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Protective effects of dexpanthenol in an experimental model of necrotizing enterocolitis☆,☆☆

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ABSTRACT

Background/purpose: In pathogenesis of necrotizing enterocolitis (NEC), both oxidative stress and inflammation are considerable risk factors. The study was designed to evaluate whether administration of dexpanthenol (Dxp) is able to attenuate intestinal injury through the antioxidant and antiinflammatory mechanisms in a neonatal rat model of NEC.

Methods: Forty newborn pups divided into four groups were included in the study: control, control + Dxp, NEC, and NEC + Dxp. NEC was induced by hyperosmolar formula and additionally the pups were exposed to hypoxia/hyperoxia and cold stress. They were sacrificed on postnatal day four, and their intestinal tissues were analyzed biochemically and histopathologically.

Results: Dxp caused a significant decrease in intestinal damage as determined by the histological score, villus height and number of goblet cells in NEC groups ($p < 0.0001$). Tissue malondialdehyde, total oxidant status, and oxidative stress indexes levels were higher in the NEC group than in the control and control + Dxp groups ($p < 0.001$). These values were reduced in the pups treated with Dxp ($p \leq 0.004$). Superoxide dismutase, glutathione peroxidase, and reduced glutathione activities were significantly reduced in the NEC group compared to the others ($p < 0.005$). Treatment with Dxp significantly reduced elevations in tissue homogenate levels of tumor necrosis factor-α and interleukin-1β in the NEC + Dxp group ($p = 0.002$ and $p = 0.01$, respectively).

Conclusions: Dexpanthenol seems to have antiinflammatory and antioxidant properties. Prophylaxis with Dxp has a potential to reduce the severity of intestinal damage in NEC in the animals.

Necrotizing enterocolitis (NEC) remains a major cause of morbidity and mortality among premature infants born at less than 1500 g. Recent data suggest 7% of these infants develop NEC, and 20–30% of them do not survive [1]. Despite many advances in the management of the critically ill neonates, the exact etiology, attempts for the prevention and the best treatment strategy for NEC have been elusive [2].

The pathogenesis of NEC is multifactorial, and was poorly understood. The premium theory in demand is local intestinal inflammation initiated by perinatal stress. Intestinal bacteria and their products adhere to the epithelium, breach the immature and fragile intestinal mucosal barrier, and activate nuclear factor-κB in lamina propria immunocytes, causing them to secrete proinflammatory mediators and cytokines, chemokines, platelet-activating factor, and nitric oxide. Inflammatory mediators including tumor necrosis factor-α (TNF-α), and interleukins (IL-6, IL-8, IL-10, IL-12, and IL-18) were also proposed to have significant roles in the pathogenesis [3]. TNF-α, secreted predominantly by polymorphonuclear neutrophils, is a proinflammatory cytokine to induce apoptosis. The pivotal role of TNF-α in the pathogenesis of NEC has been well documented [4]. Interleukin-1β (IL-1β) is another well-recognized proinflammatory cytokine, and several studies have shown that it is associated with immune activation in NEC [5]. On the other hand, reactive oxygen species (ROS) have also been reported to play an important role in NEC pathogenesis [6,7]. Miller et al. demonstrated that ROS make a substantial contribution to intestinal injury in an experimental model of NEC, and that this injury was eliminated by the addition of superoxide dismutase (SOD) [8]. Recently, we also demonstrated that *all*-trans-retinoic acid, a derivate of vitamin A, and etanercept (a TNF-α inhibitor) therapy reduce the severity of NEC in

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pups via their antiinflammatory and antioxidant properties [9,10]. The use of several antiinflammatory and antioxidants reduces the NEC severity in experimental models that were shown, as well [11,12]. Thus, we have assumed that preventing the generation of free radicals and/or inflammatory mediators, or neutralization of these factors might have a reducing effect on the severity of NEC.

Dexpanthenol (Dxp), an alcoholic analogue of pantothenic acid (PA), is oxidized to PA within tissues, which is known to protect against cell damage produced by ROS. PA that supports cellular antioxidant systems, including glutathione, glutathione peroxidase (GPx), catalase (CAT), SOD, and the other enzymatic reactions that prepare the host to encounter physiopathological conditions mediated by ROS, is well documented. PA and its derivatives increase the level of reduced glutathione, coenzyme-A and adenosine-5′-triphosphate synthesis within the cell [13–15]. All of them play a major role in cellular defense and in the repair systems against oxidative stress and the inflammatory response [16,17].

Given these characteristics of Dxp, using Dxp that might prevent or reduce inflammatory responses is suggested. Therefore, in this histopathological and biochemical study it was aimed to evaluate whether Dxp administration could attenuate intestinal injury, and prevent NEC in a neonatal rat model.

1. Materials and methods

1.1. Animals and experimental design

After the ethics committee approval, the present study was performed at the Animal Laboratory of Inonu University, School of Medicine, according to the Guidelines for the Care and Use of Laboratory Animals of the US National Institutes of Health (Washington, DC).

The pregnant rats were kept in identical cages, and were left to feed with regular laboratory chow and water. Forty newborn pups from Wistar-albino pregnant time-mated rats were divided randomly into four groups in the first day of life. Group 1 (n = 10) was assigned as control which were only nursed by their mothers; Group 2 (n = 10) was also nursed by their mothers, but received Dxp. Group 3 (n = 10) was subjected to NEC procedure, and treated with intraperitoneal physiological saline. Group 4 (n = 10) was also assigned to NEC procedure and treated with Dxp. To prevent from protective effect of mother's milk, Groups 3 and 4 were immediately separated from the mothers, and kept at 37 °C in a humidified incubator. These animals were fed orally using a plastic sheath of 24-gauge polyflon Venocath with 0.2 mL of special rodent formula prepared with 15 g Similac 60/40 (Ross Pediatrics, Columbus, Ohio, USA) and 75 mL of puppy-canine milk replacement (Beaphar BV, Raalte, Netherlands).

1.2. NEC procedure and Dxp application

Hypoxia was accomplished by placing the pups in an airtight plexiglass chamber that was perfused with 100% CO_2 for 10 min. At the end of this period, the animals became cyanotic and began gasping. After the hypoxia procedure, the animals were exposed to +4 °C cold for 5 min, and 97% O_2 for 5 min twice a day to induce NEC. The procedure was applied to the pups for three days [9,11]. The pups were weighed daily.

NEC-induced pups in Group 3 were given physiologic saline (0.2 mL) by intraperitoneal (IP) injection. Those in Groups 2 and 4 were treated with Dxp (Bepanthene ampul®, 500 mg, Bayer Corp., Istanbul, Turkey) and were administered once a day by IP injection at a dose of 500 mg/kg of body weight, starting from birth until postnatal day four. The dosage of Dxp was chosen based on the previous studies which have been reported to cause marked antioxidative effects in rats [18,19].

1.3. Tissue preparation

All rats were sacrificed on the 4th day of experiment. For each rat, the abdomen was opened, and the intestines were inspected for macroscopic evidence of NEC such as intestinal discoloration, edema, fragility, weakness of tissue integrity, ileal distension, intestinal hemorrhage, pneumatosis intestinalis, perforation, and necrosis. Three centimeters of the terminal ileum, including the caecum, was harvested for biochemical and histological evaluations. Tissue specimens were flushed with cold saline solution, and half of the distal intestine was fixed in 10% buffered formalin for histological evaluation. The other half was frozen in liquid nitrogen, and then stored at −80 °C for biochemical examination.

1.4. Biochemical analysis

On the day of analysis, phosphate buffer (pH 7.4) was added to the frozen tissues, which shortly after were homogenized on an ice cube using a homogenizer in order the mechanical process and the heat not to contribute to the oxidation of the tissue. The supernatant was used for the entire assay. The protein content of tissue homogenates was determined as described by Lowry et al. [20] by means of the standard bovine serum albumin. The malondialdehyde (MDA, one of the specific markers and end product of lipid peroxidation [LPO]) concentrations of the homogenates were determined spectrophotometrically [21] by determination of the presence of thiobarbituric acid reactive substances (TBARS). The amount of lipid peroxides was calculated as TBARS of LPO. Another oxidation marker, SOD activity, was assayed using the nitroblue tetrazolium method of Sun et al. [22]. GPx activity was assayed using the method described by Paglia and Valentine [23]. The GSH content in kidney tissue as nonprotein sulfhydryls was analyzed following a previously described method [24]. Tissue total antioxidant status (TAS), total oxidant status (TOS), and oxidative stability index (OSI) markers were assayed as described previously [25] using commercial assay kits (Rel Assay Diagnostics, Gaziantep, Turkey). Intestinal tissue levels of TNF-α and IL-1β were analyzed in duplicate with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China) according to the manufacturer's instructions.

1.5. Histological evaluation

Intestinal tissues were fixed in 10% formalin, and were embedded in paraffin. Sections of tissue were cut at 5 μm, mounted on slides, stained with hematoxylin-eosin (HE) and periodic acid Schiff (PAS). The sections were examined by a Leica DFC 280 light microscope. Intestinal injury was classified as follows: Grade 0, no injury; Grade 1, for swelling of the surface epithelial cell less than 25% of total villous; Grade 2, for swelling of the surface epithelial cell 25–75% of total villous; Grade 3, for swelling of the surface epithelial cell more than 75% and Grade 4, for loss of villi. The median height of the intestinal villi was measured from 100 villi per each of the groups. Goblet cells were counted under 40× objective magnification using Leica Q Win Image Analysis System (Leica Micros Imaging Solution Ltd. Cambridge, UK).

1.6. Statistical analysis

Statistical analysis was carried out using SPSS® for Microsoft Windows. Data are expressed as medians (minimum to maximum). We analyzed differences among groups using the Kruskal-Wallis test. Posthoc comparisons among groups with significant values were evaluated with the Bonferroni-corrected Mann-Whitney U tests. Statistical significance was defined as $p < 0.05$.

2. Results

One rat per NEC and NEC + Dxp groups died throughout the study.

Table 1
Comparison of biochemical evaluations for each group.

Groups	Control (n: 10)	Control + Dxp (n: 10)	NEC (n: 9)	NEC + Dxp (n: 9)
MDA, nmol/g protein	16.5 (13.5–20.1)	17.2 (12.3–18.7)	27.8 (22.8–33.8)[a,b]	18.1 (15.4–21.1)[c]
SOD, U/g protein	1.9 (1.2–2.1)	2.1 (1.3–2.5)	1.1 (0.9–1.5)[a,b]	1.8 (1.2–2.1)[c]
GP_X, U/g protein	218 (156–387)	220 (124–337)	121 (65–204)[a,b]	191 (144–293)[c]
GSH, μmol/g protein	14.6 (11.8–19.1)	19.1 (13.8–25.9)[a]	11 (8.4–15)[a,b]	18.7 (12.8–22.1)[c]
TOS, μmol H_2O_2/L	5.6 (3–7.1)	4.9 (4.2–8.2)	9.3 (6.1–11.5)[a,b]	4.6 (3.4–9.8)[c]
TAC, mmol Trolox Eq/L	0.84 (0.73–0.93)	0.88 (0.74–0.93)	0.88 (0.62–0.94)	0.89 (0.82–0.99)
OSI, arbitrary unit	6.6 (3.5–10.1)	5.7 (4.7–10.8)	11.3 (7.1–15.6)[a,b]	5.6 (3.5–9.9)[c]
TNF-α, μg/g protein	1.3 (1–2.8)	1.8 (1.4–2.1)	2.6 (2–7.2)[a,b]	1.7 (1.1–2.4)[c]
IL-1β, ng/g protein	11.2 (9.2–19.2)	15.1 (10–16.9)	18.9 (14–28.6)[a,b]	13.3 (9.9–23.4)[c]

Dxp, dexpanthenol; NEC, necrotizing enterocolitis; MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; TOS, total oxidant status; TAC, total antioxidant capacity; OSI, oxidative stress indexes; TNF-α, tumor necrosis factor α; IL-1β, interleukins 1β.
[a] Significantly different from the control group,
[b] Significantly different from the control + Dxp group,
[c] Significantly different from the NEC group.

2.1. Biochemical findings

Tissue MDA, TOS, and OSI levels were higher in Group 3 pups than in Groups 1 and 2 ($p < 0.001$), suggesting increased LPO and protein oxidation. These values were reduced in the pups treated with Dxp ($p < 0.0001$, $p = 0.004$, and $p = 0.001$, respectively).

Tissue SOD, GPx, GSH and TAC levels were evaluated as antioxidant enzyme activities. SOD, GPx, and GSH activities were significantly reduced in the NEC group compared to the other groups ($p < 0.005$; NEC vs other groups [for three activities]). On the other hand, GSH level was higher in the control + Dxp group than in the control group ($p = 0.01$). There were no significant differences among four groups according to TAC level.

As inflammatory mediators, TNF-α and IL-1β were analyzed. The median intestinal TNF-α and IL-1β levels were higher in Group 3 pups than in Groups 1 and 2 ($p < 0.001$). Treatment with Dxp significantly reduced elevations in TNF-α and IL-1β in Group 4 pups ($p = 0.002$ and $p = 0.01$, respectively). All biochemical values are summarized in Table 1 and Fig. 1.

2.2. Histological findings

The gross findings of NEC procedure including intestinal edema, discoloration, fragility and weakness of tissue integrity were observed in the NEC group. Less severe findings such as edema, necrosis and minimal weakness of tissue integrity were observed in the control + Dxp group. Intestinal histological appearance and mucosal integrity were observed intact in the control group (Fig. 2-A). Dxp alone treated group was similar to that of the control group (Fig. 2-B). The PAS (+) reaction showed a magenta staining where goblet cells were present among surface epithelial cells of villi. Numerous goblet cells were seen on the surface epithelium of villi in the control and control + Dxp groups (Fig. 2-C,D).

Morphological damage was observed toward from swelling of the epithelial cells (Fig. 3-A) to loss of villi (Fig. 3-B) in the NEC group. The histological score of NEC group was found to be significantly increased compared to the control group ($p < 0.0001$). On the other hand, although intestinal damage was recognized as alleviated in NEC + Dxp group, the lesions did not completely improve. Degenerative alterations such as swelling of the surface epithelial cells in some areas were still present in this group. Median villus height was found to be significantly increased in NEC + Dxp group compared with NEC group ($p < 0.0001$) (Fig. 3-C). Another remarkable finding in the NEC group was reduction in the number of goblet cells. Goblet cells were nearly lost in some areas, too (Fig. 4-A). On the other hand, in NEC + Dxp group, numbers of goblet cells were found to be significantly increased compared with NEC group ($p < 0.0001$) (Fig. 4-B). The results of semiquantitative histological grade, morphometric measure of villi and the number of goblet cells in all groups were shown in Table 2.

3. Discussion

In the present study the effects of Dxp in an experimental model of NEC in newborn rats were evaluated. The study findings showed that

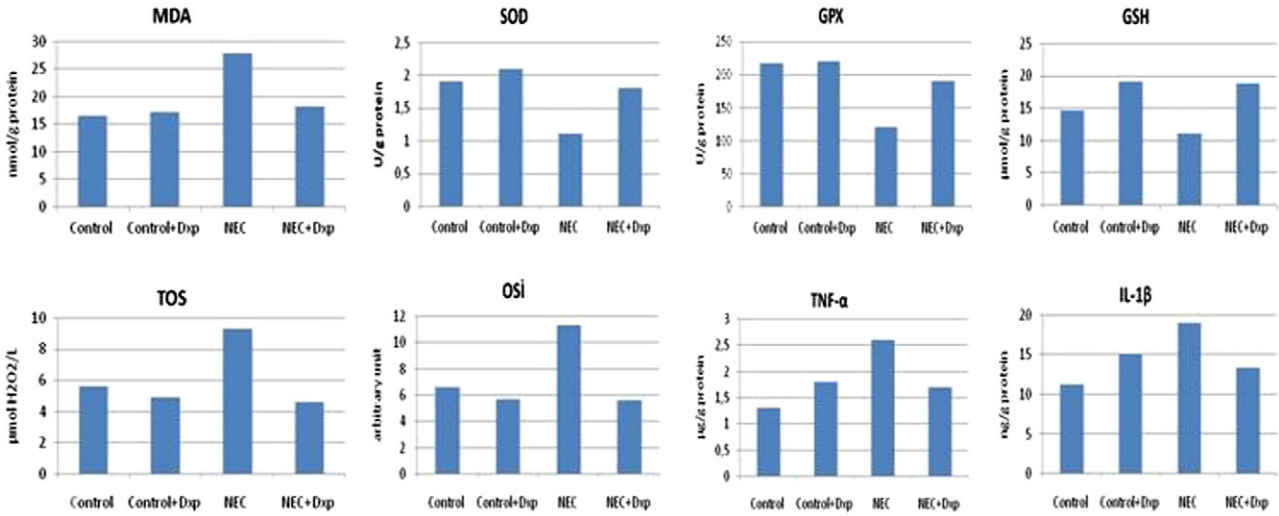

Fig. 1. Comparison of biochemical evaluations for each group.

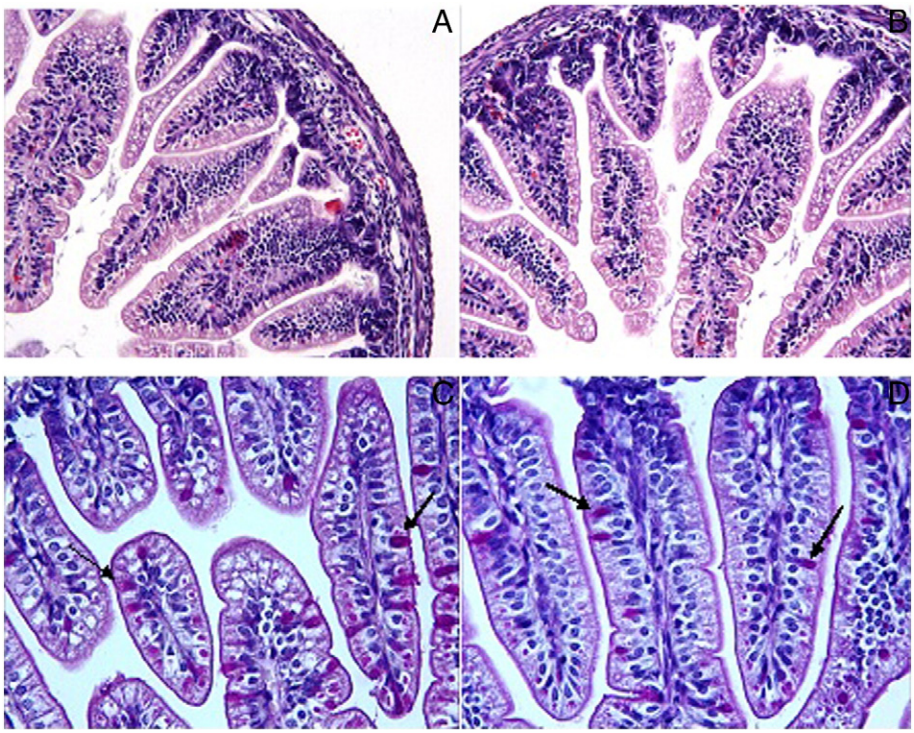

Fig. 2. Control (A) and control + Dxp (B) groups. The villi are long and normal. H-E; ×20. Control (C) and control + Dxp (D) groups. PAS (+) staining goblet cells are seen on the surface of villus epithelium (arrows). PAS; ×40.

Dxp attenuates intestinal injury, and decreases inflammation. We have also shown that Dxp therapy decreases the LPO and diminishes the oxidation, while increasing antioxidant status in this model.

ROS have been linked to the development of NEC in premature infants [26]. ROS cause cellular injury via several mechanisms including peroxidation of membrane lipids as well as oxidation of proteins and DNA, determined by MDA, TOS, and OSI, leading to tissue damage and NEC [27–29]. In the present study, it was demonstrated that significantly higher levels of intestinal MDA, TOS, and OSI showed off in the NEC group than in the control and control + Dxp groups ($p < 0.001$),

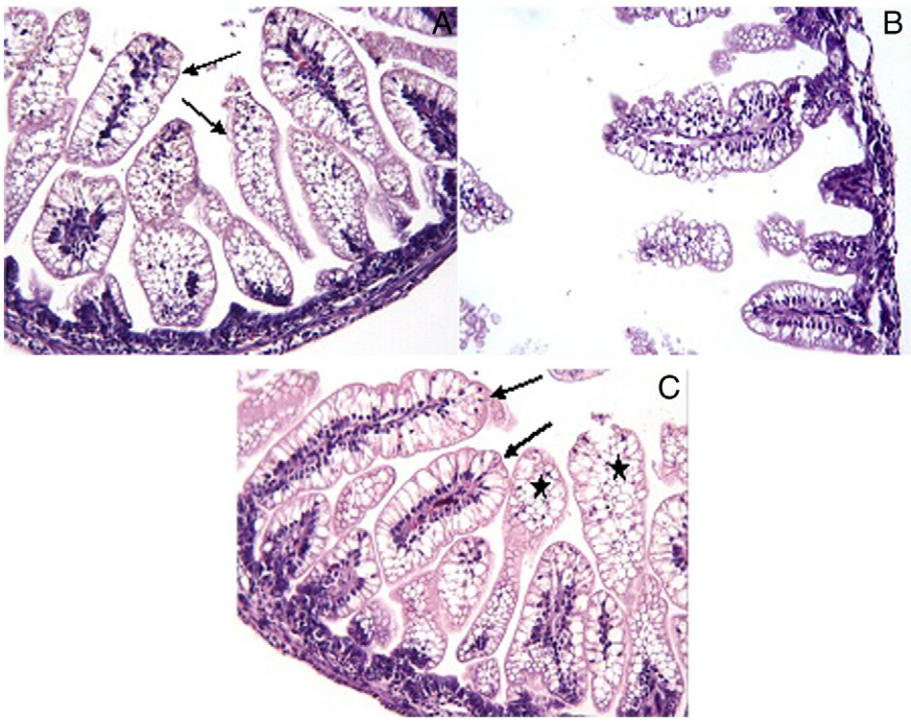

Fig. 3. NEC group. (A) Swelling of the epithelial cell (arrows). (B) Loss of villi. H-E; ×20. (C) The some of villi show nearly normal histological appearance (arrows) but swelled surface epithelium of villi is still evident (asterisk) H-E; ×20.

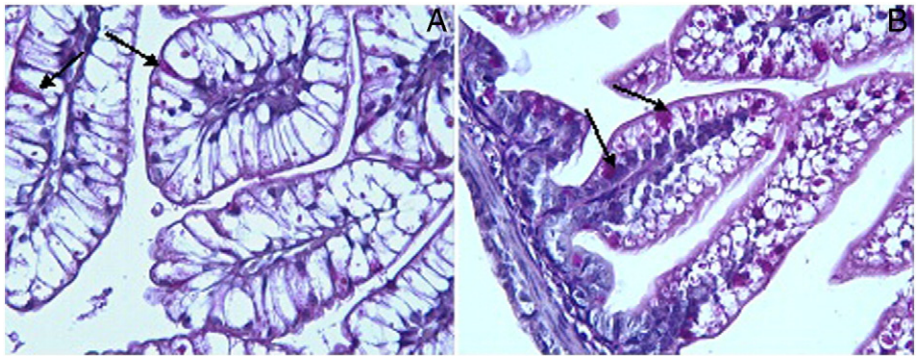

Fig. 4. NEC + Dxp group. (A) Notice the number of goblet cells severely decreased compare to control group (arrows). (B) Swelling of surface epithelium and preservation of goblet cells (arrows). PAS; ×40.

which suggests an increase in LPO. However, we found similar TAC levels in all four groups. Recently, Aydemir et al. [29] demonstrated that higher TOS and OSI levels in preterm neonates with NEC were associated with development of severe NEC. Thus, the suggestion was that antioxidant intervention might be an important part of the medical therapy of NEC in the future.

Dxp is oxidized enzymatically to PA, which is widely distributed in tissues. PA protects against cell damage produced by ROS. It has been demonstrated that PA supports cellular antioxidant systems, including GSH, GPx, CAT, SOD, and the other enzymatic reactions that prepare the host to encounter physiopathological conditions mediated by ROS [13–15,30]. In a viable intestinal tissue, reactive radicals are neutralized by the action of enzymes such as SOD, GPx, and GSH. In the presence of intestinal pathology, loss of intestinal integrity leads to a limited antioxidant enzyme capacity in neonates, which is considered an important predisposing factor for intestinal tissue injury in NEC [31]. In the present study, intestinal SOD, GPx, and GSH activities were significantly reduced in the NEC group ($p < 0.005$). However, rats treated with Dxp had normal or near-normal enzyme activities. On the other hand, GSH level was higher in the control + Dxp group than in the control group ($p = 0.01$). Previously, it was reported that PA provides an increase GSH synthesis in cells [13,30]. We hypothesize that Dxp treatment impeded antioxidant consumption and increased the use of these enzymes via an antioxidant effect and increasing the reduced glutathione in cells. We also found that Dxp treatment reduced the intestinal MDA, TOS, and OSI elevation significantly down to the levels of that of the control. This observation demonstrated the protective role of Dxp in the intestinal mucosa damaged during the NEC procedure by inhibiting ROS-induced LPO and scavenging ROS. The literature displays Dxp as a significant attenuator of serum LPO [30]. Recently, Altintas et al. [18] studied Dxp on I/R induced renal injury in a rat model, and reported beneficial effects related to the reduction in oxidative stress. In another study, it was tested in bleomycin induced pulmonary fibrosis in a rat model, and a significant protection of Dxp from lung fibrosis by reduced oxidative stress was pointed out [19]. In a testicular ischemia-reperfusion study, Etensel et al found it to be useful in reducing LPO, which translates into protection against the sequences of oxidative stress that may amplify the inflammatory response [32].

Cytokines are thought to play a central role in intestinal inflammation and injury during NEC by inducing exaggerated inflammatory responses, leading to significant intestinal injury in premature neonates [3,12]. It is widely accepted that elevated cytokines are principal modulators of the release of ROS during NEC [33]. In the present study, it is shown that TNF-α and IL-1β are increased in the NEC rats, and reduced in the NEC + Dxp rats. We postulate that the inhibitory effect of Dxp on TNF-α and IL-1β levels might have been a consequence of possible modulation of Dxp on antioxidant defenses via ROS inhibition. Furthermore, the antiinflammatory effect of Dxp has been ascribed to the inhibition of the release of TNF-α, IL-1β (like myeloperoxidase) from human polymorphonuclear neutrophils [16]. However, this hypothesis needs further investigation.

The histological score of NEC + Dxp group was found to be significantly reduced compared to the NEC group. On the other hand, median villus height and number of goblet cells in NEC + Dxp group were significantly higher than in NEC group. These findings obviously suggest a possible "Dxp shield" against newborn's NEC.

4. Conclusion

To the best of our knowledge, this is the first study to show the beneficial effects of Dxp treatment in a neonatal rat model of NEC. This study emphasizes the potential of Dxp as an antioxidant and antiinflammatory agent in the prophylaxis of NEC in premature infants. Thus, Dxp may be considered a new and potentially effective therapy in the prevention and treatment of NEC.

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Table 2
The results of semiquantitative histological assessment and morphometric analysis.

Groups	Control (n: 10)	Control + Dxp (n: 10)	NEC (n: 9)	NEC + Dxp (n: 9)
Histological score	0 (0–1)	1 (0–2)	7 (5–9)[a,b]	4 (4–6)[c]
Villus height, μm	237.6 (135–390)	222.5 (111–365)	167.9 (92–292)[d,e]	189.4 (99–329)[f]
Number of the goblet cells	121 (95–135)	111 (98–132)	44.5 (31–65)[d,e]	64.5 (55–81)[f]

[a] Significant increase ($p < 0.0001$), vs. control group.
[b] Significant increase ($p < 0.0001$), vs. control + Dxp group.
[c] Significant decrease ($p < 0.0001$), vs. NEC group.
[d] Significant decrease ($p < 0.0001$), vs. control group.
[e] Significant decrease ($p < 0.0001$), vs. control + Dxp group.
[f] Significant increase ($p < 0.0001$), vs. NEC group.

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