles (11) of hemodialysis. Multiple PCV transfusions were provided for her declining Hb.

During hospitalization 28/09/2021 to 13/10/2021, her Hb declined 7 g % to 4 g %, platelet counts declined seen from 119000 up to 35000, other investigations are shown in Table 1.

The calculated Plasmin score was 6, the value is considered as a high risk group.

Clinical and laboratory findings supported evidence of septic thrombotic MAHA with AKI. Plasmapheresis in this sepsis induced thrombotic microangiopathy case was advised and during treatment with plasmapheresis, the patient developed fatal cardiac arrhythmia, despite resuscitative measures patient succumbed to death.

Discussion

We describe our initial approach of thrombocytopenia associated with pregnancy that deteriorated during peri-partum period. The progression of the patient to pre-eclampsia (hypertension,

edema feet with albuminuria) were present. The features were prominent at term and progressed near delivery. The peripheral Smear revealed schistocytes 3-4/hpf suggesting the entity of Thrombotic Microangiopathic Hemolytic Anemia (TMA). MAHA is considered in clinical settings when presence of demonstrable schistocytes in the peripheral blood film are seen.

The causes of thrombocytopenia in pregnancy varies with duration of gestation and clinical status. Gestational thrombocytopenia is a benign, self-limiting condition. It doesn't require additional evaluation of thrombocytopenia because it gets resolved within 6 weeks of post-partum period.

In the present case, thrombocytopenia worsened during pre-eclampsia. The patient condition deteriorated as she developed abscess at episiotomy site which was excised and drained. Immuno thrombocytopenia occurs in 1-3 cases in 100000 pregnancies with 10 fold greater incidence than general population. ITP is an another cause, and autoimmune condition where antiplatelet antibody interfere with platelet production and causes destruction of circulating platelets observed in any trimester. 10% of patients of ITP known to be associated with HUS/TTP [1]. Other possibilities associated with pre-eclampsia with severe features and HELLP syndrome may have hypertension, headache and visual abnormalities along with thrombocytopenia and such patients may also present with MAHA with schistocytes in peripheral smear, raised LDH and decreased haptoglobin levels. All such features may also be found in thrombotic microangiopathic syndrome such as TTP. The present case has evidence of hemolysis, low platelets, hypertension and proteinuria, but failed to reveal elevated liver enzymes which rules out possibility of HELLP syndrome in this case.

Pre-eclampsia is common, seen in approximately 5% of pregnancy which is present with new onset hypertension, proteinuria and/or end organ dysfunction. After 20 weeks of gestation, low platelet counts are observed in 7% of cases with severe thrombocytopenia in 3% of cases [2].

DIC is yet another cause related to this entity in which coagulation and fibrinolysis become activated within vasculature. Peripheral blood smear often show depleted platelets and crescented RBCs associated with depletion of protein factors and platelets leading to bleeding as well as risk of thrombosis. There may be MAHA with schistocytes of PBS with aPTT, PT prolongation, low fibrinogen and increase plasma D-dimer levels. In the present case, PBS show schistocytes, has not shown prolongation of PT, aPTT nor shown decrease in fibrinogen. Also, there was no evidence of bleeding diathesis. Hence, DIC possibility was not considered.

The possibility of Acute Fatty Liver of Pregnancy (AFLP) which is uncommon form of liver injury observed in third trimester. This possibility is ruled out because of presence of normal liver function tests and normal PT, aPTT.

The case under discussion has evidence of a septic focus observed in peripartum period at episiotomy site. The culture revealed the presence of E.coli. Many systemic infections, bacterial, viral and fungal are known to trigger MAHA and thrombocytopenia. However, our patient who was having thrombocytopenia in pregnancy and the said infection was combated with higher antibiotics. Though, improved clinically but had persistent thrombocytopenia ,and reduced urine output (AKI) needing multiple hemodialysis.

Primary TMA can have multiple presentations with rapid onset illness or gradual onset with minimal symptoms. May have anuria (AKI) or normal kidney function. Once thrombocytopenia and MAHA are confirmed, main goal remains to identify primary TMA. It is essential as specific treatment are available for TTP and complement mediated TMA. When complement mediated TMA suspected, anticomplement therapy like Eculizumab should be started within 24-48 hours to limit kidney injury. The possibility of TTP is high in this case as plasmic score is 6 that belongs to high risk group and 72% risk of deficiency of ADAMTS13 $\leq$ 15%. It is to be emphasized that schistocytes $\geq$ 2/hpf in consultation with clinical scenario is highly suggestive of MAHA [3,4].

The complements are implicated in etiology of TMA which has been classified as primary TMA when genetics and acquired defects are observed as primary derivatives. In the secondary TMA; infections, auto-immune disease and pregnancy are recognized as co-factors. This case has shown reduction in complement C3 and C4.This patient has evidence of E.coli infection at local site as well as found in blood culture, such infections imply granulocytes, cytokines, elastase which are produced in sepsis and enclave the factors metalloprotease with thrombospondin, reducing ADAMTS13 levels in sepsis. The mechanism may contribute in development of MAHA. Sepsis induced thrombotic microangiopathic hemolytic anemia are reported in western literature. However, there is scarcity of such cases in Indian literature [5].

Sepsis is a condition with very high mortality rates >20% and the systemic review and meta-analysis done in 2014, does not show any benefit of plasmapheresis in sepsis. However, other studies denoted the fruitful efficacy of plasmapheresis in sepsis and septic shock with acute renal failure demonstrated a fourfold increase in survival compared to historic controls [6,7]. Unfortunately, our patient who was in septic shock with acute renal failure succumbed to death while on plasmapheresis developed fatal ventricular arrhythmias.

Enterotoxigenic E.coli is considered grade 3C recommendation for plasmapheresis. Some serotypes of E.coli cause direct damage to kidney epithelial, mesangial and vascular endothelial cells causing clinical manifestations of thrombotic microangiopathies with acute kidney injuries in adults [8]. Other gram negative infections resulting in TMA may not extrapolate the same results with use of plasmapheresis. In Indian scenario, gram negative organisms induced TMA and benefits of plasmapheresis need more extended studies..

Conclusion

Peripartum sepsis induced thrombotic microangiopathic hemolytic anemia should be suspected in patients with especially gram negative sepsis when the case depicts rapidly falling platelets and hemoglobin. Importance should be attached to blood smear peripheral examination for detecting schistocytes. Repeated PBS examinations for suspecting of

MAHA is warranted. Timely consideration for plasmapheresis is beneficial in septic TMA.

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Case Report

Signal Recognition Particle (SRP) Positive Necrotizing Autoimmune Myopathy: A Case Report

Anand Soni[1*], Muktaram Chate[2], Sohel Khan[2], Sagar Jaiswal[2], Srabani Tripathy[2]

[1]Department of Neurology, Electromyography (EMG), Intravenous Immunoglobulin (IVIG), Aurangabad, Maharastra, India, [2]Department of Medicine, MGM Medical College and Hospital, Aurangabad, Maharashtra, India

Corresponding author:
Anand Soni, Department of Neurology, MGMIHS University, Aurangabad, Maharastra, India, E-mail: dranandsoni@gmail.com
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Abstract

Necrotizing Autoimmune Myopathy (NAM) is a sub-type of inflammatory myopathy which is characterized by acute or subacute onset progressive weakness of the proximal muscle of the body. Recognition of this subtype is important as prognosis varies with subtype. As for other myopathies, elevated CPK-total is hallmark. On the basis of histopathology differentiation is made from others subtypes of inflammatory myopathy. Most common antibodies associated with Necrotizing Autoimmune Myopathy (NAM) are Anti-Signal Recognition Particles (anti-SRP) and anti-3 Hydroxy- 3-Methylglutaryl-Coenzyme A Reductase (anti-HMGCR) antibodies. Patients with anti-SRP antibodies often present clinically with rapidly progressive proximal muscle weakness leading to significant disability. We are here presenting a clinical case of a patient with autoimmune necrotizing myopathy with positive anti-SRP autoantibodies and typical clinical presentation, who responded to treatment on diagnosis.

Keywords: Necrotizing Autoimmune Myopathy (NAM); Signal Recognition Particles (SRP); Electromyography (EMG); Intravenous Immunoglobulin (IVIG)

Introduction

Necrotizing Autoimmune Myopathy (NAM) also called Immune-Mediated Necrotizing Myopathy (IMNM) is a rare disease grouped under idiopathic inflammatory myopathy showing signs of necrosis in muscles on histopathology. It is characterized by acute or subacute weakness in the proximal muscles such as forearms, thighs, hips, shoulders, neck and back muscles, difficulty in climbing stairs, difficulty in standing up from chair, difficulty in lifting arms over the head, falling tendency with difficulty in getting up and general feeling of tiredness [1,2]. Most commonly antibodies associated with Necrotizing Autoimmune Myopathy (NAM) are Anti-Signal Recognition Particle (anti-SRP) and anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (anti-HMGCR) antibodies [3]. Anti-signal Recognition Particle (anti-SRP) autoantibodies are myositis specific and found in about 4% to 6% of patients of idiopathic inflammatory myopathy [4,5]. The Patients with anti-SRP antibodies often present clinically with rapidly progressive proximal muscle weakness leading to significant disability which on histopathology demonstrates a necrotizing myopathy without primary inflammation [6-8]. Marked and sustained clinical response has been observed to combination of intravenous methylprednisone pulse therapy followed by oral steroid therapy, intravenous immunoglobulin therapy [9], plasma exchange and repeated courses of Rituximab [10]. In this study we report a case of necrotizing autoimmune myopathy with positive anti-SRP autoantibodies presented at our tertiary care hospital and which responded to IVIG.

Case Presentation

A 32-year-old female patient presented in medicine OPD at our hospital in February 2022 complaining of progressive weakness and fatigue of proximal muscles in upper and lower limbs. Weakness was first noticed first in proximal upper limb (left>right) which gradually progressed to involve both lower limbs proximal muscles. Weakness which was characterized by difficulty in holding neck, standing up from sitting and squatting position, combing hair, changing cloths. Comorbidities like diabetes and hypertension were absent. History was short here hence inherited myopathies was not considered. Physical examination findings showed wasting of proximal muscle groups with atrophy of girdle and arm muscles (Figure 1). Skin rashes, oral ulcers, photosensitivity, hair fall or weight loss was absent. Manual muscle strength was graded as 2/5 in the proximal lower extremities as well as in the proximal upper extremities. Physical examinations of respiratory system, cardiovascular system and gastrointestinal system were unremarkable with pulse 80 beats/min, BP 110/80 mm of Hg and SpO_2 99% on room air. No organomegaly Peripheral lymph nodes were normal. No history of consumption of any myotoxic drugs or statins.

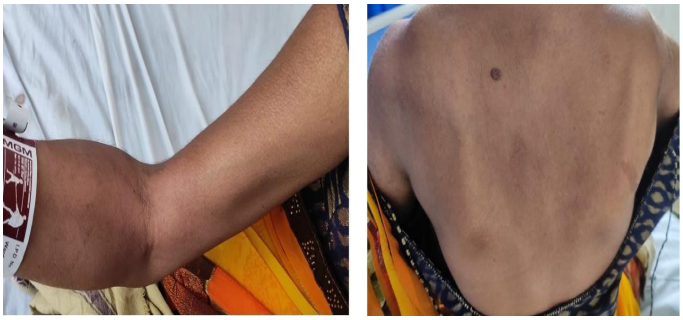

Figure 1. Gross muscle wasting.

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Investigation

Hemogram revealed haemoglobin 12.30 g/dl, total leukocyte counts 12750/dl, thrombocytes 324 × 10^9/l. Non specific inflammatory biomarker like Erythrocyte Sedimentation Rate (ESR) 7 mm/h and C-reactive protein 0.7 mg/l were normal, renal function rest results showed urea 14 and creatinine 0.4. Liver enzymes SGOT 126 U/l, SGPT 126 U/l and ALP 68 U/l were increased. Cpk- total were markedly elevated >5000 U/l. Urine test results were normal. Hepatitis B and C, HIV, were all negative. Antinuclear Antibodies (ANA) on indirect immunofluorescence were positive ANA 1:320 (normal<1:160). Myositis specific antibodies against SRP antigen were positive. EMG showed myopathic changes.

Muscle biopsy of left quadriceps revealed necrotizing changes without any evidence of significant inflammatory process and scattered muscle fibre regeneration. Necrotic altered rhabdomyocytes dominated. Echocardiography was normal. Computed Tomography (CT) scan of the chest and abdomen for any possible malignancy was negative. Nerve Conduction Studies (NCS) was characterized by the presence of normal CMAP values over right axillary, bilateral radial, bilateral peroneal and bilateral tibial nerves whereas reduced values over bilateral suprascapular, bilateral musculocutaneous, left axillary, bilateral median and bilateral ulnar nerves. F wave latencies were normal over bilateral lower and upper limb nerves. Sensory nerve parameters found normal SNAP over bilateral lower and upper limb nerves.

Differential diagnosis of Limb-girdle muscular dystrophy was rule out as muscle biopsy in limb-girdle dystrophy shows “dystrophic” triad- anisometry, muscle fiber necrosis and interstitial Fibrosis and positive result of anti-SRP in present case

Treatment

Patient initially started on IV Methylprednisolone 1 gm/day for 5 days. Pulse therapy with intravenous immunoglobulin (0.4 gm/kg/day.) for five days was given. After five days patient was started on oral steroid [Tab Omnacortil] according to body weight and dose of steroid was tapered overtime. Daily Limb Physiotherapy was given. Steroid Sparing Agent Azathioprine 100 mg/day was added to this treatment regimen. Initially patient's response to the therapy was minimal. After four months CPK Total, Hemogram SGOT, SGPT normalized with concurrent improvement in muscle weakness and muscle bulk. Patient has received another pulse therapy with intravenous immunoglobulin (0.4 gm/kg/day) given for 5 days. Prednisolone dosage was tapered slowly with Azathioprine.

Microscopic findings

Section studied from the left quadricep muscle biopsy revealed mild myopathic changes including focal myofiber size variation with small round myofibrils. At places scanty lymphocytic infiltration surrounding the non-necrotic fibres is seen. Few degenerating myofibers and scanty fibrosis is noted (Figure 2).

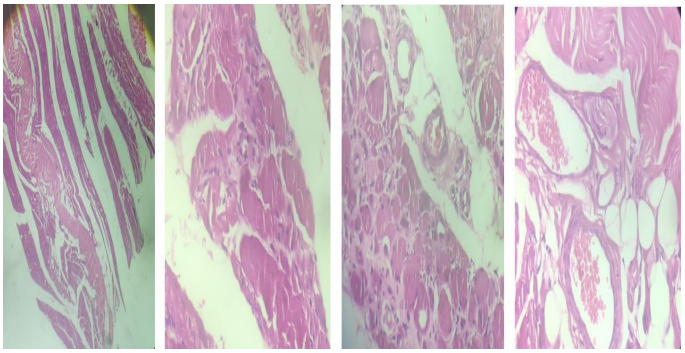

Figure 2. Microscopic findings.

Results and Discussion

Necrotizing Autoimmune Myopathies (NAM) was described for the first time in 2004. It is categorized based on the presence of different autoantibodies in the patient’s blood as cases with positive Anti-Signal Recognition Particle (anti-SRP) antibodies and cases with positive anti-3-hydroxy-3-methylglutaryl-coenzyme. A reductase (anti-HMGCR) antibodies. Signal Recognition Particle (SRP) is a cytoplasmic RNA protein consisting of 7S RNA and 6 proteins with molecular weights of 9, 14, 19, 54, 68 and 72 kD which regulates translocation of newly synthesized protein across the endoplasmic reticulum [11]. HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) is a key enzyme in production of cholesterol. Diagnosis is based on detection of anti-SRP antibodies in patient’s serum and histological diagnosis of necrotizing myopathy. Other test results including markedly elevated serum creatine kinase, electromyography and muscle images support the diagnosis. Anti-SRP antibodies were first discovered in serum of patients with clinical polymyositis by RNA immunoprecipitation with presence of 7S RNA which can also be detected by immunoassay using a 54-kD subunit protein of SRP (SRP54) [12]. Anti-SRP antibodies are regarded as myositis-specific antibodies and used as serological markers of necrotizing myopathy.

Muscle weakness is the predominant clinical feature [13]. Patients may also have complaints of dysphagia, cardiac involvement, including rhythm or conduction abnormalities as well as cardiac insufficiency [14,15]. Other extra-muscular manifestations include mild interstitial lung disease [16]. In present case progressive weakness and fatigue of proximal muscles in upper and lower limbs were found. In similar study by Kalinova, et al. [17] patient manifested with proximal muscle weakness with atrophy of quadriceps and gluteus muscles, conduction abnormalities, elevated CK levels, and myopathic EMG findings. Muscle biopsy demonstrated prominent necrotic myofibres. In present case muscle biopsy revealed necrotizing changes with scattered muscle fibre regeneration. Antinuclear antibodies on indirect immunofluorescence were positive ANA 1:320. In similar study by Allenbach, et al. [18] in muscle biopsies necrotic muscle fibres were distributed with a diffuse pattern, lymphocytic infiltration was sparse or absent and muscle fibre regeneration was scattered. When clinical phenotypes of both anti-SRP and anti-HMGCR patients were compared anti-SRP myopathy showed

more severe and with intense muscle damage. In present case combined therapy with corticosteroids, Azathioprine and IVIG was found beneficial. In similar study by Milone, et al. and Suzuki, et al. early administration of therapy of corticosteroids with immunosuppressant was found beneficial [3,14]. Kassardjian, et al. stated that the early initiation of IVIG was seen to be advantageous [19]. Arlet, et al. [10] demonstrated marked and sustained clinical response to the combination of prednisone, plasma exchange and repeated courses of Rituximab in two patients with refractory anti-SRP myopathy.

Conclusion

In conclusion SRP positive autonomic necrotizing myopathy is one of the disabling myopathies causing an initial severe muscle weakness with often poor muscle recovery even after treatment.

Hence it is necessary to identify this subtype early by myositis profile and histopathology. Aggressive combined therapy including corticosteroids and immunotherapy (Plasma exchange vs. IVIG) early in case benefits patients as seen in our case. Clinical characteristics, autoantibody status and neurological outcome study in present case suggests that anti-SRP antibodies could define a distinct subset of inflammatory myopathies.

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Analysis of Risk Factors for Mucormycosis in COVID-19 Patients Admitted in Tertiary Care Hospital Aurangabad

M. Naik [a,bφ], Shamisha Khade [a,b#*], Vinayak Bangi [a,b†] and Nilofer Banoisa Patel [a,b†]

[a] Department of Medicine, MGM Medical College and Hospital, Aurangabad, (MS), India.
[b] Constituent Unit of MGM Institute of Health Sciences, Navi Mumbai, (MH), India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Mucormycosis manifests as various syndromes in human beings, particularly in immunocompromised patients and those with diabetes mellitus. Recently, several cases of mucormycosis in people with COVID19 have been increasingly reported worldwide, especially from India. This prompted us to conduct a study in mucormycosis patients with COVID-19, to know its clinical profile of the COVID-19 patients with mucormycosis and identify of various risk factors in mucormycosis patients with COVID-19 infection.
Materials and Methods: This Cross sectional Retrospective Qualitative Descriptive study was conducted in Department of Medicine, MGM Medical College and Hospital, Aurangabad [Maharashtra]. A total of 100 patients admitted from April 2021 to August 2021 were enrolled as study participants. All COVID-19 patients admitted in MGM who are diagnosed with mucormycosis by microbiologically (KOH mount) or radiologically (CT/MRI) or by histopathology.
Observations and Results: The mean age of patients was 59.72±12.47 years. The male 73 (73.0%) predomianance than female 27(27.0%). 88(88.0%) of patients were having Diabetic Mellitus and 31(31.0%) of patients were having hypertension. All the patients were given Antibiotic

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[φ] Professor and Head;
[#] Chief Resident;
[†] Senior Resident;
**Corresponding author: E-mail: shamishakhade@gmail.com;*

& steroids during treatment of COVID-19 at hospitalisation. 15(15.0%) of patients were admitted in ICU during treatment of COVID-19. 76(76.0%) patients were required Oxygen, 03(3.0%) were on NIV/Ventilator and 08 (8.0%) patients were on HFOT during treatment of COVID-19. Overall (97.0%) of patients were recovered.

Conclusion: Diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients. Also use of steroid, duration of use of steroid, and oxygen therapy during the treatment of COVID-19 were risk factors observed in the patients with mucormycosis.

Keywords: Mucormycosis; steroid in COVID-19; post-COVID-19.

1. INTRODUCTION

Mucormycosis is manifested by a variety of different syndromes in humans, particularly in immunocompromised patients and those with diabetes mellitus. Devastating rhino-orbital-cerebral and pulmonary infections are the most common syndromes caused by these fungi.

"Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory distress syndrome. It has been associated with a wide range of opportunistic bacterial and fungal infection" [1]. "Both Aspergillosis and candida have been reported as the main fungal infections in patients with COVID-19" [2]. Recently, several cases of mucormycosis in people with COVID19 have been increasingly reported worldwide, especially from India. The main reason that appears to be facilitating mucorales spores to germinate in patients with COVID-19 is favourable conditions of low oxygen (hypoxia), high glucose(diabetes, new onset hyperglycemia, steroid induced hyperglycemia), acidic medium (metabolic acidosis, diabetic ketoacidosis [DKA]) ,high iron levels(increased ferritins) and decreased phagocytic activity of WBC due to immune suppression along with several other risk factors like prolonged hospitalization with or without mechanical ventilators.

"The genera in the order mucorales are responsible for causing Mucormycosis in humans. These organisms are universal in nature and can be found on decaying vegetations and in the soil. These fungi grow rapidly and release large numbers of spores which are air borne. Because the agents of mucormycosis are common in the environment hence they are most common contaminants in the clinical microbiology laboratory; all humans have plenty of exposure to these fungi during day to day activities. The fact that mucormycosis is a rare human infection reflects the effectiveness of the intact human immune system. This is further supported by the finding that almost all human infections due to the agents of mucormycosis occur in the presence of some underlying compromising condition" [2].

"The genera most commonly found in human infections are *Rhizopus*, *Mucor* and *Rhizomucor*; *Cunninghamella*, *Absidia Saksenaea*, and *Apophysomyces*are genera that are less commonly implicated in infection. *Rhizopus oryzae* is most common type and responsible for nearly 60% of mucormycosis cases in humans and also accounts for 90% of Rhino-orbital –cerebral (ROCM) form" [3].

"Globally, the prevalence of mucormycosis varied from 0.005-1.7 per million population, while its prevalence is nearly 80 times higher (0.14 per 1000) in India compared to developed countries in a recent estimate of year 2019-20" [4,5,6] "in other words, India has highest cases of mucormycosis in the world. Notwithstanding, India is already having second largest population with diabetes mellitus (DM) and was the diabetes capital of the world, until recently. Importantly, DM has been the most common risk factor linked with mucormycosis in India, although haematological malignancies and organ transplant takes the lead in Europe and USA" [7].

While long term use of corticosteroids has often been associated with several opportunistic fungal infections including aspergillosis and mucormycosis [8], even a short course of corticosteroids has recently been reported to link with mucormycosis especially in people with DM. A total prednisolone dose of more than 600mg or total methyl prednisolone dose of 2-7gm given during the month before, puts immunocompromised patients at risk of mucormycosis [9]. The case reports of mucormycosis resulting from even a short course

(5-14 days) of steroid therapy in patients of DM are rare [10]. “Surprisingly 46% of the patients had received corticosteroids within the month before the diagnosis of mucormycosis in the European Confederation of Medical Mycology study” [11].

“These findings need to be considered with respect to COVID-19 pandemic where corticosteroids are commonly used. There has been a rampant rise in case reports/series of mucormycosis patients with COVID-19 infection especially in India. Similarly, several case reports are being reported from various continents. These findings are unprecedented and are of great public health importance especially because there is high fatality rate with mucormycosis. Intracranial involvement of mucormycosis increases the fatality rate to as high as 90%” [12].

Moreover, rapidity of dissemination of mucormycosis is an extraordinary phenomenon and even a delay of 12 hours in the diagnosis could be fatal, the reason 50% of cases of mucormycosis have been historically diagnosed only in the post-mortem autopsy series. This prompted us to conduct a study in mucormycosis patients with COVID-19, to know its clinical profile of the COVID-19 patients with mucormycosis and identify of various risk factors in mucormycosis patients with COVID-19 infection.

1.1 Aim and Objectives

- To study the clinical profile of the COVID-19 patients with mucormycosis
- To identify the various risk factors in mucormycosis patients with COVID-19 infection.
- To study the outcome of mucormycosis patients in COVID-19 infection.

2. MATERIALS AND METHODS

2.1 Study Design

Cross sectional Retrospective Qualitative Descriptive study.

2.2 Study Area

Department of Medicine, MGM Medical College and Hospital, Aurangabad [Maharashtra], India.

2.3 Sample Size

100 mucormycosis patients

2.4 Study Duration

A total of 100 patients admitted from April 2021 to August 2021 were enrolled as study participants.

2.5 Inclusion Criteria

All COVID-19 patients admitted in MGM who are diagnosed with mucormycosis by microbiologically (KOH mount) or radiologically (CT/MRI)or by histopathology.

2.6 Exclusion Criteria

- COVID-19 patients with mucormycosis who were not willing to participate in study.

2.7 Elimination Criteria

All COVID-19 patients who are microbiologically and radiological diagnosed to have Mucormycosis but have failed to follow up.

2.8 Methodology

After getting ethical permission from ethics committee of MGM Medical College & Hospital, Aurangabad [MH], India, data was collected from COVID-19 with mucormycosis patients who satisfying inclusion and exclusion criterion of study. The purpose of the study was explained to the study participants. Only after their written consent patients were enrolled in the study. Confidentiality of the information was ensured. For the purpose of data collection a detailed proforma was prepared. The proforma was included demographic profile (Name, age, sex and BMI), Personal history, comorbidity and detailed history of COVID-19, treatment during COVID-19.

Also diagnosis method of mucormycosis patients, patients according to involvement, anti-fungals received surgical intervention and outcome of mucormycosis patients.

2.9 Statistical Analysis

The collected data was entered in Microsoft excel and analysed using SPSS version 24th. Mean and SD was calculated for quantitative variables and proportions were calculated for categorical variables.

3. RESULTS

In present study out of 100 patients, maximum patients i.e. 49 (49.0%) were from age more than 60 years, 40(40.0%) were age-group of 45-60 years and only 11(11.0%) of patients were from age-group 15-45 years. The mean age of patients was 59.72±12.47 years. The male 73 (73.0%) predominance than female 27(27.0%).

In present study, 88(88.0%) of patients were having Diabetic Mellitus, 31(31.0%) of patients were having hypertension and 12(12.0%) of patients were having CHD/IHD, one patient was having Hypothyroidism and Asthma.

In present study, all 100(100%) of patients were diagnosed on Imaging and 55(55.0%) were positive on KOH and 36(36.0%) patients were positive on histopathology.

All 100 patients were reported Sinuses involvement, 70(70.0%) patients were having ocular, 02(2.0%) Pulmonary and 03(3.0%) patients were having cerebral involvement.

All the patients were given Antibiotic & steroids during treatment of COVID-19 at hospitalisation. 15(15.0%) of patients were admitted in ICU during treatment of COVID-19. 76(76.0%) patients were required Oxygen, 03(3.0%) were on NIV/Ventilator and 08 (8.0%) patients were on HFOT during treatment of COVID-19. 67 (67.0%) patients were used steroids for COVID-19 treatment for 6–10 days, 28(28.0%) study participants used steroids for 11-15 days. Where as 5(5.0%) patients were used steroids more than 15 days.

All the 100 patients were given Posaconazole & Amphotericin, 17(17%) & 16(16.0%) patients were given Liposomal Amphotericin and Lyophilized Amphotericin respectively. 67(67.0%) of patients were given Lipid Emulsion Amphotericin.

All the patients required Functional endoscopic sinus surgery (FESS), 78 (78.0%) of patients were done Endoscopic Debridement where as 16(16.0%) patients done Maxillectomy.

Out of 100 patients 97(97.0%) of patients were recovered and 03(3.0%) were died during treatment of mucormycosis.

4. DISCUSSION

In present study out of 100 patients, maximum patients i.e. 49 (49.0%) were from age more than 60 years, 40(40.0%) were age-group of 45-60 years and only 11(11.0%) of patients were from age-group 15-45 years. The mean age of patients was 59.72±12.47 years. Similar findings was reported by Bhagyashri Jadhav et al. [13] “the mean age was 54.46±13.13, years ranging from 28 to 77 years”. Also Ganesh Lokhande et al [14] observed “mean age of the patient was 52.47 ±12.84 years with a minimum age of 26 and maximum age of 83 years”. Study conducted by Sen et al. [15] observed that “the mean age of the study participants was 51.9”. A study conducted by Gupta [16] revealed that “the mean age of the study participants was 50 years. Maximum study reported mean age of mucormycosis patients were above 50 years”.

Table 1. Distribution of patients according to demographic profile of patients

		No. of patients	Percentage
Age-group in years	15-45	11	11.0
	45-60	40	40.0
	>60	49	49.0
	Total	100	100%
	Mean±SD	59.72±12.47 years	
Gender	Male	73	73.0
	Female	27	27.0

Table 2. Distribution of patients according to co-morbidities

Comorbidities	No. of patients (n=100)	Percentage
Hypertension	31	31.0
Diabetic Mellitus	88	88.0
IHD/CHD	12	12.0
Hypothyroidism	01	01.0
Asthama	01	01.0

Table 3. Distribution of patients according to diagnosis method

Diagnosis method	No. of patients (n=100)	Percentage
KOH	55	55.0
Hisopathology	36	36.0
Imaging	100	100.0

Table 4. Distribution of patients according to involvement

Involvement	No. of patients (n=100)	Percentage
Sinuses	100	100.0%
Occular	70	70.0%
Pulmonary	02	02.0%
Cerebral	03	03.0%

In present study the male 73 (73.0%) predominance than female 27(27.0%). Similar male predominance was observed by Patel et al [17] 69.5% of participants affected by mucormycosis were men. Sen et al. [15] observed 71% of the male. Bhagyashri Jadhav et al [13] Observed 75% of male patients. , Lokhande GS et al [14] also reported 61.34% were males.

In present study, 88.0% of mucormycosis patients were having Diabetic Mellitus. John et al. [18] observed that 94% of the patients with mucormycosis were diabetic. In 73.5% of cases with mucormycosis, diabetes was observed as a risk factor in India [19]. Sen et al. [15] observed that 78% of the patients with mucormycosis were having diabetes. 77% found by Priya et al. [20] In contrast to the Findings in this study, Lokhande GS et al [14] reported (57%) were diabetic. COVID-19 cases with a history of diabetes are at increased risk of developing the severe disease and these patients are also at higher risk of fungal infections. Globally diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients [21].

In present study 76.0% patients were required Oxygen, 3.0% were on NIV/Ventilator and 8.0% patients were on HFOT during treatment of COVID-19. Similarly Sen et al. [15] observed that 79% of the patients with mucormycosis received O2 therapy for the treatment of COVID-19. Whereas Afroze SN et al [22] reported 80.22%. Whereas Bhagyashri Jadhav et al. [13] reported 18.75% patients gave the history of receiving oxygen or mechanical ventilation during the treatment of COVID-19.

In present study, all 100 patients were given steroids during treatment of COVID-19 at hospitalisation. Lokhande, et al [13] found that more than 90% of patients had a history of steroid use for the treatment of COVID-19. Also Sen et al. [15] revealed a history of use of steroids in 87% of patients admitted with mucormycosis. Use of corticosteroids was observed in 88% of the study participants with mucormycosis in the study conducted by John et al. [18]. In present study (67.0%) patients were used steroids for COVID-19 treatment for 6–10 days, (28.0%) study participants used steroids for 11-15 days. Where as (5.0%) patients were used steroids more than 15 days. Lokhande, et al [14] reported "(77.11%) study participants used steroids for COVID-19 treatment for 7–14 days, whereas (20.48%) study participants used steroids for less than 7 days. The National Institute of Health recommends the use of dexamethasone (6 mg per day for a maximum of 10 days) in patients who are ventilated or require supplemental oxygen but not in milder cases. 17 The guidelines specifically mention the risk of developing a secondary infection".

In present study, all (100%) of patients were diagnosed on Imaging and (55.0%) were positive on KOH and (36.0%) patients were positive on histopathology. Lokhande, et al. [14] reported 57.14% of patients found positive on KOH.

In this study, All 100 patients were reported Sinuses involvement, (70.0%) patients were having ocular, (2.0%) Pulmonary and (3.0%) patients were having cerebral involvement. Singh et al. [22] found that 88.9% of Sinuses involvement, ocular (1.0%), Pulmonary (7.9%) and Cerebral (22.2%).

Table 5. Distribution of patients according to treatment during COVID-19

		No. of patients(n=100)	Percentage
Antibiotic		100	100.0%
Steroid		100	100.0%
ICU admission		15	15.0%
Oxygen requirement		76	76.0%
NIV/ventilator		03	3.0%
HFOT		08	8.0%
No. of days steroid given in COVID-19	0-5 Days	00	00
	6-10 Days	67	67.0%
	11-15 Days	28	28.0%
	>15 Days	5	5.0%

Table 6. Distribution of patients according to anti-fungals received

Antifungals received	No. of patients (n=100)	Percentage
Posaconazole	100	100.0
Amphotericin	100	100.0
Liposomal amphotericin	17	17.0
Lipid emulsion amphotericin	67	67.0
Lyophilized amphotericin	16	16.0

Table 7. Distribution of patients according to surgical intervention

Surgical intervention	No. of patients (n=100)	Percentage
Functional Endoscopic Sinus Surgery (FESS)	100	100.0%
Endoscopic debridement	78	78.0%
Maxillectomy	16	16.0%

Table 8. Distribution of patients according to Outcome

Surgical intervention	No. of patients (n=100)	Percentage
Recovered	97	97.0%
Death	03	3.0%
Total	**100**	**100.0%**

In present study All the patients required Functional endoscopic sinus surgery (FESS), 78 (78.0%) of patients were done Endoscopic Debridement where as 16(16.0%) patients done Maxillectomy. Whereas contrast finding was reported by Bhagyashri Jadhav et al [13] that in "(25%) patients only medical line of treatment was sufficient whereas (62.5%) patients required surgical debridement during the treatment. In our study, (97.0%) of patients were recovered and (3.0%) were died during treatment of mucromycosis". Bhagyashri Jadhav et al. [13] reported Overall survival was 90.62%.

5. CONCLUSION

In Post COVID-19 patients, Mucormycosis is one of the complications observed in the later stage of the disease. Diabetes mellitus is identified as the leading underlying comorbidity in cases diagnosed with mucormycosis in post COVID-19 patients. Also use of steroid, duration of use of steroid, and oxygen therapy during the treatment of COVID-19 were risk factors observed in the patients with mucormycosis. A high clinical suspicion and early and accurate diagnosis of AIFR in COVID-19 patients are essential for better prognosis.

6. LIMITATIONS OF STUDY

The study doesn't do justice in the aspect that it doesn't include all the cases of Mucormycosis on a single based criteria, i.e some are included on the basis of histopathology, while some on the basis of microbiology and the rest on basis of radiological diagnosis.

CONSENT

As per international standard or university standard, patient (s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The study is subjected for approval to "Ethical Committee" of MGM Medical College & Hospital Aurangabad [MH], India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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ORIGINAL ARTICLE

Efficacy and Safety of Biosimilar Romiplostim Versus Innovator Romiplostim in Patients with Chronic Immune Thrombocytopenia

S. Chandrakala[1] · Manoj Toshniwal[2] · Mitesh Halvawala[3] · Namita Padwal[4] · Neeraj Sidharthan[5] · Pankaj Malhotra[6] · B. Prashantha[7] · Riya Ballikar[8] · Sandip Shah[9] · Shashikant Apte[10] · T. Kasi Viswanathan[11] · Vijay Ramanan[12] · Akhilesh Sharma[13] · Dattatray Pawar[13] · Roshan Pawar[13] · Vinayaka Shahavi[13]

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Abstract Romiplostim is a Food and Drug Administration (FDA)-approved therapy for immune thrombocytopenia (ITP). Biosimilar is a biological product that has no clinical meaningful difference from an existing FDA-approved reference product. It has a potential of lowering health-care-related cost. Biosimilar of romiplostim can be made available to patients with ITP at a low cost and can be beneficial in providing the best therapy. Thus, the efficacy and safety of biosimilar romiplostim (ENZ110) was compared with innovator romiplostim (Nplate) with respect to platelet response in patients with chronic ITP. This was a prospective, multicenter, randomized, and double-blind clinical trial. Patients with chronic ITP, aged 18–65 years, were enrolled in a study and were randomized to receive either ENZ110 or Nplate in a 3:1 ratio for a treatment period of 12 weeks, respectively. After completion of the treatment period, the patients were followed-up for one week to evaluate the platelet response and to monitor the adverse events (AEs). Over the duration of 12 weeks, platelet response of $> 50 \times 10^9$/L was achieved in 85.3% patients treated with ENZ110 and in 75.0% patients treated with Nplate in per protocol population. In intent-to-treat population, 83.8% patients with ENZ110 and 76.9% patients with Nplate achieved a platelet response of $> 50 \times 10^9$/L. In the ENZ110 group, 111 AEs were recorded in 66.7% patients, while 18 AEs were reported in 61.5% patients in the Nplate group. The study demonstrated non-inferiority with comparable efficacy and safety between biosimilar romiplostim and innovator romiplostim in patients with chronic ITP.

Trial registration number and date of registration: CTRI/2019/04/018614.

Keywords Immune thrombocytopenia · Biosimilar romiplostim · Efficacy endpoint · Adverse event

✉ Roshan Pawar
roshan.pawar@alkem.com

1 Seth G. S. Medical College and KEM Hospital, Mumbai, India

2 Ishwar Institute of Health Care, Aurangabad, India

3 Nirmal Hospital Pvt Ltd, Surat, India

4 LTMMC and General Hospital, Sion, India

5 Amrita Institute of Medical Sciences, Kochi, India

6 Postgraduate Institute of Medical Education and Research, Chandigarh, India

7 Kasturba Medical College (KMC) Hospital, Mangalore, India

8 Criticare Hospital and Research Institute, Nagpur, India

9 Hemato-Oncology Clinic, Ahmedabad, India

10 Sahyadri Super Specialty Hospital, Pune, India

11 Meenakshi Mission Hospital and Research Centre, Madurai, India

12 Grant Medical Foundation Ruby Hall Clinic, Pune, India

13 Medical Affairs Department, Alkem Laboratories Limited, Alkem House, Senapati Bapat Marg,Lower Parel, Mumbai, India

Introduction

Immune thrombocytopenia (ITP) is a prevalent hematologic disorder that affects people of all ages, genders, and ethnicities [1]. Idiopathic ITP is a condition of having a low platelet count (thrombocytopenia) of no known cause (idiopathic) [2]. Chronic ITP is described as a disease that lasts for more than 12 months [1]. Adults are most commonly affected by chronic ITP. These individuals require treatment since the condition seldom cures independently, and there is a risk of severe consequences [3].

Adult patients with ITP have higher rates of morbidity and mortality than the general population, especially those who cannot maintain a hemostatic platelet count > 30×10^9/L despite treatment [4, 5]. The objective of therapy is to produce a hemostatic platelet count of at least 20–30×10^9/L while producing the least amount of harm possible [4]. Current treatments aim to increase platelet counts in ITP patients, mainly by reducing platelet destruction [6].

First-line treatment includes IV immunoglobulin, steroids, anti-D-immunoglobulin, and lastly, splenectomy. Rituximab is an alternative treatment in patients who are at increased risk of bleeding after the failure of above treatments. Recombinant versions of human thrombopoietin (TPO) were the first generation of thrombopoietic agents [7]. TPO is the endogenous ligand for the TPO receptor, expressed on the surface of platelets and megakaryocytes, and is the key cytokine involved in thrombopoiesis [8, 9]. Because neutralizing autoantibodies cross-react with endogenous TPO, the development of these agents was stopped. As a result, second-generation thrombopoiesis-stimulating compounds with no sequence similarity to endogenous TPO were developed [8]. Romiplostim (ROM) and eltrombopag (ELT) has been authorized by the Food and Drug Administration (FDA) in 2008, European Medicines Agency (EMA), and the UK's Medicines and Healthcare Products Regulatory Agency (MHRA) for the treatment of primary ITP in adult patients who are refractory to other treatments [e.g., corticosteroids, immunoglobulins (Igs)] Romiplostim and eltrombopag have no sequence homology with TPO, thus decreasing the risk of antibody formation. [10–12].

Romiplostim is a peptibody or Fc-peptide fusion protein. It is made up of two identical single-chain subunits, each with two human IgG1 Fc domains covalently bonded at the C-terminus to a peptide with two TPO receptors (c-Mpl)–binding domains (four total binding sites) [13, 14]. Romiplostim is a recombinant DNA product made in *Escherichia coli* (*E. coli*) that resembles human TPO. By attaching to the TPO receptor, romiplostim activates intracellular transcriptional pathways, resulting in enhanced platelet synthesis. TPO receptor binding stimulates the development of bone marrow megakaryocyte colony-forming cells, resulting in enhanced platelet synthesis via the Janus kinase 2 (JAK2) and signal transducers and activators of transcription 5 (STAT5) kinase pathways [3].

ROM is a subcutaneously administered peptide mimetic binding to the extracellular TPO-receptor, while ELT is an oral non-peptide binding to a transmembrane site of the TPO-receptor. An indirect comparative study between two concluded that romiplostim significantly improved overall platelet response compared with eltrombopag, however the durable platelet response of the two was similar. Another indirect study concluded that overall response, the incidence of adverse events, durable response, the incidence of overall bleeding and clinically significant bleeding, and the proportion of patients receiving rescue treatment were similar between eltrombopag and romiplostim. However, studies had concluded that ELT is more cost effective than romiplostim [15].

A biosimilar is a biological product similar to an approved and marketed biological product known as the reference product. It has no clinically meaningful differences in terms of safety and effectiveness from the reference product. Biosimilars are believed to have a positive impact on drug pricing. Health-care experts and physicians are optimistic that the use of biosimilars will lower the cost of biologics and, as a result, improve patient's access to these life-saving drugs. The biosimilar of Romiplostim, ENZ110, would accomplish the unmet need in the niche Indian market in patients with ITP, its introduction would be an effective treatment option due to its affordability [16].

In the present study, we compared the efficacy and safety of biosimilar romiplostim i.e., ENZ110 with innovator romiplostim i.e., Nplate in terms of platelet response in patients with chronic ITP.

Materials and Methods

Study Design and Population

Male and female patients diagnosed with chronic ITP, aged 18–65 years were enrolled in a prospective, multicenter, randomized, double-blind clinical trial. After signing the informed consent form, these patients were selected. Subjects diagnosed with idiopathic ITP according to the American Society of Hematology guidelines, with a bone marrow biopsy report consistent with an ITP diagnosis if over 60 years old, having received at least one prior therapy for ITP, having a single platelet count of $\leq 30 \times 10^9$/L at any time during the screening period, had splenectomy/non-splenectomy and willingly and ably providing written informed consent were included in the study.

Subgroup analysis was not done & was not considered while calculating sample size. This phase III study was conducted as per the regulatory requirements for marketing authorization to address the pre-market regulatory requirements including comparability exercise for quality, preclinical and clinical studies. The comparative Pharmacodynamic, Pharmacokinetic, Immunogenicity of the product and clinical trials are critical to demonstrate the similarity in safety and efficacy profiles between the Similar Biologic and Reference Biologic for the manufacturing and marketing authorization approval. The study was conducted as per the principles and requirements of Declaration of Helsinki, and International Conference on Harmonization-Good Clinical Practice (ICH-GCP) guidelines along with the

local regulatory requirements of Good Clinical Practices for Clinical Research in India (2004, CDSCO), Indian Council of Medical Research (ICMR) guidelines for Biomedical Research on Human Subjects (2017), and New Drugs and Clinical Trial Rules, 2019 (CDSCO).

The study protocol was approved by the independent ethics committee at all the participating 14 Centre before any patient enrollment in the study at that site. Written informed consent was obtained from the patient before the patient underwent any protocol-specific screening or study procedures. The trial was registered with Clinical Trials Registry-India (CTRI).

Subjects with a history of haematological malignancy, myeloproliferative disorder, myelodysplastic syndrome (MDS), bone marrow stem cell disorder, congenital thrombocytopenia, systemic lupus erythematosus, Evans syndrome, autoimmune neutropenia, antiphospholipid antibody syndrome, disseminated intravascular coagulation, haemolytic uremic syndrome, thrombotic thrombocytopenic purpura, infection with *Helicobacter pylori*, chronic liver disease (Child–Pugh score ≥ 7), any thromboembolic disease or were known to be positive for lupus anticoagulant, or positive for hepatitis B, hepatitis C, or human immunodeficiency virus at screening were excluded from the study. Also, subjects with previous use of romiplostim, pegylated recombinant human megakaryocyte growth and development factor, Eltrombopag, recombinant human TPO, or any platelet producing agent, or having known hypersensitivity to any recombinant *E. coli*-derived product, or of reproductive potential and was not using adequate contraceptive precautions, in the judgment of the investigator, or was pregnant or breastfeeding, or was unable to comply with the protocol procedures, were excluded from the study.

Treatment Plan

The study consisted of a screening period (up to one week), a treatment period (12 cycles-each of 7 days), and a follow-up period (one week after cycle 12). Upon fulfilment of the selection criteria, subjects were randomized to receive either biosimilar romiplostim or innovator romiplostim in a 3:1 ratio to enter into the treatment period of 12 weeks, respectively. During the treatment period, romiplostim (ENZ110 or Nplate) was administered subcutaneously to all the eligible patients once a week.

Romiplostim dose during the study week was adjusted according to the protocols. Suppose the platelet count was $< 50 \times 10^9$/L, the dose was increased by 1 mcg/kg, if platelet count was $> 200 \times 10^9$/L for two consecutive weeks, the dose was reduced by 1 mcg/kg, if platelet count was $> 400 \times 10^9$/L, no dose was administered, and platelet count was assessed weekly, and if platelet count decreased to $< 200 \times 10^9$/L, romiplostim was resumed at a dose reduced by 1 mcg/kg against the last received dose. The weekly dose did not exceed more than 10 mcg/kg. Responders were defined as patients achieving platelet count $\geq 50 \times 10^9$/L, i.e., sufficient platelet count to avoid clinically significant bleeding at the maximum weekly dose of 10 mcg/kg.

Rescue medications were permitted at the investigator's discretion, or when the subjects experienced bleeding, wet purpura, were at immediate risk for haemorrhage, or when the platelet count did not increase to a level sufficient to avoid clinically significant bleeding at the maximum weekly dose of 10 mcg/kg. Rescue medications administered were corticosteroid, intravenous immune globulin, anti-D Ig, and platelet transfusions.

Study Assessments

After completion of the treatment period, the patients were followed up for one week to assess platelet response and monitor the adverse events (AEs). During the clinical trial, complete blood count (CBC), including peripheral blood smear (PBS), platelet count, prothrombin time/international normalized ratio (PT/INR), activated partial thromboplastin time (aPTT), were evaluated every week for ensuring patient safety as it not only play a role in coagulation but also in host defence against infection, and aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin, and serum creatinine were assessed every 4 weeks before the dose of romiplostim.

For the pharmacokinetic (PK) sub-study, blood samples of volume 5 mL in BD vacutainer were collected pre-injection within 30 min before the start of romiplostim injection (0 min) at cycle 1 and post-injection at 1 h ($\pm$5 min), 2 h ($\pm$5 min), 4 h ($\pm$5 min), 6 h ($\pm$5 min), 12 h ($\pm$5 min), 15 h ($\pm$5 min), 18 h ($\pm$5 min), 21 h ($\pm$5 min), 24 h ($\pm$1 h), 48 h ($\pm$1 h), and 72 h ($\pm$1 h). Biological assay was done with standard Enzyme linked Immunosorbent assays (ELISA) method at Enzene bioscience limited.

Anti-romiplostim antibodies were assessed across both the treatment groups. Blood samples were collected before the start of the romiplostim administration at cycle one and at the end of study for efficacy endpoints and after week 24 (post-study for immunogenicity assessment).

Efficacy Endpoints

The primary endpoint included a proportion of patients achieving platelet response (achievement of a weekly platelet count $\geq 50 \times 10^9$/L) in both the study groups, and secondary endpoints were single-dose truncated PK parameters (C_{max}, T_{max}, $AUC_{0\text{-}t}$) and incidence of treatment-emergent AEs (TEAEs) in both the treatment groups during the study period, and presence of anti-romiplostim antibody at baseline, end of study (EOS) visit, and after week 24 (post-study) visit.

Statistical Analysis

A formal non-inferiority test was conducted for the primary endpoint. The null and alternative hypotheses for non-inferiority testing are given below:

H0: p[test]—p[reference] > Δ

HA: p[test]—p[reference] < Δ

H0 is the null hypothesis whereas HA is the alternative hypothesis, Δ is the margin of non-inferiority, which is already defined in the protocol (i.e., 20%), *p*[test]: proportion of patients achieving platelet response in the test group, *p*[reference]: proportion of patients achieving platelet response in the reference group.

A response rate was defined as the proportion of patients achieving a weekly platelet count $\geq 50\times10^9$/L within 12 weeks of romiplostim treatment in both the study groups. The chi-square test was used for the comparison of two proportions from the two treatment groups. In addition to a *p*-value of the test, two-sided 90% confidence interval (CI) for the difference were calculated. If the lower limit of the 90% CI is more significant than zero, the proportion was estimated with sufficient precision. Proportion of patients with platelet count $\geq 50\times10^9$/L for six or more times during the last 8 weeks of treatment study in both the study groups were summarized using counts (N: number of subjects per treatment group, n: number of subjects with non-missing values) and percentages.

Results

Patient Population and Demographic

In this multicentre study, a total of 67 subjects were screened from 14 clinical sites in India, and 52 were randomized (39 in biosimilar romiplostim arm [ENZ110], 13 in innovator romiplostim arm [Nplate]). Out of these, 46 (88.5%) patients [ENZ110, 34 (87.2%); Nplate, 12 (92.3%)] completed the study. The patient disposition is described in Fig. 1. No significant differences in the baseline characteristics were observed between the two treatment groups (Table 1).

Efficacy Analysis

Summary of Proportion of Patients Achieving a Weekly Platelet Count (Per Protocol [PP] and Intention-to-Treat [ITT] Population)

For the PP population, 46 patients were considered (34 patients in ENZ110 group; 12 patients in Nplate group); whereas for the ITT population, 50 patients were evaluated (37 patients in ENZ110 group; 13 patients in Nplate group) (Fig. 2). In over 12 weeks, a response of $> 50\times10^9$/L platelet count was achieved in 29 (85.3%) patients with ENZ110 and 9 (75.0%) patients with Nplate in the PP population and 31 (83.8%) patients with ENZ110 and 10 (76.9%) patients with Nplate in the ITT population (Fig. 2). Statistically significant response was noted in both the groups from baseline to week 12 in both the populations (*p* value for response in both groups was < 0.0001).

The other evaluation including CBC, AST, ALT were within normal range and showed no significant difference from baseline to the end of study.

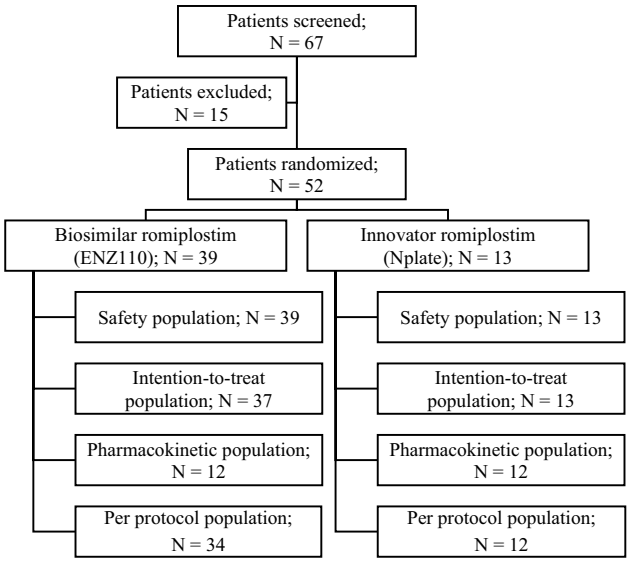

Fig. 1 Patient disposition

Table 1 Baseline demographic characteristics of patients

Baseline characteristics	ENZ110 (n = 39)	Nplate (n = 13)	Overall (n = 52)
Age (years), mean (SD)	37.97 (13.19)	33.54 (10.81)	36.87 (12.68)
Height (cm), mean (SD)	157.75 (9.53)	157.65 (3.80)	157.72 (8.43)
Weight (kg), mean (SD)	63.76 (13.94)	60.93 (12.01)	63.05 (13.43)
Gender			
Female, n (%)	25 (64.1)	10 (76.9)	35 (67.3)
Male, n (%)	14 (35.9)	3 (23.1)	17 (32.7)

SD standard deviation, *ENZ110* biosimilar romiplostim, *Nplate* innovator romiplostim

Pharmacokinetic and Immunogenicity Endpoints

The research enrolled a total of 24 individuals as planned. After subcutaneous injection of 0.1, 0.3, and 1.0 mcg/kg romiplostim, no measurable quantities were detected, according to the innovator's PK investigations. Only two

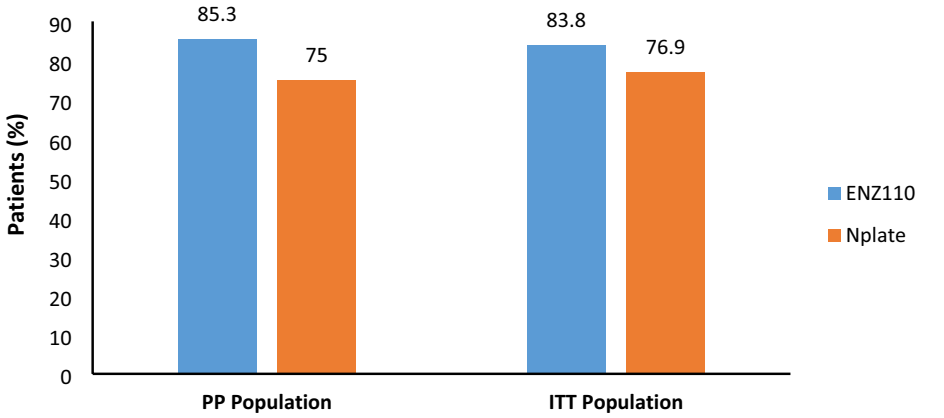

Fig. 2 Proportion of patients responding to the treatment during the 12 weeks of romiplostim treatment. PP–per protocol; ITT–intention-to-treat; ENZ110 = biosimilar romiplostim; Nplate = innovator romiplostim

Abbreviations: PP - per protocol; ITT - intention-to-treat; ENZ110 = biosimilar romiplostim; Nplate = innovator romiplostim

out of eight individuals exhibited measurable amounts at dosage 2.0 mcg/kg. Based on this finding, a larger dosage of 3 mcg/kg subcutaneous was used in a later PK investigation in chronic ITP patients. Two patients in the ENZ110 group tested positive for anti-drug antibody (ADA), whereas one patient in the Nplate group tested positive.

Safety Analysis

A total of 129 TEAEs were reported by 34 (65.4%) individuals (Table 2). Five serious AEs (SAEs) were reported out of which one SAE led to death (Table 3). Out of 129 TEAEs, 83 AEs were possibly related to the study medication, 4 AEs were probably related, and 42 AEs were unlikely to be related. In the ENZ110 group, 111 AEs were recorded in 26 (66.7%) patients, while 18 AEs were reported in 8 (61.5%) patients in the Nplate group. The proportion of patients experiencing at least one AE were similar between the groups.

Discussion

The study conducted on ITP patients over a duration of 12 weeks showed non-inferiority between biosimilar

Table 2 Summary of TEAEs

	ENZ110 (N = 39)	Nplate (N = 13)	Overall (N = 52)
All TEAEs, n (%) E	26 (66.7) 111	8 (61.5) 18	34 (65.4) 129
p-value for difference in the incidence of AEs between treatment	0.8679		
TEAEs related to investigational product, n (%) E			
Possible	15 (38.5) 71	5 (38.5) 12	20 (38.5) 83
Probable/Likely	2 (5.1) 3	1 (7.7) 1	3 (5.8) 4
Unlikely	20 (51.3) 37	3 (23.1) 5	23 (44.2) 42
Severity of TEAEs, n (%) E			
Mild	21 (53.8) 76	8 (61.5) 16	29 (55.8) 92
Moderate	12 (30.8) 29	1 (7.7) 2	13 (25.0) 31
Severe	6 (15.4) 6	0 (0.0) 0	6 (11.5) 6
Seriousness of TEAEs, n (%) E			
Hospitalization or prolongation of hospitalization	4 (10.3) 4	0 (0.0) 0	4 (7.7) 4
Death	1 (2.6) 1	0 (0.0) 0	1 (1.9) 1
TEAEs leading to discontinuation of IMP	3 (7.7) 3	0 (0.0) 0	3 (5.8) 3

E number of events, *N* number of subjects dosed with each treatment, *n* number of subjects with adverse event with particular category, % calculated using the number of subjects treated with each treatment as the denominator (n/N)*100, *TEAEs* treatment-emergent adverse events, *ENZ110* biosimilar romiplostim, *Nplate* innovator romiplostim, *IMP* investigational medicinal product

Table 3 Summary of TEAEs reported by at least 5% subjects

System organ class	ENZ110	Nplate	Overall
Preferred term N (%) E	(N = 39)	(N = 13)	(N = 52)
Blood And lymphatic system disorders			
Anemia	3 (7.7) 3	1 (7.7) 1	4 (7.7) 4
Cardiac disorders			
Sinus tachycardia	0 (0.0) 0	1 (7.7) 2	1 (1.9) 2
Gastrointestinal disorders			
Abdominal pain upper	2 (5.1) 4	0 (0.0) 0	2 (3.8) 4
Constipation	2 (5.1) 3	0 (0.0) 0	2 (3.8) 3
Diarrhea	2 (5.1) 4	0 (0.0) 0	2 (3.8) 4
Dysphagia	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Gastritis	2 (5.1) 4	0 (0.0) 0	2 (3.8) 4
Vomiting	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
General disorders and administration site conditions			
Chills	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Non-cardiac chest pain	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Pyrexia	6 (15.4) 6	1 (7.7) 1	7 (13.5) 7
Infections and infestations			
Pharyngitis	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Upper respiratory tract infection	10 (25.6) 13	2 (15.4) 2	12 (23.1) 15
Investigations			
Liver function test abnormal	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Musculoskeletal and connective tissue disorders			
Arthralgia	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Muscle spasms	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Musculoskeletal pain	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Pain in extremity	3 (7.7) 4	1 (7.7) 1	4 (7.7) 5
Pain in jaw	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Nervous system disorders			
Headache	5 (12.8) 7	0 (0.0) 0	5 (9.6) 7
Renal And urinary disorders			
Dysuria	0 (0.0) 0	1 (7.7) 1	1 (1.9) 1
Reproductive system and breast disorders			
Menorrhagia	3 (7.7) 3	0 (0.0) 0	3 (5.8) 3
Skin and subcutaneous tissue disorders			
Acne	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Dry skin	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Pruritus	4 (10.3) 5	1 (7.7) 2	2 (3.8) 2
Vascular disorders			
Ecchymosis	3 (7.7) 7	0 (0.0) 0	3 (5.8) 7
Petechiae	2 (5.1) 2	0 (0.0) 0	2 (3.8) 2
Vaginal hemorrhage	0 (0.0) 0	1 (7.7) 2	1 (1.9) 2

E number of events, *N* number of subjects dosed with each treatment, *n* number of subjects with adverse event with particular category, *%* calculated using the number of subjects treated with each treatment as the denominator (n/N)*100, *TEAEs* treatment-emergent adverse events, *ENZ110* biosimilar romiplostim, *Nplate* innovator romiplostim

romiplostim and innovator romiplostim. The platelet response of $> 50 \times 10^9$/L was achieved in 85.3% in patients treated with ENZ110 and 75% in patients treated with Nplate with no statistically significant difference in the incidence of TEAE between the two groups.

With respect to PK assessment, the majority of the samples were reported below the lower limit of quantitation (LLOQ) (40 pg/mL) of the assay. Hence, the statistical analysis was not performed.

No statistically significant difference observed in the incidence of TEAEs between the two treatment groups. These reported AEs were expected and consistent with reference to the romiplostim (Nplate) [12]. Treatment-emergent ADA was detected in two patients (5.71%) from ENZ110 group and one patient (9.09%) from Nplate group, which was consistent with the Summary of Product literature [12].

Romiplostim is an approved treatment in ITP. However, in India, due to cost constraints, the majority prefer immunosuppression therapy. The biosimilar of romiplostim, ENZ110, would come as a huge relief to patients with ITP, as its affordable cost would fulfill an unmet need in patients requiring the best treatment [17].

Two multicenter, placebo-controlled phase III trials were conducted simultaneously. These studies included 63 splenectomized and 62 non-splenectomized patients who had chronic ITP and a mean of three platelet counts of up to 30×10^9/L. The overall platelet response rate was noted in 88% of non-splenectomized and 79% of splenectomized patients given romiplostim compared with 14% of non-splenectomized and no splenectomized patients given placebo ($p < 0.0001$) [18].

Another prospective, multicenter, randomized, double-blind study compared the efficacy and safety of biosimilar romiplostim with innovator romiplostim in patients with chronic ITP. Non-inferiority was statistically demonstrated for the primary efficacy endpoint between the biosimilar romiplostim and the innovator romiplostim. Proportion of patients achieving a weekly platelet count $\geq 50 \times 10^9$/L was 85.3% with ENZ110 and 75.0% with Nplate over 12 weeks. The study achieved its non-inferiority efficacy endpoint as lower bound of the 90% two-sided CI (− 8.36, 28.94%) was greater than − 20%.

In a PK study conducted for innovator Nplate, maximum romiplostim serum levels in ITP patients were attained after 7–50 h following subcutaneous dose of 3–15 mcg/kg romiplostim (median 14 h). Patients' blood concentrations varied and were not related to the dose given and Romiplostim's elimination half-life in ITP patients ranged from 1 to 34 days. No accumulation in serum concentrations was observed after six weekly doses of 3 mcg/kg [10]. Since our study used 1 mcg/kg dose of ENZ110, there was no statistically significant difference observed in change from baseline to week 12 for

hematology and biochemistry laboratory parameters in both the treatment groups.

Conclusion

This study established non-inferiority, along with comparable safety and immunogenicity between biosimilar romiplostim and innovator romiplostim in patients with chronic ITP.

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Authors' Contributions All authors participated in the interpretation of study results and in the drafting, critical revision, and approval of the final version of the manuscript. R Pawar, D Pawar, V Shahavi, and A Sharma were involved in the study design and/or data analyses. B Prashantha, N Sidharthan, S Shah, M Toshniwal, S Chandrakala, V Ramanan, N Padwal, P Malhotra, TK Viswanathan, S Apte, R Ballikar, M Halvawala were investigators in the study.

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Declarations

Conflicts of interest None.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication The participant has consented to the submission of the case report to the journal.

Ethics Approval The study protocol was approved at all the institutional ethics committees.

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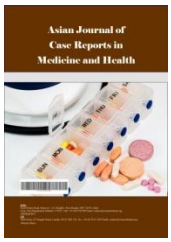

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Alkaline Diuresis and Pre-emptive Hemodialysis as Treatment for 2, 4-Dicholorophenoxy Acid Herbicide Intoxication

Manjiri Naik [aω], Shamisha Khade [a#*] and Siddhiraj Paramshetti [a#]

[a] Department of Medicine, MGM Medical College and Hospital, Aurangabad, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Case Report

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ABSTRACT

Introduction: Chlorophenoxy herbicides poisoning is very rare. It is used widely to control broad-leaved weeds. 2, 4-D is a Chlorphenoxy herbicide which has no antidote. The mission of this case report is to emphasize the role of accurate diagnosis and management of 2, 4-D herbicide intoxication.

Case Report: 19 year old male was admitted 6h after the deliberate ingestion of the contents of a half bottle (300 ml) of weedkiller named Dallas contents of which were 59% w/w 2,4-D acid tech, 30% w/w Di-methylamine. Soon after ingestion the patient gave history of vomiting, after which patient became unconscious.

On examination, RR was 35/min with a saturation of 90% on room air requiring 5-6L/min of oxygen. The pupils were small, 1.5 mm in diameter, reactive. Arterial blood analysis showed mild Metabolic acidosis. Gastric aspiration and lavage were performed. Patient was unresponsive to deep painful stimulus. He was sweating profusely with a temperature of 39 degree Celsius. Patient was electively intubated for airway protection.

Patient was admitted in the ICU. The total leukocyte counts were 17000/cmm. Liver function and kidney function tests were within normal limits. CPK total was 3150. Over the next 7 hours, patient's condition worsened and the blood pressure started dropping to 80/50 mmHg. Patient was started on vasopressor support. Alkaline diuresis was started by giving 1 meq/kg sodium

[ω] Professor and HOD;
[#] Resident;
**Corresponding author: E-mail: shamishakhade@gmail.com;*

bicarbonate in 0.9% normal saline, 100 ml iv within the next 30 minutes and then added with 75meq of sodium bicarbonate and 25 meq of potassium in 500 ml 5% dextrose within 8 hours. Inj Furosemide 20 mg was given every 12hours intravenously. On the second day, patient's laboratory investigations showed a rising trend in TLC, creatinine rose to 2.4 mg/dl from 1.1mg/dl on the day of admission. Patient's metabolic acidosis also worsened. Patient's output dropped to 5-10 ml/hr. Patient was then taken on Haemodialysis. Patient's urine output improved and GCS also improved. Three cycles of haemodialysis were done, the TLC showed a falling trend with normalising creatinine, metabolic acidosis also improved; pH normalised and patient was extubated on the fourth day. Patient was then shifted to ward and discharged subsequently on clinical improvement.

Conclusion: 2,4-Dimethylamine intoxication is uncommon, doesn't have an antidote with very high morbidity and mortality. Alkaline diuresis as a life-saving treatment and must be supplemented by other therapies including decontamination of the gastrointestinal system, initial emergency resuscitation and supportive treatment with haemodialysis as and when required.

Keywords: Alkaline dieresis; hemodialysis; dicholorophenoxy acid; herbicide intoxication.

1. INTRODUCTION

Chlorophenoxy herbicides poisoning is rare. It is used mainly for the control of broad-leaved weeds. These Compounds exhibit broad spectrum of mechanisms of toxicity which includes dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation, and disruption of acetylcoenzyme A metabolism [1]. 2, 4-D is a Chlorphenoxy herbicide which doesn't have any antidote [2]. Forced alkaline diuresis and Hemodialysis is the treatment of choice and if initiated on time may improve the otherwise very poor prognosis in severe intoxication with 2, 4-D and related weed killers. The mission of this study is to draw to attention the role of accurate diagnosis and the management of 2,4-D herbicide intoxication.

2. CASE REPORT

19 year old male was admitted 6h after the deliberate ingestion of the contents of a half bottle(300ml) of weedkiller named Dallas contents of which were 59% w/w 2,4-D acid tech,30% w/w Di-methylamine,1% w/w lignin sulphonate. Soon after ingestion the patient gave history of vomiting, which was followed by aggressive behaviour and then a confused state. Within 1 hour patient became drowsy and was followed by unconsciousness.

Patient was brought to MGM in unconscious state. On examination in Casualty, the pulse rate and blood pressure were normal but the respiratory rate was 35/min with a saturation of 90% on room air requiring 5-6L/min of oxygen. The pupils were small, 1.5mm in diameter, reactive, muscle tone was normal and deep tendon reflexes were normal, plantars were flexors. Arterial blood analysis showed mild Metabolic acidosis and hypoxia (H+ 48 nmol/l, paCO2 37 kPa, bicarbonate 15 mmol/l and paO2 70 kPa). Patient was unconscious, not responding to Deep Painful Stimuli. Ryles tube no.14 was inserted and Gastric aspiration and lavage were performed. Over the next few hours his condition deteriorated. Patient still remained unresponsive to painful stimuli, the tendon reflexes disappeared, the pulse rate increased to 140/min and the temperature rose to 39 degree Celsius. He was vasodilated and sweating profusely. Cyanosis was evident despite a respiratory rate of 40-50/min and the Chest was clear clinically and radiologically. A normal arterial oxygen tension could only be maintained with 8L/min of oxygen through a mask and the temperature remained at 39 degree Celsius despite cold sponging. Patient was electively intubated for airway protection.

Patient was admitted in the Intensive care unit, routine laboratory investigations were performed. The haemoglobin of the patient was 16.2g/dl, total leukocyte counts were 26250/cmm. Liver function and kidney function tests were within normal limits. Over the next 7 hours, the condition of patient deteriorated and the blood pressure started dropping to 80/50 mmHg. Fluid resuscitation was given but blood pressure didn't reach the target and hence was started on vasopressor support to maintain systolic blood pressure >100 mmHg. Pantoprazole was given in loading dose and was continued with maintenance dose via infusion. Alkaline diuresis was initiated by giving 1 meq/kg sodium bicarbonate in 0.9% normal saline, 100 ml iv

within the next 30 minutes and then supplemented with 75 meq of sodium bicarbonate and 25 meq of potassium in 500 ml 5% dextrose within 8 hours. Inj Furosemide 20 mg was given every 12 hours intravenously. Iv dexaamethsone was also given to treat cerebral edema. On the second day, patient's laboratory investigations showed a rising trend in total leukocyte count upto 30,000, creatinine rose to 2.4 mg/dl from initial creatinine 1.1 mg/dl on the day of admission. Patient's metabolic acidosis also worsened. Patient's output also dropped to 5-10ml/hr. A decision was then made to start the patient on Haemodialysis. Patient's urine output improved and GCS also improved. Three cycles of haemodialysis was done, patient was extubated on the fourth day, the total leukocyte count showed a falling trend with normalising creatinine with metabolic acidosis also improved; pH normalised. Patient was then shifted to ward and discharged subsequently on clinical improvement.

3. DISCUSSION

"Anticholinesterase compounds are the commenest method of poisoning in India but herbicide poisoning is also a method of suicide and is usually associated with high morbidity and mortality" [3]. "Among different herbicidal poisonings we come across the most predominantly found poisonings are paraquat and glyphosate" [4] "The incidence of 2, 4-dichlorophenoxy acetic acid poisoning are few and rarely cases are reported from India" [5]. "2, 4-dichlorophenoxy acetic acid commonly known as 2, 4-D is a plant herbicide. It is secondarily a plant growth regulator" [6]. It was developed in the 1940s. It is the most commonly used pesticide in the non-agricultural sector at the same time one of the top ten most commonly used in the agricultural sec.

"There is no discrete antidote available for 2,4-D dimethylamine intoxication. Chlorophenoxy is a weak acid (pKa 2.6 for 2,4-D) that is excreted in the urine in the same form. Intravenous sodium bicarbonate has its mechanism of action to act by increasing urine pH. Renal excretion is better in alkaline urine conditions (63 ml/min at pH 8.3) than in acidotic conditions (0.14 ml/min at pH 5.1) of urine" [2]. "For each unit increase in urine pH, the clearance of 2,4-D by the kidney is estimated to increase nearly five-fold" [3]. "Therefore its necessary to note that the administration of sodium bicarbonate with a target urine flow of 4–6 ml/minute increases the excretion of 2, 4-D dimethylamine. In this scenario, urine pH after alkaline diuresis was not assessed, so the renal clearance and half time of 2,4-D in this patient could not be stated. Hypokalaemia may occur during alkaline diuresis, according to the literature, hence sodium bicarbonate should be followed by potassium injection" [2]. "Haemodialysis is more efficient than alkaline diuresis in that it can cause the release of hazardous chemicals without changing the pH of the urine or requiring huge volumes of intravenous fluids. However, the treatment strategy chosen is ultimately determined by the availability of facilities. There are case reports describing plasmapheresis as a therapy for intoxication in relation to haemodialysis, but there is very little evidence to support this strategy in the treatment of severe 2,4-D dimethylamine intoxication" [3,4]. "In cases of mild intoxication, supportive therapy might be ample, but, in cases of severe intoxication, treatment with alkaline diuresis or haemodialysis is a necessity" [4]. "However, there is no severity classification as a reference to determine the most appropriate therapy. Shock and loss of consciousness in cases of 2,4-D dimethylamine intoxication are indicators of poor prognosis. However, timely and adequate administration of an alkaline diuresis can be life-saving. Recovery can be achieved in weeks to months despite initial severe toxicity" [2,4,5].

4. CONCLUSION

2,4-Dimethylamine intoxication is uncommon, has no antidote, and has a significant morbidity and fatality rate. Alkaline diuresis is a life-saving therapy that needs to be combined with additional treatments such first emergency resuscitation, gastrointestinal system purification, and supportive care.

CONSENT

As per international standard or university standard, patient(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Case Report

A Rare Case of Primary Mediastinal B-Cell Lymphoma – The Great Masquerade

Shamisha Shashank Khade[1], Manjiri Rajiv Naik[1]

[1]Department of Internal Medicine, MGM Medical College and Hospital, Aurangabad, Maharashtra, India.

***Corresponding author:**
Shamisha Shashank Khade, Department of Internal Medicine, MGM Medical College and Hospital, Aurangabad, Maharashtra, India.

shamishakhade@gmail.com

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ABSTRACT

Primary mediastinal B-cell lymphoma (PMBCL) is a relatively rare lymphoma subtype affecting mainly seen in young adults with female predominance. It constitutes approximately 2–4% of all non-Hodgkin's lymphomas (NHLs). No risk factors for this type have been identified but it may be related to 5533 C>A mutation in the MLL gene. Its molecular signature and clinical features resemble classical Hodgkin's lymphoma. PMBCL belongs to a group of aggressive diffuse large B-cell lymphomas. 2008 WHO classification distinguishes this lymphoma as a separate entity due to its specific clinical features and pathological features. Gene expression profile studies showed that it shares common features with classical Hodgkin's lymphoma. The optimal chemotherapy for this lymphoma subtype has not been established. Furthermore, no convincing data are supporting the use of radiotherapy. Relatively low patient numbers are the main obstacle in conducting randomised prospective trials. Hence, therapeutic decisions have been based mainly on retrospective studies.

Keywords: Primary mediastinal B-cell lymphoma, Non-Hodgkin's lymphoma, Positron emission tomography scan, Chemotherapy, Radiotherapy, MLL gene, Immunohistochemical examination

INTRODUCTION

Primary mediastinal B-cell lymphoma (PMBCL) is a relatively rare lymphoma subtype affecting mainly seen in young adults with female predominance.[1-5] It constitutes approximately 2–4% of all non-Hodgkin's lymphomas (NHLs). No risk factors for this type have been identified but it may be related to 5533 C>A mutation in the MLL gene. Its molecular signature and clinical features resemble classical Hodgkin's lymphoma. PMBCL belongs to a group of aggressive diffuse large B-cell lymphomas.[2,5,6] 2008 WHO classification distinguishes this lymphoma as a separate entity due to its specific clinical features and pathological features. Gene expression profile studies showed that it shares common features with classical Hodgkin's lymphoma. The optimal chemotherapy for this lymphoma subtype has not been established. Furthermore, no convincing data are supporting the use of radiotherapy. Relatively low patient numbers are the main obstacle in conducting randomised prospective trials. Hence, therapeutic decisions have been based mainly on retrospective studies.[6-9]

CASE REPORT

We present the case of a 19-year-old female who came to MGM Hospital with complaints of breathlessness on exertion and cough for 2-weeks. On enquiring further, she gave a history of weight loss of around 10 kg in 3 months and on and off fever. On doing a Skiagram of the chest,

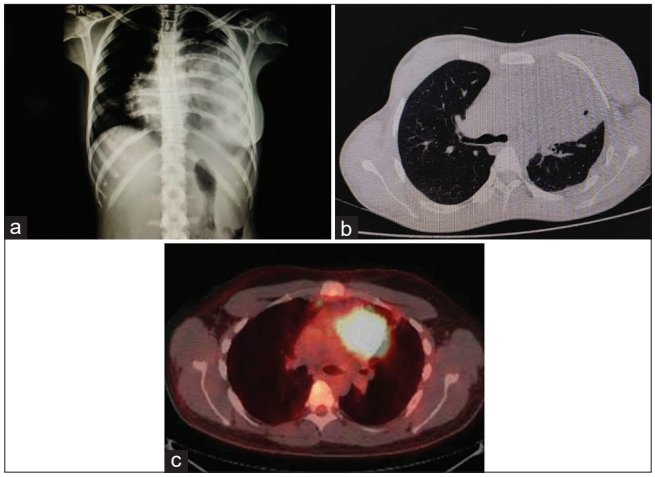

Figure 1: (a) Chest X-ray, (b) contrast-enhanced computed tomography chest, (c) positron emission tomography scan.

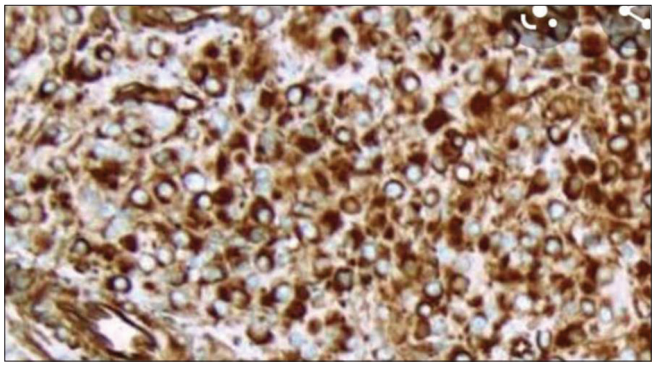

Figure 2: Vimentin-positive IHC biopsy specimen.

left sides shadow with blunting of costophrenic angle on the left side was seen with opacity in the left upper lobe suggestive of the left-sided pleural effusion and left upper lobe mass. An ultrasound (USG)-guided diagnostic and therapeutic pleural tap was done and around 200 ml of pleural fluid was tapped out. Infective aetiology was ruled out by running tests on the pleural fluid collected. This was followed by a contrast-enhanced computed tomography abdomen and chest for evaluation of the mass [Figure 1]. It revealed a neoplastic carcinomatous lesion of size 11.4 × 8.2 × 14.6 cm involving left upper lobe lung parenchyma with extension into the anterior mediastinum and enlarged necrotic prevascular and supraclavicular lymph nodes. The abdomen was clear suggestive of no metastasis to the abdomen. A USG-guided biopsy of the mass was performed and the sample underwent histopathological and immunohistochemical (IHC) examination which revealed a low-grade neoplasm and on running the IHC markers on it [Figure 2], the diagnosis of mediastinal B-cell NHL was made. A positron emission tomography (PET) scan was performed which showed increased uptake of FDG in the mediastinum and supraclavicular region [Figure 1]. The patient was started on an R-CHOP regimen for NHL and is on the same treatment currently with 2 cycles of chemotherapy completed. A repeat PET scan will be performed after 6 cycles of chemotherapy to check the progression of the tumour.

CONCLUSION

Because PMBCL is uncommon, its clinical management varies across centres. There is no standard protocol for the treatment of PMBCL but chemotherapy R-CHOP and dose-adjusted E-POC regimen are shown to be beneficial. The role of radiotherapy is unclear. Recent research has brought new insight into molecular mechanisms contributing to the malignant phenotype of PMBCL and this could direct the development of targeted therapies.

Declaration of patient consent

Patient's consent not required as patient's identity is not disclosed or compromised.

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Conflicts of interest

There are no conflicts of interest.

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Efficacy and Safety of Empagliflozin as Add on in Patients with Type II Diabetes Mellitus (DM) Inadequately Controlled on Triple Drug Combination

Deepak Bhosle[1], Snehal Chavan[2], Shraddha Kardile[3]

Abstract

Objectives: Diabetes mellitus (DM) refers to a group of metabolic disorders characterized by hyperglycemia resulting from insulin resistance, insulin action or both. Despite availability of various treatment modalities it is difficult to achieve the desired glycemic control in many patients. In such patients new class of anti-diabetic agent sodium-glucose co-transporter II (SGLT2) inhibitors has been approved by FDA. SGLT-2 inhibitor Empagliflozin has been associated with HbA1c reduction and weight loss in a broad range of patients with type 2 Diabetes Mellitus (T2DM).

Methods: An open label, interventional, single arm, 24 weeks study was done on 120 patients who were inadequately controlled on three oral hypoglycaemic agents and reluctant to take insulin therapy. Empagliflozin 25 mg once a day was added to ongoing triple drug therapy. Changes in glycemic parameters like fasting blood sugar levels, post-prandial blood sugar levels, HbA1C, body weight, systolic and diastolic blood pressure and safety profile were assessed at baseline, three months and sixth months. Study was conducted at MGM medical college and hospital, Aurangabad in collaboration with Department of Medicine.

Results: Our study revealed Empagliflozin 25 mg once daily when used as add on to ongoing triple drug therapy has shown 3.02 % reduction in HbA1c and 3.83 kg reduction in bodyweight.

Conclusion: Empagliflozin a SGLT 2 inhibitor is a promising drug for reduction in HbA1c value and body weight in patients with T2DM who are inadequately controlled on triple drug therapy and are reluctant to insulin therapy.

Introduction

Diabetes mellitus (DM) refers to a group of metabolic disorders characterized by hyperglycemia resulting from insulin resistance, insulin action or both. Chronic hyperglycemia in Diabetes mellitus is associated with long-term dysfunction and failure of various organs, micro vascular disorders like diabetic neuropathy, diabetic nephropathy, diabetic retinopathy and macro vascular disorders like cardiovascular diseases, peripheral vascular disease and cerebrovascular accidents.[1,2] Different types of Diabetes mellitus are caused by a complex interaction of genetic and environmental factors. Predominant types of Diabetes mellitus include Type 1 Diabetes mellitus, Type 2 Diabetes mellitus and Gestational Diabetes mellitus.[3]

[1]Consultant Diabetologist, Deogiri Diabetes Care Center, Professor and HOD, [2]Tutor, [3]Asst. Professor, Department of Pharmacology, MGM Medical College, Aurangabad, Maharashtra; *Corresponding Author
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The Worldwide prevalence of Diabetes mellitus is increasing alarmingly. In 2019, the prevalence was estimated to be 9.3% (463 million people) across the globe, and this is estimated to rise to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045.India is one of the seven countries included in the International Diabetes Federation (IDF) South-east Asia (SEA) region. Currently, 88 million people suffer from diabetes in the SEA region, which is expected to rise to 153 million by 2045. As per IDF SEA estimates, Diabetes mellitus is a growing challenge among the Indian population with a prevalence of 8.9%.[4]

An important role in glucose homeostasis is played by kidneys as they cause the reabsorption of glucose from the glomerular filtrate. Glucose reabsorption in the kidney is mediated by two sodium glucose co transporter (SGLT) proteins, SGLT1 and SGLT2.The majority of glucose reabsorption ~90% is mediated by SGLT2 and occurs in the first part of the proximal convoluted tubule while ~10% is reabsorbed distally in the proximal convoluted tubule by the action of SGLT1.[5,6]

SGLT2-mediated glucose transport inhibition in the kidney leads to loss of glucose in the urine and a reduction in hyperglycemia. In addition SGLT-2 inhibitors action does not depend on a functioning pancreatic β-cell, thus they are effective in any degree of β-cell function and also provide additional glucose lowering when combined with other classes of antihyperglycemic agents.[7] The urinary glucose excretion results in loss of calories which causes significant weight loss and the osmotic diuretic effect reduces blood pressure. [(7,8)] SGLT-2 inhibitors are approved in the treatment of Type 2 Diabetes mellitus in adults. Canagliflozin, dapagliflozin, and empagliflozin have approval in the United States and European Union and also in India.[9,10]

Empagliflozin is currently approved SGLT2 inhibitors for the use of Type 2 Diabetes mellitus.The drug received US FDA approval in August 2014 to reduce Type 2 Diabetes mellitus associated cardiovascular risk in adult patients.[11] In India, Central Drug Standards Control Organization has approved the drug Empagliflozin at the dose of 10mg and 25mg doses, to improve glycemic control in adults with T2DM. In addition to its glucose-lowering effects, empagliflozin has been shown to reduce body weight and blood pressure without increase in heart rate.[11,12]

Empagliflozin is the first glucose-lowering agent to demonstrate cardiovascular risk reduction in patients at high risk of cardiovascular disease. In a prospective outcome trial, a 14% reduction in risk of the 3-point composite endpoint of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke.[13] Recently, the EMPA- REG study showed that patients, with a high-risk for cardiovascular diseases receiving empagliflozin had a lower rate of deaths from cardiovascular diseases.[14,15] Triple drug therapy has been introduced in India following the global acceptance of two drug fixed dose combinations. Studies related to triple drug treatment for Type 2 Diabetes mellitus patients have shown that the therapy provides significant reduction in HBA1C levels.[16]

This analysis evaluated changes in the glycemia parameters such as FBS, PPBS, HbA1, body weight, systolic and diastolic blood pressure in patients who were inadequately responding to maximum dose of three oral hypoglycaemic agents and reluctant to take insulin therapy along with therapeutic safety of the patients.

Methods

24 weeks prospective, open label, single centre, single arm, interventional, clinical study was conducted at MGM medical college and hospital, Aurangabad in collaboration with Department of Medicine. Patients aged 18 to 65 years (N=120) who were inadequately controlled on triple drug therapy for Type 2 Diabetes Mellitus (T2DM). Inclusion criteria was T2DM patients of either sex (male or female) on maximum dose of three OHA with inadequate response, HbA1c > 8.5% and BMI > 25 kg/m^2. Newly diagnosed T2DM patients, type 1 diabetes mellitus, gestational diabetes, patients with eGFR value less than 45 ml/min/1.73 m^2 calculated by MDRD formula, patients on insulin therapy, patients with recurrent UTI and patients with history of diabetic ketoacidosis or other co-morbid cardiac, hepatic and renal diseases were excluded.

All the patients participating in the study were explained clearly about the purpose and nature of the study in the language they can understand. They were included in the study only after obtaining a written informed consent form (ICF)

The study was commenced following the approval of the Institutional Ethics Committee. All information pertaining to the patient visiting Out Patient Department, such as patient's age, gender, occupation, relevant history, past history and drug therapy will be recorded in a Case Record Form (CRF).

Details of the prescribed drugs for Diabetes mellitus, and all other drugs used in the patient during treatment were recorded. They include the dose, duration, type of dosage form used, frequency of drug administration etc. and necessary information was recorded in a structured Case Record Form.

Empagliflozin 25 mg (1 tablet) once daily was administered as an add-on therapy to triple drug treatment and patients were asked to take it in the morning with ample amount of water. Study assessment was done by evaluating the study visit checklist which included informed consent, screening for inclusion criteria & exclusion criteria, general & physical examination. Blood sugar – fasting & post prandial, glycosylated haemoglobin level (HbA1C), blood pressure and body weight with safety assessment were performed at baseline and follow-up visits. Total 3 visits were planned. First visit at the baseline, Second visit at 12 weeks and third visit at 24 weeks, i.e. at the end of the study.

Primary end point was change in Hb A1c (%) from baseline up to 24 weeks. Secondary end point was change in body weight from baseline up to 24 weeks. Safety assessment was performed by general and systemic examination and as per ADR reported by patients. The study was performed on 120 patients of which 76 were males and 44 were females. Data were collected at the baseline and at 12 weeks and 24 weeks for estimation of FBS, PPBS, HbA1c value and body weight and blood pressure. Paired t test was applied to this data and result was derived by using SPSS v.24

Results

The study was performed on 120

Table 1: Triple drug therapy prescribed to study patients

Triple Drug Combination	Number of Patients
Metformin + Glimeperide +Voglibose	23
Metformin + Glimeperide +Teneligliptin	31
Metformin + Teneligliptin +Voglibose	17
Metformin + Glimeperide +Vildagliptin	13
Metformin + Glimeperide +Pioglitazone	23
Metformin + Teneligliptin +Pioglitazone	2
Metformin + Vildagliptin + Voglibose	11

patients of which 76 were males and 44 were females. Among 120 patients recruited, 116 patients completed the study (96.67%), 3 were withdrawn due to ADR (2.5%) and there was 1 drop out (0.8%). After 24 weeks of study, 3.02% reduction in HbA1c was observed from baseline and 3.83 kg reduction in body weight was recorded, P value= 0.001. 3 out of 120 patients (2.5%) reported UTI and were withdrawn from study. All the three patients were female and treatment for UTI was provided as required. 116 patients tolerated Empagliflozin 25 mg once daily well. The mean duration of Diabetes mellitus was **7.24 ± 2.29 years.** The average duration of triple drug therapy in patients was **2.87 ± 0.83 years.** Most frequently prescribed triple drug combination was Metformin+Gli meperide+Teneligliptin (N=31), while Metformin+Teneligliptin+Pioglitazo ne was the least commonly prescribed triple drug therapy (N=2) in our study (Table 1). At week 24, Empagliflozin 25 mg provided significant reductions in glycemia parameters and body weight from baseline (p<0. 001) (Table 2). Significant reduction was observed in the values of FBS with mean difference from baseline to 24 weeks of – 46.76 mg/dl (P< 0.001). Reduction was recorded in baseline PPBS value by – 87.41 mg/dl at 24 weeks (P< 0.001). Differences in mean changes in HbA1c were −3.02 % (P< 0.001) with Empagliflozin 25 mg given additionally with three drug combination therapy. Significant dose-related reductions from baseline in body weight were observed (p<0.001) with empagliflozin 25 mg resulting in mean weight loss of 3.83 kg from baseline at 24 weeks. Males have shown more reduction in values of glycemia Parameters like FBS, PPBS and HbA1C as compared to females but weight loss was observed more in females as

Table 2: Mean values of all parameters at baseline, 12 and 24 weeks

Parameters	Duration	Mean ± SD	Mean difference	P value
FBS	Baseline vs. 12 weeks	193.03 ± 61.69 vs 167.88 ± 43.95	25.15	P<0.001
	12 weeks vs. 24 weeks	167.88 ± 43.95 vs 146.27 ± 31.80	21.61	P<0.001
	Baseline vs. 24 weeks	193.03 ± 61.69 vs 146.27 ± 31.80	46.76	P<0.001
PPBS	Baseline vs. 12weeks	299.50 ± 95.94 vs 245.16 ± 60.16	54.34	P<0.001
	12 weeks vs. 24 weeks	245.16 ± 60.16 vs 212.09 ± 45.87	33.07	P<0.001
	Baseline vs. 24 weeks	299.50 ± 95.94 vs 212.09 ± 45.87	87.41	P<0.001
HbA1C	Baseline vs. 12weeks	11.90 ± 2.16 vs 10.34 ± 1.72	1.56	P<0.001
	12 weeks vs. 24 weeks	10.34 ± 1.72 vs 8.88 ± 1.34	1.46	P<0.001
	Baseline vs. 24 weeks	11.90 ± 2.16 vs 8.88 ± 1.34	3.02	P<0.001
Body Weight	Baseline vs. 12weeks	87.09 ± 13.19 vs 85.20 ±12.92	1.89	P<0.001
	12 weeks vs. 24 weeks	85.20 ±12.92 vs 83.26 ± 12.66	1.94	P<0.001
	Baseline vs. 24 weeks	87.09 ± 13.19 vs 83.26 ± 12.66	3.83	P<0.001

P <0.05 is considered statistically significant

Table 3: Gender wise reduction in FBS, PPBS, HbA1C and Body weight

Parameters		Males Mean ± SD	Females Mean ± SD	P value
FBS (mg%)	Baseline	190.12±58.14	**192.04±63.24**	**0.8663**
	12 weeks	**168.43±42.93**	**167.13±43.91**	**0.8743**
	24 weeks	**145.93±32.19**	**147.39±30.82**	**0.8083**
PPBS(mg%)	**Baseline**	**299.11±94.13**	**298.65±96.98**	**0.9797**
	12 weeks	**244.98±58.13**	**245.94±60.94**	**0.9319**
	24 weeks	**211.87±44.96**	**212.73±45.13**	**0.9199**
HbA1c(%)	**Baseline**	**11.49±2.47**	**12.09±2.03**	**0.1734**
	12 weeks	**10.57±1.62**	**10.77±1.57**	**0.5111**
	24 weeks	**8.67±1.47**	**8.89±1.32**	**0.4142**
Body weight(Kg)	**Baseline**	**87.14±13.49**	**86.57±13.52**	**0.8240**
	12 weeks	**85.29±12.87**	**85.09±12.75**	**0.9345**
	24 weeks	**83.37±12.69**	**83.11±12.59**	**0.9138**

P <0.05 is considered statistically significant

compared to male population. Though continuous reduction was observed in values of all the parameters in both males (N=76) and females (N=44); no statistically significant difference was recorded amongst both genders (Table 3). A reduction of 3.8 mm of Hg in systolic and a reduction of 2 mm of Hg in diastolic blood pressure were observed in the patients at the end of 24 weeks of study from the baseline.

Discussion

In healthy individuals, about 180mg of glucose is filtered and reabsorbed daily through the kidneys and maximal transport rate (Tmax) is 300mg/min. This rate is about 20% higher i.e. 352 mg/min (19.5mmol/l/min) to 419mg/min (23.3mmol/l/min)[5] in patients with poorly controlled T2DM. This pertains to the increased expression of SGLTs in persons with diabetes which represents a physiological response to increased glucose delivery to the nephrons that is ultimately maladaptive.[6,7] Antagonizing these transporters with SGLT2 inhibitors is an insulin-independent mechanism that offers a considerable advantage of increasing urinary glucose excretion without inducing hypoglycaemia and promoting weight loss due to loss of 300-400 kcal/day.[8] Empagliflozin is currently approved SGLT2 inhibitors for the use of Type2 Diabetes mellitus The drug has gained US FDA approval in August 2014 to reduce T2DM associated cardiovascular risk in adult patients. CDSCO has approved the drug in India at the dose of 10mg and 25mg doses on May 2015, to improve glycemic control in adults with T2DM. In addition to its glucose-lowering effects, empagliflozin has been shown to reduce body weight and blood pressure without increase in heart rate.[9-11]

According to the latest American Diabetes Association (ADA) and European Association of Study of Diabetes (EASD) joint statement released in October 2018 use of newer cardio friendly drugs for treatment of type II DM has been highly recommended.[12] Empagliflozin is the first glucose-lowering agent to demonstrate cardiovascular risk reduction in patients with diabetes at high risk of cardiovascular disease in a prospective outcomes trial: a 14% reduction in risk of the 3-point composite endpoint of death from

cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke has been reported.. Recently, the EMPA- REG study showed that patients, with a high-risk for CVD, receiving empagliflozin had a lower rate of deaths from CVD. S.R Pattanaik et al. (2018) demonstrated the long-term tolerability, glycemic efficacy and safety of empagliflozin as an add-on to triple drug treatment.[12-14]

In this study, at week 24, Empagliflozin 25 mg provided significant reductions in glycemia parameters from baseline (p<0. 001). Significant reduction was recorded in the values of FBS with mean difference from baseline to 24 weeks of – 46.76 mg/dl (P< 0.0001). Reduction was recorded in baseline PPBS value by –87.41 mg/dl at 24 weeks (P< 0.0001). Differences in mean changes in HbA1c were −3.02 % with Empagliflozin 25 mg given additionally with three drug combination therapy. In all the reduction was more from baseline to 12 weeks period than from 12 weeks to 24 weeks duration

Significant dose-related reductions from baseline in body weight were observed at week 24 (p<0.001). Empagliflozin 25 mg provided mean changes of −3.83 kg from baseline at 24 weeks.Weight loss with Empagliflozin 25 mg occurred rapidly through week 12; a progressive decrease in weight loss over the remaining treatment period was seen. Our results correlate with studies done on T2DM patients who were administered Empagliflozin 25 mg as monotherapy, or other regimens like with metformin, other two OHA and insulin. A reduction of 3.8 mm of Hg in systolic and a reduction of 2 mm of Hg in diastolic blood pressure were observed in the patients at the end of 24 weeks of study from the baseline.

In conclusion, Empagliflozin a SGLT-2 inhibitor is a promising new drug; when administered in a dose of 25 mg (one tablet) as an add on to patients with inadequately controlled type II DM who were receiving triple drug OHAs and were reluctant for an insulin therapy, it provided a significant reduction in HbA1c and body weight over a period of 24 weeks.

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ORIGINAL ARTICLE

Evaluation of Teneligliptin a DPP4 Inhibitor in Terms of Efficacy and Safety with Respect to QT/QTc Prolongation in Patients with Type II Diabetes Mellitus (T2DM)

Deepak Bhosle[1*], Bhakti Chandekar[2], Shaikh Alimuddin[2]

Abstract

Introduction: Low risk of hypoglycemia and weight neutrality have increased the administration of dipeptidyl peptidase 4 (DPP-4) inhibitors in patients with T2DM in clinical practice.Currently Teneligliptin is prescribed as a second or third add on to the standard treatment with other classes of oral hypoglycemic agents (OHAs) to achieve targeted glycemic control in type 2 DM patients.

Methods: An open label, interventional, single arm, 12 weeks study was conducted on160 patients with type 2 DM at MGM Medical College, Aurangabad with Teneligliptin 20 mg once a day as add on to the ongoing standard treatment with other classes of OHAs. Changes in glycemia parameters like FBS, PPBS HbA1C, body weight were assessed and twelve lead ECG was recorded with safety assessment at baseline and follow-up visits.. The QTc was calculated by using the Bazett's formula (QTc=QT/√RR).The study was conducted with an objective to assess efficacy and safety of Teneligliptin with respect to QT/QTc prolongation in patients with T2DM.

Results: A significant reduction was seen in the glycemic parameters like FBS, PPBS HbA1C from the baseline values (P<0.001) but no significant change in the QT interval (P=0.9563) and QTc interval (P=0.5594) from the baseline to the end of study at12 weeks.

Conclusion: Teneleglitpin is a promising new drug to help to achieve targeted glycemic control in patients with T2DM without prolonging the QT/QTc interval.

Introduction

Diabetes mellitus, a heterogeneous group of metabolic syndromes is characterized by an elevation in blood glucose. A variety of pathogenic mechanisms involving insufficient insulin secretion, reduced responsiveness to endogenous or exogenous insulin, increased glucose production, and /or abnormalities in fat and protein metabolism have been recognized.[1] The resulting prolonged hyperglycemia is the major cause of chronic long term microvscular complications of diabetes such as retinopathy, neuropathy, nephropathy, and macrovascular complications like cardiovascular diseases, cerebrovascular accidents and peripheral vascular diseases.[2]

The worldwide prevalence of diabetes mellitus is increasing alarmingly and it is estimated to rise to 10.2% (578 million) by 2030.[3] As per International Diabetes Federation (IDF) South-east Asia (SEA) estimates, DM is a growing challenge among Indian population with a prevalence of 8.9%.[3]

The choice of glucose lowering agent must be made carefully, particularly when a diverse range of pharmacological agents (consisting of at least 12 drug classes) are available for the treatment of T2DM.[4] Of these, biguanides, sulfonylureas (SUs), meglitinides, dipeptidyl peptidase - 4 inhibitors (DPP4i), thiazolidinediones, alpha glucosidase inhibitors, and sodium glucose co-transporter 2 (SGLT2) inhibitors are the commonly used oral antidiabetic agents (OADs) both as mono and combination therapy inT2DM patients.[4]

According to the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) guidelines metformin has been suggested to be used as a first-line drug treatment along with life style modifications (LSMs). Both agencies recommend an addition of a second drug if monotherapy with metformin along with LSMs fails to achieve glycemic control within the target levels as laid down by ADA.[5] According to the latest ADA-EASD joint statement released in october 2018 use of the newer cardio-friendly drugs for treatment of T2DM has been highly recommended. Differing from the recommendations of EASD-ADA, Japanese Diabetes Society (JDS) emphasizes more on the pathophysiology of patients' diabetes and recommends use of any antidiabetic drugs that are appropriate to it.[6] Thus the incretin-based drugs especially DPP-4 inhibitors are considered to be the first choice therapy in Japanese type 2 diabetes patients according to the recommended guidelines.[7]

Dipeptidyl peptidase 4 (DPP-4) inhibitors considered as a relatively new category, produce their effects by increasing the concentration of active forms of incretin, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).[8] DPP-4 inhibitors show marked structural heterogeneity, despite their common mechanism of action.[8] A novel DPP-4 inhibitor, Teneligliptin, produces a potent and long-lasting effect by virtue of it's unique structure exhibiting five consecutive rings.[9] Teneligliptin was

[1]Professor and HOD, Department of Pharmacology, [2]Tutor, Department of Pharmacology, MGM Medical College, Aurangabad, Maharashtra; *Corresponding Author
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originally synthesized by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan) and was the first drug of its kind to be synthesized in Japan.[10] Since September 2012, teneligliptin has been commercially sold in Japan. Since the introduction of teneligliptin, DPP-4 inhibitors have been consistently prescribed in India.[10] Currently Teneligliptin is prescribed as a second or third add on to mono therapy or dual therapy with other classes of OHAs to achieve targeted glycemic control in patients with type 2 DM. From the results of various clinical trials it has been observed that along with effective glycemic control, teneligliptin, as monotherapy or add-on therapy, was generally well tolerated in patients with T2DM.[11]

In the recent past all the gliptins were questioned for development of pancreatitis especially Sitagliptin and regarding cardiac safety in terms of QT/QTc prolongation especially teneligliptin. Efficacy and safety evaluation with respect to QT/QTc assessment studies associated with use of teneligliptin in patients with type 2 diabetes were conducted in Japan and other countries.[12] The maximal dose of teneligliptin is considered to be 20 mg/day or 40 mg/day depending upon the glycemic status of the patient of teneligliptin, and no adverse events (AEs) related to QT prolongation were detected with these doses[12] But, during initial safety assessment studies, teneligliptin at 160 mg/day dose, was associated with changes in QT interval. [13] As diabetic patients are more prone to develop cardiovascular diseases, cardiac safety of antidiabetic drugs must be ensured and demonstrated. In India not much published data is available regarding the safety of teneligliptin, with respect to QTc prolongation at therapeutic doses, in patients with type 2 DM. [13]

The present study was conducted to assess teneligliptin in terms of efficacy and safety with respect to QT/QTc prolongation in patients with T2DM.

Material and Methods

12 weeks prospective, open label, single center, single arm, interventional, clinical study, was conducted at MGM Medical College, Aurangabad in collaboration with Department of Medicine. Patients aged 18 to 65 years (N=160) who were either on mono therapy or on combination therapy of oral hypoglycemic agents (OHAs) other than DPP4 inhibitors for Type 2 Diabetes Mellitus (T2DM), T2DM patients of either sex (male or female) with HbA1c > 7.0% were included in the study. Newly diagnosed patients of T2DM, Type 1 DM, Gestational DM, Patient on insulin therapy, Patients with marked baseline prolongation of QT/QTc interval, having history of additional risk factors for Torsades de pointes (TdP), Patients with the use of concomitant medications that have the potential to prolong the QT/QTc interval, Patients with a history of seizures, history of stroke, and cardiovascular events, Patients with history of DKA, Patients with history of hepatic diseases and renal diseases were excluded.

All the patients participating in the study were explained clearly about the purpose and nature of the study in the language they can understand. They were included in the study only after obtaining a written informed consent form (ICF)

The study was commenced following the approval of the Institutional Ethics Committee. All information pertaining to the patient visiting out patient department, such as patient's age, gender, occupation, relevant history, past history and drug therapy given will be recorded in a Case Record Form (CRF).

Details of the prescribed drugs for diabetes mellitus, and all other drugs used in the patient during treatment were recorded. They include the dose, duration, type of dosage form used, frequency of drug administration etc. and necessary information was recorded in a structured CRF.

Patients were given Teneligliptin 20 mg once a day as an add on to the standard treatment. Study assessment was done by evaluating the study visit checklist which included informed consent, screening for inclusion criteria and exclusion criteria, general and physical examination. Blood sugar – fasting and post prandial, glycosylated haemoglobin level (HbA1C), body weight assessments were performed and twelve lead ECG was recorded with safety assessment at baseline and follow-up visits.. The QTc was calculated by using the Bazett's formula ($QTc=QT/\sqrt{RR}$).

Total 4 visits were planned for ECG recording. First visit before starting the therapy (baseline), Second visit on day 1 (4 hours after Teneleglipti administration), visit 3 after 6 weeks, and visit 4 after 12 weeks i.e. at the end of the study. The glycemia parameters and body weight were assessed at baseline and at visit 4 at the end of study.

Primary end point was change in QT/QTc interval from baseline up to 12 weeks. Secondary end point was change in glycemic parameters like FBS, PPBS and HbA1c (%) and body weight from baseline up to 12 weeks. Safety assessment was performed by general and systemic examination and as per ADR reported by patients. The study was performed on 160 patients of which 118 were males and 42 were females. Data were collected at the defined visits. Paired t test was applied to this data and result was derived by using SPSS v.24.

Results

The study was performed on 160 patients of which 118 were males and 42 were females. The average age of patients enrolled was 48.2±7.1 years The average duration of diabetes was 3–4 years, average body weight was 81.84**±14.07 kg**.

All the patients were prescribed with Teneligliptin 20 mg once a day as an add on to the standard treatment. Metformin was the most common drug prescribed as mono therapy and combination of Glimepiride and Metformin was the preferred combination as a dual therapy along with teneligliptin.

The mean QT interval at screening visit 1(Day 0, baseline ECG) was 344.68 ± 20.07 milliseconds (msec), while at visit 2 (Day 1, 4 hours after Teneligliptin dosing) it was 344.48 ± 22.21 msec, at visit 3 (6 weeks) it was 344.50 ± 21.97 msec, and at visit 4 (12 weeks) it was 344.63 ± 22.13 msec. (table 1). No significant difference was seen in the QT interval (P=0.9563) at the end of 12 weeks (Table 1). The mean QTc interval at baseline was 395.27 ± 25.09 msec, while at visit 2 it was 396.71 ± 25.39 msec, at visit 3 it was 395.26 ± 24.52 msec, and at visit 4 it was 396.93 ± 25.51 msec. (Table 2). There was no statistically significant difference in QTc interval from baseline to any subsequent follow-up visits. Therefore

Table 1: Values of QT interval (in msec) at subsequent visits

Baseline value 344.68 ± 20.07(Visit 1)			
Number of visits	Mean+ SD (Standard Deviation)	Mean difference	P value
(Visit 2) 4 hours after Teneleglipti n dosing	344.48 ± 22.21	0.2	0.7243
(Visit 3) At 6 weeks	344.50 ± 21.97	0.18	0.817
(Visit 4) At 12 weeks	344.63± 22.13	0.03	0.9563

Table 2: Values of QTc interval (in msec) at subsequent visits

Baseline value 395.27±25.09 (Visit 1)			
Number of visits	Mean+ SD	Mean difference	P value
(Visit 2) 4 hours after Teneleglipti n dosing	396.71 ± 25.39	1.441	0.6283
(Visit 3) At 6 weeks	395.26 ± 24.52	0.008625	0.9975
(Visit 4) At 12 weeks	396.93± 25.51	1.652	0.5594

there was no significant change in the QTc interval (P=0.5594) at the end of study (Table 2).

A significant reduction was seen in glycemic parameters like fasting blood sugar

(P<0.001), postprandial blood sugar (P<0.001), and HbA1c (P<0.001) at the end of 12 weeks, from the baseline values (Table 3) The average reduction of 32.8 mg% was marked in FBS, a reduction by 48.7 mg %was observed in PPBS and HbA1C was reduced by 1.03 % at the end of study duration (Table 3). The body weight was reduced by an average of 0.44 kg at the end of 12 weeks which was not significant (P= 0.5819) (Table 4).

Discussion

Type 2 diabetes mellitus (T2DM) is a chronic disease that develops as a result of defective insulin secretion and is frequently associated with obesity-related insulin resistance.[1] Involvement of multiple physiological pathways and complex pathogenesis of diabetes explains the multifaceted morbidity noted in individuals with T2DM.[2]

The reduction in fasting and postprandial blood glucose levels by DPP-4 inhibitors is attributed to their effects on entero insular axis consisting of GLP and GIP which consequently increase the sensitivities of both β-and α- cells to glucose levels.[14] Because of the low risk of hypoglycemia and being

Table 3: Values of glycemic parameter like FBS, PPBS, HbA1C

Parameters	Baseline value Mean+ SD (Visit 1)	At 12 weeks Mean+ SD (Visit 4)	Mean difference	P value
FBS (mg%)	172.80± 17.23	140.40± 17.23	32.8	P<0.001
PPBS (mg%)	255.88± 12.01	206.89± 12.02	48.7	P<0.001
HbA1C (%)	9.0±0.94	7.96±0.94	1.03	P<0.001

Table 4: Values of body weight

Parameters	Baseline value Mean+ SD (Visit 1)	At 12 weeks Mean+ SD (Visit 4)	Mean difference	P value
Body Weight (Kg)	81.84±14.07	81.40±14.46	0.4281	P= 0.5819

weight neutral the administration of DPP-4 inhibitors in patients with T2DM has been markedly increased in clinical practice.[15]

Teneligliptin, a DPP4 inhibitor, was approved for the management of type 2 diabetes mellitus in Japan (2012), in South Korea (2014), and in India (2015).[11] In adults, usually teneligliptin is orally administered at a dosage of 20 mg once daily, which can be increased up to 40 mg per day depending upon the values of glycemia parameters.The elimination of metabolic products via renal and hepatic excretion, patients with renal impairment need no dose adjustment.[11] Teneligliptin has a similar safety profile as compared with other available DPP-4 inhibitors. However, caution must be exercised while administering teneligliptin to patients who are prone to QT prolongation.[12]

To determine threshold pharmacologic effect of a drug on cardiac repolarization "thorough QT/QTc study" has been explained.[16] The risk of development of Torsades de pointes is linked with the prolongation of QT interval. According to the USFDA level of regulatory concern for cardiac safety of any drug, drugs prolonging the mean QT/QTc interval by >20 ms are considered to be having proarrhythmic potential ; and those which prolong the mean QT/QTc interval by around 5 ms or less are usually considered to be nonarrhthmogenic.[16]

According to the teneligliptin data submitted to PMDA, (Pharmaceuticals and Medical Devices Agency) Japan, based on the thorough QT/QTc study, clinically recommended doses (20 mg and 40 mg), of teneligliptin do not cause QTc prolongation.[17] Patients taking teneligliptin along with drugs having known potential to cause QT prolongation on their own, should be carefully observed.[13] Hypoglycemia considered as one of the strongest QTc prolongators, should also be watched for when a combination therapy with other hypoglycem drugs is administerd.[18]

In this study at week 12 the average change in the mean QT interval from baseline ECG, was 0.03msecs.. The average change in the mean QTc interval from baseline ECG, was 1.652 msecs at the end of 12 weeks. No significant difference was seen in the QT as well as QTc interval at each visit subsequent to the baseline visit and there was no significant change in the QT interval (P=0.9563) and QTc interval (P=0.5594) at the end of study.

At the end of 12 weeks a significant reduction (P<0.001) was seen in glycemic parameters such as fasting blood sugar, postprandial blood sugar and HbA1c. The body weight was also reduced but it was not a significant reduction (P= 0.5819).

A significant reduction in glycemic parameters with teneligliptin daily 20 mg as a monotherapy was observed in a 3 months study by Kutoh et al,13 which was done in 31 japanese patients with type 2 DM who had never received teneligliptin.[19] TREAT INDIA study also observed similar significant reduction in parameters like FBS, PPBS and HbA1c at the end of 3 months from the baseline values with teneligliptin therapy.[20] Q SET study performed over a period of 3 months by S Erande et al also concluded that Teneligliptin at a therapeutic dose of 20 mg/day or 40 mg/day improved glycemic parameters significantly and did not cause QT/QTc interval prolongation.[21]

In our study patients were given Teneligliptin 20 mg once a day as an add on to the standard treatment with other OHAs such as biguanides, sulfonylureas, glitazones either as mono therapy or combination therapy. The drug was well tolerated by the patients except 14 patients in the study reported hypoglycemia but did not withdraw and completed the study. The main drawback of the study was it was an open label study and the study duration

was less to observe the effects of the drug.

Conclusion

Teneligliptin 20 mg once a day as an add on to the standard treatment with other OHAs was well tolerated and did not prolong QT/QTc interval. It provided a significant reduction in FBS, PPBS and HbA1c over 12 weeks and body weight was also reduced but not significantly.

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Serum Magnesium Levels in Critically Ill Patients on Admission in ICU and its Correlation with Outcome

Jivabhai Solanki[1], Kiran Runwal[2], Nandkumar Beke[2], Arun Bahulikar[3], Deepak Phalgune[4*]

Abstract

Background: Many studies found that only hypomagnesemia, but not hypermagnesemia is linked with increased mortality. However, reports of mortality due to magnesium dysregulation in the critical care setting are controversial.

Objectives: To study serum magnesium levels in critically ill patients on admission in intensive care unit (ICU) and its correlation with patient's need and duration for ventilator support, duration of ICU stay, incidence of cardiac arrhythmias and mortality.

Methods: Two hundred forty six critically ill patients admitted in ICU with Acute Physiology and Chronic Health Evaluation (APACHE) II scores>10, were included for this prospective observational study. Serum total magnesium level was measured at the time of admission to ICU. Primary outcome measure was ICU mortality whereas, secondary outcome measures were patient's need and duration for ventilator support, duration of ICU stay, and incidence of cardiac arrhythmias. Categorical and continuous variables were tested using Chi-square/Fisher's exact test and analysis of variance respectively. Multivariate logistic regression analysis was carried out to determine association of serum magnesium levels with ICU mortality.

Results: Incidence of ICU mortality was significantly higher in group of patients with hypomagnesemia compared to those with normal magnesium levels. Hypomagnesemia was associated with need and longer duration of ventilator support, longer duration of ICU stay, higher APACHE II score, QTc prolongation, higher incidence of cardiac arrhythmias compared to patients with normal magnesium levels. Hypomagnesemia was an independent and statistically significant determinant of ICU mortality.

Conclusions: Hypomagnesemia was associated with higher mortality rate, longer duration of ventilator support and ICU stay, and higher APACHE II score in critically ill patients.

[1]Senior Resident, [2]Consultant Physician, [3]HoD and Consultant Physician, Dept. of Medicine, [4]Research Consultant, Poona Hospital & Research Centre, Pune, Maharashtra; *Corresponding Author
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ORIGINAL ARTICLE

A Comparative Study of efficacy and safety of different Sodium Glucose Co-transporter 2 (SGLT-2) Inhibitors in the Management of Patients with Type II Diabetes Mellitus

Deepak Bhosle[1*], Sanjeev Indurkar[2], Umar Quadri[3], Bhakti Chandekar[4]

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Abstract

Background: There are a handful of sodium glucose co-transporter 2 (SGLT2) inhibitors available in the global and Indian markets to manage type II diabetes mellitus (T2DM). However, head-to-head comparison between different SGLT2 inhibitors is scarce. Therefore, the present study was aimed to analyze the effect of different SGLT2 inhibitors on glycemic control and body weight in Indian patients with T2DM.

Methods: This was a prospective, interventional, nonrandomized study that included patients (N = 480) of either sex, aged ≥30 years, with inadequately controlled T2DM having HbA1c > 8.5%, and were receiving either Canagliflozin, Empagliflozin, Dapagliflozin or Remogliflozin on the background of triple-drug therapy. In this study, patients were evaluated for HbA1c, fasting blood sugar (FBS), post-prandial blood sugar (PPBS), body weight, and systolic and diastolic blood pressure at baseline, 12 and 24 weeks.

Results: A total of 480 patients who received either Canagliflozin (*n* = 120), Empagliflozin (*n* = 120), Dapagliflozin (*n* = 120), or Remogliflozin (*n* = 120) were included in this study. There was a significant reduction in levels of HbA1c, FBS, PPBS, body weight, systolic blood pressure (SBP), and diastolic blood pressure (DBP) at week 12 and 24 in all treatment groups. The difference in mean values of glycemic parameters and body weight was comparable across the treatment groups at week 12 and 24 but was not significant. Out of all 480 patients, 10 patients (2.08%) reported urinary tract infection (UTI), and five (1.04%) reported genital mycotic infection. All the five patients were females and treatment for UTI and mycotic infection was provided as required. Rest of the patients tolerated the therapy well.

Conclusion: Overall observations indicate that all the four SGLT2 inhibitors are effective in reducing HbA1c, FBS, PPBS, body weight SBP, and DBP. Therefore, gliflozins can be the best choice to start early in patients with inadequately controlled T2DM receiving triple-drug therapy which helps in controlling the parameters of glycemia and significantly reducing the body weight. Hence SGLT2 Inhibitors could be considered as an add-on to all antidiabetic agents currently used for the management of diabetes in Indian setting.

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Introduction

Sodium glucose co-transporter 2 (SGLT2) inhibitors are recently approved oral anti-hyperglycemic agents by US Food and Drug Administration (FDA) (2013). Owing to their non-pancreatic action SGLT2 inhibitors have demonstrated efficacy and safety in the management of T2DM through the reduction of hypoglycemia risk. These agents are recommended along with diet and exercise by various international as well as Indian guidelines for diabetes management. In a recent update of the ADA 2020 guideline of diabetes management, SGLT2 inhibitors are recommended especially in patients with diabetes with high cardiovascular risk.[1] Likewise, the use of SGLT2 inhibitors in patients with type II diabetes has been recommended by the Research Society for the Study of Diabetes in India.[2]

Unlike the other oral hypoglycemic agents, SGLT2 inhibitors have a novel mechanism of action that reduces blood glucose levels without triggering insulin secretion.[3] In addition, several SGLT2 inhibitors have benefits in terms of reduction in body weight, blood pressure, and cardiovascular risk. Assessment of the safety profile indicates genitourinary infection is more commonly observed among patients with diabetes receiving treatment of SGLT2 inhibitors followed by mycotic infection, polyuria, volume depletion, hypotension, and dizziness.[4–6]

Currently, there are a handful of SGLT2 inhibitors including Canagliflozin, Empagliflozin, Dapagliflozin, and Remogliflozin available in the global and Indian market to manage type II diabetes either as monotherapy or with concomitant medication.[7] Recent real-world studies from Ireland and Southern Europe on the clinical efficacy of SGLT2 inhibitors reported a significant reduction in HbA1c and body weight in patients with type II diabetes.[8,9] Similarly, real-world experience from India reported the effectiveness of SGLT2 inhibitor in terms of significant improvement in glycemic control and weight reduction with the insignificant incidence of adverse events.[5]

Aim of the study: To analyze the efficacy and safety of different SGLT2 inhibitors in patients with T2DM.

Objective: To study the effect of different SGLT2 inhibitors on glycemic parameters, body weight, and blood pressure in patients with T2DM.

Methods

This was a prospective, interventional, nonrandomized study conducted in MGM Medical College and Hospital, in collaboration with the Department of Medicine and Deogiri Diabetes Care Centre, Aurangabad, Maharashtra, India, between November 2019 and November 2020.

The study protocol was approved by MGM Ethics Committee for Research on Human Subjects (MGM. ECRHS).

Study Population and Data Collection

Patients (N = 480) of either sex, aged ≥30 years, with inadequately controlled T2DM having HbA1c > 8.5%, and BMI > 25 kg/m^2 who were receiving either Canagliflozin (100 mg OD), Empagliflozin (25 mg OD), Dapagliflozin (10 mg OD) or Remogliflozin (100 mg BD) (N = 120 for each group) on the background of triple-drug therapy were included in this study. Newly diagnosed T2DM

[1]Deogiri Diabetic Care Centre; [2]Madhumeha Chikitsalaya, Rachanakar Colony; [3,4]MGM Medical College and Hospital, Aurangabad, Maharashtra, India; *Corresponding Author

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patients, type I diabetes mellitus, gestational diabetes, patients with eGFR value less than 45 ml/min/1.73 m^2 calculated by MDRD formula, patients on insulin therapy, patients with recurrent UTI, and patients with a history of diabetic ketoacidosis or other comorbid cardiac, hepatic, and renal diseases were excluded.

Data collection included body weight in Kgs measured with the electronic weighing machine, and laboratory data included parameters determining glycemic control [HBA1c % measured using High Performance Liquid Chromatography method (Bio-Rad D 10), FBS, PPBS in mg% were analyzed with fully automated Vitros 250 Dry Chemistry analyzer], SBP and DBP measured with a sphygmomanometer in mm of Hg. These parameters were recorded at different time points, at baseline, at week 12 and 24. Safety assessment was performed by general and systemic examination and as per adverse drug reaction reported by patients.

Statistical Analysis

Data were analyzed using Statistical Package for The Social Sciences (SPSS) software, version 24.0. Quantitative data were presented as mean [standard deviation (SD)] while qualitative data were presented as number. We have applied paired *t*-test for within-group comparison (before and after therapy) ANOVA test for intergroup comparison and a comparison of two groups was done using *post hoc* test of LSD (Latin Square Design). A *p*-value < 0.05 was considered statistically significant

Results

A total of 480 patients who received either Canagliflozin ($n = 120$), Empagliflozin ($n = 120$), Dapagliflozin ($n = 120$), or Remogliflozin ($n = 120$) were included in this study. The mean (SD) age of the patients was 52.1 (9.35) years in Canagliflozin (C), 51.8 (10.74) years in Empagliflozin (E), 52.0 (12.33) years in Dapagliflozin (D), and 51.9 (12.19) years in Remogliflozin (R) groups. All four groups were having comparable ages (*p*-value 0.361) with a slightly higher proportion of men than women in each group.

Though the difference in mean values of glycemic parameters like FBS, PPBS, HbA1c, and other parameters such as body weight, SBP and DBP was comparable across the treatment groups at 12-week and 24-week follow-up from baseline; the intergroup comparison between all four groups did not demonstrate a significant difference (Tables 1 and 2).

Similarly, two groups comparison using a *post hoc* test of LSD observed nonsignificant differences in the mean values of all the parameters except C vs R and E vs R groups where a significant reduction was observed in mean FBS values at 24 weeks.

A significant reduction was observed within the groups in the HbA1C values at the end of 24 weeks with a total mean reduction of 3.08, 2.87, 2.74, and 2.79% in Canagliflozin, Empagliflozin, Dapagliflozin, and Remogliflozin groups, respectively (Table 3). An overall highly significant reduction was recorded in the mean values of other glycemia parameters like FBS and PPBS within all four groups (Table 3). Similarly, body weight reduction was also observed in all the patients along with a reduction in SBP and DBP with highly significant differences within individual groups from baseline to the end of 24 weeks (Table 3). Out of a total of 480 patients enrolled in all the four groups 10 patients (2.08%) reported UTI and 5 (1.04%) patients reported genital mycotic infection. All the five patients were females

Table 1: Comparison of mean values of glycemic parameters in all groups (ANOVA)

Glycemia Parameters	*No. of visits*	*Canagliflozin (Mean ± SD) (C)*	*Empagliflozin (Mean ± SD) (E)*	*Dapagliflozin (Mean ± SD) (D)*	*Remogliflozin (Mean ± SD) (R)*	*p-value*
Fasting blood sugar (FBS) (mg%)	Baseline (visit 1)	195.94 ± 23.69	193.93 ± 22.52	198.58 ± 27.52	200.42 ± 27.17	0.201
	12 weeks (visit 2)	164.11 ± 25.14	161.62 ± 23.77	167.50 ± 30.32	167.31 ± 29.90	0.291
	24 Weeks (Visit 3)	140.32 ± 24.99	139.77 ± 24.71	143.82 ± 36.51	148.31 ± 29.72	0.095
Post prandial blood sugar (PPBS) (mg%)	Baseline (visit 1)	291.37 ± 62.54	287.72 ± 65.02	289.23 ± 61.36	287.91 ± 63.37	0.968
	12 weeks (visit 2)	246.41 ± 65.92	245.62 ± 66.18	248.63 ± 65.72	246.37 ± 65.90	0.987
	24 weeks (visit 3)	205.94 ± 70.31	203.71 ± 68.65	205.44 ± 70.32	206.8 ± 70.27	0.988
HbA1C (%)	Baseline (visit 1)	11.7 ± 1.79	11.6 ± 1.76	11.5 ± 1.80	11.6 ± 1.81	0.854
	12 weeks (visit 2)	10.23 ± 1.62	10.31 ± 1.68	10.83 ± 1.75	10.3 ± 1.52	0.747
	24 weeks (visit 3)	8.62 ± 1.57	8.73 ± 1.70	8.76 ± 1.67	8.81 ± 1.74	0.837

Two groups comparison using a *post hoc* test of LSD observed nonsignificant differences in the mean values of all the parameters except C Vs R (0.036*) and E vs R (0.025*) groups where the significant reduction was observed in mean FBS values at 24 weeks

Table 2: Comparison of mean values of other parameters in all groups (ANOVA)

Other Parameters	*No. of visits*	*Canagliflozin (Mean ± SD) (C)*	*Empagliflozin (Mean ± SD) (E)*	*Dapagliflozin (Mean ± SD) (D)*	*Remogliflozin (Mean ± SD) (R)*	*p-value*
Bodyweight (Kg)	Baseline (visit 1)	72.81 ± 9.88	73.12 ± 13.06	71.82 ± 12.15	72.74 ± 13.11	0.879
	12 weeks (visit 2)	70.43 ± 14.52	71.24 ± 14.54	69.13 ± 13.82	70.24 ± 14.48	0.724
	24 weeks (visit 3)	68.22 ± 13.87	68.43 ± 13.78	67.62 ± 12.34	69.23 ± 13.38	0.828
Systolic blood pressure (SBP) (mm of Hg)	Baseline (visit 1)	138.81 ± 5.31	139.32 ± 5.78	139.11 ± 5.36	138.94 ± 5.68	0.940
	12 weeks (visit 2)	136.90 ± 7.46	137.44 ± 6.40	136.63 ± 5.66	136.0 ± 6.67	0.345
	24 weeks (visit 3)	134.71 ± 8.35	135.21 ± 6.64	134.94 ± 6.38	134.82 ± 7.32	0.863
Diastolic blood pressure (DBP) (mm of Hg)	Baseline (visit 1)	89.31 ± 5.90	87.63 ± 6.17	87.53 ± 7.92	87.64 ± 7.75	0.163
	12 weeks (visit 2)	88.14 ± 6.06	86.01 ± 5.92	86.34 ± 8.35	86.23 ± 8.09	0.084
	24 weeks (visit 3)	86.61 ± 6.14	85.22 ± 4.85	85.11 ± 8.56	85.32 ± 8.96	0.343

Table 3: Comparison of mean difference in values of all parameters within groups (Paired *t*-test)

Parameters	*No. of visits*	*Canagliflozin (C)*	*Empagliflozin (E)*	*Dapagliflozin (D)*	*Remogliflozin (R)*
FBS (mg%)	Baseline vs 24 weeks	55.62 ($p < 0.001^{**}$)	54.16 ($p < 0.001^{**}$)	54.79 ($p < 0.001^{**}$)	52.12 ($p < 0.001^{**}$)
PPBS (mg%)	Baseline vs 24 weeks	85.43 ($p < 0.001^{**}$)	84.01 ($p < 0.001^{**}$)	83.79 ($p < 0.001^{**}$)	81.11 ($p < 0.001^{**}$)
HbA1C (%)	Baseline vs 24 weeks	3.08 ($p < 0.001^{**}$)	2.87 ($p < 0.001^{**}$)	2.74 ($p < 0.001^{**}$)	2.79 ($p < 0.001^{**}$)
Bodyweight (Kg)	Baseline vs 24 weeks	4.59 ($p = 0.0035^{**}$)	4.69 ($p = 0.0073^{**}$)	4.20 ($p = 0.0084^{**}$)	3.51 ($p = 0.0412^{*}$)
SBP (mm Hg)	Baseline vs 24 weeks	4.10 ($p < 0.001^{**}$)	4.11 ($p < 0.001^{**}$)	4.17 ($p < 0.001^{**}$)	4.12 ($p < 0.001^{**}$)
DBP (mm Hg)	Baseline vs 24 weeks	2.70 ($p < 0.001^{**}$)	2.41 ($p < 0.001^{**}$)	2.42 ($p < 0.001^{**}$)	2.32 ($p < 0.001^{**}$)

Within group comparison using a *paired t* test observed significant differences ($p < 0.001^{**}$) in the mean values of all the parameters except body weight in all groups where the reduction was non significant at 24 weeks

Table 4: Adverse drug reactions (ADRs) in each group

ADR	*Canagliflozin*	*Empagliflozin*	*Dapagliflozin*	*Remogliflozin*
Genital mycotic infection	2	1	1	1
Urinary tract infection	3	3	2	2

and treatment for UTI and mycotic infection was provided as required. The rest of the patients tolerated the therapy well (Table 4).

Discussion

An extensive literature search has revealed that there is a scarcity of data that compared the efficacy of several available gliflozins in a single study at the global as well as national levels. The use of gliflozins varies widely due to varied clinical inertia toward a marketed drug.[8,9] A real-world study from Southern Europe carried out the clinical effectiveness of dapagliflozin in various countries and reported geographical diversity may have a significant impact on gliflozins on glycemic control.[8]

In view of this lacuna, the present study attempted to evaluate the effect of SGLT2 inhibitors on glycemic control and body weight in Indian patients with diabetes. The overall observations of this study suggest a reduction in glycemic level at all visits in all the treatment groups indicating the effectiveness of gliflozins on glycemic control. At 12 and 24 weeks of follow-up, all the four gliflozins in this study showed a significant reduction in HbA1c, blood glucose levels, and body weight from baseline indicating the efficacy of these drugs in achieving good glycemic control and weight reduction. These findings corroborate with the previous studies where each of these gliflozins has shown improvement in glycemic control and better influence on weight reduction.[8–10]

Empagliflozin is the first gliflozin approved by USFDA followed by Canagliflozin and Dapagliflozin. However, Remogliflozin is recently approved SGLT2 inhibitor by USFDA for the management of diabetes. In the present study, the mean difference in HbA1c at 6-months was comparable across the treatment groups. Similarly, a real-world observational study of 120 Indian patients with uncontrolled type II diabetes that compared Remogliflozin 100 mg with Canagliflozin 300 mg reported similar effectiveness between these two agents in terms of reducing HbA1c level, PPBS, FBS, and body weight.[11] India is a developing country with a large proportion of the patient population from lower socioeconomic classes, and the cost-effectiveness of drugs is a crucial factor attributable to drug compliance. Remogliflozin and dapagliflozin were more cost-effective and can be used as alternative SGLT2 inhibitor options.[11] SGLT2 inhibitors have also been observed to address cardiovascular and renal outcomes in terms of safety and efficacy through various global cardiovascular outcome trials. Another previous clinical trial (open-labeled, 52-week study) comparing Empagliflozin with dapagliflozin as add-on therapy in patients with uncontrolled type II diabetes showed both SGLT2 agents as effective as previous antidiabetic agents. However, the authors further demonstrated Empagliflozin is more effective in improving glycemic control and other cardiometabolic outcomes along with a reduction in body weight compared to dapagliflozin.[12] On the contrary present study reported all the four gliflozins are comparable in terms of achieving glycemic control and weight loss. A recently published randomized active-controlled trial compared Remogliflozin vs dapagliflozin for 6 months in patients with uncontrolled type II diabetes demonstrated noninferiority of Remogliflozin over dapagliflozin in terms of reducing HbA1c, FBS, PPBS, and body weight.[13] Similarly, in the present study, the mean difference of HbA1c, FBS, PPBs, and weight were comparable between Remogliflozin and dapagliflozin at 12 and 24 weeks follow-up from baseline.

Sodium glucose co-transporter 2 inhibitors are associated with significant weight reduction in patients with diabetes.[14] Likewise, a previous real-world study conducted on 30 Irish patients with diabetes reported a reduction in HbA1c and body weight over 15 months of exposure to SGLT2 inhibitors.[9] This is in accordance with the present study that reported all the four gliflozins to have significant weight loss at 12 and 24 weeks follow-up from baseline.

Several limitations of this study should be considered and observations should be interpreted vigilantly. The most important limitation of our study was the small sample size and duration of the study. More prospective clinical studies with head-to-head comparisons of SGLT2 inhibitors will be helpful in validating these observations.

Conclusion

Overall observations indicate all the four gliflozins (Canagliflozin, Empagliflozin, Dapagliflozin, and Remogliflozin) were similarly effective in achieving target glycemic levels and reduction in body weight. A reduction was also observed in blood pressure with the use of all the four gliflozins. Therefore, gliflozins can be a possible choice for the management of diabetes in Indian settings.

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ORIGINAL ARTICLE

Impact of COVID-19-related Stress on Glycaemic Control in Hospitalized Patients with Type 2 Diabetes Mellitus

Rohini Patil[1,2], PhD candidate;
Kamal Shah[3], PhD;
Deepak Bhosle[4], MD

[1]Department of Information Technology, Thakur college of Engineering and Technology, Mumbai, Maharashtra, India
[2]Department of Computer Engineering, Terna Engineering College, Navi Mumbai, Maharashtra, India
[3]Department of Information Technology, Thakur College of Engineering and Technology, Mumbai, Maharashtra, India
[4]Department of Pharmacology, MGM Medical College, Aurangabad, Maharashtra, India

Correspondence:
Rohini Patil, PhD candidate;
Department of Information Technology, Thakur college of Engineering and Technology, Mumbai, Maharashtra, India
Tel: +91 98 20167350
Email: rohinipatil@ternaengg.ac.in
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Abstract

Background: Evaluation of the impact of stress on glycaemic control in hospitalized type-2 diabetes (T2DM) patients with coronavirus disease (COVID-19).

Methods: In this retrospective study conducted at a single centre in Maharashtra from May to July 2020 on hospitalized COVID-19 patients with T2DM who reported having stress of pandemic; they were selected using purposive sampling. DASS-12 stress sub-scale was used to estimate the severity of their stress. Fasting blood glucose (FBG) and post-prandial blood glucose (PPBG) before admission and at the time of discharge were compared.

Results: One hundred and ninety-nine patients (mean age 54 years; 61.30% females) were included. Mean±SD FBG before admission was 168.4±30.6 mg/dl which increased to 195.9±28.8 mg/dl at the time of discharge (P<0.001). Also, Mean±SD PPBG before admission was 312±62.3 mg/dl which increased to 351.6±61.9 mg/dl (P<0.001). A total of 73 (36.7%) participants had perceived stress. Moderate and severe/extremely severe stress was found in 44 (27.1%) and 19 (9.6%) patients, respectively. A significant difference was observed in the mean FBG before and during discharge in patients who had no stress and those with moderate stress (P<0.001). There was no difference in FBG in patients with severe/extremely severe stress (P=0.43). Similar observations were seen for PPBG (no stress P<0.001; moderate stress P<0.001; severe/extremely severe stress P=0.06).

Conclusion: There was a rise in the glucose level in T2DM patients discharged after COVID-19 treatment. The increase was significant in T2DM without stress and those with moderate stress. In addition to traditional treatment, measures for psychological stress control should also be taken for such patients.

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Introduction

Diabetes mellitus is a challenging and prevalent chronic metabolic disorder from psychosocial and behavioural perspective. Untreated diabetes can result in serious short-term or long-term complications resulting in significant morbidity and mortality. According to global report of International Diabetes Federation (IDF 2017), there are 463 million people with diabetes.[1] In 2019, the global prevalence of diabetes was 9.3%. It is expected to rise to 10.2% and 10.9% by 2030 and 2045, respectively.[2] Overall type 2 diabetes (T2DM) prevalence in India is 8.9%.[1] Uncontrolled T2DM can result in several acute and chronic complications. Although traditional risk factors contribute to the high risk of T2DM development, its incidence continues to increase despite strategies to

control traditional risk factors.[3]

Stress contributes to many pathological conditions and hormonal imbalance; during stress, situations can adversely affect the normal glycaemic control in people with diabetes.[4] Perceived stress can contribute to the risk of T2DM development.[3] A 12- year longitudinal study on women showed three years later stress levels were associated with a higher risk of diabetes.[3] Apart from traditional stress factors, during the last year corona virus disease (COVID-19) emerged and caused a significant stress on people, families and community. Diabetes is expected to have poor outcomes after COVID-19 infection.[5] Patients with diabetes are reported to have COVID-19-specific concerns regarding their disease.[6] A cross-sectional study from South India reported unhealthy dietary habits, mental stress, and sleep disturbances during COVID-19 lockdown period. However, the same study reported no major difference in overall glycaemic control among patients with T2DM during lockdown.[7] Therefore, we aimed to study the effect of stress on T2DM patients. The objective was to evaluate the impact of stress on glycaemic control in T2DM patients hospitalized for COVID-19 treatment.

Methods

A retrospective single-centre questionnaire based on a study in Maharashtra, with patients of both gender with T2DM who received anti-diabetes medications with a history of hospitalization for the treatment of COVID-19 from May to July 2020 and those whose readings for glycaemic parameters, i.e. fasting blood glucose (FBG) level and post-prandial blood glucose (PPBG) level were available were included in the study. Type 1 diabetes patients, newly diagnosed cases of T2DM who did not receive any anti-diabetic medication, patients with T2DM with no history of hospitalization for COVID-19 treatment, and those with gestational diabetes were excluded. Demographic details [gender, age, weight, body mass index (BMI)] and duration of T2DM were noted. DASS-12 stress sub-scale was used to estimate the severity of stress.[8] There are four items in the stress sub-scale which are rated as "Never (0), Sometimes (1), Often (2), and Almost Always (3)". Based on the stress scores, the patients were classified as (0-4) normal, (5) moderate, (6) severe and (≥7) extremely severe.[8] Face validity and content validity of the questionnaire were checked with an expert.

Difference in glycaemic parameters (FBG and PPBG) before and at the time of discharge was estimated. Gender-wise and age-wise (<34 years, 35-49 years and >50 years) comparison was done for estimating the difference in the severity of stress.

The data were entered into MS-EXCEL sheet. Number and percentages are provided for categorical data whereas Mean±SD are provided for continuous data. With the use of paired t-test, the difference in glycaemic parameters before and after COVID-19-related admission was compared. Unpaired t-test was used to estimate the statistical difference in glycaemic parameters between different groups. Chi-square test was used for comparing the categorical variables among the two groups. Results were found statistically significant (P<0.05).

Results

A total of 199 patients with a Mean±SD age of 54±12.8 years were included, of whom 122 (61.30%) were female and 77 (38.7%) were male. The Mean±SD weight and BMI of patients were 78.1±14.6 kg and 30.9±7.5 kg/m^2. A total of 116 (58.3%) patients were from urban areas, whereas 83 (41.7%) were from rural areas (Table 1). A total of 114 (57.3%) patients were housewives.

Table 1: Demographics characteristics of the study participants

Parameter	Result
Mean±SD age	54±12.8 years
Age range (minimum, maximum)	30-92 years
Gender n (%)	
Male	77 (38.7%)
Female	122 (61.3%)
Mean±SD weight	78.1±14.6 kg
Range of weight	30-114 kg
Mean±SD BMI	30.9±7.5 Kg/m^2
Range of BMI	10.6-51.5 Kg/m^2
Residence n (%)	
Rural	83 (41.7%)
Urban	116 (58.3%)
Profession n (%)	
Business	7 (3.5%)
Service	10 (5.03%)
Housewife	114 (57.3%)
Other	68 (34.2%)
Mean±SD duration of diabetes	5±3.6 years
Range of duration of diabetes (minimum, maximum)	0.08-25 years

Mean±SD FBG before admission was 168.4±30.6 mg/dl which increased to 195.9±28.8 mg/dl after discharge (P<0.001). Similarly, Mean±SD PPBG before admission was 312±62.3 mg/dl which increased to 351.6±61.9 mg/dl (P<0.001), as shown in Table 2.

Overall, out of 199 participants, 54 (27.1%) patients had moderate stress. Severe or extremely severe stress was observed in 19 (9.6%) patients (Figure 1). Thus, out of 199 participants, 73 (36.7%) had perceived stress.

In the group of moderate stress, 23 out of 54 patients (42.6%) were females and 31 (57.4%) were male. In the group of patients with severe/extremely severe stress, 11 out of 19 patients (57.9%) were females and 08 (42.1%) were male. Gender-wise as well as age-wise comparison showed a significant difference in the severity of stress (P<0.05).

Comparison of glycemic parameters, weight, age, and BMI between the groups is shown in Table 3.

Table 2: Glycaemic parameters before and at the time of hospital discharge after COVID-19 treatment

	Before hospital admission	After discharge	P value
Mean±SD fasting blood glucose mg/dl	168.4±30.6	195.9±28.8	<0.001
Mean±SD post prandial blood glucose mg/dl	312±62.3	351.6±61.9	<0.001

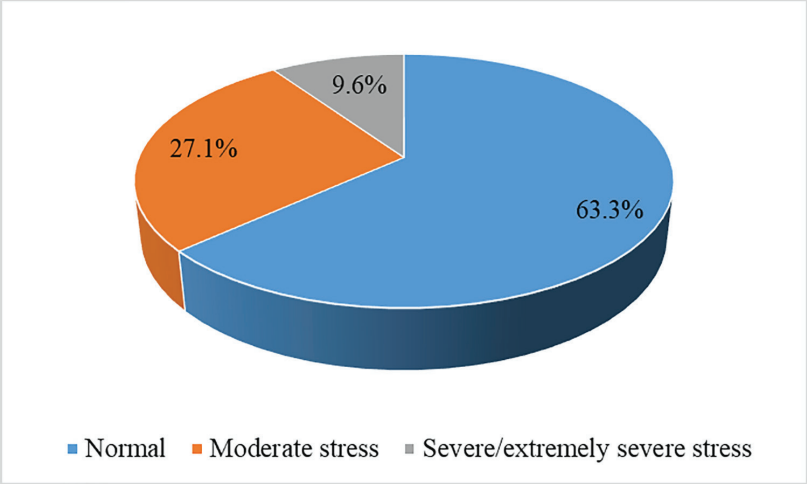

Figure 1: Distribution of patients based on the severity of stress as assessed by DASS-12 score (n=199)

Table 3: Comparison of glycemic parameters based on the severity of stress

	No stress (n=126)	Moderate stress (n=54)	Severe/Extremely severe stress (n=19)
Mean±SD age in years	52.6±13	57.3±12.5	54.2±10.6
Mean±SD weight in Kg	80±13.9	74±15.1	77.2±16.5
Mean±SD BMI in kg/m^2	32.6±7.3	27.8±6.7	28.3±7.5
Gender n (%)			
Male	38 (30.16%)	23 (42.59%)	8 (42.11%)
Female	88 (69.84%)	31 (57.41%)	11 (57.89%)
Mean±SD fasting blood glucose (mg/dl)			
Before admission	164.7±30	171.3±24.5	185±43.2
After admission	195.1±27.8	198.3±30.8	194.2±30.8
P value	<0.001	<0.001	0.43
Mean±SD post-prandial blood glucose (mg/dl)			
Before admission	309.2±62.3	315.8±59	319.7±72.8
After admission	347.3±64.7	359±52.6	358.7±68.1
P value	<0.001	<0.001	0.06

Table 4: Comparison of glycemic parameters in three groups with different levels of stress

	Without stress versus moderate stress	Moderate versus severe/ extremely severe stress	Without stress versus severe/ extremely severe stress
Fasting blood glucose before admission	0.15	0.09	0.01
Fasting blood glucose at the time of discharge	0.497	0.68	0.98
Post-prandial blood glucose before admission	0.51	0.82	0.50
Post-prandial blood glucose at the time of discharge	0.24	0.99	0.48

There was a significant difference in the mean FBG before and after admission in patients without stress and those with moderate stress. There was no significant difference in the mean FBG in patients with severe/extremely severe stress (P=0.43). Similar observations were seen for PPBG (without stress P<0.001; moderate stress P<0.001; severe/extremely severe stress P=0.06) (Table 3).

There was no difference in the FBG or PPBG in patients without stress versus moderate stress, moderate versus severe/extremely severe stress, no stress versus severe/extremely severe stress before admission or after discharge (Table 4).

In patients with stress, there was significant difference in the FBG and PPBG based on their residence, i.e. urban versus rural population (P<0.001).

Discussion

COVID-19 pandemic has impacted every individual's life, resulting in significant changes in their lifestyle. A study on 435 patients has reported increase in perceived

stress among patients with diabetes.[9] In the current study, we investigated the impact of perceived stress on glycaemic control in 199 T2DM patients discharged after their COVID-19 treatment. Generally, male patients are at higher risk of complications related to COVID-19 disease as compared to female. Similarly, male predominance is observed among hospitalized diabetic patients with COVID-19.[10] However, in our study there was female predominance. Our observations are in accordance with those of Yoshida et al. who conducted a study to evaluate the gender differences in clinical presentations and outcomes in hospitalized patients for COVID-19. In their study, there were 61.4% females, and women had a significantly higher prevalence of diabetes as compared to males (38.2% vs. 31.8%).[11] We focused only on patients with diabetes.

In our study, out of 73 patients with stress, 46.6% patients were female. A study from urban slums of Bangalore reported higher prevalence of diabetes in females as compared to males.[12] In our study, the prevalence of severe stress was more common in females.

Negative effect of stress on glycaemic control in patients with diabetes is known. Direct as well as indirect effects both contribute to the glycaemic impairment in patients with diabetes. Direct effects are related to the stress hormones, and indirect ones are due to changes in the lifestyle and behaviour.[4] Chronic stress can lead to neuroendocrine changes and dysregulation of physiological systems.[13]

A study from South India reported no major change in the overall glycaemic control among patients with T2DM due to lockdown after COVID-19 pandemic.[7] We observed a significant increase in the mean FBG and PPBG in T2DM patients at the time of discharge as compared to before admission for COVID-19 treatment. Suboptimal glycaemic control during infectious diseases is known.[14] However, we analysed the data at the time of discharge from the hospital.

Depending on the duration of exposure to stressors, patients with diabetes may be exposed to acute or chronic stress. Acute stress, because of its short duration, may not affect HbA1c which indicates glucose control over several weeks.[13] Considering this, we did not focus on changes in HbA1c. However, a retrospective study from Japan reported a significant rise in HbA1c levels after the outbreak of COVID-19 as compared to before the pandemic. There have been changes in the physical and psychological health of patients during this period. Behavioural changes have been suggested to affect the level of HbA1c in these patients.[15] Although not specifically examined, stress contributing to glycaemic derangement cannot be ruled out. In the same study, when compared by age, a significant increase in HbA1c was observed in patients with age more than 65 years. Also, there was a significant increase in HbA1c in patients with BMI more than 25 kg/m^2, but not in those with lower BMI. We focused on T2DM patients hospitalized for the treatment of COVID-19, unlike outpatients in a study by Tanji et al.[15]

Faulenbach et al. evaluated the effect of acute stress on glycaemic control in 30 patients with T2DM with a mean age of 60 years. In this study, experience of stress after the meals resulted in a significant increase in the post-prandial blood glucose level.[16] Another cross-sectional study from Chennai, India, has reported a positive correlation between both FBS and PPBG levels and the stress levels.[17] Another study has reported the association of increased stress with difficulty in glycaemic control.[9]

Perceived intensity of stress can also vary between different individuals. To categorise the patients into different levels of stress, we used DASS-12 stress scale. In our study, 36.7% had stress, of whom 74% had moderate stress and 26% had severe or extremely severe stress. In our study, a significant difference was observed in the mean FBG before admission and at the time of discharge in patients with moderate stress. However, there was no significant difference in the mean FBG in patients with severe/extremely severe stress. Similar observations were seen for PPBG (moderate stress P<0.001; severe/extremely severe stress P=0.06). We could not findany study on the effects of severity of stress on glycaemic control in T2DM patients. Furthermore, deterioration in HbA1c values has been reported, in particular among women, patients aged more than 65 years, those with body mass index of more than 25 kg/m^2, and those that were not using insulin.[15] Further studies on evaluation of the effect of stress on glycaemic parameters in these subgroups are recommended.

Intergroup analysis showed no difference in the FBG or PPBG in patients without stress versus moderate stress, moderate versus severe/extremely severe stress, no stress versus severe/extremely severe stress before admission or after discharge.

Diabetes is a known risk factor for hospitalization and mortality due to infections.[18] It has also been reported as a risk factor for severity and mortality in patients with COVID-19.[19, 20] Patients with diabetes may get frustrated with experience of hyperglycemia despite the lifestyle modifications.[21] Stress may further add to the impairment of glycaemic control. Thus, it is essential to address psychological issues of vulnerable groups during the COVID-19 pandemic.[22] Considering the adverse impact on glycaemic control, patients with diabetes should be counselled effectively to control stress.

This was a retrospective study; hence, a definite cause and effect relationship between stress and glycaemic parameters cannot be ascertained. The single centre study with limited sample size is

another limitation. COVID-19 may contribute to the development of hyperglycaemia.[23] Moreover, steroids used in the treatment of COVID-19 can also contribute to the hyperglycaemia. Because of lack of pharmacotherapy details in these patients, we could not conduct separate analysis of patients who received steroids versus those who did not. Larger prospective studies are recommended to be conducted to confirm our observations.

Conclusion

Overall, the study population showed a rise in fasting and postprandial glucose level in T2DM patients discharged after COVID-19 treatment. The rise was significant in T2DM without stress and those with moderate stress. Studies with larger sample size on T2DM patients with stress may be needed to provide more insights regarding the difference between those without stress and moderate to severe/extremely severe stress. In addition to traditional treatment of diabetes, measures for control of psychological stress should also be taken in patients with COVID-19.

Conflicts of interest: None declared.

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