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The Possible Ameliorating Effect of Lactoferrin as Adjuvant to Ceftriaxone on Induced Intestinal Sepsis in Adult Male Albino Rats (Histological and Immunohistochemical Study)

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Abstract:

Background: Intra-abdominal infections can be seriously complicated to abdominal sepsis causing injury of various organs in the abdomen. Lactoferrin could be utilized in combination with Ceftriaxone to treat intestinal sepsis. Objective: to assess the possibility of Lactoferrin to ameliorate induced intestinal sepsis in adult male albino rats when used in combination with Ceftriaxone. **Materials and methods:** Fifty male rats were utilized in this study. The rats were divided into five groups: Group I (Control), Group II (Abdominal Sepsis model group): Rats received a single intraperitoneal injection of 1ml fresh fecal suspension (FSI) at a 50 mg/ml concentration. Group III (Abdominal Sepsis + Ceftriaxone group): Rats were treated with an intramuscular injection of Ceftriaxone, Group IV (Abdominal Sepsis + Lactoferrin group): Rats were treated with oral Lactoferrin, Group V (Abdominal Sepsis + Ceftriaxone + Lactoferrin group): Rats were treated with a combination of both. Colon samples were obtained and processed for histological examination. **Results:** Group II exhibited epithelial injury, detachment, and shedding; some crypts were damaged, obliterated, and lost parallelism; there was obvious depletion of goblet cells; and the lamina propria was markedly infiltrated with inflammatory cells. Furthermore, a significant increase in TNF-α, COX-2, and Caspase-3 immunoexpression was detected. Groups III and IV exhibited some histological attenuation, while group V showed histological architecture with more improvement. **Conclusion:** Ceftriaxone can treat intestinal sepsis, but Lactoferrin can give better results when combined with Ceftriaxone.

Key words: Alcian blue, Caspase-3, COX-2, TNF-α.

Introduction

Sepsis is a potentially fatal syndrome of organ dysfunction arising from a dysregulated host response to infection. Sepsis remains a major global health challenge. Current evidence implicates excessive inflammation, oxidative stress, and immune dysregulation as central drivers in both the onset and progression of sepsis [1]. Intra-abdominal infections (IAIs) rank as the second leading source of sepsis after pulmonary infections [2]; when pathogens breach the peritoneal cavity, they provoke localized or diffuse peritonitis, a principal trigger of severe sepsis in surgical intensive care units. Diffuse peritonitis is further categorized into primary, secondary, or tertiary forms [3]. Although standard therapeutic options, comprising aggressive fluid resuscitation, anti-endotoxin agents, vasopressors, and broad-spectrum antibiotics, can stabilize many patients, no definitive medicine for sepsis has yet been established, underscoring the urgent need for novel therapeutic approaches [4].

Ceftriaxone, a third-generation cephalosporin produced in the 1980s, has antibacterial action against most Gram-negative and Gram-positive bacteria. It acts principally against Gram-negative bacteria, proving significant effectiveness against Gram-negative bacilli [5].

Lactoferrin (LF) is a multifunctional glycoprotein that binds iron and plays a vital role in various biological processes [6]. By chelating iron, LF not only regulates systemic iron homeostasis, absorption, and transport within the gastrointestinal tract but also exerts bacteriostatic effects by depriving microbes of this essential nutrient and by increasing bacterial membrane permeability, leading to cell death. Proteolytic cleavage of LF by pepsin releases lactoferricin (LFcin), a highly potent antimicrobial peptide [7].

This research aimed to assess the possibility of Lactoferrin to ameliorate induced intestinal sepsis in adult male albino rats when used in combination with Ceftriaxone.

Material and methods:

Animals: Fifty adult male albino rats, each weighing between 250 and 300 grams, were obtained from the Animal House of the Faculty of Veterinary Medicine, Moshtohor, Benha University. The rats were housed in standard cages, ensuring ample space for movement. They had unrestricted access to tap water and were provided with commercial rodent chow. Environmental conditions were carefully controlled to ensure the well-being of the animals. These conditions adhered to the ethical guidelines established by the Benha University Faculty of Medicine to ensure proper animal care and welfare during the course of the experiment.

Ethical approval: Prior to the commencement of any experimental procedures, a comprehensive review of all animal-related protocols was conducted and received formal authorization from the Institutional Animal Care and Use Committee (IACUC). Ethical approval for all animal procedures was granted under protocol number Ms 27-6-2023. The routine husbandry and welfare monitoring of the research animals were overseen by the Laboratory Animal House of the Pharmacology Department at Benha Faculty of Medicine.

Drugs and Chemicals:

- **Ceftriaxone** was purchased in a white powder from (NOVARTIS PHARMA S.A.E, El Sawah st., El Amirya, Cairo - Egypt).
- **Lactoferrin** was purchased from (Hygint Pharmaceuticals, 49 Bahaa El-

Deen Al-Ghatoury Street – Smouha, Alexandria, Egypt) as light rose powder.

Induction of Abdominal Sepsis Model by Fecal Suspension Injection (FSI) technique: -

The fecal suspension injection (FSI) method was utilized to induce abdominal sepsis in the rats. Fresh stool samples, collected within 24 hours of excretion, were dissolved in a saline solution. The fecal mixture was then filtered through a Falcon 70 µm cell strainer to remove large particles. A total of 500 mg of the collected feces was dissolved in 10 ml of saline, resulting in a fecal suspension with a concentration of 50 mg/ml. This fecal suspension was injected intraperitoneally (1 ml per rat) to induce sepsis. This specific dose was chosen because it has been established as a sub-lethal dose in previous studies [8].

Experimental design: -

An experimental study was done from 1st August 2023 to 13 August 2023 in the Faculty Veterinary Medicine, Moshtohor, Benha University. After one week of housing, 50 adult male albino rats were randomly distributed into five distinct experimental groups, each containing 10 rats:

Group I (Control Group, n=10): The rats in this group were subdivided into five subgroups:

Ia (n=2): Rats in this subgroup were left untreated, without any medication.

Ib (n=2): Rats received a single intraperitoneal injection of saline (1 ml).

Ic (n=2): Rats were given an intraperitoneal injection of saline (1 ml) and intramuscular saline injection (1 ml) for 5 consecutive days.

Id (in=2): Rats were administered intraperitoneal saline (1 ml) once, followed by daily oral saline administration (1 ml) for 5 days.

Ie (n=2): Rats received both intraperitoneal saline (1 ml) and intramuscular saline (1 ml) injections, then daily oral saline administration (1 ml) for 5 days.

Group II (Abdominal Sepsis Model Group, n=10):

In this group, rats were induced with abdominal sepsis through the intraperitoneal injection of 1 ml of fresh fecal suspension (FSI) at a concentration of 50 mg/ml as previously mentioned [8].

Group III (Abdominal Sepsis + Ceftriaxone Group, n=10): Sepsis was induced as group II, then Ceftriaxone was administered (100 mg/kg) intramuscularly into the quadriceps muscle of all rats 2 hours post-sepsis induction. The administration continued every 24 hours for 5 days [9].

Group IV (Abdominal Sepsis + Lactoferrin Group, n=10): Sepsis was induced as group II, then all rats in this group received Lactoferrin at a dose of 200 mg/kg/day, orally. Treatment commenced 6 hours before sepsis induction, was repeated 2 hours after sepsis induction, and continued daily for 5 days [10].

Group V (Abdominal Sepsis + Ceftriaxone + Lactoferrin Group, n=10): Sepsis was induced as group II, then rats received both Ceftriaxone and Lactoferrin in the same doses and via the same routes as described in Groups III and IV, respectively for 5 days.

Mortality: During the course of the experiment, 4 rats from group II, 1 rat from group III, and 2 rats from group IV unfortunately died. These animals were promptly replaced with healthy and living rats, which then received the same treatment regimen.

Sampling: Twenty-four hours after the final dose of the assigned treatment, all rats were anesthetized using ether. The colon tissues were carefully excised from all animals. To ensure proper

tissue preparation, the collected colon samples were thoroughly washed with saline to remove any fecal remnants and then cut into small 1 cm sections. These specimens were subsequently processed for histological and immunohistochemical analysis, which were crucial for examining the effects of the treatments on intestinal injury and inflammation.

Histological and Immunohistochemical Studies:

Tissue samples were first fixed in formalin, then processed and embedded in paraffin wax, and subsequently cut into serial sections measuring 5–7 μm in thickness. The sections were mounted onto standard glass slides for staining and analysis. For histological evaluation, the sections were stained with hematoxylin and eosin to examine the general tissue morphology, and with Alcian blue to highlight the acidic mucins present in the goblet cells. Additional sections were mounted on positively charged slides to carry out immunohistochemical analysis [11]:

The antigen detection was performed using the avidin-biotin-peroxidase method with diaminobenzidine (DAB) serving as the chromogen. The slides were then counterstained with hematoxylin. Three primary antibodies were utilized for the immunohistochemical study:

1. **TNF-α (inflammatory marker):** A mouse monoclonal antibody (BSB 141, cat. no. BSB 3708 7, MMab), which stains both the cytoplasm and membrane of the cells with a brown color.
2. **COX-2 (oxidative stress marker):** A rabbit monoclonal antibody (SP21, cat. no. ab16701, Abcam), which stains the cytoplasm of the cells with a brown color.
3. **Caspase-3 (apoptosis marker):** A mouse monoclonal antibody (2G4B2, cat. no. 50 173 6893, Proteintech), which stains the cytoplasm of the cells with a brown color.

Morphometric and statistical analysis:

The mean area percentage of Alcian blue staining and the immunoexpression of TNF-α, COX-2, and Caspase-3 was carried out using the Image-Pro Plus version 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA). The analysis involved evaluating five randomly selected, non-overlapping fields per group, with five images taken from each field. All collected measurements were aggregated and subjected to statistical evaluation using IBM SPSS Statistics (Version 22; IBM Corp., Armonk, NY, USA). Intergroup differences were assessed via one-way analysis of variance (ANOVA), with subsequent pairwise comparisons conducted using the Least Significant Difference (LSD) post hoc test. Data were presented as mean (M) ± standard deviation (SD), with a *P*-value < 0.01 denoting statistical significance.

Results:

H & E Stain results (Fig. 1):

Group I (Control Group): All subgroups within Group I demonstrated consistent histological characteristics, maintaining normal tissue architecture with no visible pathological changes. The colon's structure appeared normal, with the mucosa composed of simple absorptive columnar epithelium that was intact and continuous. The underlying lamina propria exhibited infiltration by lymphoid cells. The Lieberkühn crypts extended into the lamina propria, running parallel to each other and were rich in goblet cells filled with secretions. Beneath the epithelial crypts lies the muscularis mucosa; a thin band of smooth muscle fibers that demarcates

the mucosal layer from the underlying submucosa. The submucosa itself was composed of dense connective tissue with blood vessels and lymphatic channels. External to this, the muscularis externa was organized into two distinct smooth muscle layers, an inner circular layer and an outer longitudinal layer. (Fig. 1a & 1b).

Group II (Abdominal Sepsis Model): This group exhibited severe epithelial injury, with detachment and shedding of the epithelium. The lamina propria was heavily infiltrated by neutrophils and other inflammatory cells. The crypts of Lieberkühn were distorted, lost their parallel arrangement, and many goblet cells were absent. Some crypts were completely obliterated, and several cells exhibited darkly stained nuclei. Both the submucosa and musculosa were infiltrated by inflammatory cells, indicating significant inflammation and tissue damage (Fig. 1c & 1d).

Group III (Abdominal Sepsis Model + Ceftriaxone): In this group, a moderate degree of epithelial injury alleviation was observed. While some areas of the epithelial lining were still damaged and discontinuous, the lamina propria showed infiltration by neutrophils and other inflammatory cells. Some crypts appeared distorted, and others were completely obliterated, with a loss of goblet cells. Additionally, several cells exhibited darkly stained nuclei, indicating cell stress and apoptosis (Fig. 1e & 1f).

Group IV (Abdominal Sepsis Model + Lactoferrin): Mild improvement in epithelial injury was observed in this group. While areas of epithelial discontinuity and shedding persisted, the lamina propria was still heavily infiltrated with neutrophils and other inflammatory cells. A large number of goblet cells were lost, and some crypts were completely obliterated. Many crypt cells exhibited darkly stained nuclei, and both the submucosa and muscularis mucosa were infiltrated with inflammatory cells (Fig. 1g & 1h).

Group V (Abdominal Sepsis Model + Ceftriaxone + Lactoferrin): This group exhibited significant recovery of the epithelium and crypts. The crypts appeared relatively normal, with restored parallelism, and there was a notable reduction in inflammation within the lamina propria. This combined treatment showed a marked alleviation of the sepsis-induced damage to the tissue (Fig. 1i & 1j).

Alcian Blue staining results (Fig. 2):

1. **Group I (control group)**: showed normal structure of intestine and the goblet cells mucus was stained blue.
2. **Group II** (**Abdominal sepsis model):** showed minimal blue staining indicating marked loss of goblet cells in crypts of Lieberkühn.
3. **Group III (Abdominal sepsis model + Ceftriaxone):** showed moderate blue staining.
4. **Group IV (Abdominal sepsis model + Lactoferrin):** showed mild blue staining.
5. **Group V (Abdominal sepsis model + Ceftriaxone + Lactoferrin):** showed marked intense blue staining indicating recovery of goblet cells in crypts of Lieberkühn.

TNF-α staining results (Fig. 3):

1. **Group I (Control group):** showed weak immunoreaction for TNF-α in colon tissue (Fig.3a).
2. **Group II (Abdominal sepsis model):** showed marked cytoplasmic and membranous immunoreaction for TNF-α in colon tissue indicated massive inflammatory reaction (Fig. 3b).
3. **Group III (Abdominal sepsis model + Ceftriaxone):** showed

mild cytoplasmic and membranous immunoreaction TNF-α in colon tissue (Fig. 3c).

4. **Group IV (Abdominal sepsis model + Lactoferrin):** showed moderate cytoplasmic and membranous immunoreaction for TNF-α in colon tissue (Fig. 3d).
5. **Group V (Abdominal sepsis model + Ceftriaxone + Lactoferrin):** showed minimal cytoplasmic and membranous immunoreaction for TNF-α in colon tissue (Fig. 3e).

COX-2 staining results (Fig. 4):

1. **Group I (Control group):** showed weak immunoreaction for COX-2 in colon tissue (Fig. 4a).
2. **Group II (Abdominal sepsis model):** showed severe cytoplasmic immunoreaction for COX-2 enzyme indicating strong oxidative stress in colon tissue (Fig. 4b).
3. **Group III (Abdominal sepsis model + Ceftriaxone):** showed mild cytoplasmic immunoreaction for COX-2 enzyme in colon tissue (Fig. 4c).
4. **Group IV (Abdominal sepsis model + Lactoferrin):** showed moderate cytoplasmic immunoreaction for COX-2 enzyme in colon tissue (Fig. 4d).
5. **Group V (Abdominal sepsis model + Ceftriaxone + Lactoferrin):** showed minimal cytoplasmic immunoreaction for COX-2 enzyme in colon tissue (Fig. 4e).

Caspase-3 staining results (Fig. 5):

1. **Group I (Control group):** showed weak immunoreaction for Caspase-3 in colon tissue (Fig. 5a).
2. **Group II (Abdominal sepsis model):** showed marked cytoplasmic immunoreaction for Caspase-3 in colon tissue indicating that many cells undergo apoptosis process (Fig. 5b).
3. **Group III (Abdominal sepsis model + Ceftriaxone):** showed mild cytoplasmic immunoreaction for Caspase-3 in colon tissue (Fig. 5c).
4. **Group IV (Abdominal sepsis model + Lactoferrin): showed** moderate cytoplasmic immunoreaction for Caspase-3 in colon tissue (Fig. 5d).
5. **Group V (Abdominal sepsis model + Ceftriaxone + Lactoferrin):** showed minimal cytoplasmic immunoreaction for Caspase-3 in colon tissue (Fig.5e).

Morphometric and statistical results:

Tables (1:4) and Histograms (1:4) show the mean area percentage and standard deviation (SD) of Alcian blue staining goblet cells and TNF-α, COX-2, and Caspase-3 immunostaining for all groups. When comparing group V to groups II, III, and IV, the mean area percentage of Alcian blue stained goblet cells increased significantly ($P < 0.01$). Additionally, compared to groups II, III, and IV, group V's mean area percentage of TNF-α, COX-2, and Caspase-3 immunostaining was significantly lower ($P < 0.01$).

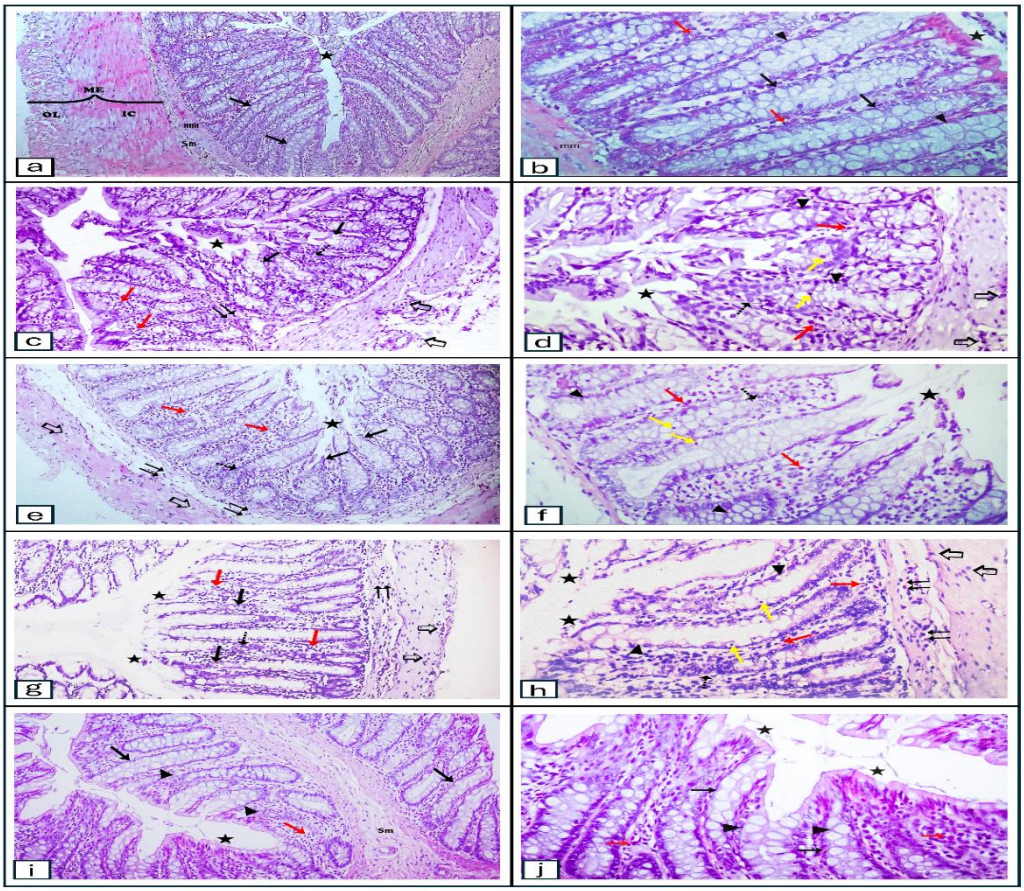

Fig. (1). A photomicrograph of colon of experimental rats showing:

(a) group I (control group): showing the normal structure of colon: mucosa appears folded (plicae circularis) with a healthy simple columnar epithelium lining (★), Lamina propria is penetrated by normal parallel tubular crypts (→), Their bases rest on muscularis mucosa (**mm**); a thin layer of smooth muscle fibers. Submucosa is a layer of connective tissue containing blood and lymphatic vessels (**Sm**). Muscularis Externa (**ME**) is formed of two layers of smooth muscle fibers; inner circular (**IC**) and outer longitudinal (**OL**). Serosa isn't obvious **(H&EX100).**
(b) shows crypts of Lieberkühn (→) lined with simple columnar cells with basal oval nuclei (★) and plenty of goblet cells with vacuolated cytoplasm (▶), lamina propria fills the space between glands and shows some lymphocytes (→). **(H&E X200).**
(c) group II: showing severe injury, detachment and shedding of the epithelium into the lumen (★), lamina propria is markedly infiltrated with inflammatory cells (→), crypts of Lieberkühn are distorted, lost parallelism (→), some crypts are completely obliterated (⇡), submucosa is relatively thin and infiltrated with inflammatory cells (⇉), musculosa is distorted and infiltrated with inflammatory cells (⇨)**(H&EX100).**
(d) showing severe injury, detachment and shedding of the epithelium into the lumen **(★)**, lamina propria is markedly infiltrated with inflammatory cells **(→)**, some crypts are completely obliterated **(⇡),** some cells of the lining epithelium show darkly stained nuclei (pycnotic nuclei) **(→)**, many goblet cells appear small in size losing their secretions (▶), musculosa is infiltrated with inflammatory cells **(⇨) (H&EX200)**.
(e) group III: showing improved histological structure of moderate degree, some areas of epithelial lining still injured and discontinued **(★)**, lamina propria is infiltrated with inflammatory cells **(→)**, some crypts are distorted (→) and others are completely obliterated **(⇡)**, submucosa is still infiltrated with inflammatory cells **(⇉)**, musculosa is partially detached from submucosa and infiltrated with inflammatory cells**(⇨)(H&EX100).**
(f) showing that some areas of epithelial lining still injured and discontinued **(★),** lamina propria is infiltrated with inflammatory cells (→), some crypts are completely obliterated (⇡), some cells show

darkly stained nuclei (→), some goblet cells still small in size (▶) **(H&E X200).** **(g)** group IV shows mild alleviation of epithelial injury in this group, areas of discontinuity and shedding still appear **(★)**, lamina propria is highly infiltrated with inflammatory cells **(→)**. some crypts are distorted **(→)** and others are completely obliterated **(⇑)**, submucosa **(⇉)** and musculosa **(⇨)** are infiltrated with inflammatory cells **(H&E X100).**
(h) showing areas of discontinuity and shedding still appear **(★)**, lamina propria is highly infiltrated with inflammatory cells **(→)**, some crypts are completely obliterated **(⇑)**, many goblet cells are still small, losing their secretions **(▶),** many darkly stained nuclei appear in crypts cells **(→)**, submucosa **(⇉)** and musculosa **(⇨)** are infiltrated with inflammatory cells **(H&E X200).**
(i) group V shows the mucosa appears mostly intact, epithelium restores its continuity **(★)**, there is regression of inflammation in lamina propria **(→)**, crypts are relatively normal and restore their parallelism **(→)**, submucosa appears nearly healthy (**Sm**) **(H&E X100).**
(j) showing the mucosa appears mostly intact, the simple columnar epithelium restored its

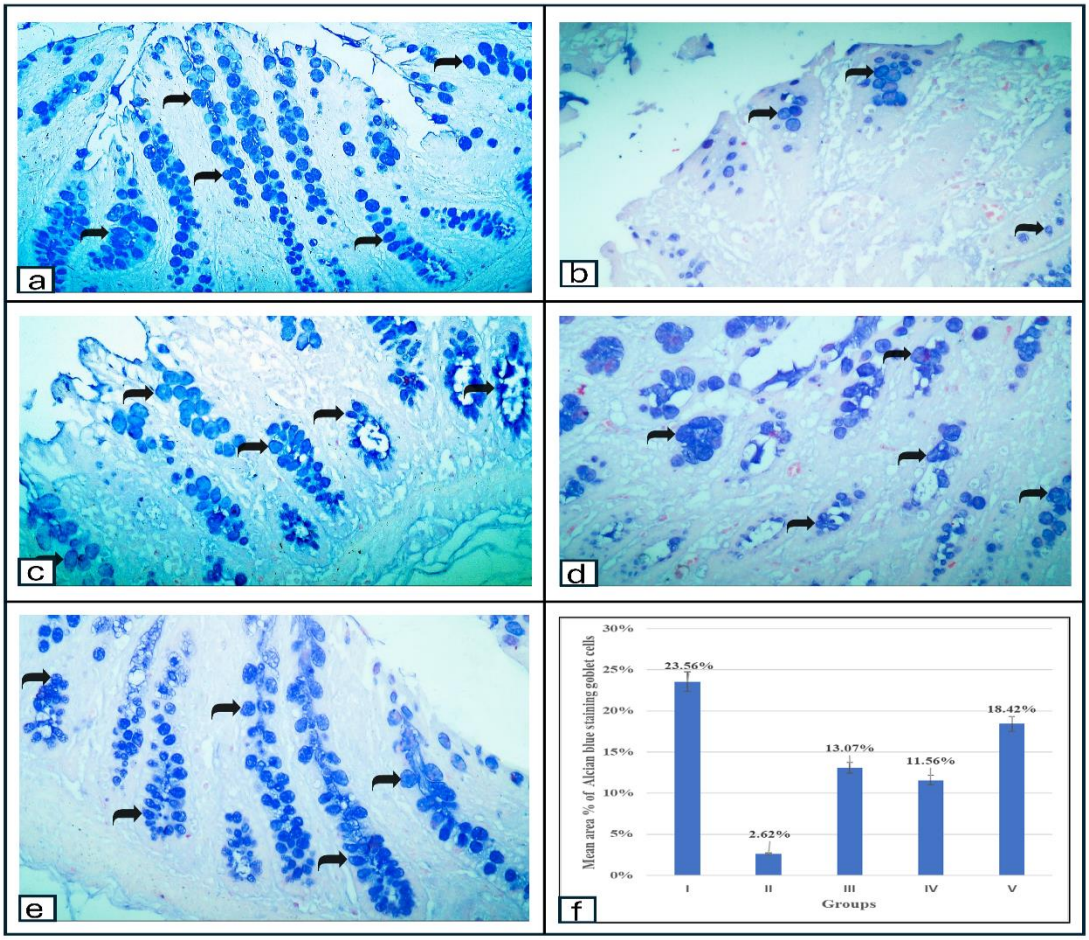

Fig. (2): A photomicrograph of colons of experimental rats showing:
(**a) group I:** normal structure of intestine with the goblet cells mucus is stained blue along the crypt especially in its base (➦).
(b) group II: minimal blue staining indicating marked loss of goblet cells in crypts of lieberkühn which appeared small with little amount of mucin, little mucin appeared in the base of destructed crypts (➦**).**
(c) group III: moderate blue staining of goblet cells especially at the bases of crypts (➦**).**
(d) group IV: mild blue staining of goblet cells especially at the bases of crypts (➦**).**
(e) group V: intense blue staining indicating observed recovery of goblet cells in crypts of Lieberkühn (➦) **(Alcian Blue; X200)**.
(**f)** Histogram showing the mean area % of Alcian blue staining goblet cells in groups I, II, III, IV and V.

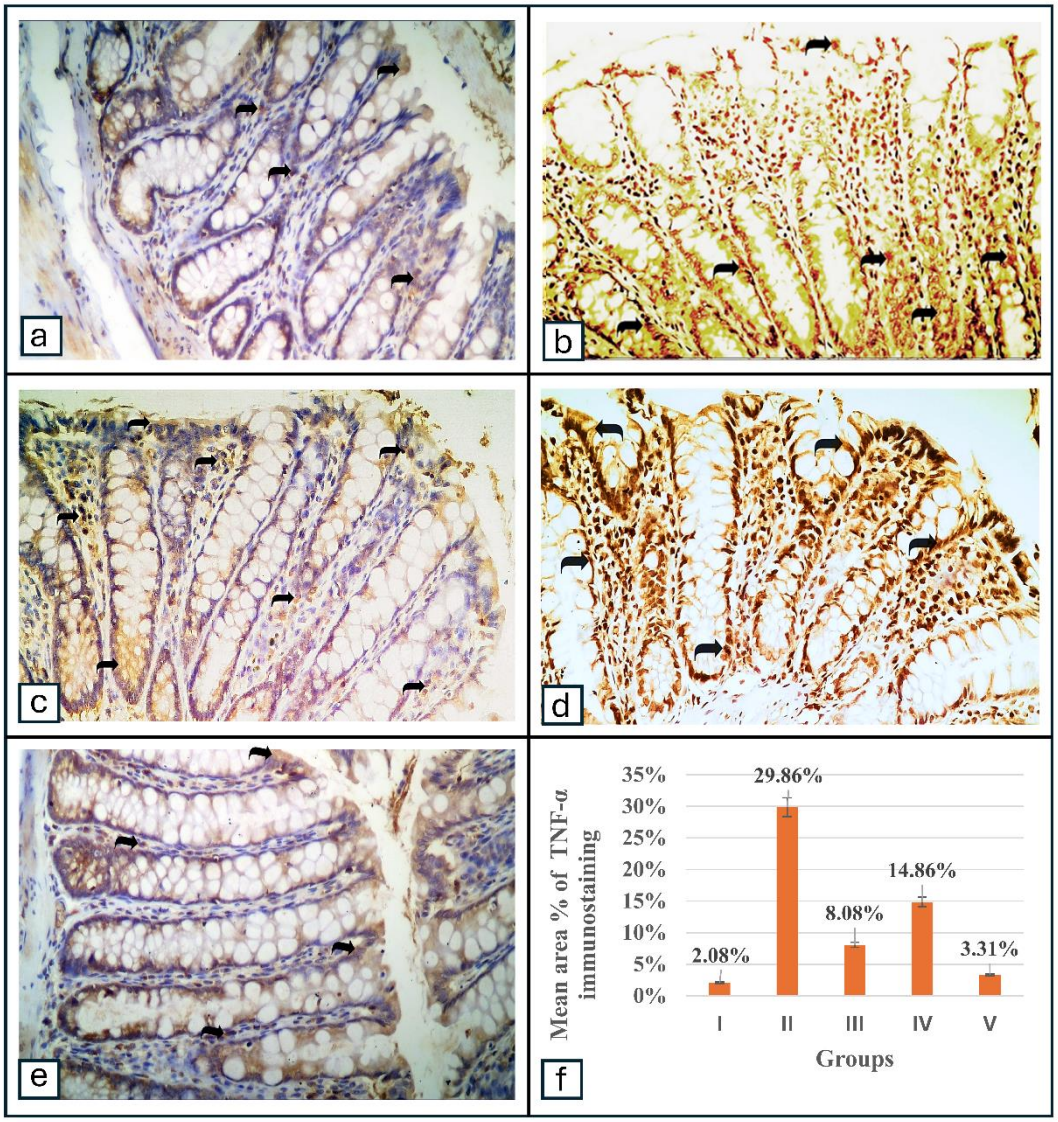

Fig. (3): a photomicrograph of a section in the colon of a rat of:
(a) group I (control group): showing weak immunoreaction for TNF-α in the epithelial lining and connective tissue cells (➦**).**
(b) group II: showing marked cytoplasmic and membranous immunoreaction for TNF-α in the epithelial lining and connective tissue cells (➦).
(c) group III: showing mild cytoplasmic and membranous immunoreaction for TNF-α in the epithelial lining and connective tissue cells (➦).
(d) group IV: showing moderate cytoplasmic and membranous immunoreaction for TNF-α in the epithelial lining and connective tissue cells (➦).
(e) group V: showing minimal cytoplasmic and membranous immunoreaction for alpha TNF-α in the epithelial lining and connective tissue cells (➦) (**Anti- TNF- α X200)**.

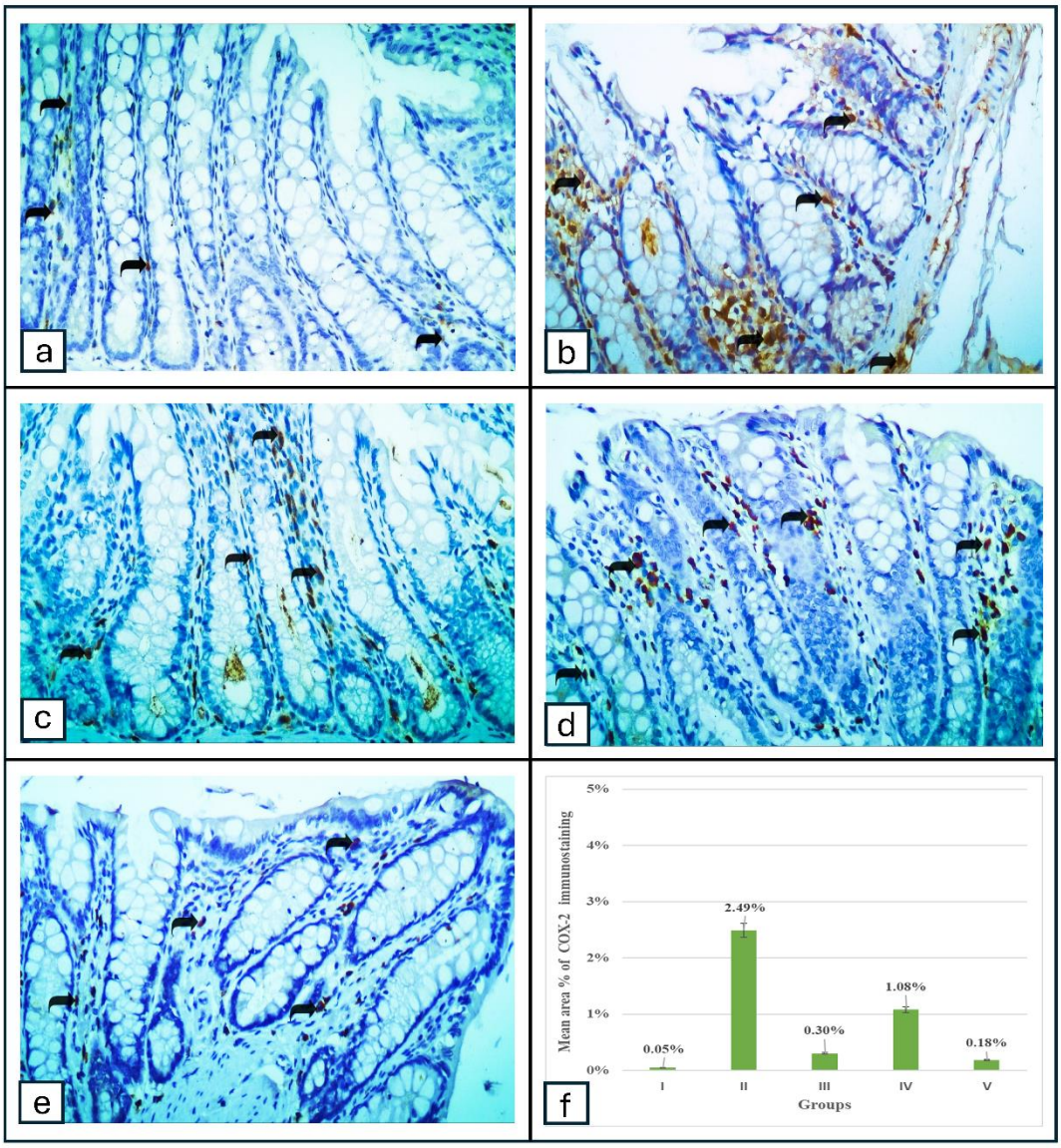

Fig. (4): A photomicrograph of a section in the colon of a rat of:
(**a)** group I (control group): showing weak immunoreaction for COX-2 enzyme (➦).
(b) group II: showing marked cytoplasmic immunoreaction for COX-2 enzyme (➦).
(c) group III: showing mild cytoplasmic immunoreaction for COX-2 (➦).
(d) group IV: showing moderate cytoplasmic immunoreaction for COX-2 (➦).
(e) group V: showing minimal cytoplasmic immunoreaction for COX-2 (➦) **(Anti- COX-2, X200).**
(f) histogram showing the mean area % and SD of COX-2 immunostaining in groups I, II, III, IV and V.

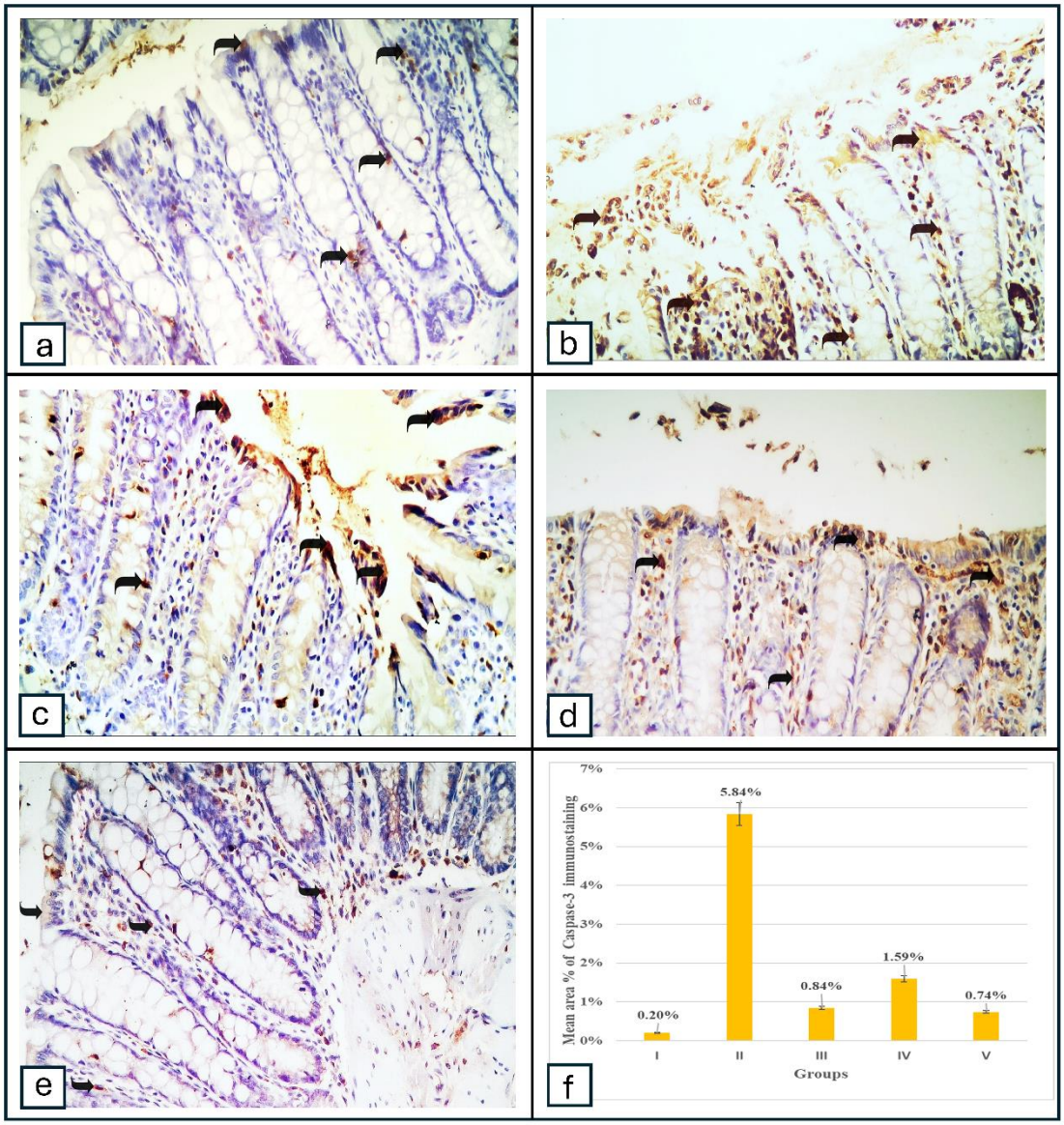

Fig. (5): A photomicrograph of a section in the colon of adult male albino rat of**:**
(a) group I: showing weak immunoreaction for Caspase-3(➦).
(b) group II: showing marked cytoplasmic immunoreaction for Caspase-3(➦).
(c) group III: showing mild cytoplasmic immunoreaction for Caspase-3(➦).
(d) group IV: showing moderate cytoplasmic immunoreaction for Caspase-3(➦).
(e) group V: showing minimal cytoplasmic im
(f) Histogram: showing the mean area % of Caspase-3 immunostaining in groups I, II, III, IV and V.

Table 1: Alcian blue–positive goblet cell area % (mean ± SD) across all experimental groups, with pairwise comparisons determined by one-way ANOVA and LSD post hoc testing ($P < 0.01$).

	Group I	Group II	Group III	Group IV	Group V
Mean area %	23.56%	2.62%	13.07%	11.56%	18.42%
SD	0.7265	0.4306	0.1555	0.3721	0.4504
Significance (sig) at P < 0.01	2,3,4,5	1,3,4,5	1,2,4,5	1,2,3,5	1,2,3,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV

Table 2: TNF-α immunostaining area percentage (mean ± SD) in all experimental groups

	Group I	Group II	Group III	Group IV	Group V
Mean area %	2.08%	29.86%	8.08%	14.86%	3.31%
SD	0.1656	1.2180	0.4637	0.4616	0.4595
Significance (sig) at P < 0.01	2,3,4,5	1,3,4,5	1,2,4,5	1,2,3,5	1,2,3,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV

Table (3): COX-2 immunostaining area % (mean ± SD) for each experimental group

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0.05%	2.49%	0.30%	1.08%	0.18%
SD	0.0281	0.3868	0.1146	0.0851	0.0282
Significance (sig) at P < 0.01	2,3,4	1,3,4,5	1,2,4	1,2,3,5	2,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV

Table (4): Caspase-3 immunostaining area % (mean ± SD) across experimental groups

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0.20%	5.84%	0.84%	1.59%	0.74%
SD	0.0202	0.4218	0.1276	0.1636	0.0357
Significance (sig) at P < 0.01	2,3,4,5	1,3,4,5	1,2,4,5	1,2,3	1,2,3,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group

Discussion:

Intra-abdominal sepsis is widely recognized as one of the most difficult and complex surgical complications, frequently presenting as peritonitis. A particularly common and life-threatening surgical emergency associated with intra-abdominal sepsis is gastrointestinal perforation, which occurs when digestive contents leak into the peritoneal cavity. If not identified and treated in a timely manner, gastrointestinal perforation significantly increases the risk of mortality due to subsequent sepsis [12]. In the context of animal modeling, the fecal suspension injection (FSI) model has been chosen in our research due to its simplicity, standardization, and the ability to control disease severity by adjusting the fecal dosage

administered. This model is also particularly advantageous as it is less invasive and allows for precise control over individual variables, thus reducing inter-animal variability, and making it ideal for focutilized research on the pathophysiology of sepsis once it has been induced **[8]**. This model is also particularly advantageous as it is less invasive and allows for precise control over individual variables, thus reducing inter-animal variability, and making it ideal for focutilized research on the pathophysiology of sepsis once it has been induced **[14]**.

In Group II, induction of abdominal sepsis caused deterioration in the histological structure of colon with severely damaged lining epithelium, crypts, submucosa and musculosa. Also inflammation, oxidative stress and many apoptotic cells appeared markedly in all layers, these findings align with the results reported in numerous other studies that have studied similar models of sepsis and inflammatory injury [15, 16, 17, 18].

To explain the pronounced colonic inflammatory response observed in our septic cohort, Lei et al [1] underscored the pivotal roles of inflammation, excessive oxidative stress, and deranged immune regulation in both the initiation and amplification of sepsis. These interrelated processes are widely recognized as the principal drivers that precipitate the cascade of tissue injury, systemic cytokine release, and progressive organ dysfunction characteristic of severe sepsis.

In our Group II animals, the marked reduction in Alcian blue–positive goblet cell area ($P < 0.01$) mirrors findings of some researchers [19, 17] who attributed this depletion to epithelial erosion and apoptosis secondary to overwhelming inflammatory insult. The loss of mucin-producing goblet cells compromises the mucosal barrier, further exacerbating bacterial translocation and perpetuating the inflammatory milieu.

Consistent with the patterns reported by Conesa et al [20] and Chen et al [15], we documented a dramatic upsurge in TNF-α immunoreactivity in Group II. As Mao et al [21] elucidated, TNF-α—secreted predominantly by activated macrophages—serves as a potent chemoattractant for neutrophils, which in turn generate reactive oxygen species (ROS) and secrete proteolytic enzymes, amplifying local oxidative injury and epithelial cell death. Moreover, systemic TNF-α translocation into the circulation initiates Caspase-3 activation, triggering the execution phase of apoptosis via proteolytic cleavage of intracellular substrates [22]. Our observation of elevated Caspase-3 levels in Group II aligns with the work of Liu et al [23] and Ho et al [16], confirming that oxidative stress strongly modulates Caspase-3–mediated apoptotic pathways [24].

As an indicator of this oxidative stress, we utilized the COX-2 immune marker. Increased COX-2 expression in this group confirmed high oxidative stress in sepsis. These results align with those of previous studies, supporting the reliability and validity of our findings [25, 26, 18]. TNF-α is one of several proinflammatory cytokines that cause the release of COX-2. The prostaglandins generated by COX-2 attract inflammatory cells to infection sites, increasing vascular permeability and resulting in acute inflammation [27].

In sepsis and septic shock, timely antibiotic administration remains the cornerstone of therapy. Empirical antibiotic intervention typically begins immediately after diagnosis and culture collection, aligning with

therapeutic guidelines [28]. Ceftriaxone, a third-generation β-lactam cephalosporin, is favored due to its broad-spectrum activity, excellent pharmacokinetics, and low toxicity. It acts by inhibiting the transpeptidation step in bacterial cell wall synthesis, leading to bacterial death [29]. Its selection in this research was supported by its potency and favorable safety profile, as noted by Ayele et al [30].

In the current research, Group III (sepsis + Ceftriaxone) demonstrated moderate histological improvement. Although some epithelial regions, some crypts remained damaged. Inflammatory infiltration in the lamina propria was moderately reduced. Also, there was a reduction in inflammatory, oxidative, and apoptotic activity. Collectively, these findings suggest that Ceftriaxone exerted a moderate protective effect against sepsis-induced intestinal injury.

We desperately need to develop new strategies to boost the immune system and manage inflammation because of the increasing emergence of antibiotic resistance. When treating serious infections, adjuvant treatments may be a useful complementary therapy to antibiotics [31]. Some researchers as Dhanda et al [32] encouraged combination therapy by combining two or more drugs.

The cationic glycoprotein LF, which is produced by neutrophils and exocrine glands, is widely known for its ability to bind two ferric irons per molecule. Due to its established immuno-modulatory, anti-inflammatory, anti-toxic, and anti-microbial properties [33], we utilized LF in our research. Another study by Shini et al [34] explained that LF exhibits antimicrobial activity against a wide range of both Gram-positive and Gram-negative bacteria. In addition, LF specifically stimulates beneficial bacteria growth in the gut, including Lactobacillus and Bifidobacterium. On the basis of these considerations, we aimed to use LF as adjuvant therapy.

In our research, we found morphological changes in Group IV (Abdominal sepsis model + Lactoferrin) as mild alleviation of the epithelium and crypts with slight regression of inflammatory cell infiltration in lamina propria. Also, there was a mild decrease in inflammatory, oxidative, and apoptotic processes. These results agreed with [35].

Many studies explained this ameliorating effect of LF as Yami et al [36] who stated that LF inhibits the release of pro-inflammatory cytokines such (TNFα), an essential mediator in immunological and infectious responses. Also, Cao et al [37] explained the bactericidal and bacteriostatic effects of LF. The bacteriostatic effect of LF is mediated by its ability to bind free iron, a critical nutrient for bacterial growth and reproduction, effectively depriving bacteria of essential resources. Additionally, LF enhances the permeability of the bacterial outer membrane through its strong cation-binding region at the amino terminus, leading to the leakage of bacterial lipopolysaccharides and other cellular components, thus exerting a bactericidal effect.

LF has the ability to break down pathways that bacteria utilize to attach to and penetrate host cells, and even affect the development of virulence proteins. Moreover, LF uses molecular and cellular processes to control the innate immune system. On a cellular level, LF shares in cellular responses such migration, maturation, and proliferation. LF receptors are present on lymphocytes, macrophages, and dendritic cells. At

the molecular level, LF interacts with numerous soluble and membrane molecules involved in the inflammatory response [20].

Some researchers, such as Xue et al [38] discovered that LF acts as an anabolic agent, primarily by promoting cell proliferation and exerting anti-apoptotic effects. This explained the anti-Caspase 3 activity in this search.

Our findings are in agreement with [39] results on murine models of colitis and IBD. MacManus et al [39] concluded that; LF administration led to a decrease in the inflammatory score and an improvement in the intestinal histological structure. This resulted in reduced tissue damage, inflammatory cell infiltration, loss of crypts, and preservation of epithelial integrity in the small intestine and colon, along with an increase in goblet cell numbers.

Lactoferrin achieved mild alleviation of intestinal sepsis when utilized alone in Group IV. So, In Group V, we combined (Ceftriaxone and LF), that already showed better results than group III and group IV, it showed marked alleviation of intestinal epithelial injury, it restored its continuity and appeared with less inflammation signs, crypts restored their normal structure and parallelism, also, there was significant regression of inflammation, oxidative stress and apoptosis. Our findings parallel reports of [40,41].

In this group, TNF-α expression reduced markedly in comparison to group III and group IV. This ensures the better anti-inflammatory effect of LF when combined with Ceftriaxone. LF interacts with the negatively charged groups on immune cell surfaces, activating signaling pathways that promote physiological anti-inflammatory reactions [42]. This anti-inflammatory effect of Lactoferrin was agreed with [36].

Furthermore, the LF and Ceftriaxone combination in this group reduced oxidative stress. That was confirmed by decreased COX-2 expression significantly. That was in agreement with some studies [43,44]. Phagocytes release ROSs during inflammation, which neutralizes microbes. Regrettably, ROSs also cause necrosis in live tissue because of their detrimental effects. Subsequently, tissues release iron, which produces further free radicals. Due to its ability to bind iron, LF helps to decrease oxidative stress [33].

Our results of Group V showed that the combination of LF and Ceftriaxone had a synergistic effect in treatment of induced intestinal sepsis. LF can reduce bacterial load by its bacteriostatic and bactericidal actions, as mentioned detailed before, giving better results when combined with strong antibiotic [45].

Moreover, Redwan et al [46] reported that the antimicrobial efficacy of various, though not all, classes of antibiotics can be diminished in the presence of iron. However, this inhibitory effect is mitigated by LF, which acts by sequestering free iron, thereby restoring or enhancing the antibacterial activity. Also, LF exerts its antimicrobial effect by sequestering iron, a critical nutrient for microbial proliferation, and by binding to lipopolysaccharides on the surface of Gram-negative bacteria. This interaction disrupts and destabilizes the bacterial outer membrane, thereby enhancing the permeability and efficacy of concurrently administered antibiotics [42]. LF can also potentiate the effectiveness of certain antibiotics by increasing bacterial susceptibility, thereby allowing for a reduction in the required therapeutic dose and

enhancing overall bactericidal activity [42]. In parallel with our findings, murine model of typhoid fever in previous search treated with ciprofloxacin combined with LF showed that, LF enhanced intestinal bacterial clearance and significantly reduced bacterial translocation to the liver and spleen, thereby preventing the establishment of chronic systemic infection [47].

Conclusion:

At the end, we can conclude that LF has a powerful ameliorating effect when combined to Ceftriaxone on intestinal sepsis in albino rats. We recommend further studies on adjusting its doses and exploring its probable side effects.

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