



## **The Effect of Restraint Stress on the Reactivation of Colitis in Acetic Acid-Induced Ulcerative Colitis Rat**

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Author SFI designed the study, performed the statistical analysis and wrote the protocol. Author OTA wrote the first draft of the manuscript. Authors SFI and OTA managed the literature searches. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aim:** To evaluate the effect of restraint stress on the reactivation of ulcerative colitis in rats that have been chemically-induced with ulcerative colitis after allowing progression of natural healing to take place.

**Study Design:** 36 animals were used for this study, and were divided into three groups; control (group 1), colitis alone (group 2) and colitis + restraint stress (group 3).

**Methodology:** 36 healthy female wistar rats of weight of 170±20 were used. Colitis was induced by intra-rectal administration of 1 mL/100 g body weight of 7% acetic acid. Fourteen days post colitis induction, colitis + restraint stress group animals were exposed to restraint stress for six consecutive days while animals in colitis alone group were not. Colon levels of Malondialdehyde (MDA), Glutathione (GSH) concentration, Superoxide dismutase (SOD), Catalase (CAT) and Myeloperoxidase (MPO) activities were measured spectrophotometrically. Histological assessment was also done on the colon tissues.

**Place and Duration of Study:** Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria, between April 2019 and August 2019.

**Results:** Colitis caused significant increase in the levels of GSH, SOD, CAT and MPO on the 3<sup>rd</sup>

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day. On the 14<sup>th</sup> day, group 2 showed lowered level of antioxidant activities (SOD and CAT) when compared with the 3<sup>rd</sup> day, while on the 20<sup>th</sup> day, group 3 animals exhibited increased levels of antioxidant activities (as well as increase in MDA and MPO) when compared to group 2 and group 1. Histological examination revealed cyst in the mucosal layer, infiltration of inflammatory cells and dilation of vessels in the colitis + restraint stress on the 20<sup>th</sup> day post colitis induction. Results are considered significant when  $p \leq 0.05$ .

**Conclusion:** The results of this study showed that restraint stress is capable of reactivating and inhibiting colitis healing in acetic acid-induced ulcerative colitis rats.

**Keywords:** *Ulcerative colitis; restraint stress; inflammation; antioxidants.*

## 1. INTRODUCTION

Although, the etiology of inflammatory bowel disease (IBD) has not been fully elucidated, there are evidences that the pathogenesis involves interaction among genetic susceptibility, the immune system, and the environment. Colonic inflammation has been associated with oxidative damage in humans [1] and in several animal models of IBD [2]. This damage is mediated by pro-inflammatory mediators, for example reactive oxygen metabolites (ROM). It has been shown that IBD is accompanied by a decrease in colonic low-molecular-weight antioxidants (LMWAs) [3]. These LMWAs are water-soluble compounds, for example glutathione, ascorbic acid, and uric acid, and lipid soluble compounds, for example tocopherol and ubiquinone-10, which act as scavengers in the tissue. Previously, it has been demonstrated that in the normal colon, glutamine has a protective role by increasing the antioxidant capacity of the mucosa [4]. However, tissue levels of malondialdehyde have been known to serve as an index of lipid peroxidation [5].

With regard to the effect of stress in precipitating in the exacerbation of IBD, studies of humans have yielded conflicting results. Bitton et al. followed 60 patients with ulcerative colitis prospectively for one year and discovered that the number of stressful life events (as evaluated by the Psychiatric Epidemiology Research Interview Life Events Scale) was significantly higher in the period preceding exacerbation [6]. Duffy et al. have also shown positive temporal association between stress and exacerbation of IBD. Stress-exposed patients were at increased risk of clinical exacerbation compared with unexposed patients, and in a multiple regression analysis stress was the most significant indicator of disease activity [7]. In contrast, North et al. found no correlation between life events and relapses of IBD. For a small group of IBD patients who had at least one relapse in a two-year period mood changes occurred concurrently with the exacerbation but there were no indications that stressful life events

or depressed mood precipitated these events [8].

Studies of rodent models of IBD have revealed significant effects of stress on inflammation in the bowel. Restraint and noise exposure facilitate the development of intestinal inflammation in colitis induced by 2, 4-dinitrobenzene sulfonic acid (diinitrobenzene sulfonate, DNBS) [9], 2,4,6-trinitrobenzene sulfonic acid (trinitrobenzene sulfonate, TNBS) [10] and dextran sulfate sodium (DSS) [11]. Intestinal inflammation can be induced by water deprivation [12] or by housing in hectic surroundings [13]. Early weaning predisposes rats to development of intestinal inflammation when the animals are exposed to stressors in adulthood [12]. According to Qiu et al.; Collins et al. and Milde and Murison [9,10,11], amongst many others, exposure to restraint stress and noise has been seen to worsen the formation of chemically-induced ulcerative colitis however the effect of restraint stress on the reactivation of ulcerative colitis has not been elucidated and this is the principal focus of this study.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Animals

A total of 36 healthy female wistar rats with weight of  $170 \pm 20$  g as at the time of acquisition and that have not being previously used for any experimental procedure were used. They were reared in the Animal House of Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria. The rats were allowed to acclimatize for 10 days before colitis was induced and were fed with standard pelletized rat feed and water *ad libitum*, with 12 hours night/day cycle, all through the course of the research.

### 2.2 Animal Housing

Animals were kept in large plastic cages with dried wood shaving as bedding to provide the

needed comfort for the rats. The animal bedding, in each cage, was changed 2 times per week.

### 2.3 Animal Grouping

Animals were grouped into Group 1 (Control), Group 2 (Colitis alone) and Group 3 (Colitis + restraint stress), each consisting of 12 animals. Group 1 animals served as control, while group 2 and 3 animals were induced with colitis. While group 2 animals were allowed to heal naturally, group 3 animals were exposed to restraint stress.

### 2.4 Induction of Colitis

The animals were fasted for 24 hours prior to the administration of 7% acetic acid, while water was made available *ad libitum*. Colitis was induced by a single instillation of 1 mL/100 g body weight of 7% acetic acid intra-rectally. The control animals were only given an equal quantity of distilled water. Afterwards, the animals were given their feed again.

### 2.5 Period of Exposure to Restraint Stress

After confirming that colitis healing has significantly progressed naturally, which, in this case, was considered so on the 14th day post colitis induction. Group 3 animals were exposed to restraint stress by placing them in a hollow perforated transparent plastic tube, while making sure that gross blood flow to the head and limbs is not affected [14].

Each rat was allowed to completely enter in to their individual plastic tubes where no movement of the limbs was permitted. This was done for 5 hours daily for 6 consecutive days.

### 2.6 Method of Sacrifice

Animals were sacrificed from each of the groups on the 3<sup>rd</sup>, 14<sup>th</sup> and 20<sup>th</sup> day post colitis induction. Each rat was sacrificed using the cervical dislocation method. After the dissection of the rats, the colon of each animal was cut 6cm proximal to the anal opening to assess the severity of colonic tissue damage.

### 2.7 Assessment of Colitis

After animals were sacrificed and the colon excised, the colonic tissues were cut open longitudinally and washed in chilled normal saline solution and weighed. A large portion of each rat's colonic tissue was homogenized in

phosphate buffer solution (PBS) and then centrifuged at a speed of 16,000 RPM at 40C for 20 mins. The supernatant was kept between 20C-40C and appropriately used for the determination of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) activity, malondialdehyde (MDA) and myeloperoxidase (MPO) levels. The level of colonic superoxide dismutase and catalase activities, glutathione and Malondialdehyde were determined as described in previous studied [15-18]. Myeloperoxidase (MPO) was determined via activity assay kits (Elabscience Biotechnology Inc) following the manufacturers protocols.

A smaller portion of the harvested colonic tissue was also cut and then fixed in 10% formalin for histological studies.

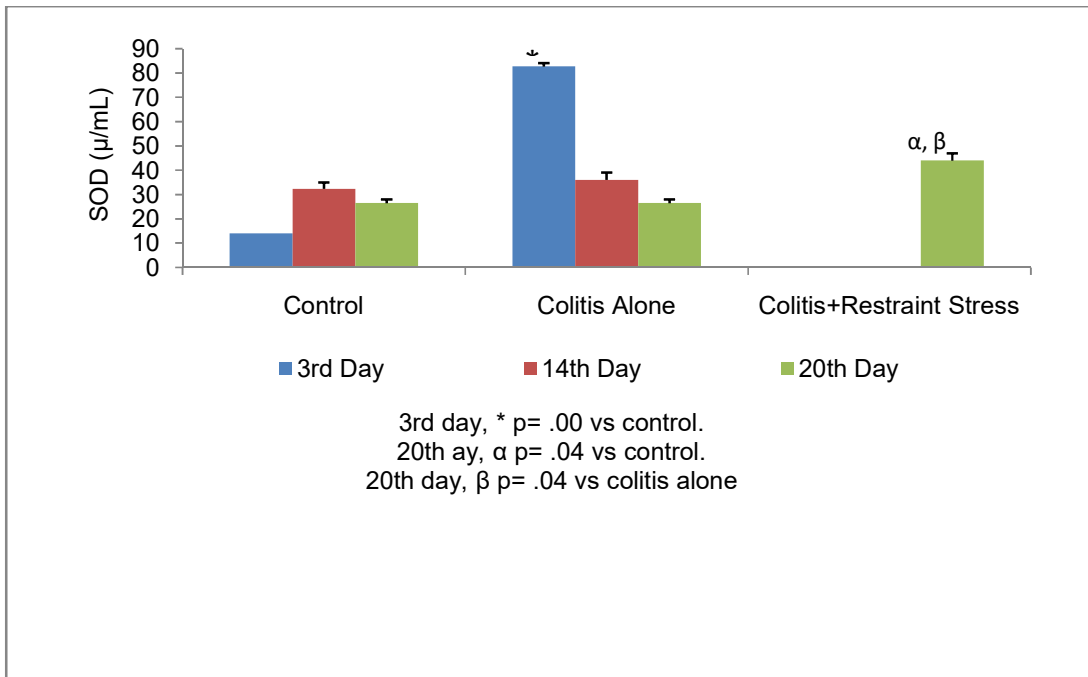
## 3. RESULTS AND DISCUSSION

### 3.1 Superoxide Dismutase Activities in the Colons of Rats on day 3, 14 and after Exposing Some of them to Restraint Stress from the 14th to 20th day Post Colitis Induction

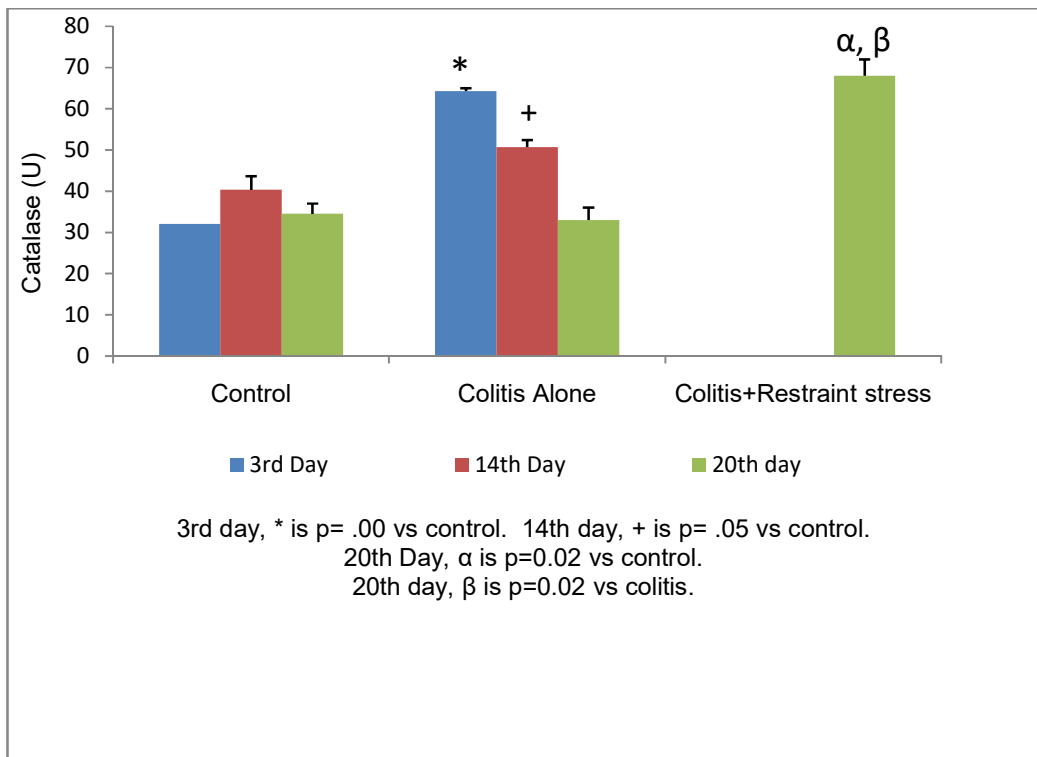
Fig. 1 shows the level of the activity of superoxide dismutase (SOD) in colon of animal in their various groups and on different days after the induction of colitis. On the 3<sup>rd</sup> day, there was significant increase in colitis group when compared with the control. There was also significant increase in SOD activity in the restraint group when compared colitis alone group and the control group on the 20<sup>th</sup> day.

### 3.2 Activities of Catalase in the Colons of Rats on Day 3, 14 and after Exposing Some of them to Restraint Stress from Day 14th to 20th Day Post Colitis Induction

In Fig. 2, the activity of catalase (CAT) in colon of animal in their various groups and on different days after the induction of colitis is shown. There was significant increase when comparing colitis with control group on the 3<sup>rd</sup> and 14<sup>th</sup> day while there was no significant difference on day 20. However, when comparing the restraint stress group with the colitis alone group and when comparing the restraint stress group and control group, there was significant increase in the activity of CAT on the 20<sup>th</sup> day.



**Fig. 1. Superoxide dismutase activities in the colons of rats on day 3, 14 and after exposing some of them to restraint stress from the 14th to 20th day post colitis induction**



**Fig. 2. Activities of catalase in the colons of rats on day 3, 14 and after exposing some of them to restraint stress from day 14th to 20th day post colitis induction**

### 3.3 Glutathione Concentration in the Colons of Rats on Day 3, 14 and After Exposing Some of Them to Restraint Stress from Day 14th to 20th Day Post Colitis Induction

The result in Fig. 3 shows the concentration of glutathione (GSH) in colon of animal in their various groups and on different days after the induction of colitis. There was only significant increase in the concentration of GSH on the 3<sup>rd</sup> day when comparing colitis group and control group.

### 3.4 Myeloperoxidase Activities in the Colons of Rats on Day 3, 14 and After Exposing Some of Them to Restraint Stress From Day 14 to 20 Day Post Colitis Induction

As seen in Fig. 4, the result shows the activity of myeloperoxidase in the colon of animals in their various groups and on different days after the induction of colitis. When the control group was

compared with the colitis group, there was significant increase in MPO activity on the 3<sup>rd</sup> day post colitis induction. There was no significant difference when comparing the control group with colitis group on the 14<sup>th</sup> day. However, when compared with the control group there was increase in MPO activity in animals exposed to restraint stress on the 20<sup>th</sup> day.

### 3.5 Malondialdehyde Concentration in the Colons of Rats on Day 3, 14 and After Exposing Some of them to Restraint Stress from Day 14 to 20 Day Post Colitis Induction

When the control group was compared with the colitis group on the 3<sup>rd</sup> day, there was an increase in the MDA level from 31.33 nmol/g to 32.5 nmol/g. There was significant decline in the level of MDA concentration as the natural healing continued into the 20<sup>th</sup> day in colitis alone group. However, there was significant increase in MDA concentration in the animals that were exposed to restraint stress when compared with the colitis alone and control groups on the 20<sup>th</sup> day.

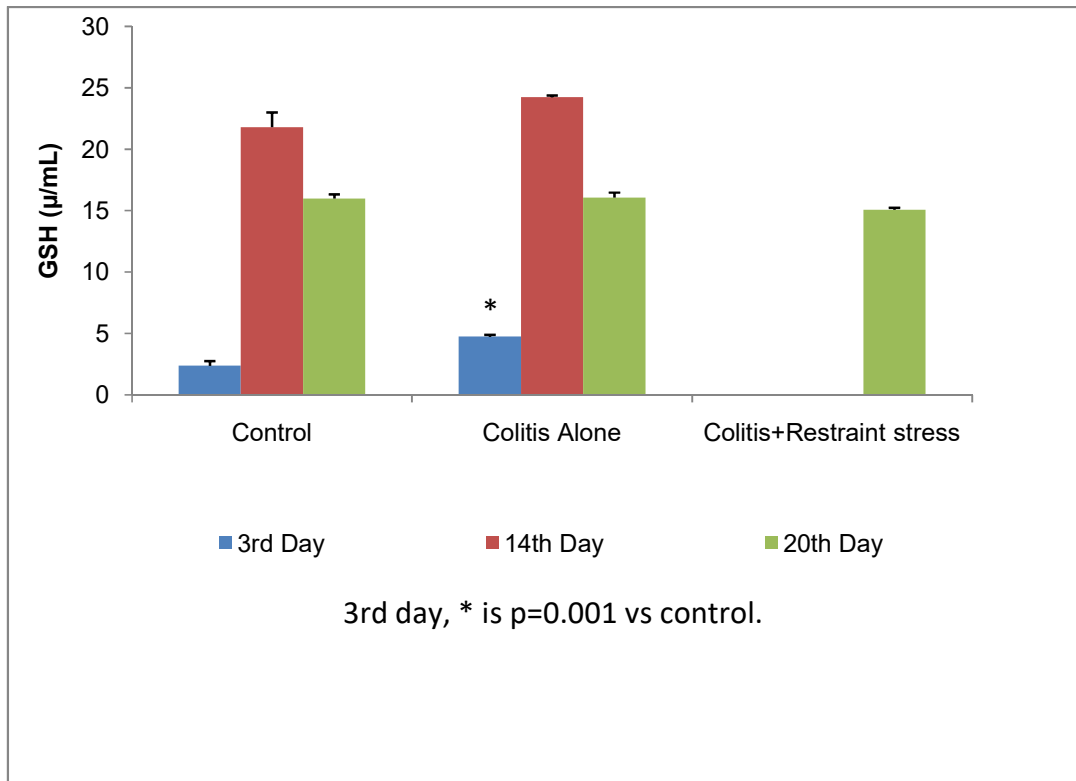
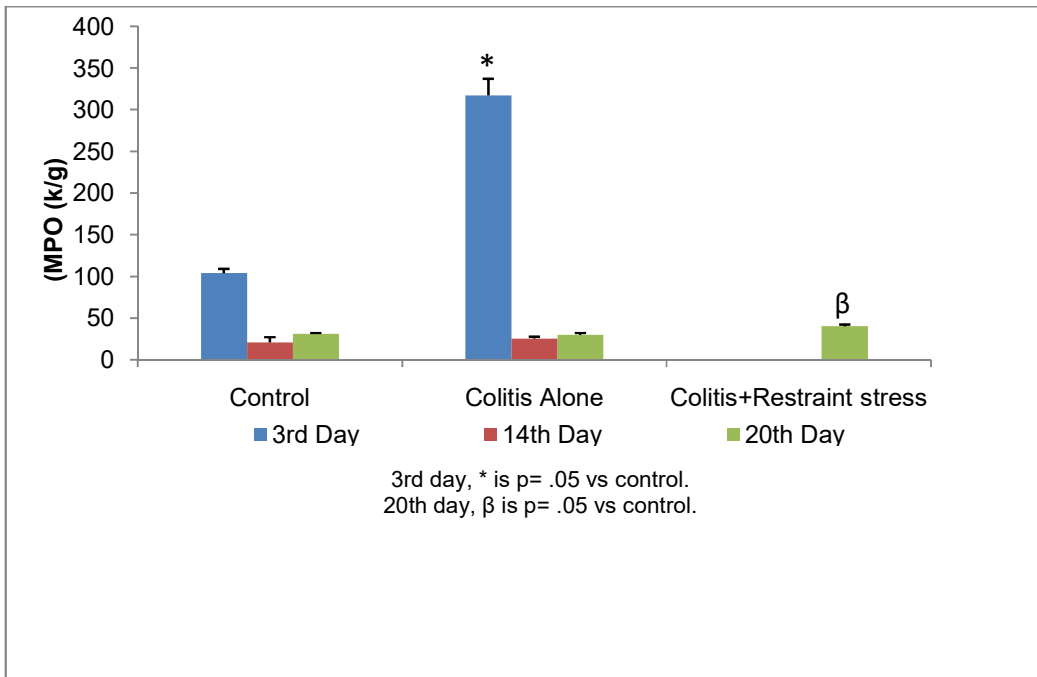
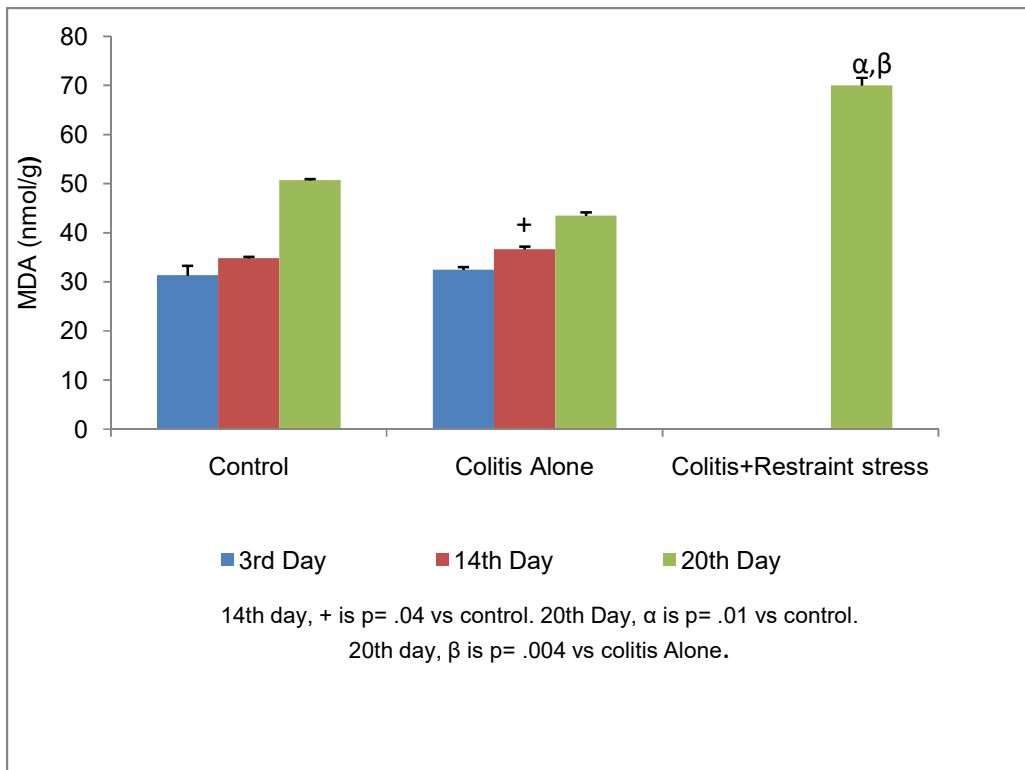


Fig. 3. Glutathione concentration in the colons of rats on day 3, 14 and after exposing some of them to restraint stress from day 14th to 20th day post colitis induction



**Fig. 4. Myeloperoxidase activities in the colons of rats on day 3, 14 and after exposing some of them to restraint stress from day 14 to 20 day post colitis induction**



**Fig. 5. Malondialdehyde concentration in the colons of rats on day 3, 14 and after exposing some of them to restraint stress from day 14 to 20 day post colitis induction**

### 3.6 Histological Sections of Rats Colons on Day 3, 14 and After Exposing Some of Them to Restraint Stress From Day 14 To 20 Day Post Colitis Induction

Photomicrograph of colonic section in this study showed; colitis alone, inflammation of the mucosal layer and lamina propria with neutrophil accumulation and abscess. there is necrosis in the mucosal glands with extension to severely infiltrated submucosal layer on 3<sup>rd</sup> day, colitis alone-14<sup>th</sup> day, moderately preserved mucosal epithelium, focal area of mucosal ulceration, the lamina propria is moderately infiltrated by

inflammatory cells, the edematous submucosal layer is moderately infiltrated by inflammatory cells. colitis alone - 20<sup>th</sup> day, well preserved mucosal epithelium, the lamina propria and the glands show mild infiltration of inflammatory cells which include mostly the lymphocytes and few polymorphs, the submucosal layer appear normal. 20<sup>th</sup> day of colitis + restraint stress, fairly preserved mucosal epithelium, there is cyst seen in the mucosal layer, the lamina propria and the glands is infiltrated by inflammatory cells, the submucosal layer show moderate dilation of vessels, (Fig. 6).

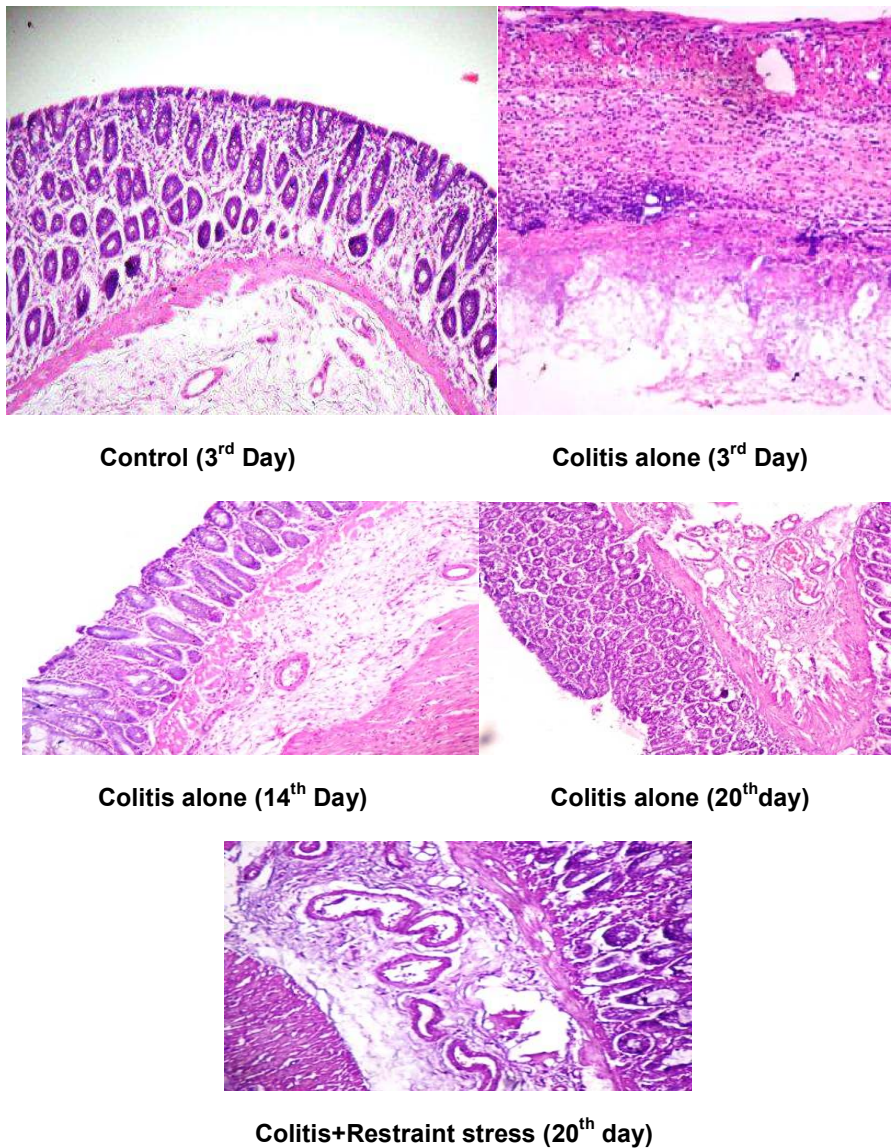


Fig. 6. Photomicrograph of colonic sections, (X40)



#### 4. DISCUSSION

Neutrophils are an important source of free oxygen radicals and therefore, are considered major effectors in the tissue damage that occurs in many inflammatory disorders [19]. The histological section of the colitis colon (3rd Day) in this study is consistent with this and this confirms the formation of colitis on the 3rd day.

Products of lipid peroxidation may lead to change in biological membranes, therefore these changes result in serious cellular (tissue) injury. Malonaldehyde (MDA), being a by-product of tissue peroxidation, has been known to serve as an index of lipid peroxidation [5], such that the higher the level of MDA in the tissue, the more pronounced the tissue damage. As shown in Fig. 5, the level of MDA, after exposure to restraint stress (colitis+restraint stress bar) significantly increased when compared with the control and the colitis alone group that has not been exposed to stress.

Hagar et al. have confirmed that reactive oxygen metabolites (ROM) play a critical role in the accumulation of neutrophils in tissues after colitis, whereas activated neutrophils are also a potential source for ROMs [20]. Myeloperoxidase (MPO) plays a basic role in the production of oxidants by neutrophils, thus, in this study, it was observed that on the 20th day after colitis induction, there was significant increase in MPO activity in animals that were exposed to restraint stress when compared to the control and colitis alone animals that has not been exposed to stress, as seen in Fig. 4.

Superoxide dismutase (SOD) is crucial for the redox balance, dismuting the superoxide anion into the hydrogen peroxide ( $H_2O_2$ ), and Catalase (CAT) degrades into water and oxygen, thereby avoiding the formation of HO radical [21] protecting against oxidative damage. In 2009, Yildiz et al. reported that SOD and CAT activities were significantly increased in the colitis group when compared with the control [22]. This study agrees with this; as it shows that the levels of SOD and CAT were elevated on the 3rd day of colitis induction as well as on the 20th day of restraint stress animals [22].

It is to be noted that increase in SOD may result in increase in formation of  $H_2O_2$ , which may cause its accumulation in the tissues and consequent increase in HO through the Fenton and Haber-Weiss reaction, leading to more

oxidative stress [23]. This infers that continuous exposure to restraint stress may lead to a vicious cycle of inflammation and the eventual necrosis of the entire distal colon.

Decline in level of reduced glutathione (GSH) has often been considered to be indicative of increased oxidative stress [24]. Results of this study shown in Fig. 3 agrees with this fact, since there is decline in GSH level in the colitis+restraint stress group on the 20th day when compared with the control group. Low level of GSH observed in ulcerative colitis was due to increased oxidative stress. The results of this present study were similar to that reported by Nieto et al. where it was said that in TNBS-induced ulcerative colitis, the level of GSH was lower in colitis group when compared with the control group [25].

#### 5. CONCLUSION

Looking at various oxidative stress markers and antioxidants (using both histopathological and biochemical study) in this study such as the level of malondialdehyde, myeloperoxidase, superoxide dismutase, catalase, and glutathione, after healing has been allowed to take place in animals without restraint stress, it can be confirmed that restraint stress has led to the reactivation (relapse) of ulcerative colitis in acetic-acid-induced rats.

#### 6. LIMITATION

This study has limitation of the healing period which can be extended to 21 days instead of the 14 days that was used.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

Authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. All experiments have been examined and approved by Faculty of Basic Medical Sciences, LAUTECH, ethics committee.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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