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ORIGINAL ARTICLE

A Histological Study of The Protective Role of Intermittent Fasting and Probiotics on Indomethacin Induced Colitis in Adult Male Albino Rats

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ABSTRACT

Background: Inflammatory bowel diseases (IBD) are considered as one of the commonest gastrointestinal disorders. Probiotics and intermittent fasting (IF) were reported to improve various inflammatory disorders. So, the present work aimed to study the histological changes in indomethacin induced colitis and to evaluate the protective role of probiotics alone and in combination with IF.

Methods: 48 rats were divided into 4 groups; Control, Colitis, Probiotic- Colitis and Combined IF& probiotics - Colitis groups. Colitis was induced by subcutaneous injection with indomethacin (7.5 mg/kg /day) for 2 days then rats were sacrificed after the last injection. Probiotic- Colitis rats were given probiotic powder (135 mg/kg) for 21 days then colitis was induced. Rats of combined IF& probiotics - Colitis group were fed for only 8 h per day and were given probiotics for 21 days then colitis was induced. At the end of the experiment, colon sections were examined by light and electron microscopy.

Results: Colitis group showed deformed crypts, loss of the epithelium, few goblet cells, deformed intestinal barrier and infiltration with several types of inflammatory cells. A significant increase in TNF-α immunoexpression and serum malondialdehyde (MDA) was observed. In the probiotics- colitis and combined IF and probiotics groups, there was an improvement in the histological and biochemical alterations.

Conclusion: Indomethacin led to histological alterations in the rat colon which was accompanied by an increase in serum MDA. Probiotics could protect the colon from these alterations. Also, combination of IF and probiotics might provide more protection than probiotics alone.

Keywords: IBD, Indomethacin, Probiotics, Intermittent Fasting.

INTRODUCTION

Inflammatory bowel diseases (IBD) are considered as one of the commonest gastrointestinal disorders with a rising incidence in the Arab world and worldwide over the past decade **[1&2]**. IBD also has significant impacts on patient health, quality of life, mental health and work productivity **[3]**.

Inflammatory bowel disorders include ulcerative colitis (UC) and Crohn's disease (CD). They are considered two highly debilitating, incurable, persistent, immune-arbitrated inflammatory pathologies causing inflammation and ulceration of the digestive system **[4]**. The etiology of IBD is multifactorial, including genetic tendencies, dysfunctional immunity, intestinal barrier dysfunction and environmental risk factors **[5].** Food intake is an important risk factor that affects the progress of IBD. So, intake of fast foods with high fat and sugar content may exacerbate the development of Crohn's disease **[6].** Also, artificial food flavorings frequently used in western diets may foster intestinal inflammation by interfering with the intestinal barrier function **[7].**

Indomethacin is one of the nonsteroidal anti-inflammatory drugs (NSAIDs). It is commonly used for its anti-inflammatory and analgesic properties. Gastrointestinal injury is one of the

most common side effects of NSAIDs use. The injury is not limited to the stomach and duodenum but may affect the distal intestine **[8]**. So, indomethacin is commonly used in rat models to mimic the picture seen in IBD and to examine the various protective and therapeutic agents **[9].**

Several therapies are used for the management of IBD, such as anti-inflammatory drugs e.g sulfasalazine or corticosteroids, and immunosuppressive agents as azathioprine. Some antibiotics as amoxicillin, ciprofloxacin and metronidazole may have beneficial effects on Crohn's disease. However, these drugs resulted in adverse effect in addition to high relapses rates **[10].**

Probiotics including bacteria and yeast are living microorganisms, they are beneficial to the body. They are naturally found in fermented foods e.g Yogurt, kimchi, pickles, Sour cream, cottage cheese, aged cheese, and buttermilk. They also could be taken as supplements produced by pharmaceutical companies **[11]**.

Probiotics contain a larger variety and number of microorganisms, ranging from 108 to more than 1010 organisms. Most strains of probiotics were developed for their capacity to resist low gastric pH, giving rise to a number of variants with unknown physiological properties **[12]**

Many studies highlight their effect on the intestinal epithelium including the intestinal barrier, the immune cells and the resident microbiota. Probiotics bind with the host epithelium to produce antimicrobial peptides (AMP) and metabolites with potential anti-inflammatory immunomodulatory activities, and ability to reduce microbial growth **[13].**

Intermittent fasting (IF) is a term including a group of dietary patterns using periodic energy restriction to reduce caloric intake. IF includes alternate-day fasting (ADF), time-restricted fasting (TRF), and intermittent energy restriction (IER) **[14].**

Intermittent fasting has become popular over the last decade as a regimen for weight loss. It is effective for lowering body weight, improving insulin sensitivity, lowering blood pressure, and reducing markers of oxidative stress in obese individuals **[15]**. Also, intermittent fasting (IF) was reported to have influence in various inflammatory diseases **[16]**.

Combination of probiotics and intermittent fasting may have a beneficial effect in IBD. So, this research was performed to mimic IBD in a rat model using indomethacin to induce colitis. The histological changes in indomethacin induced colitis were observed. The protective effects of probiotics alone and in combination with intermittent fasting before the induction of colitis were also examined.

METHODS

Animals

The present study was conducted on forty-eight adult healthy male albino rats (4-6 months) weighting 180-200 grams each. They were obtained from the Breading Animal House, Faculty of Medicine, Zagazig University.

Ethical approval and care of experimental animals:

The rats had free access to food and water. They were housed at room temperature and were on a 12-hour light/dark cycle. Rats adapted to this environment for one week before starting the experiment. To ensure ethical treatment throughout the investigation, all procedures were carried out in compliance with Zagazig University's IACUC rules for the use and care of laboratory animals. The Zagazig University IACUC Committee reviewed and approved the experimental protocol, which had the approval number of **(ZU-IACUC/3/F/106/2023).**

Chemicals

A. Indomethacin: was obtained in the form of Liometacen ampules produced by NILE pharmaceuticals (Cairo), each ampule contains 77.2 Meglumine indomethacinate which is equivalent to 50 mg indomethacin**.**

B. Probiotics: were obtained in the form of powder from i herb California united states which is mixture of spores as bacillus clausii, bacillus subtilis, bacillus coagulnus and other spores**.**

Experimental design

A total of 48 rats were divided into four main groups:

- **Group I (control):** It included 24 rats that were subdivided into 4 subgroups 6 rats each:

-***Subgroup Ia (negative control):*** the rats didn't receive any treatment throughout the experiment.

-***Subgroup Ib (positive control):*** the rats were received 0.5 ml volume of 5% sodium bicarbonate (the vehicle of indomethacin) for 2 consecutive days.

-***Subgroup Ic (positive control):*** the rats received probiotics powder at a dose of (0.0128 x 10^9) CFU (colony forming unit) per gram of rat body weight, which is equivalent to (135 mg/kg), dissolved in drinking water and received orally by oro-gastric tube for 21 days **[17].**

- ***Subgroup Id (positive control***): the rats were exposed to time restricted fasting, they were fed for only 8 h per day from 12pm to 8pm while, the rest of the day they were only allowed drinking water for 21 days **[18].**

The animals from each subgroup were sacrificed with their corresponding experimental group.

- **Group II (Colitis group):** It included 8 rats that received freshly prepared 7.5 mg/kg/day of indomethacin dissolved in 0.5 ml volume of 5% sodium bicarbonate and were administered subcutaneously for 2 consecutive days **[19]**. This dose was prepared by dissolving the whole indomethacin ampule in 16.6 ml of 5% sodium bicarbonate and injecting each rat with ≈ 0.5 of this solution then rats were sacrificed 4 hours after the last dose of indomethacin.
- **Group III (Probiotic- Colitis group):** It included 8 rats that were given probiotic powder as subgroup 1c for 21 days **[17]**. Colitis was induced by indomethacin as group II starting from the 20th day for 2 consecutive days**.** Rats of this group were sacrificed 4 hours after the last dose **[19]**.
- **Group IV (Combined intermittent fasting (IF) & Probiotics- Colitis group):** It included 8 rats that were fed for only 8 h per day from 12pm to 8pm like group Id **[18]** and simultaneously were given probiotic powder as subgroup 1b for 21 days **[17]**. Colitis was induced by indomethacin as group II starting from the 20th day for 2 consecutive days **[19]**. Rats from this group were sacrificed 4 hours after the last dose.

Sampling

At the end of the experimental period after 21 days, rats from all groups were anesthetized by intraperitoneal injection of 200 mg/kg sodium pentobarbital solution **[20]**, then blood samples were obtained from the retro-orbital veins and collected in tubes. Rats were sacrificed, the abdominal wall was incised, the proximal part of colon was carefully dissected and promptly washed with saline then prepared for the histopathological procedures.

Biochemical study:

Estimation of malondialdehyde (MDA) level in the blood: [21]

Malondialdehyde (MDA) was determined as an oxidative stress marker. Briefly, 0.5 mL serum was added to 2.5 mL of 20% trichloroacetic acid (TCA) and also, one mL of 0.67% tissue plasminogen activator) TPA) in a 10 mL centrifuge tube. Then, blood serum was centrifuged for 10 minutes at 8000 rpm. Measurement of MDA level was carried out using the MDA standard curve. The results were expressed as nanomole MDA/mg protein. The samples were examined in Biochemistry department, Faculty of Medicine, Zagazig University. using MDA 586™ (R&D, Europe Ltd, Abingdon, Oxon, UK).

Histological study:

Colon samples (the proximal 5 cm of colon) were carefully dissected and processed for:

i. Light microscope examination:

Following the standard processing procedure, colon samples (1 cubic centimeter) were taken shortly after sacrifice, fixed in 10% formalin, dehydrated, cleared and embedded in paraffin wax. Slices of 5µm thickness were stained with:

1- Hematoxylin and Eosin (H&E): samples were examined by light microscope **[22]** to investigate the histological structure.
2- Alcian blue stain: as a method for demonstration of mucus in the goblet cells **[22].**
3- Immunohistochemical detection of tumor necrosis factor alpha (TNF-α) immunostaining: was used as a marker for inflammation. By using the streptavidin–biotin complex immune-peroxidase technique, an immunohistochemical reaction for TNF-α antibody was carried out. Primary and secondary antibodies were applied to the 5µm-thick sections. Rabbit monoclonal anti-TNF-α (Abclonal, USA catalogue number: A21265) were used **[23].** A brown cytoplasmic reaction is the result of a positive reaction.

ii. ***Electron microscopy examination:***

Specimens of the colon (1 cubic millimeter) were cut and immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h at 4◦C. Then the specimens were processed for electron microscope examination. Ultrathin sections (70-90 nm) were stained with uranyl acetate and lead citrate **[24]** and photographed in the Electron Microscopy Unit of the Faculty of Agriculture, El Mansoura University in Egypt, JEOL JEM 2100 transmission electron microscope (Jeol Ltd, Tokyo, Japan)

Histomorphometric study:

The mucosal thickness of the colon in H&E-stained sections, the goblet cells number in sections stained with alcian blue and the area percentage of TNF-α immunoreaction were measured.

The image analyzer consisted of a colored video camera, colored monitor and hard disc of IBM personal computer connected to the Olympus microscope (CX 41) microscope and controlled by Leica QWin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units.

The measurements were done using the interactive measuring menue, the measurement in ten random non-overlapping fields were done. It was done by measuring twenty readings from three sections for each rat in each group.

Statistical analysis

The data from biochemical and morphometrical analysis were expressed as mean ± SD (standard deviation). The "Statistics for Windows SPSS" version 20 software was used to conduct statistical analysis. One-way analysis of variance (ANOVA) and the LSD test were used for data analysis. The differences were classified as non-significant if $p > 0.05$, highly significant if $p < 0.001$, and statistically significant if $p < 0.05$ **[25].**

RESULTS

The biochemical, histological results of the control subgroups showed similar results. So, the subgroup Ia was used as control group in the interpretation of the results.

A) Light microscope results:

1) H & E results:

H&E-stained sections of the **Group I (control group)** showed the normal colon structure; mucosa, submucosa, **musculosa** and serosa. The colonic mucosa appeared as crypts lined by epithelium, lamina propria and muscularis mucosa **(Fig. 1 a).** By higher magnification, the crypts were lined by surface columnar epithelial cells, with oval basal nuclei and acidophilic cytoplasm. Goblet cells were flask shaped cells with basal flat nuclei and vacuolated cytoplasm. Most of them were found at the basis of the crypts and few were found among the surface epithelium **(Fig. 2 a). Group II (Colitis group)**

revealed the effect of indomethacin on the colonic wall. All layers of the colon especially mucosa and submucosa were highly infiltrated by inflammatory cells **(fig 1 b).** The mucosa was the most affected layer, many of the colonic crypts were deformed and some of them were highly attenuated, the surface epithelium and the goblet cells were lost in many areas while the intact epithelium appeared deformed with vacuolated cytoplasm. Intraepithelial lymphocytes were frequently seen **(fig. 2 b,c.d). Group III (Probiotic-Colitis group**) showed that administration of probiotics partially ameliorated the indomethacin induced colitis. H&E-stained sections revealed that the general architecture of the colonic wall was partially restored. However, moderate cellular infiltration was observed in the mucosa and the submucosa **(fig 1 c).** The colonic crypts had apparently normal contour. The surface epithelium was lost in a few areas but in many areas cuboidal to low columnar epithelium was seen **(fig 2 e). Group IV (Combind IF & probiotic- Colitis group)** demonstrated that combination of intermittent fasting and probiotics significantly improved the indomethacin induced colitis. In H&E-stained sections, the colonic wall had apparently normal organization, but the cellular infiltration was still present in some sections **(fig 1 d).** The lining epithelium was cuboidal to low columnar with oval nuclei, some cells had vacuolated cytoplasm and intraepithelial lymphocytes were observed. cellular infiltrations were seen in the lamina propria underlying the surface epithelium and around the crypts **(fig 2 f).**

2) Alcian blue results:

In alcian blue stained sections, the goblet cells were lining the crypts and found in between the surface epithelial cells **in the control group (fig 3 a).** While in the **colitis group** the goblet, few cells were detected in the basis of the crypts **(fig, 3 b).** Examination of the **probiotic-colitis group** revealed that the goblet cells number was increased, and they were observed in-between the surface epithelium and lining the crypts **(fig 3 c).** In the **Combind IF & probiotic- Colitis group** many goblet cells were lining the crypts and found in between the surface epithelium **(fig 3 d).**

3) Immunohistochemical results:

Immunohistochemical staining of tumor necrosis factor alpha **(TNF-α)** performed on colon sections from the **control group** revealed faint expression in few cells of the lamina propria **(fig 4a).** TNF-α -stained sections from the **colitis group** showed strong expression in many cells of the lamina propria and in the submucosa **(fig 4b).** Sections from the **Probiotic- Colitis group** revealed moderate expression of TNF-α in the lamina propria around the crypts **(fig 4 c).** TNF-α -stained section showed mild expression in fewer cells in the lamina propria in **Combind IF & probiotic- Colitis group (fig 4 d).**

B) <u>Electron microscope results:</u>

In the **control group,** the lining epithelium was columnar cells with oval nuclei, plenty of mitochondria, microvilli, and well-developed intercellular junction **(fig 5 a).** The goblet cells were flask shaped; their cytoplasm was filled with well-defined electron lucent granules **(fig 6 a).** The lamina propria contained fibroblasts having elongated nuclei, blood capillaries, smooth muscle cells of the muscularis mucosa and fine collagen bundles **(fig 7a).** In **the Colitis group,** the surface epithelium was lost in many areas. The underlying lamina propria was rarified, contained wide interstitial spaces and many apoptotic cells **(fig 5b).** Goblet cells were smaller in size, and their mucin granules were ill defined. Many apoptotic nuclei were seen among the surface epithelium **(fig 6b).** The lamina propria contained many interstitial amorphous areas, fine collagen bundles and several types of inflammatory cells. Mast cells were frequently seen; they had heterochromatic nuclei and large electron dense granules. Eosinophils appeared with their characteristic dense core granules. Macrophages had multiple lysosomes. Activated plasma cells had a cartwheel nucleus and wide cisternae of RER. Lymphocytes with rounded nuclei and scanty cytoplasm were also detected. Other areas had

wide interstitial spaces and degranulated mast cells **(fig 7 b, c, d).**

In the **Probiotic- Colitis group**, the surface epithelium in many areas appeared normal but in others lost microvilli and intracytoplasmic spaces were detected. Intercellular junctions were lost in many of the observed sections **(fig 5c).** Goblet cells appeared normal with electron lucent granules **(fig 6c).** The lamina propria had fewer inflammatory cells, they were mostly mast cells and eosinophils **(fig 7e).** In the **Combined IF & probiotic- Colitis group,** the epithelial cells appeared normal with highly organized microvilli, frequent mitochondria and well-developed intercellular junctions. However, intracytoplasmic spaces were still observed in some cells **(fig 5d).** Goblet cells appeared normal with electron lucent granules **(fig 6d).** In the lamina propria, mast cells, eosinophils and other inflammatory cells were still found but they were fewer in number **(fig 7f).**

Morphometric and statistical results:

1. **Biochemical Results**

The control group had the lowest serum MDA level (1.33 ± 0.08), which was lower than all other groups. The colitis group had the highest MDA level (3.77±0.38) that was significantly higher than all the groups. The probiotic - Colitis (1.82±0.18) and the combined IF &Probiotic groups (1.55±0.20) showed significantly reduced MDA level when compared to the colitis group. There was no significant difference between these two groups and the control group (Table 1s) (significant difference, p value <0.05).

2- Morphometric Results

- **The mucosal thickness of the colon in the different studied groups**

The control group (246 ± 61.4 μm) had higher mucosal thickness than all other groups. The colitis group (142 ± 34.4 μm) had significantly lower mucosal thickness than the other groups. The probiotic - Colitis (222 ± 30.4 μm) and the combined IF& Probiotic - colitis groups (236 ± 54.2 μm) did not differ significantly from each other, but both were significantly thicker than the colitis group and they showed no significant difference when compared to the control group (Table 1s) (significant difference, p value <0.05).

- **The goblet cells number in the different groups**

The control group had the highest goblet cell count (177 ± 72.6), which was higher than all the other groups**.** The colitis group exhibited the lowest goblet count (54.7 ± 10); significantly lower than all other groups**.**

The probiotic - colitis (130 ± 33.8) and Combined IF & Probiotic - Colitis groups (151 ± 25.2) had a moderately increased goblet count with no significant difference between them, but both were significantly higher than the colitis group and they showed no significant difference when compared to the control group (Table 1s) (significant difference, p value <0.05).

- **The area percentage of TNF-α immuno-expression in the different groups**

The control group had the lowest TNF-α expression ($0.25 \pm 0.07\%$). The colitis group exhibited the highest TNF-α expression ($2.71 \pm 0.99\%$) which is significantly higher than all other groups**.**

The probiotic - colitis ($1.21 \pm 0.13\%$) and the combined IF&Probiotic - colitis groups (0.84±0.52%) showed a significantly reduced TNF-α expression, with no statistically significant differences among them, but both were significantly lower than colitis group and significantly higher than control group (Table 1s) (significant difference, p value <0.05)**.**

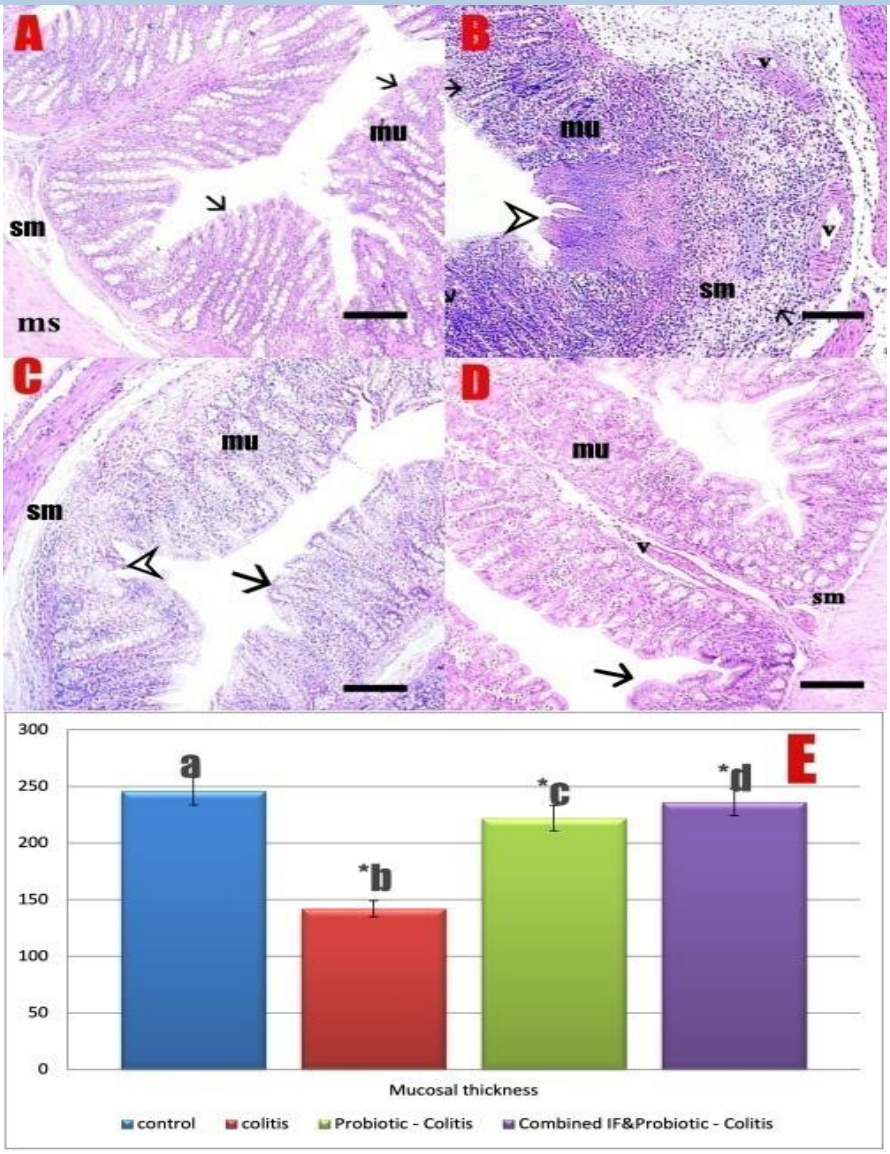

Fig (1): Photomicrographs show the general architecture of the colon wall in all groups. **A) Control group:** The mucosa (mu) is formed of crypts lined by surface epithelium (arrows) and goblet cells, normal submucosa (sm) and musculosa
(ms) are seen. **B) Colitis group:** All the layers are heavily infiltrated with inflammatory cells (arrows). The mucosa (mu) shows loss of epithelium and attenuated crypts (arrowhead). The submucosa (sm) contains thick wall blood vessels (v). **C) Probiotic - Colitis group:** Cellular infiltration is detected in the submucosa (sm), the epithelium is lost in some areas (arrowhead) and intact in other areas (arrow). **D) Combined IF& Probiotic - Colitis group:** The mucosa (mu) is formed of crypts lined with epithelium and goblet cells(arrow) and mild cellular infiltration is seen in the mucosa (mu), the submucosa (sm) contains engorged blood vessel (v), **E)** Bar chart of the mean level of mucosal thickness of different studied groups.
(* significant difference, p value <0.05); (b) group 2 versus group 1, (a) group 1 versus group 3& 4, (c) group 3 versus group 2. (d) group 4 versus group 2.
(H&E X 100, Scale bar10um).

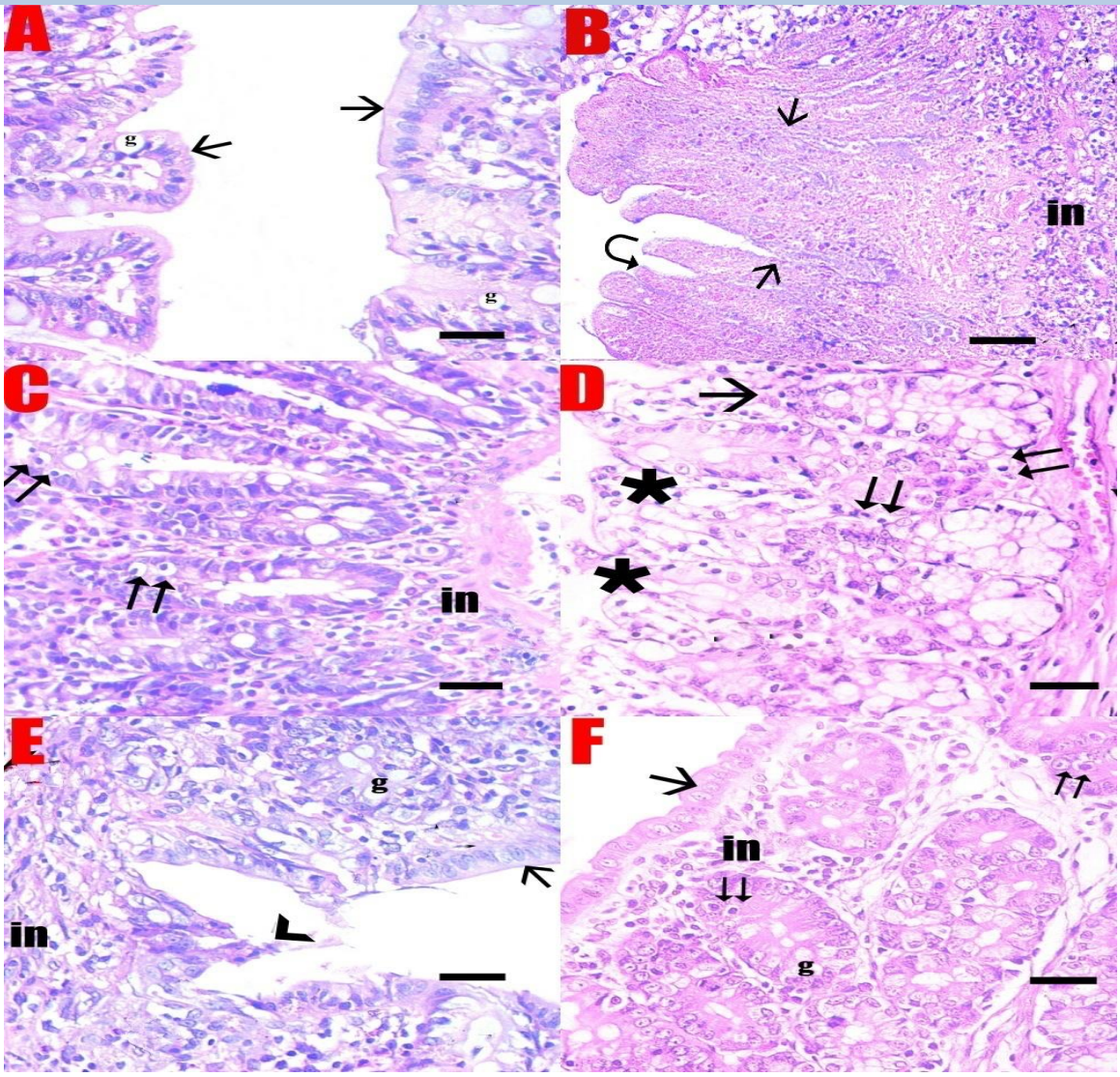

Fig (2): A) Control group: The apices of the crypts are lined by surface epithelium with oval nuclei and acidophilic cytoplasm (arrows), goblet cells (g) with foamy cytoplasm. **B) Colitis group:** A Higher magnification of figure 1 B showing attenuated crypts (arrows) with complete loss of the surface epithelium (curved arrow) and cellular infiltration (in) are seen. **C) Colitis group:** The mucosa has cellular infiltration in the lamina propria (in) and intraepithelial lymphocytes (double arrows). **D) Colitis group:** Highly deformed crypts are lined by vacuolated epithelium (*), intraepithelial lymphocytes (double arrows) and cellular infiltration (in) are seen. **E) Probiotic - Colitis group:** Alternating areas of intact surface epithelium (arrow) and lost or deformed epithelium (arrow head) are seen. Cellular infiltration (in) is seen. **F) Combined IF&Probiotic - Colitis group:** The crypts are lined by low cuboidal epithelium (arrow), goblet cell(g), intraepithelial lymphocytes (double arrow) and cellular infiltration (in) in the lamina propria are seen.
(H&E X 400, scale bar 20um)

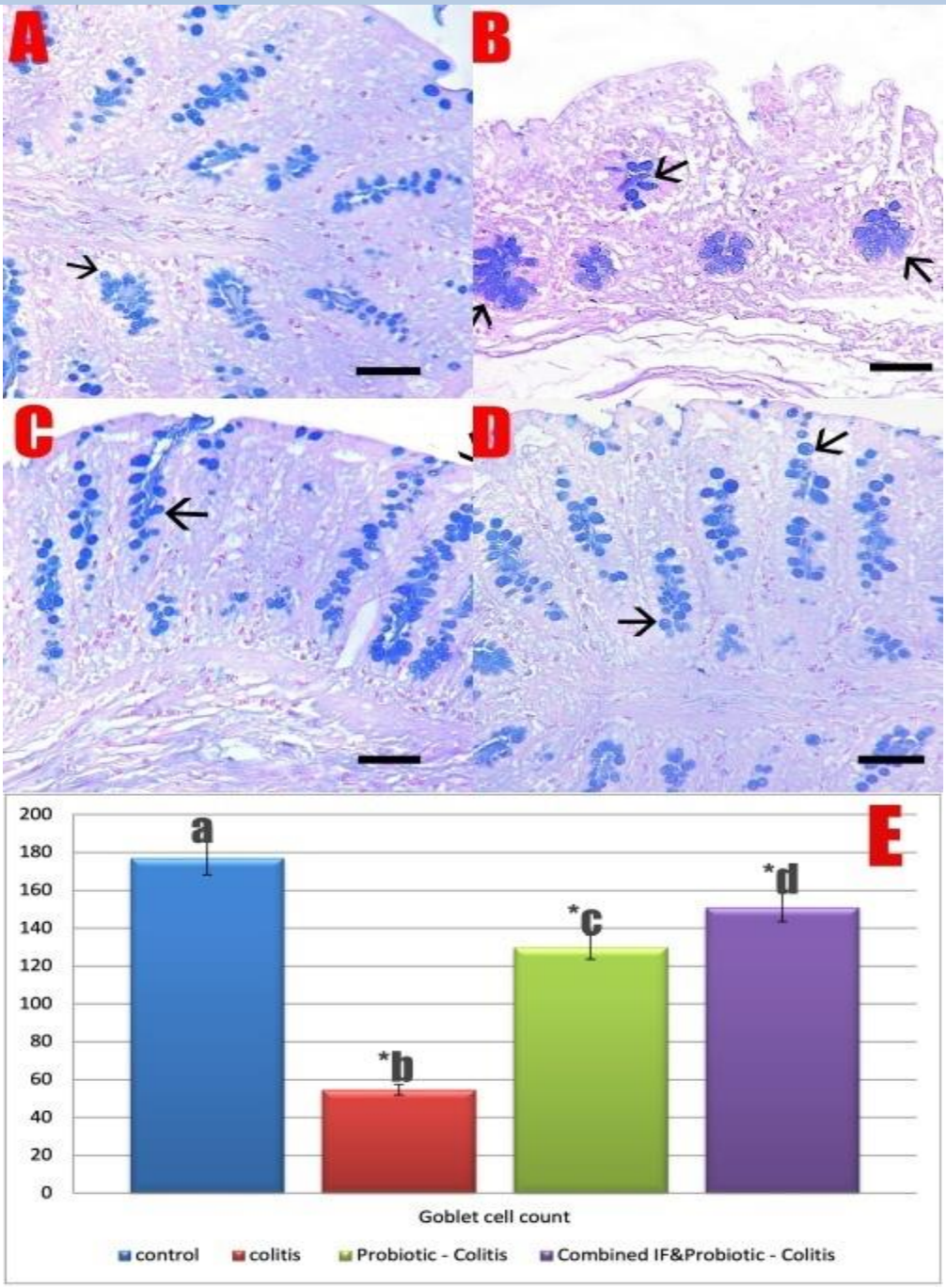

Fig (3): photomicrographs of alcian blue stained sections of all the groups; **A) Control group:** Goblet cells (arrows) are seen in the bases of the crypts and fewer cells in between the surface epithelium. **B) Colitis group:** Deformed crypts with fewer goblet cells (arrows) in the bases of the crypts are seen. **C) Probiotic - Colitis group:** Increased number of goblet cells (arrows) are seen along the crypts and in between the surface epithelium. **D) Combined IF&Probiotic - Colitis group:** Moderate number of goblet cells (arrows) are seen lining the crypts and in between the surface epithelium. **E)** Bar chart of the mean level number of goblet cell number in different studied groups. **(* significant difference, p value <0.05); (b) group 2 versus group 1, (a) group 1 versus group 3& 4, (c) group 3 versus group 2. (d) group 4 versus group 2.**

(Alcian blue X 200,scale bar 50um).

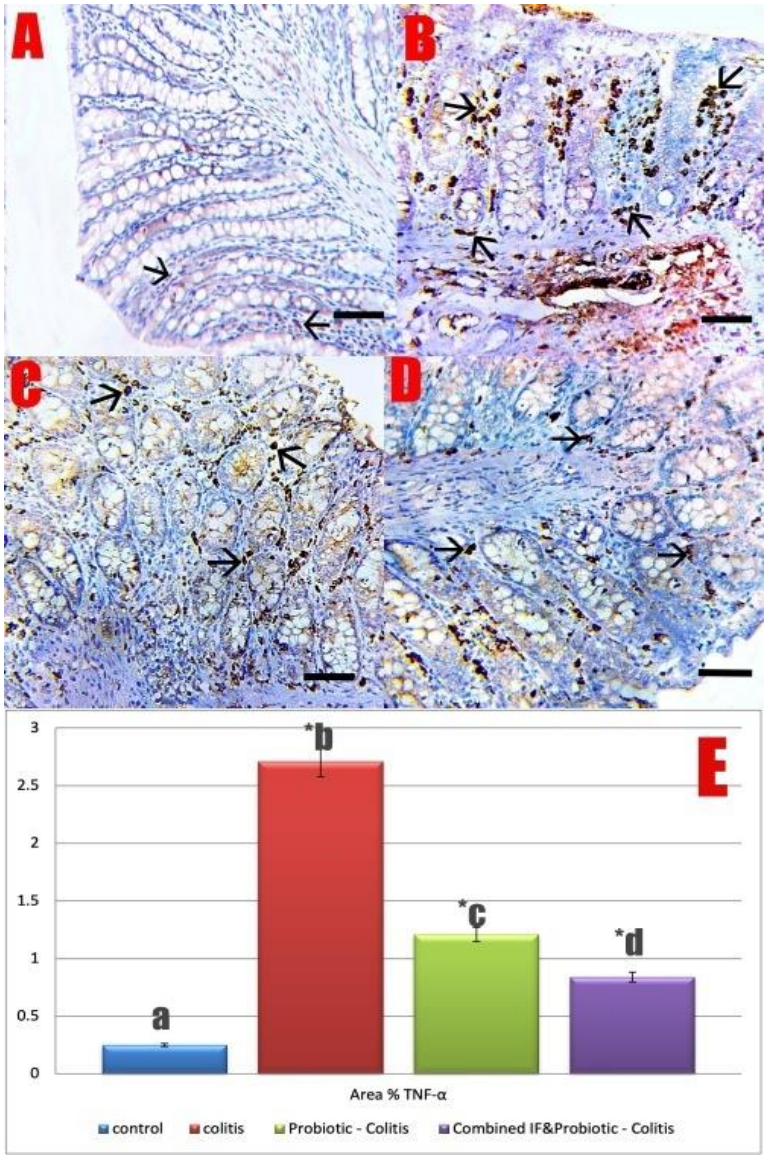

Fig. (4): Photomicrographs of **TNF-α** immune-stained sections of all groups; **A) Control group:** Faint expression is seen in the lamina propria (arrows). **B) Colitis group:** Strong expression is seen in the cytoplasm of many cells in the lamina propria surrounding the crypts (arrows). **C) Probiotic - Colitis group:** Moderate expression is detected in cells of the lamina propria (arrows). **D) Combined IF&Probiotic - Colitis group:** Mild expression is seen in the cells of the lamina propria (arrows). **E)** Bar chart of the mean level of TNF-a (Area%) of different studied groups. **(* significant difference, p value <0.05); (b) group 2 versus group 1, (a) group 1 versus group 3& 4, (c) group 3 versus group 2. (d) group 4 versus group 2.**

(anti- TNF-α X 200, scale bar 50um).

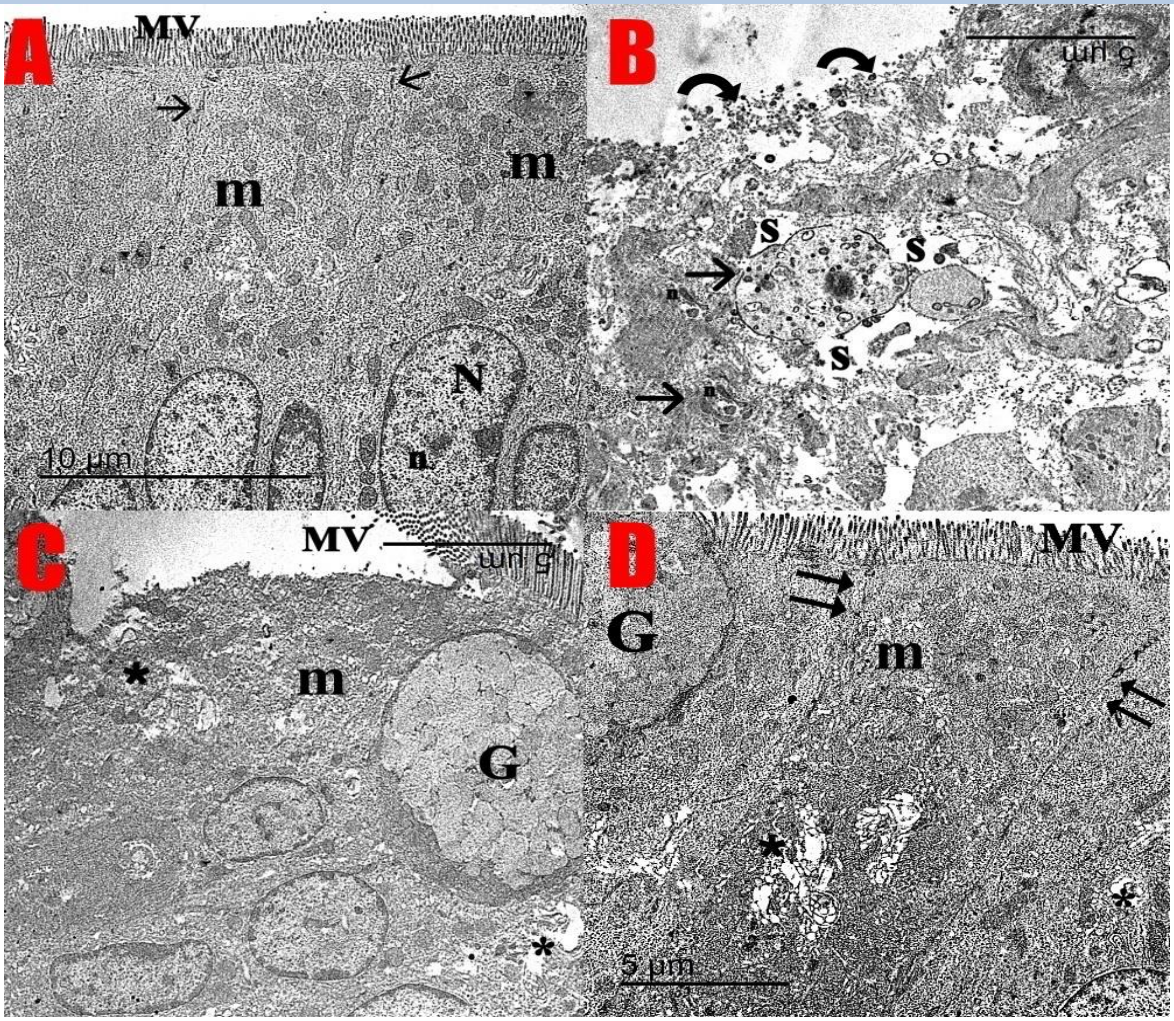

Fig. (5): Electron micrographs show ultrathin sections of the surface epithelium from all the groups; **A) Control group:** The columnar cells have prominent microvilli (MV), well developed cell junctions (arrows) and multiple mitochondria (m). A part of the nucleus (N) with its nucleolus (n) is seen. **B) Colitis group:** The surface epithelium is lost (curved arrows); the underlying lamina is rarified with wide interstitial spaces (s) and many apoptotic cells (arrows). **C) Probiotic - Colitis group:** The apical part of a surface epithelial cell shows partial loss of microvilli (mv), dilater ER cisternea (*) and mitochondria (m). A part of a goblet cell (G) is seen. **D) Combined IF&Probiotic - Colitis group:** The apical parts of surface epithelial cells show intact microvilli (MV), mitochondria (m), dilated cisternae of ER (*) and well-developed cell junctions (double arrows) are seen. A part of a goblet cell (G) is seen.

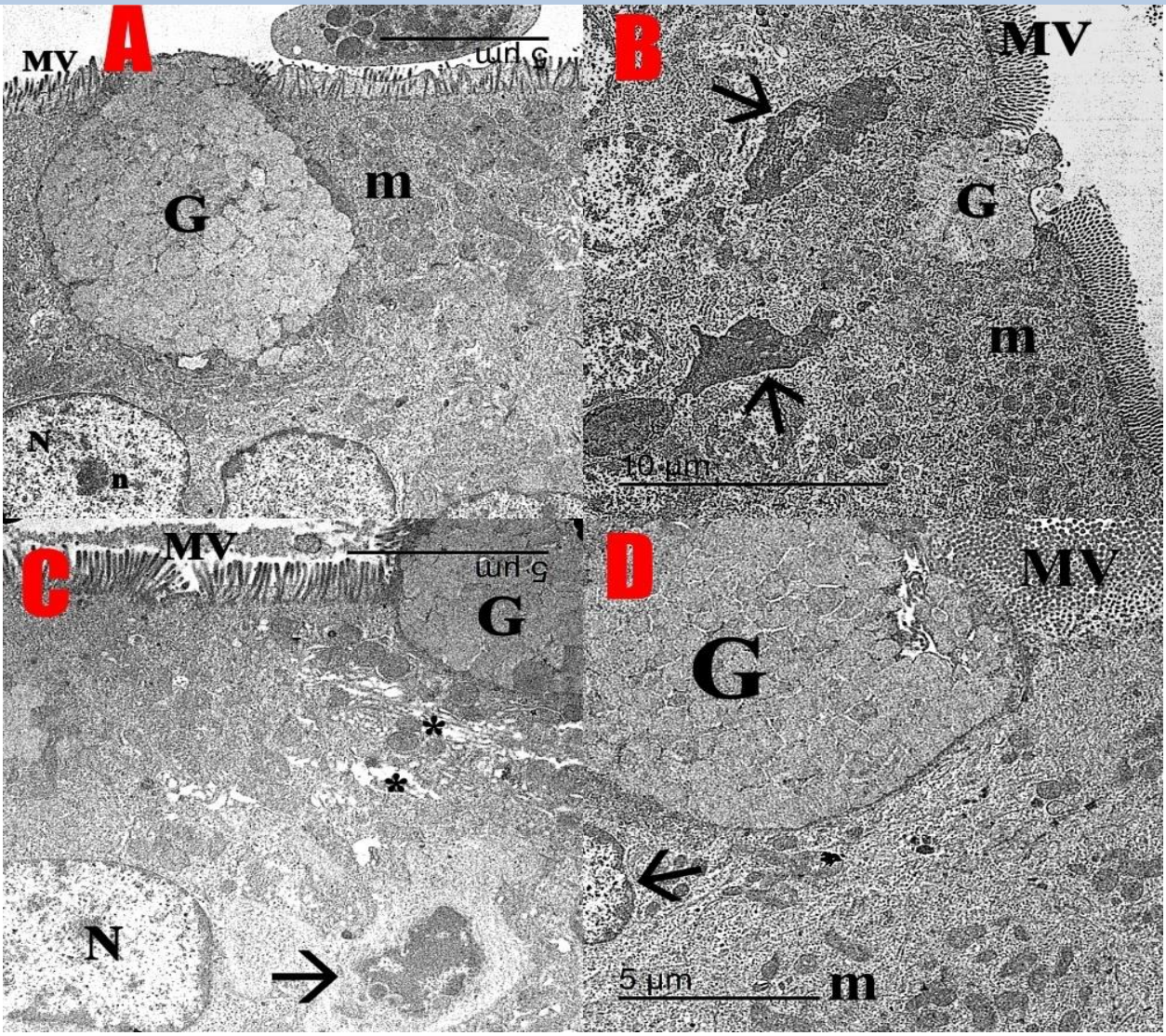

Fig. (6): Electron micrographs of ultrathin sections of the goblet cells from all groups; **A) Control group:** A goblet cell (G) opens into the lumen, the cytoplasm is filles with electron lucent granules, the surrounding epithelial cells have microvilli (MV) and plenty of mitochondria (m)and intact nucleus (N) with prominent nucleolus (n). **B) Colitis group:** A part of a small goblet cell (G) opens in the lumen, the cytoplasm contains ill-defined electron lucent granules. Wide electron dense areas are seen between the epithelial cells most probably pyknotic nuclei (arrows). **C) Probiotic - Colitis group:** A goblet cells (G) with electron lucent ill-defined granules are seen among the apical part of epithelial cells. These cells have dilated cisternae of ER (*), rounded mitochondria (m) and intact nuclei (N). An apoptotic cell with pyknotic nucleus (arrow) is seen. **D): Combined IF&Probiotic - Colitis group:** A goblet cell (G) opens into the lumen, the cytoplasm is filles with electron lucent granules, the surrounding epithelial cells have microvilli (MV), plenty of mitochondria (m) and part of intact nucleus (N).

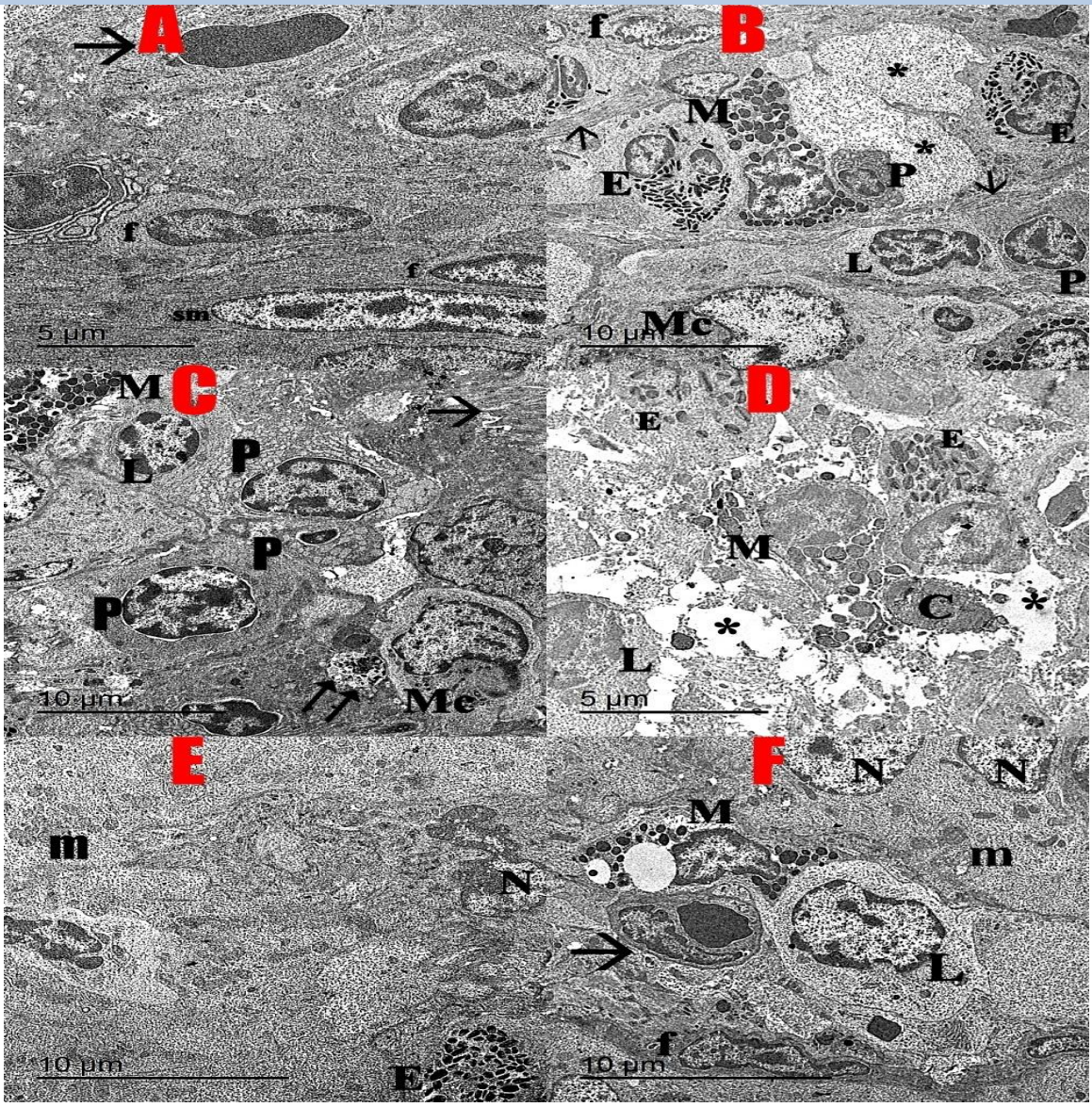

Fig. (7): Electron micrographs of ultrathin sections of the lamina propria from all the groups; **A) Control group:** Nuclei of fibroblasts (f), smooth muscle cells (sm) of the muscularis mucosa and a blood capillary (arrow) are seen. **B) Colitis group:** The lamina propria contains amorphous electron lucent areas (*) and collagen fibers (arrows). Inflammatory cells are seen; mast cells (M) with electron dense large granules, eosinophils (E) with dense core granules, plasma cells (P) with cartwheel nuclei and RER cisternae and a macrophage (Mc). **C) Colitis group:** another area of the lamina propria containing collagen fibers (arrow), Mast cells (M), lymphocytes (L), macrophage (Mc) and apoptotic cell (double arrow) are seen. **D): Colitis group:** A highly rarified lamina propria is seen, it contains wide interstitial spaces (*), Eosinophils (E), lymphocytes (L) and a degranulated mast cell (M). **E) Probiotic - Colitis group:** The lamina propria contains; an eosinophil (E) with dense core granules and cells with irregular nuclei (N). The basis of epithelial cells contains plenty of mitochondria (m). **F) Combined IF& Probiotic - Colitis group:** The basis of epithelial cells contains plenty of mitochondria (m) and intact nuclei (N). The lamina propria contains; a mast cell (M) with electron dense granules and large cytoplasmic vacuoles, a lymphocyte (L), a fibroblast (f) and a blood capillary (arrow).

Table (1s): Mean values (± SD) of MDA and morphometrical analysis (mucosal thickness, number of goblet cells and area % of TNF-α immuno-reaction in the studied groups:

Variables	Group Ⅰ (Control group)	Group Ⅱ (Colitis group)	Group Ⅲ (Probiotic - Colitis group)	Group Ⅳ (Combined IF& Probiotic - Colitis group)	P-value
MDA level (nmol) in the blood	1.33±0.08[a]	3.77±0.38[b*]	1.82±0.18 [c*]	1.55±0.20[d*]	<0.05
Mucosal thickness µm)	246±61.4[a]	142±34.4[b*]	222±30.4 [c*]	236±54.2[d*]	<0.05
Number of goblet cells	177±72.6[a]	54.7±10[b*]	130±33.8 [c*]	151±25.2[d*]	<0.05
Area % of TNF-α immuno-reaction	0.25±0.07[a]	2.71±0.99[b*]	1.21±0.13[c*]	0.84±0.52[d*]	<0.05

This table presents the mean ± standard deviation for various quantitative biochemical and histological parameters measured in all studied groups. For each parameter, the overall statistical test (F-statistic from ANOVA) and its corresponding p-value are provided, followed by post-hoc Least Significant Difference (LSD) tests. Differences between groups, as indicated by different superscripts (a, b, c), signify statistical significance, A p-value of less than 0.05 denotes a statistically significant difference among the groups. **(* significant difference, p value <0.05)**; (b) group 2 versus group 1, (a) group 1 versus group 3& 4, (c) group 3 versus group 2. (d) group 4 versus group 2.

DISCUSION

Indomethacin induced colitis is a common animal model for studying IBD because it is easily administered, available, and a clinically relevant compound. It induces acute and chronic phases of IBD that involve both the small and large intestine **[9].**

Administration of subcutaneous (SC) injections of indomethacin was reported to induce colitis in rats similar to human Crohn's disease. The damaged areas usually appear as inflamed patches that are next to areas of healthy tissue **[26]** through inhibition of cyclooxygenase (COX)-1-mediated prostaglandin synthesis. This leads to intestinal barrier dysfunction which allows enterobacteria to invade the mucosa triggering inflammatory cascades and induce excessive expression of cytokines resulting in intestinal ulceration**[27].**

In the colitis group of the present work, the level of Malondialdehyde (MDA), which is an oxidative stress marker, was significantly higher than that of control group. High MDA levels were reported to reflect excessive lipid peroxidation in the tissue **[28].** This finding agrees with **Aly et al. [29]** who observed that MDA levels increased by 4 folds in his experiment indomethacin induced gastric ulcerations.

In the colitis group the mucosal thickness was reduced, the surface epithelium was lost in many areas, highly deformed crypts and submucosal congested blood vessels. These findings are in line with **Ghafarzadeh et al.**

[30] who observed that indomethacin induced intestinal ulcer which was characterized by bleeding, inflammation, and crypt loss in rats. They suggested that these findings could be caused by increased reactive oxygen species (ROS) production with increased lipid peroxidation impairing cellular homeostasis and function. The high level of MDA reported in this work supports this explanation. Also, indomethacin was proved by **Fan et al. [31]**to cause physical epithelial barrier dysfunction through damaging intestinal epithelial cells.

Electron microscope examination revealed more details of the indomethacin induced injury, loss of microvilli, deformed mitochondria, intracytoplasmic spaces most probably dilated cisternae of ER and impaired or lost intercellular junctions between the surface epithelia. **Hoang et al. [9]** suggested mitochondrial alterations in both IBD models and patients and observed that mitochondrial-targeted therapy (mitoTEMPO) improved the disease and colon mitochondrial function.

Kohzuki et al. [32] explained that indomethacin also caused increased Heme Carrier Protein 1 (HCP1) expression, resulting in defective heme transport which led to more mitochondrial damage. In addition, **Ballard et al. [33]** suggested that mitochondrial dysfunction triggers inflammation through mtROS (mitochondrial reactive oxygen species) that targets the commensal bacteria leading to gut dysbiosis, decrease substrate availability for the epithelial cells and decreased ATP production.

Intracytoplasmic spaces most probably dilated cisternae of endoplasmic reticulum (ER) appeared. Prior studies already showed a direct link between the manifestation of chronic ER stress and the development of IBD **[34].** Also, **Sano and Reed [35]** concluded that ER stress due to accumulation of misfolded proteins promotes cell apoptosis.

The ultra-structural examination revealed apoptotic cells with pyknotic nuclei, and degenerated organelles. **Zhang et al. [36]** declared that oxidative stress and apoptosis are considered the main characteristics of inflammatory bowel disease. Also, **Chen et al. [37]** stated that indomethacin induced hepatocyte apoptosis through both mitochondrial dysfunction and ER stress. Also, an experimental study done by **Schulz et al. [38]** showed that defective tight junctions in IBD lead to epithelial barrier dysfunction and epithelial detachment.

The colitis group showed significant reduction in the goblet cells number. Electron microscope examination revealed small goblet cells with ill-defined mucin granules. Similar findings were observed by **Ghafarzadeh et al. [30]** and could be explained by **Aly et al. [29]** who suggested that indomethacin is acting as anti-cyclooxygenase (COX) enzyme lowering prostaglandins which has an important role in mucin production. Also, **Zhang et al. [39]** attributed the indomethacin induced reduction in mucin secretion to be due to ER stress in goblet cells.

In the current study, light and electron microscopic examination from the colitis group revealed massive inflammatory cellular infiltrations in the lamina propria and submucosa with intraepithelial lymphocytes. These inflammatory cell infiltrations were mostly eosinophils, mast cells and plasma cells. Similarly, **Lin et al. [40]** observed that indomethacin caused epithelial injury, mucosal and submucosal edema with infiltration of inflammatory cells.

This is in consistence with previous studies that considered eosinophilic infiltration as a prominent histological feature associated with increased disease severity in IBD **[41]**. Also, recruitment of eosinophils in the mucosa is mediated by several cytokines e.g IL-5, IL-13, IL-33, and chemokines released by WBCs, endothelial, and epithelial cells in both animal and human models of colitis **[42].**

Eosinophils were reported to be responsible for intestinal tissue injury through their degranulation. Several factors are released e.g eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), major basic proteins (MBP-1 and MBP-2), and eosinophil peroxidase (EPO). ECP is a ribonuclease

protein that produces apoptotic signals. MBPs alter cell membrane functions, leading to increased intestinal permeability in the inflamed intestine **[43].**

Also, the lamina propria of colitis group revealed mast cells with electron dense granules. Mast cells reside in the gastrointestinal tract and have a profound significance in maintaining the function of the mucosal surface and regulating mucosal immunity **[44].** However, abnormal aggregation and activation of mast cells due to inflammation, injury, or other immune responses can disrupt immune balance, resulting in allergic reactions or barrier damage **[45].** So, abnormal aggregation of mast cells was related to the pathological process of IBD. One of the products of activation degranulation of intestinal mast cells is tryptase, which can disrupt intestinal barrier integrity **[46].**

In the current work, the colitis group showed a significant increase in TNF-α immune-expression. This finding is consistence with **El Naggar et al. [47]** who stated that TNF-α has a role in the pathogenesis of IBD as a proinflammatory mediator. They added that there is a genetic association between TNF-α and ulcerative colitis and its level is high on IBD patients. Also, mast cells produce proinflammatory TNF-α and oxidative stress (ROS) mediators promoting intestinal damage **[48].**

The human intestines contain approximately 1000 species of intestinal bacteria, with a total number up to 100 trillion bacteria**.** Dysbiosis is an imbalance between the beneficial microbiome and the commensal microflora harboring in the human gut. Dysbiosis plays an important role in IBD pathogenesis **[49].**

Clinical probiotics contain beneficial bacteria that compete for nutrients and starve harmful bacteria. They produce butyrate, immunoglobulin A (IgA), and short-chain fatty acid (SCFA). They reduce secretion of pro-inflammatory cytokines and increased mucin-2 expression. They also lead to increased autophagy and increased production of defensins **[49].**

In the present work, administration of probiotics prior to indomethacin treatment improved the changes observed in the colitis group. In the probiotic-colitis group; the MDA level, mucosal thickness, goblet cell number and TNF- α expression were significantly improved. Also, the histological structure, inflammatory cellular infiltration and the electron microscopic picture were improved. These findings were in agreement with **Mangin et al. [50]** who found that oral administration of some strains of probiotics modified colonic microbiota and increased mucous production by increasing MUC2 expression.

Probiotics also produce Short-chain fatty acids (SCFA) which have been proven to increase MUC2 expression **[51].** The production of SCFA lower the pH of the intestinal environment, thereby inhibiting the growth of potentially pathogenic microorganisms **[52].**

Some strains of probiotics as Bifidobacterium can adhere well to mucus by surface adhesion and use it as nutrition source and their ability to adhere to the mucous; serves as a gateway for crosstalk between host and microbiota enabling them to deliver health promoting molecules and metabolites **[53].** They also influence the composition of the gut microbiota inhibiting pathogenic colonization **[54]** and having synergistic interactions with other beneficial **[55].**

Barbosa et al. [56] observed that some strains of probiotics decreased TNF- α immunoreactions caused by 5-fluorouracil induced colitis**.** Another study showed that certain strains of probiotics as lactobacillus could affect cell signaling pathways that regulate TNF-α expression **[57].** Other strains of bifidobacterium lowered levels of pro-inflammatory cytokines in general e.g TNF-α, IL-2, IL-1β, IL-13, IL-17A, IL-21, IL-23, IFN-γ and MCP-1and promoted anti-inflammatory cytokine IL-10 **[58].** This is explained their ability to inhibit the pro-inflammatory NFκB (nuclear factor kappa B) pathway while upregulating PPARα (Peroxisome proliferator-activated receptor alpha) signaling **[59].**

Studies showed that probiotics protect against ER stress caused by colitis **[53&60]**. **Zheng et al. [61]** also declared that administration of probiotics mixture has a role in promoting intestinal barrier function**.**

Moreover, probiotics affect tight junction proteins increasing occludin and zonula occludens-1 (ZO-1) and significantly decreased claudin-2 by increasing the protein level and enzymatic activity of T-cell **[59].** Another study stated that tested strains of probiotics prevent the destruction of tight junction proteins by decreasing the number of lipopolysaccharides (LPSs) which have important role in inflammation **[62].**

In the present study, the combination of intermittent fasting and probiotics in the IF and probiotic- colitis group greatly improved the histological structure of the colon. These findings were supported by the morphometric and statistical analysis which revealed a non-significant difference between IF & probiotic-colitis group and the control group.

A study done on the rabbit liver showed that combining a fasting program with oral probiotics significantly improved MDA level compared to probiotics alone and decreased the expression of ROS-related genes **[63].**

Scientific studies showed that fasting improved intestinal epithelium health and goblet cell abundance by decreasing microbiome dysbiosis. Fasting elevated Lactobacillus number and decreases intestinal permeability and can stimulate goblet cell mucus production and replication **[64]. Teker et al. [65]** who worked on ileum and colon of the rat and observed that combining probiotic administration and IF regimen exert a more pronounced impact improving intestinal epithelium and significantly decreasing TNFα immune expression.

Okada et al. [66] found that a fasting refeeding cycle induced two phases in animal model , a phase of arrest of cell division during fasting and a phase of hyperproliferation after refeeding with three-fold increase in the number of mitotic cells. Also, Lactate that was produced by intestinal microbiota Lactobacillus induced intestinal cell proliferation **[67].**

Intermittent fasting can induce autophagy and reduce oxidative stress in rat models which can alleviate ER stress. IF also was suggested to reduce the accumulation of harmful lipids that can exacerbate ER stress **[68].** In addition, **Wolska et al. [69]** concluded that IF can activate pathways like AMPK (5' adenosine monophosphate-activated protein kinase) and inhibit mTOR, which are involved in regulating autophagy and cell growth. This process helps cells adaptation to metabolic stress and clear damaged components including components related to ER stress **.**

In conclusion, this study demonstrated that indomethacin administration in male albino rats led to histological alterations in the colon tissue which was accompanied with an increase in serum oxidative stress marker MDA and the pre adminstration of probiotics could protect the colon from these alterations. However, the combination of both intermittent fasting and probiotics might provide more protection than probiotics alone.

Recommendation

It is recommended to consider the combination of intermittent fasting and probiotics as a new protective strategy against IBD. However, applying these measures to human patients needs large-scale controlled and double-blinded clinical trials with different sizes, doses and durations before this line becomes a trustful method of protection.

Conflict of Interest

The authors report no conflicts of interest. The authors along are responsible for the content and writing of the paper.

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Availability of data

All the raw data is available upon request.

Author contributions: Suggesting the topic of the study, valuable assistance in reviewing,

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