The protective effects of Anakinra in a neonatal rat model of necrotizing enterocolitis

Abstract

Background: Necrotizing enterocolitis (NEC) is a commonly seen life-threatening condition in newborns characterized by ischemic necrosis. This study aimed to investigate anakinra's effects, an interleukin-1 receptor antagonist, on oxidative stress, inflammation, and tissue necrosis in an NEC rat model.

Materials and methods: Forty Wistar albino pups were divided into four groups randomly as follows; group 1, control group; group 2, anakinra-treated control group; group 3, NEC group, group 4, NEC and anakinra treatment group. The rats were given hyperosmolar formula feeding, and they were exposed to hypoxia after cold stress at +4° C and oxygen in order to create the NEC model. On the fourth day of the experiment, the pups were decapitated, and the intestinal tissues were resected for biochemical and histopathologic examination.

Results: Microscopic injury scores and apoptotic indexes were higher in group 3 than the control group ($p<0.001$, $p=0.002$, respectively), and there was a significant decrease after anakinra. Interleukin 1β and caspase-3 levels increased with NEC and decreased significantly after administration of anakinra ($p=0.006$, $p=0.004$, respectively). Malondialdehyde and glutathione peroxidase levels also increased compared with the control group ($p=0.019$, $p=0.002$, respectively).

Conclusion: In this experimental study, we found that anakinra had anti-inflammatory and antioxidant effects and was protective against intestinal injury and apoptosis.

Keywords: Anakinra, apoptosis, caspase-3, interleukin 1β, necrotizing enterocolitis, rat

1. Introduction

Necrotizing enterocolitis (NEC) is a severe condition with fatal risks, especially in preterm infants. The progressive development of neonatal intensive care units has increased the survival rates of premature infants but has also increased the risk of encountering this disease [1]. The etiology consists of various factors such as hypoxia, intestinal ischemia, prematurity, enteral nutrition, and bacterial colonization, which develops based on intense inflammation and necrosis [2]. Ischemia-reperfusion injury in the intestines has a pivotal role. There are some inflammatory mediators released at the beginning of the mucosal damage and tissue ischemia such as interleukin (IL) 1-β, IL-6, IL-12, IL-18, tumor necrosis factor (TNF)-α, platelet-activating factor, and leukotrienes [3-5]. Ischemia and necrosis occur in the intestinal wall through the numerous reactive oxygen-derived free radicals released, which causes tissue damage by lipid peroxidation in both the cellular and mitochondrial membranes. Despite this increased oxidative stress, the weak antioxidant system of preterms, which is still immature, cannot adequately eliminate these increased reactive radicals, so the intestines are exposed to oxidant-induced damage [5, 6]. As medical treatments are generally inadequate, and preventive interventions are less effective on the development of NEC, the search for new treatments continues [7].

IL-1β is one of the inflammatory cytokines derived from tissue analysis of infants with inflammatory bowel disease and NEC [8]. It was also shown in NEC experiments on rats that this cytokine level increased 6-fold higher than in controls [9]. Counter regulatory cytokines such as IL-1 receptor antagonist (IL-1ra), TNF-α, and IL-10 have been shown to release more slowly than in acute inflammatory cytokines in intestinal inflammatory conditions [8]. The increase in IL-1ra in humans and animal models is associated with improved outcomes [10]. Anakinra, which can block IL-1α and IL-1β activity, is a

recombinant IL-1 receptor antagonist [11]. According to the known features of anakinra, we aimed to examine the effects of anakinra in preventing or reducing the inflammation or necrosis in neonatal rats' intestines with an induced NEC model. Additionally, the effect of anakinra on oxidative status is examined.

2. Materials and Methods

2.1. Animals and experimental design

This experimental study was approved by the Experimental Animal Ethics Committee of Kahramanmaraş Sütçü İmam University (Protocol No: 18). Forty simultaneously born pups of Wistar albino rats fed freely in different cages were randomly divided into four groups. Group 1 (n = 10), the control group, were left with their mothers and allowed to breastfeed freely. Group 2 (n = 10) received breast milk with and additional anakinra treatment at a dose of 1 mg/kg of anakinra per day intraperitoneally in order to reveal the effects of anakinra on healthy intestine independent of NEC. [12]. Group 3 (n = 10) pups underwent the NEC procedure and were given 0.2 ml/day intraperitoneal saline. Group 4 (n = 10) pups were given anakinra daily following the NEC protocol.

2.2. NEC procedure

Inconsistent with the Özdemir R et al. [6] study, Groups 3 and 4, who underwent NEC procedure, were separated from the mother's side due to breast milk's protective effect and kept in a humidified incubator at 37 °C. These groups were fed with 0.2 ml of special rodent formulation (15 g Similac 60/40 [Ross Pediatrics, Columbus, OH]) and 75 ml of puppy canine milk (Beaphar-Bogena BV, Hedel, The Netherlands) three times a day orally with a 24 G catheter. NEC model was created according to the description of Güven et al. [13]. In order to accomplish the defined NEC model, Group 3

and 4 were placed in an airtight plexiglass cage and were exposed to 100% CO_2 perfusion for 10 minutes. At the end of the process, the rats were observed to be cyanotic and had dyspnea. Animals were exposed to +4℃ cold and 97% O_2 following hypoxia in order to induce NEC [14]. These procedures were repeated twice a day for three days.

2.3. Anakinra application and tissue preparation

Pups in groups 2 and 4 received 1 mg/kg of anakinra (Kineret; Sobi, Stockholm, Sweden) once a day for four days via intraperitoneal injection starting from the postnatal first day.

On the 5th day of the experimental study, all animals were sacrificed (deep anesthesia provided by 200 mg/kg pentobarbital sodium, intraperitoneally). The abdominal cavity was opened surgically, and the presence of discoloration, edema, tissue integrity, hemorrhage, ileal distension, perforation, and necrosis, which are important intestinal NEC findings, were examined. For histopathological and biochemical examinations, 3 cm of the terminal ileum, including the cecum, was excised. All tissue samples were washed with cold saline solution. Half of the distal intestine was fixed with formalin for histopathological examination and apoptosis scoring. The intestine's remaining half was separated for biochemical analysis and frozen in liquid nitrogen and stored at -70 ° C.

2.4. Apoptosis evaluation

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end-labeling staining (TUNEL) method

After 4-6–µm thickness sections from the paraffin blocks were taken into polylysine slides, cells leading to apoptosis were determined using the ApopTagPlus

Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) following the manufacturer's instructions. The sections were deparaffinized with xylene, passed through graded alcohol series, and washed with phosphate-buffered saline (PBS). The tissues were incubated with 0.05% proteinase K for 15 minutes and then incubated with 3% hydrogen peroxide for 10 minutes to prevent endogenous peroxidase activity. After this procedure, the tissues washed out with PBS and were incubated with equilibration buffer for 6 minutes and incubated for 60 minutes with a working solution at 37°C in a moist environment. The tissues were then kept in Stop / Wash Buffer for 10 minutes. The tissues were then treated with anti-digoxigenin-peroxidase for 40 minutes, instilled with diaminobenzidine (DAB) substrate, and apoptotic cells were visualized. The tissues were contrasted with Harris hematoxylin, covered with Entellan, and examined and photographed via a light microscope. Averagely, 500 normal and apoptotic cells were counted in sections at ×10 magnifications. The proportion of apoptotic cells to the total (normal-apoptotic) number of cells determined the apoptotic index (AI).

2.5. Histopathologic examination

An experienced pathologist who was blinded to the experiment performed the tissue histopathologic examinations. The bowel was fixed in a 10% formalin solution for 24 hours after dissection. Then, samples were placed in cassettes and processes on a Leica ASP 300 and embedded in paraffin wax. Four-micron-thick sections were obtained using a Leica RM 2145 microtome. Routine hematoxylin-eosin staining was performed to the sections, and morphologic differences between the four groups of small intestine tissue were evaluated under a light microscope. Hemorrhage, submucosal edema, ulceration, and focal necrosis were examined and scored between 0 and 3 as normal, mild, moderate, and severe. All tissues were graded according to their histopathological features as follows:

grade 0, normal; grade 1 (mild), villous nucleus separation only; grade 2 (moderate), submucosal edema and epithelial shedding with villous nucleus separation; Grade 3 (severe), loss of villous pattern, full-thickness necrosis and perforation and peeling of the epithelium [15]. Grade 2 and higher damage was accepted as NEC.

2.6. Biochemical analysis

The saline washed up tissue samples were stored at -20°C until biochemical analysis. Before analysis, tissues were homogenized at 14,000 rpm for 30 minutes with 50 mM phosphate buffer, pH 7.4. The supernatants were separated by 10,000 *g* centrifuging for 30 minutes at + 4°C.

Intestinal IL1β levels of intestinal tissues were determined by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey), an automatic ELISA microplate reader (Thermo Scientific, Finland), and a computer program (Skanlt for Multiscan FC 2.5.1). The sensitivity was 15.18 pg/ml, and the detection range was 30-9000 pg/mL. Intra-assay coefficient of variation (CV) was < 8% and inter-assay CV was <10%. The results were observed as pg/mL.

Ohkawa method was used for determining tissue lipid peroxidation [total malondialdehyde (MDA)] concentration with slight modifications [16]. The reaction mixture contained 0.1 mL of supernatant, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, and 1.5 mL of a 0.8% aqueous thiobarbituric acid solution. The mixture pH was adjusted to 3.5. The volume was set up to 4 ml with distilled water, and a mixture of 5 ml n-butanol and pyridine (15: 1, v / v) was added. The mixture was shaken and centrifuged at 4000 rpm for 10 minutes. The absorbance of the organic layer was measured at 532 nm.

Glutathione peroxidase (GSH-Px) activity measurements were performed with the Beutler method [17]. GSH-Px is the catalyzer in the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) via H_2O_2. In the presence of H_2O_2 t-butyl hydroperoxide, GSSG formed by GSH-Px is reduced to GSH with the help of glutathione reductase and NADPH. The activity of GSH-Px activity can be determined during the oxidation of NADPH to NADP by reading the absorbance difference at 340 nm spectrophotometrically.

Lowry method was used to determine the tissue protein levels [18].

Rat cysteinyl aspartate-specific proteinases 3 (caspase 3) levels were determined using ELISA with a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey), an automatic ELISA microplate reader (Thermo Scientific, Finland), and a computer program (Skanlt for Multiscan FC 2.5.1). The sensitivity was 0.022 g/mL, and the detection range was 0.05-10 ng/mL. The intra-assay CV was <8%. The inter-assay CV was <10%. The results were determined as ng/mL.

2.6. Statistical analysis

Power analysis was used to calculate the sample size of the study. α: 0.05 for the first type error level, β: 0.10 for the second type error level, 0.90 for the MDA variable in the study which is taken as reference Ozdemir et al [1], the mean values of the groups for Group1: 0.36, Group 2: 0.68 and Group 3: 0.40, and the number of rats required for each group to be included in the study with an effect size of 0.82 was determined as n: 8. Considering the possible loss of rats during the study, it was planned to include 2 more rats in each group and n: 10 rats in each group, and n: 40 rats in total were included in the study.

The data were evaluated using the R project 3.3.2 and IBM SPSS version 22 program. Shapiro-Wilk test was used for determining the suitability of the variables to normal distribution. Group comparisons of abnormally distributed variables were performed using the Kruskal-Wallis H test. Binary comparisons (post-hoc) between the groups were performed using the Dunn-Sidak test and the Bonferroni test. The findings are expressed as median (min-max). A p level of <0.05 was set as the statistical significance level.

3. Results

Thirty-five of the forty pups survived at the end of the experiment. During the study period, one pup from group 2, two pups from group 3, and two pups from group 4 died. The median weight of all groups at birth was similar, but weight gains were different among the groups at the end of the study. All pups gained weight at the end of the study; however, the NEC group's increase was less compared to the other groups (Table 1).

3.1.Histopathologic examination

Histopathologically, groups 1 and 2 had normal intestinal structures (Figure 1a, 1b). Group 3 had the macroscopic findings of NEC, such as intestinal discoloration, edema, fragility, and tissue integrity weakness (Figure 1c). Less severe findings such as edema, necrosis, and minimal tissue integrity weakness were observed in group 4 (Figure 1d), but control groups had no evidence of lesions (groups 1 and 2). The microscopic injury scores were significantly different between the groups, and the median microscopic injury scores were higher in group 3 compared to the other groups. Group 3 and group 4 had significantly different microscopic injury scores ($p<0.001$). In terms of hemorrhage, ulceration, and focal necrosis, the NEC protocol-applied group (group 3) and the group treated with anakinra (group 4) had significant differences ($p=0.040$, $p<0.001$, and

p<0.001, respectively) (Table 2). None of the pups in groups 1 and 2 had microscopic injuries. Histological findings of groups are shown in Figure 1.

As a result of the TUNEL staining examination, which was performed for the determination of apoptotic cells under light microscopy, TUNEL positivity was similar in the control groups, group 1 (Figure 2a) and group 2 (Figure 2b) (p=0.699). Compared with the control group, TUNEL positivity was found to be statistically significantly increased in group 3 (Figure 2c) (p=0.002). Compared with group 3, TUNEL positivity decreased significantly in group 4 (Figure 2d) (p=0.002). The apoptotic index (%) is shown in Table 3.

3.2.Biochemical analysis

The levels of MDA, GSH-Px, IL-1β, and caspase-3 activities in intestinal tissues of the groups are presented in Table 1.

The differences between the groups regarding MDA, tissue GSH-Px, and IL1β values were found significant. In terms of MDA values, the difference between the control group (group 1) and group 3 was significant (p=0.016). However, although the MDA value decreased in group 4 compared to group 3, no statistical difference was found (p=0.799).

Similarly, compared with group 3, GSH-Px levels decreased in group 4, but the difference was not significant (p=0.066).

The median IL-1β levels were low in both control groups (group 1, 2), but with the NEC procedure, the levels were increased in group 3 (p<0.05), and a significant decrease was present the anakinra-treated group (group 4) (p=0.006).

The caspase3 levels were increased in the NEC group compared with the control groups, and a significant decrease was seen in group 4, which was treated with anakinra ($p=0.004$).

4. Discussion

In this study, we examined the effect of anakinra in an experimental NEC model in newborn rats. With this experiment, we have shown that anakinra ameliorated intestinal damage and reduced intestinal IL-1β levels. In addition to these effects, we have found that anakinra reduced apoptosis in the rats' intestinal epithelial cells. To the best of our knowledge, this is the first study examining the effects of anakinra in an experimental NEC model.

In NEC pathogenesis, the inflammatory response is impaired, and an imbalance is present between inflammatory and proinflammatory cytokines. Some inflammatory mediators such as Toll-like receptor (TLR) 4, nuclear factor-κB, and TNF, platelet-activating factor (PAF), IL-18, and interferon-gamma, IL-6, IL-8, and IL-1β, have been associated with NEC [9]. During ischemia, IL-1α is released from cells leading to necrosis. This is the first step of inflammation, and ischemic inflammation continues with the accumulation of neutrophils in the damaged cell region. IL-1 regulates the destructive effects of neutrophils and the increase in tissue macrophages and blood monocytes, which will be effective in the subsequent process in the release of IL-1β. IL-1 is the leading cytokine in both the local and systemic inflammation. It also has a major role in the production of other pro-inflammatory mediators, tissue damage, and fever [11].

Similar to our study, studies showed that IL-1 levels were increased in NEC. With the blockade of this cytokine, the organism is protected from the damage of the excessive inflammatory response [8, 19]. Anakinra, a recombinant IL-1 receptor antagonist, blocks the activity of both IL-1α and IL-1β. Özçiçek et al.[20] demonstrated that anakinra treatment reduced IL-1β levels in methotrexate-induced intestinal mucositis in rats. Nayki et al. [21] reported that anakinra suppressed IL-1β and histopathologic damage in ovarian ischemia-reperfusion injury in a rat model. Similarly, there was a significant decrease in IL-1β levels in the NEC group treated with anakinra in our study.

When NEC's pathogenesis is examined, it is seen that hypoxic-ischemic necrosis and the events triggered by it initiate NEC. The mucosal injury is mediated by reactive oxygen radicals, which causes tissue damage by lipid peroxidation of cellular membranes. MDA, which is an interval metabolite of lipid peroxidation, is a sensitive tissue injury indicator. In most of the studies, MDA levels have been shown to be elevated under ischemia-reperfusion injury or tissue necrosis [21,22]. Similarly, studies on NEC noted increased MDA levels [1,5,6]. In accordance with the literature, we concluded that the MDA levels in the NEC group increased significantly compared to the control group, and there was a decrease compared with the group that was given anakinra.

Insufficient antioxidant systems in preterm infants also make elimination of these free oxygen radicals difficult and increase the tendency to NEC [12,23]. GSH-Px is one of the antioxidant enzymes. In most experimental NEC studies, it has been observed that there are decreases in antioxidant enzyme levels in the groups given the NEC protocol [13,24,25]. Conversely, the GSH-Px levels of the NEC group increased compared with the control groups and the anakinra-treated group in our study. Similarly, Erdener et al. [23], Yurttutan et al.[5], and Özdemir et al. [6] found that GSH-Px levels increased in NEC

groups. This situation can be interpreted as an indicator of increased antioxidant demand caused by high inflammation and NEC's oxidative stress. When anakinra is given, it may be thought that this anti-oxidative requirement may have decreased rather than suppressed antioxidant enzymes because we found that increased GSH-Px levels did not suppress antioxidant levels in the healthy control group given anakinra.

The immature of anti-inflammatory responses of preterms despite increased oxidant stress increases the tendency to intestinal apoptosis, which is one of the initiating and important steps in NEC pathogenesis [2,7,26]. In histopathological examinations of the intestines of newborn babies with NEC, intense intestinal apoptosis was observed. It is assumed that the earliest histopathologic changes before the advanced stages of NEC are the apoptosis of epithelial cells [27,28]. IL-1β is known to accelerate inflammation as well as apoptosis [21,29]. Caspases, also induced by cytokines, play an important role in apoptosis. Among the caspases, caspase-3 is the one that triggers cell death and is important in the early stages of cell viability loss and the formation of apoptotic bodies [30]. In the present study, we revealed that anakinra reduced both TUNEL staining and caspase-3 activities, and therefore apoptosis in intestinal epithelial cells of pups subjected to NEC.

Additionally, anakinra improved the intestinal injury scores of pups given the NEC procedure. Nayki et al. [21] also showed that anakinra ameliorated oxidative-induced apoptosis in the ovarian tissue of rats with ischemia-reperfusion injury. Another ischemia-reperfusion injury model conducted by Hirik et al. [22] on rats' testicular tissue demonstrated that anakinra recovered the histopathologic injury. Therefore, anakinra may have a protective effect on the intestinal epithelium against apoptosis and may be recommended as a protective option for preterm infants at risk for NEC.

In our study, we also gave anakinra to a healthy control group. Our aim in doing this was to see the effects of the drug on healthy individuals. Histopathologically, there was no difference in this group compared with the healthy control group given saline. However, MDA levels were found to increase significantly. This can be considered as an indication that suppression of a healthy immune response and that has not been activated for any reason can actually harm the organism. Accordingly, one should be very careful when giving anakinra; unnecessary use should be avoided.

Our study has some limitations and strengths. One of our limitations is the lack of anakinra administration in different doses, thus the consequences of which could not be evaluated. Not evaluating IL-1 levels can also be considered as a limitation. Another limitation is the number of subjects enrolled in this study. On the other hand, one of our study's strengths is that anakinra was also given to healthy controls, in whom drug effects were also shown.

5. Conclusion

As a result, we showed that anakinra had positive effects on the experimental NEC model and reduced cell death and tissue damage. Also, we demonstrated that anakinra protected the organism from the harmful effects caused by inflammation by creating positive effects on the inflammatory response.

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Table 1. Comparison of the biochemical findings of the groups

	Group1 (contol, n=10)	**Group 2** (control+ anakinra, n=9)	**Group 3** (NEC, n=8)	**Group 4** (NEC+anakinra, n=8)	***p***
Weight at start, g	5.00(4.80-5.30)	5.20(4.70-5.40)	4.90(4.70-5.40)	5.05(4.80-5.30)	0.934
Weight at the end, g	8.95(8.40-9.50)[c,d]	8.80(8.40-9.30)[c.d]	6.00(5.80-6.30)[a.b.d]	6.90(6.00-7.00)[a.b.c]	**<0.001***
MDA, nmol/mg protein	0.91(0.62-1.52)[b,c]	5.59(2.80-15.53)[a]	5.48(0.74-12.39)[a]	2.97(1.00-9.49)	**0.006***
GSH-Px, U/mg protein	1,60(0.66-4.35)[c]	10.63(5.98-43.67)	25.97(5.99-95.60)[a]	9.67(5.18-23.16)	**0.003***
IL1β, pg/µg protein	3.01(2.12-7.77)[c]	11.32(5.57-55.30)[c]	49.64(8.51-87.71)[a,b,d]	14.58(7.38-30.48)[c]	**<0.001***
CASPASE-3, ng/mg protein	13.11(3.71-17.79)[c]	14.42(5.38-39.49)[c]	41.55(14.03-113.58)[a,b,d]	17.96(7.32-24.31)[c]	**<0.001***

Data are given as median (min.-max.), Kruskal Wallis H test;Post-hoc:DunnSidak test;bonferroni test α:0.05,

* The difference between the groups is significant, ; [a] the difference with Group 1 is significant; [b] the difference with group 2 is significant; [c] the difference with group 3 is significant; [d] difference with group 4 is significant.

CASPASE 3; cysteinyl aspartate specific proteinases 3, GSH-Px; glutathione peroxidase, IL1-β; interleukin 1β, MDA; malondialdehyte

Table 2. Comparison of the histopathological findings of the groups

	Group1 (control, n=10)	**Group 2** (control+ anakinra, n=9)	**Group 3** (NEC, n=8)	**Group 4** (NEC+anakinra, n=8)	***p***
Microscopic injury score	0.00(0.00-0.00)[c]	0.00(0.00-2.00)[c]	2.00(1.00-3.00)[a,b,d]	0.00(0.00-1.00)[c]	**<0.001**
Hemorrhage	0.00(0.00-0.00)[c,d]	0.00(0.00-0.00)	0.00(0.00-1.00)[a]	0.00(0.00-2.00)[a]	**0.040***
Submucosal edema	0.00(0.00-0.00)	0.00(0.00-1.00)	0.50(0.00-3.00)	0.00(0.00-1.00)	0.054
Ulceration	0.00(0.00-0.00)[c]	0.00(0.00-0.00)[c]	2.00(1.00-2.00)[a,b,d]	0.00(0.00-1.00)[c]	**<0.001***
Focal necrosis	0.00(0.00-0.00)[c]	0.00(0.00-1.00)[c]	2.00(1.00-3.00)[a,b,d]	0.00(0.00-1.00)[c]	**<0.001***
Polymorphonuclear leukocyte	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	1.000
Microorganism	0.00(0.00-0.00)	0.00(0.00-1.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.409

Data are given as median (min.-max.), Kruskal Wallis H test; Post-hoc: Dunn Sidak test; bonferroni test α: 0.05

* The difference between the groups is significant; [a] the difference with Group 1 is significant; [b] the difference with group 2 is significant; [c] the difference with group 3 is significant; [d] difference with group 4 is significant.

Table 3. Apoptotic index of the groups

	Apoptotic index (%) Median (min-max)
Group 1 (n=10)	1.66 (1.00-3.00)
Group 2 (n=9)	1.83 (1.00-3.00)
Group 3 (n=8)	16.00 (12.00-21.00)[a]
Group 4 (n=8)	2.83 (1.00-5.00)[b]

[a] compared to Group 1, [b] compared to group 3, $p < 0.05$. Kruskal Wallis H test; Post-hoc: Dunn Sidak test α:0.05

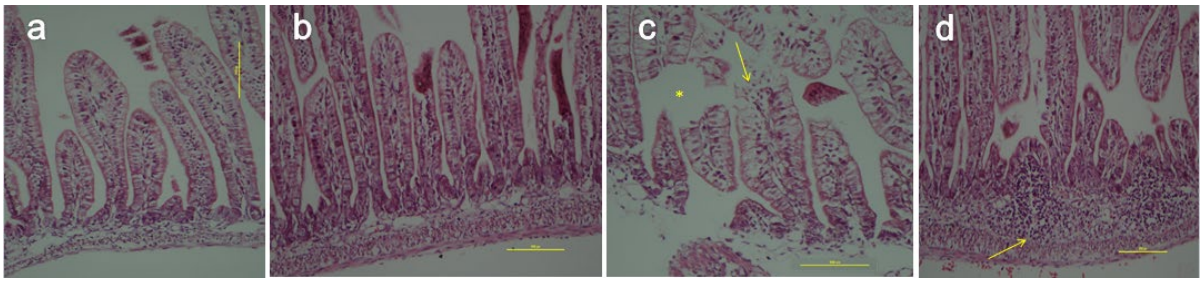

Figure 1. a Representative histopathological evaluation of the terminal ileum from each experimental group. Intestinal architecture of a rat from the control group (Group 1) revealed normal histologic feature of villi. **b** Normal histologic feature of villi of a rat from Group 2 (control+Anakinra). **c** The NEC group (Group 3) displayed severe injury findings consisting of complete loss and necrosis in villi (*arrow)*and diffuse mucosal and submucosal edema (*asterisk*). **d** The NEC+ Anakinra group (Group 4) displayed normal crypt indicating preservation and/or restoration of villus**,** submucosal leukocyte infiltration *(arrow)*. (For all pictures, scale bar represents 500 μm.)

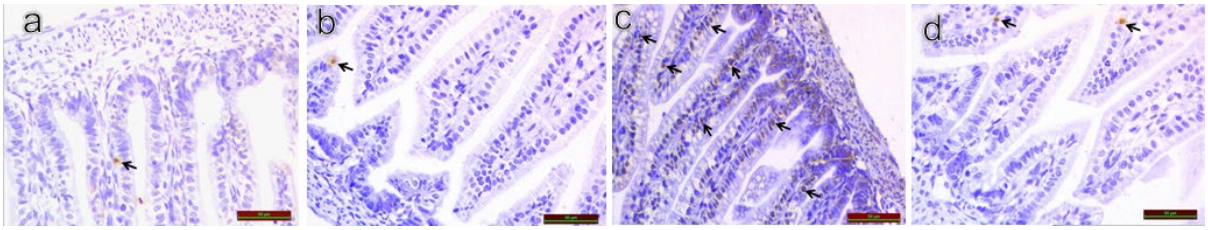

Figure 2. a Representative histopathological TUNEL staining evaluation of the terminal ileum from each experimental group. Normal intestinal architecture of a rat from control group (Group 1) apoptotic nuclei of intestinal cell *(arrow)*. **b** Normal intestinal architecture of a rat from Group 2 (control+Anakinra) apoptotic nuclei of intestinal cell *(arrow)*. **c** Intestinal tissue of the NEC group (Group 3) with increased apoptotic cells *(arrows)*. **d** Notice the improved intestinal tissue of Group 4 (NEC+Anakinra) with reduced apoptotic cells. (For all pictures, scale bar represents 50 μm.)

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