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RESEARCH ARTICLE

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The decreasing effect of troxerutin on the level of pro-inflammatory cytokines in rats with sepsis caused by the experimental cecal puncture

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ABSTRACT

Sepsis is the main mortality factor in patients undergoing surgery and its treatment currently includes cardiac resuscitation and reducing the immediate risk of infection. Troxerutin is a common compound in vegetables, fruits, and seeds and has several biological activities, including anti-platelet, anti-serotonin, antioxidant, and anti-inflammatory effects. Accordingly, we hypothesized that it can decrease interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α) levels in the serum of rats with sepsis. Twenty-four adult male Sprague-Dawley rats were used in this study. The rats were equally and randomly divided into 3 groups: sham operation group, control group, and treatment group. Both the control and treatment groups underwent surgical cecal ligation and perforation. Troxerutin (130 mg/kg) was injected subcutaneously twice a day to the animals of the treatment group for 3 days or until the animals' death. Surviving rats were euthanized after 1.5 ml of blood samples were taken 3 days after the cecal ligation and perforation. IL-1 and TNF-α were measured by the blood serum ELISA assay. The differences in mortality rates were significant between the control group and the other two groups ($p = 0.008$). The results showed a significant increase in IL-1 and TNF-α in the control group compared to the sham group ($p < 0.05$). In addition, the levels in the treatment group significantly decreased compared to the control group ($p < 0.05$). In conclusion, our results indicate that troxerutin could increase survival in rats developing septic shock by reducing pro-inflammatory cytokines including IL-1 and TNF-α.

Keywords

Immune response, laboratory animals, peritonitis, septic shock, troxerutin

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Abbreviations

CLP: Cecal ligation and perforation
IL-1: Interleukin 1
IL-1β: Interleukin 1-beta
TNF-α: Tumor necrosis factor-alpha
IL-6: Interleukin 6
IL-12: Interleukin 12
IFN-γ: Interferon-gamma
NO: Nitric oxide

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Introduction

Sepsis is caused by bacteria or their endotoxins and is the main contributor to mortality in patients undergoing surgery. When endotoxins enter the body, significant changes occur in the immune response and organ function [1]. Endotoxins are the lipopolysaccharide membrane of gram-negative bacteria. They are released during bacterial cell lysis, creating a strong response in the body that leads to various components of immune cell activation with subsequent septic shock [2]. The interaction of lipopolysaccharides with receptors on different cells induces the production of many pro-inflammatory factors including interferon-gamma (IFN-γ) nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), as well as interleukins 1-beta (IL-1β), 6 (IL-6), and 12 (IL-12) [3]. Studies have shown that TNF-α and IL-1 are the first cytokines to be produced in large quantities in response to lipopolysaccharides, and this is the main cause of the many effects of lipopolysaccharides. These cytokines and chemokines enhance the host response to bacterial infection [4]. In particular, TNF-α, which is released by macrophages, acts as a stimulant for cytokine cascade and ultimately leads to lethal septic shock. However, anti-inflammatory cytokines are also abundant during sepsis. The latter cytokines reduce pro-inflammatory cytokines production so that they could help the animal to overcome sepsis and endotoxin-related shock to some extent [3]. However, if cytokines are released too much, they could have detrimental consequences for the body. Recent studies have shown that large amounts of cytokines, particularly IL-1, TNF-α, and IFN-γ, are produced in septic shock and related injury, which mediate many of the detrimental effects of septic shock [5].

Treatment for sepsis currently includes cardiac resuscitation and management of the immediate risk of infection. Intravenous fluid therapy, vasopressor medications, and oxygen therapy are the main resuscitation options. Immediate intravenous antibiotic therapy is performed to reduce potential pathogens. The only currently available immunotherapeutic treatment is the short-term use of hydrocortisone in patients with resistant septic shock [6]. The use of corticosteroids in septic shock has been extensively studied [7]. Preliminary studies have shown that high doses of corticosteroids are not helpful for septic shock and can be harmful. In a study on 499 patients, hydrocortisone did not improve survival in patients with septic shock, and it was concluded that discontinuation of corticosteroids if the patient is unresponsive to treatment should be considered due to the potential risks of infection, hypoglycemia, and myopathy [8].

The failure to find new effective treatments, together with advances in finding the biological characteristics of sepsis, has been one of the greatest desperations of the past few decades [9]. Two types of materials could be used for this purpose: materials that disrupt the primary cytokine cascade (such as anti-inflammatory cytokines and anti-lipopolysaccharides) and those that prevent dysregulated coagulation (including activated protein C and antithrombin) [10]. Activated protein C was recently approved despite being withdrawn from the market by the manufacturer due to concerns about safety and efficacy [11]. There is currently no comprehensive evidence for the effectiveness of anti-cytokines in the treatment of sepsis [6].

Troxerutin (C33H42O19), or vitamin P4, is a compound derived from rutin flavonoid. This substance is available in vegetables, fruits, and seeds with a range of biological activities [12]. It has anti-serotonin and anti-platelet properties. The use of troxerutin in the treatment of vascular diseases such as phlebitis or capillary hemorrhage has been extensively studied [13,14]. Antioxidant and anti-inflammatory effects of troxerutin have also been reported in other studies. It crosses the blood-brain barrier and thus affects the central nervous system. Troxerutin can prevent the activation of nuclear factor kappa B (NF-κB) signaling [15]. NF-κB could increase pro-inflammatory cytokines expression and intensify the inflammatory response. It has been suggested that the anti-inflammatory effect of troxerutin in ischemia/reperfusion injuries of diabetic myocardium may be because of a reduction in TNF-α activity, resulting in NF-κB blocking [16]. A recent in vivo study suggests that troxerutin reverses the inflammatory response by inhibiting elastase. This protease activates by TNF-α and contributes to inflammation [17]. Due to the good solubility of troxerutin in water, it is easily absorbed from the gastrointestinal tract and exerts its protective effects on tissues without cell toxicity [18]. Troxerutin has a potential effect on the treatment of diabetes mellitus and Alzheimer's disease, which is partly due to its antioxidant activity. It has been reported that it reduces damage to various tissues such as the brain, liver, and kidneys by improving anti-

Abbreviations-Cont'd

NF-κB: Nuclear factor kappa B
ELISA: Enzyme-Linked Immunosorbent Assay
TMB: Tetramethylbenzidine
ANOVA: Analysis of variance
SEM: Standard error of the mean
S: Sham
C: Control
T: Treatment

oxidant levels [19]. The immunoprotective and anti-aging effects of troxerutin are also known [20]. Consequently, we hypothesized that troxerutin might prevent elevated serum levels of IL-1 and TNF-α serum levels in rats with sepsis.

Results

None of the rats of the sham (S) group (0%, n = 0) died during the 3 days. 25% (n = 2) of the animals in the control (C) group died a day after surgery and 37.5% (n = 3) died after two days, thus, only 37.5% (n = 3) in this group survived after 3 days. In addition, the remaining rats were secluded and lethargic, with no desire to move or eat. Lack of grooming and response to the external stimuli were the other clinical symptoms. These manifestations indicated peritonitis-induced septic shock [21]. Only 12.5% (n = 1) of the treatment (T) group died on postoperative day 2 and 87.5% (n = 7) of them survived. Survivors were alert and ambulatory. The differences in mortality rates were significant between groups C and the other two groups ($p = 0.008$). Because the survived rats were euthanized 3 days after cecal ligation and perforation (CLP), we considered these animals as censored after experiment termination. Therefore, the mean survival days presented in Table 1 are calculated for 3 days after CLP, and the actual survival of the animals cannot be determined. As seen in Table 1, most of the control group rats died during the experiment, and they also died earlier (with lower survival days) than the rats in the treatment group.

The results of pro-inflammatory cytokines analysis showed a significant increase in IL-1 and TNF-α in group C (1438.66 ± 23.81 pg/ml and 1358.42 ± 21.89 pg/ml, respectively) compared to the group S (17.32

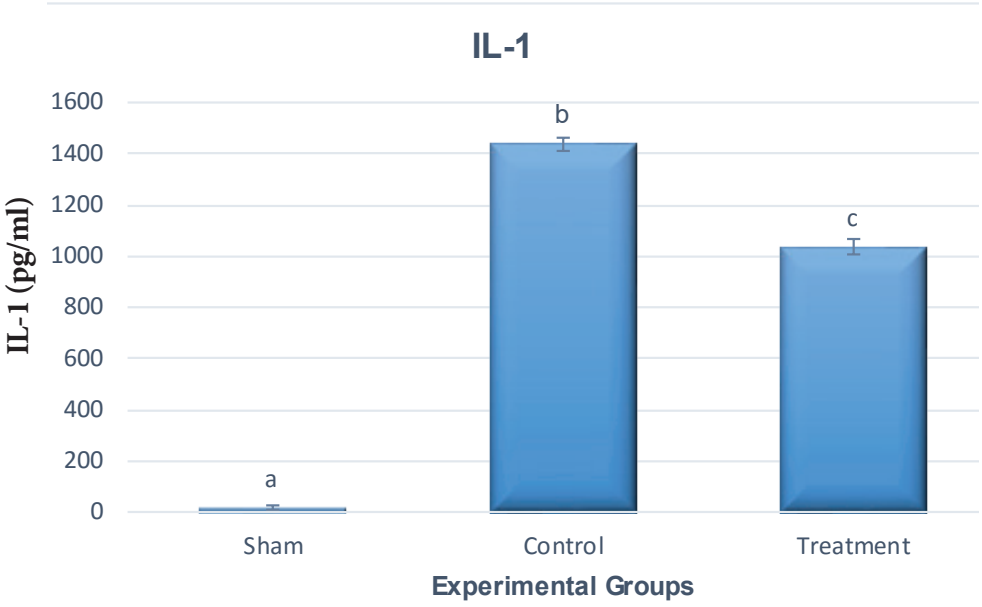

Figure 1.
IL-1 levels (mean ± SEM) in the serum of rats in three experimental groups. Different letters indicate a significant difference ($p < 0.05$).

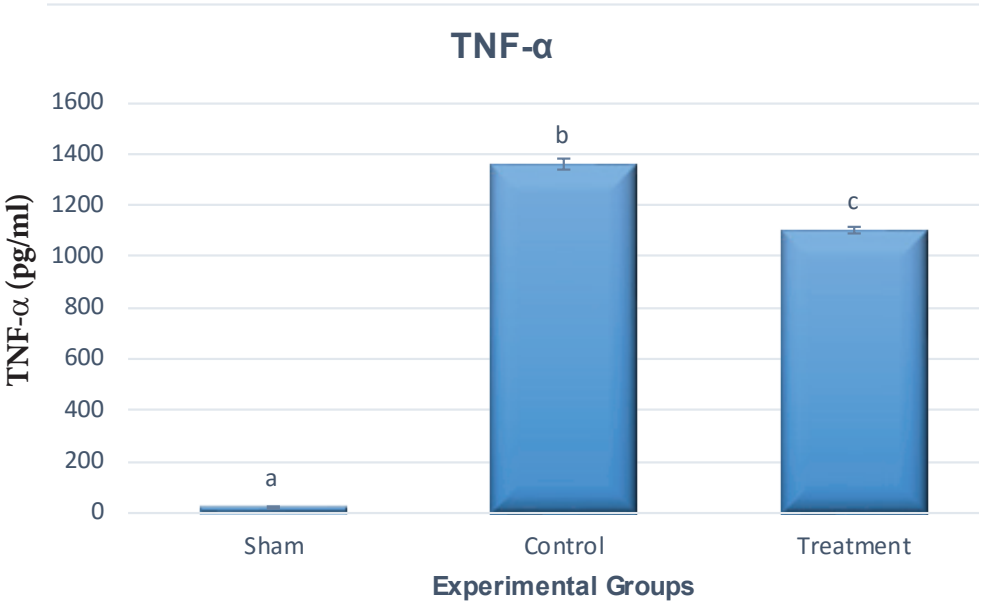

Figure 2.
TNF-α levels (mean ± SEM) in the serum of rats in three experimental groups. Different letters indicate a significant difference ($p < 0.05$).

± 7.93 pg/ml and 20.31 ± 4.72 pg/ml, respectively) ($p < 0.05$). In addition, the measured cytokine levels in group T (1036 ± 27.72 pg/ml and 1100.42 ± 15.22 pg/ml, respectively) were significantly lower than in those in group C ($p < 0.05$) (Figures 1 and 2).

Table 1.
Survival days and survial rates of the rats in different experimental groups 3 days after cecal ligation and perforation (CLP).

Groups	Survival days (mean ± SEM)	Survival rate (%)
Sham	3.00 ± 0.00	100
Control	2.12 ± 0.29*	37.5*
Treatment	2.87 ± 0.12	87.5

*: significant difference between groups ($p < 0.05$).

Discussion

Although short-term mortality in patients with septic shock has decreased in recent years, it remains a major problem. The mortality rate could vary from 35% to 70%, depending on several factors such as age, gender, comorbidities, acute respiratory involvement, or renal failure [22]. However, there is very little data for the long-term mortality in patients with septic shock. One report estimated that about 20% of hospital survivors die within the first year [23]. The short-term mortality rate in our study was also 62.5% in the control group which is in line with other studies [24,25,26] but the survival of the treatment group was significantly improved and the short-term mortality in this group was significantly improved by 12.5%. This indicates that troxerutin could prevent septic shock-related death, at least in the early stages of peritonitis development. Severe septic shock affects the central nervous system and causes drowsiness or delirium. Non-focal encephalopathy, polyneuropathy, and myopathy are common findings in these patients [6]. This could explain why the surviving rats in group C were recluse and lethargic while the others were alert and ambulatory.

Although severe inflammation was previously believed to be the cause of the clinical symptoms of sepsis, Bone et al. (1997) reported that compensatory anti-inflammatory response syndrome can arise from an initial inflammatory response [27]. On the other hand, we currently know that a more complex and prolonged host response is provoked by infection. Both the infection elimination and the tissue recovery, and secondary infection can be the result of pro-and anti-inflammatory mechanisms [28]. Pro-inflammatory responses to kill invading pathogens are liable for tissue damage, organ dysfunction, and early mortality, while anti-inflammatory reactions that limit local and systemic tissue damage are involved in the development of secondary infections in sepsis [6]. Some types of receptors (namely C-type lectin, toll-like, nucleotide-binding oligomerization domain-like, and retinoic acid-inducible gene 1–like receptors) could bind to bacterial lipopolysaccharides, resulting in immune cell activation and upregulation of inflammatory gene transcription [29] and the release of pro-inflammatory cytokines including IL-1 and TNF-α [3]. These cytokines stimulate the synthesis of phospholipase A2, inducible cyclooxygenase, 5-lipoxygenase, and acetyltransferase, which contribute to the synthesis of prostaglandins and leukotrienes and platelet-activating factor which lead to further inflammation, vasomotor tone alteration, and increasing blood flow and vascular permeability [22]. Additionally, these cytokines attract monocytes, neutrophils, T cells, and macrophages to the area to confront infection, but they also play a role in the pathogenesis of severe inflammation at the same time. Tamayo et al. (2011) reported that both pro-and anti-inflammatory (IL-10) cytokines are present in septic shock [30].

Troxerutin reduces enzymes and proteins inside cells including cyclooxygenase in some tissues [31]. The anti-inflammatory properties of troxerutin in a wide variety of organs such as kidneys, liver, brain, and heart are well understood. For instance, Najafi et al. (2018) found that troxerutin prevents myocardial arrhythmias in rats by inhibiting inflammatory cytokines and reducing inflammation arising from ischemia/reperfusion [16]. More recently, Jafari-Khataylou and coworkers (2021) reported that troxerutin reduced inflammation and histopathological lesions and improved antioxidant activity and survival rate in mice injected with lipopolysaccharides [32]. Despite some methodological differences, our results are in agreement with this study. Although their study is the only report available in the literature on the influence of troxerutin on peritoneal sepsis, there is extensive data on its protective and anti-inflammatory properties in a wide variety of organs. Shan et al. (2018) had previously found that this substance improves kidney function against inflammatory damage by blocking certain signaling pathways [33]. The results of Hoseinidoost and coworkers (2019) suggested that troxerutin can hinder the adverse effects of maternal high fat diet on their offspring through pro-inflammatory cytokines inhibition [34]. Lu et al. (2013) had recommended troxerutin for the prevention and therapy of cognitive deficits resulting from brain inflammatory response [35]. Zhang and coworkers (2015) found that troxerutin can be beneficial in the prevention and treatment of liver inflammation

[36]. All of the reports mentioned had emphasized the ameliorating effect of troxerutin against inflammatory reactions. Troxerutin could mediate its anti-inflammatory function by changing inflammation-related microRNAs (miRs) expression, including miR-146a and miR-155 [37]. A study by Yavari et al. (2016) in diabetic rats demonstrated that the anti-inflammatory effect of troxerutin on the NF-κB-mediated pathway is related to its effect on the miR-146a restoration [38].

Our results are also in accordance with other studies that investigate the effects of other natural substances in peritonitis or septic shock. Ozer and coworkers (2010) reported that *Nuphar lutea* leaf extract (NUP) has an anti-inflammatory effect in two acute septic shock models in mice by inhibiting the NF-κB pathway, modulating cytokine production and ERK phosphorylation [39]. Qin et al. (2016) investigated the effects of Micheliolide, a sesquiterpene lactone isolated from *Michelia compressa* with anti-inflammatory effects in the acute peritonitis mouse model and found that this plant terpenoid inhibits lipopolysaccharide-induced inflammatory response via NF-κB and PI3K/Akt pathways [40]. More recently, the anti-inflammatory effect of Xuebijing, a Chinese herbal medicine, in murine CLP model has been reported [41]. Accordingly, it seems that natural products may play a significant role in ongoing and future studies on discovering new treatments for peritonitis and septic shock.

We did not use laboratory diagnostics or organ dysfunction tests to confirm peritonitis, which could be a limitation of this study. It is now accepted that polymorphonuclear leukocytes in the peritoneal fluid are the cornerstone of diagnosing peritonitis, while the role of other biochemical tests is quite controversial [42]. However, we used a standard animal model for peritonitis and subsequent septic shock. In addition, the cytokines measured in the control group convinced us that peritonitis and septic shock had occurred in the rats. Clinical manifestations also demonstrated this condition.

In summary, our results demonstrate that troxerutin could increase patient survival in rats developing peritonitis and septic shock by reducing pro-inflammatory cytokines including IL-1 and TNF-α. Further studies are needed to find out whether troxerutin also prevents the synthesis of other anti-inflammatory cy-

Materials & Methods

Animals

this research was approved by the Regional Research Ethics Committee of the University of Tabriz (approval ID: IR.TABRIZU.REC.1398.008). The study complies with the Declaration of Helsinki (DoH). Twenty-four adult (2 months of old) male Sprague-Dawley rats were used in this study. The animals were given separate cages. The environment had a 12-hour light/dark cycle and controlled temperature and humidity conditions. The rats were allowed to stay in their new place for a week to acclimate, with free access to the semi-synthetic pellets and tap water. Individual rats were randomly divided into three equal groups (n = 8) based on online random number generator (available at:https://www.graphpad.com/quickcalcs/randomN1.cfm) including sham (S), control (C), and treatment (T).

Experimental sepsis and treatment

General anesthesia was induced by intraperitoneal injection of 10% ketamine (100 mg/kg, Alfasan, Woerden, Netherlands) and 2% xylazine (20 mg/kg, Alfasan, Woerden, Netherlands) in all rats, and their ventral abdomen was shaved from the xiphoid process of the sternum to the pubis. Celiotomy was performed through routine midline skin and linea alba incisions. The cecum of group S was just exteriorized, manipulated, and returned to the peritoneal cavity. In contrast, cecal ligation and perforation (CLP) was performed in groups C and T. For this purpose, the cecum was identified and exteriorized from the peritoneal cavity. Next, a simple ligature was placed just below the ileocecal valve using 3-0 silk suture material (SUPA, Tehran, Iran). The procedure was completed by piercing the cecum twice with an 18 G needle [43,44] (Figure 3a). Slight pressure was applied to the perforated cecum to ensure leakage of its contents (Figure 3b). The cecum was returned into the peritoneal cavity and the abdominal wall and skin were closed in two layers.

Troxerutin (Merck, Darmstadt, Germany) was dissolved in sterile normal saline and injected subcutaneously into the animals of group T twice daily at a dose of 130 mg/kg [20] for 3 days or until the animals died. At the same time, the rats in groups S and C received the same volume of normal saline solution. The animals were closely monitored for their general status and all symptoms related to peritonitis and septic shock (including loss of appetite and lethargy) were noted [45]. Because rats died on different days after surgery, mean survival days were also calculated in addition to the ultimate survival rate. The longer the mean survival days, the longer the animals survive in the three days of the study. On the other hand, lower survival days indicate that the rats have died earlier in that period. However, the survival rate indicates the percentage of animals that were still alive after 3 days of the experiment, without reflecting when the lost ones have died. Surviving rats were generally anesthetized and weighed 3 days after CLP. They were euthanized under deep general anesthesia after taking approximately 1.5 ml of blood samples from their heart and subjected to a subsequent cervical dislocation. Blood samples were also taken from dying rats on day 2 immediately after death.

Estimation of cytokine levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were collected from individual rats in Eppendorf tubes and allowed to clot by leaving them undisturbed at room temperature for about 30-60 minutes. The clot was removed by centrifuging at 3200 rpm for 10 minutes. The resulting supernatant serum was kept in a refrigerator at -80 °C for future ELISA assay for pro-inflammatory cytokines IL-1 and TNF-α. The Sandwich ELISA was carried out according to the manufacturer's instructions (Bender Med Systems GmbH, Vienna, Austria). Briefly, the capture antibodies specific for IL-1 and TNF-α of the rat were coated on 96-well plates. The plates were incubated overnight at 4 °C. The day after, the plates were washed 5 times with the buffer solution (0.01 M PBS, pH = 7.0 and 0.05% Tween 20). They were blocked for one hour with assay diluent of the kit and washed again 5 times. After the samples and the respective standards had been poured into the wells, the plates were incu-

bated for two hours at room temperature. In the next step, the antibodies were added. Then, the detection antibodies were added for one hour. The wells were washed again. Avidin-HRP was added for 30 minutes and washed. Adding TBM substrate and 15 minutes of incubation developed the color. Finally, the reaction was stopped with 2N sulfuric acid and the amount of absorbance was determined at 540 nm using a microplate reader (Hiperion, Model: MPR4+, Medizintechnik GmbH & Co. KG, Roedermark, Germany). A standard graph was used to calculate the relative levels of cytokines in the samples. The examiner was blind about the grouping of the samples.

Statistical analysis

The data were analyzed with the Minitab statistical software (version 16.2.0, Minitab Inc, State College, PA, USA). The examination of normal distribution was accomplished with the Kolmogorov-Smirnov test and the assumption of equal variance by Levene's test. Cytokines data were analyzed with one-way analysis of variance (ANOVA) followed by *Tukey's* post hoc test. Survival was analyzed with the *Chi*-Square method using the survival analysis tool of the software. Results are presented as percentage (%) for survival rate and mean ± standard error of the mean (SEM) for survival days and ELISA tests. The *p*-value less than 0.05 was considered statistically significant.

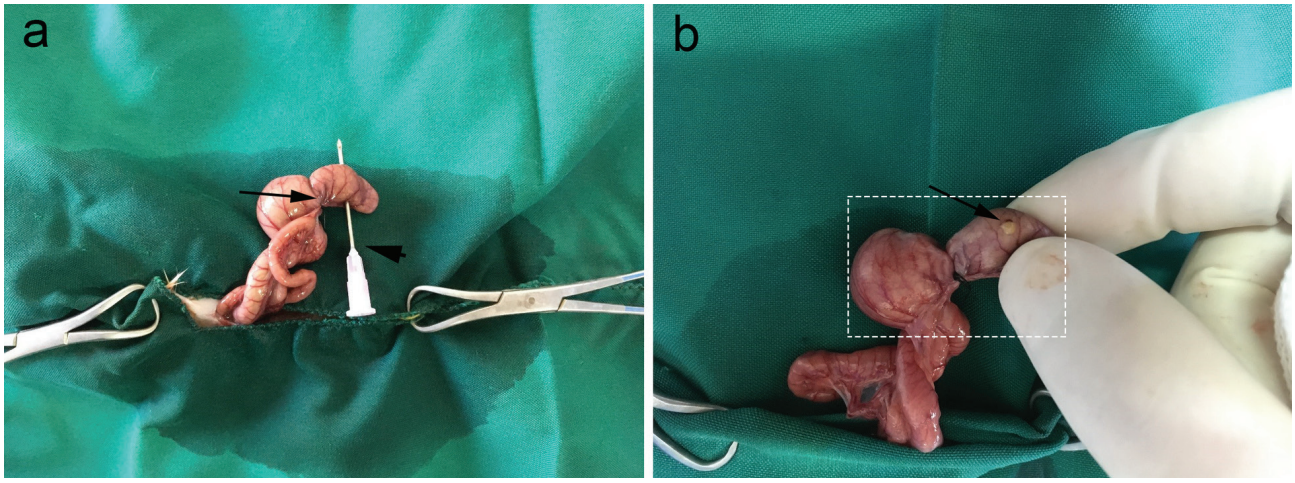

Figure 3.
a. Cecal ligation and perforation (CLP) in rats of control and treatment groups. A simple ligature is placed just below the ileocecal valve (arrow) using 3-0 silk suture material after exteriorizing the cecum from the peritoneal cavity and the cecum is perforated twice by an 18 G needle (arrowhead).
b: Slight pressure is applied to the perforated cecum of the rat to ensure leakage of its contents (arrow) after cecal ligation and perforation. The rectangle shows the cecum.

Authors' Contributions

S.K.D. and Y.J.K. conceived and planned the experiments. S.K.D. and A.S. carried out the experiments. S.K.D. and A.S. planned and carried out the simulations. Y.J.K and A.S. contributed to sample preparation. S.K.D. and Y.J.K. contributed to the interpretation of the results. S.K.D. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript

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Competing Interests

The authors declare that there is no conflict of interest.

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