Effects of Boric Acid and 2-Aminoethoxydiphenyl Borate on Necrotizing Enterocolitis

*Selçuk Yazıcı, †Hasan Akşit, ‡Oğuzhan Korkut, §Bahar Sunay, and ||Tanju Çelik

ABSTRACT

Objective: The aim was to study the effects of boric acid (BA) and 2-aminoethoxydiphenyl borate (2-APB) on oxidative stress and inflammation in an experimental necrotizing enterocolitis (NEC) rat model.

Methods: Experimental NEC was induced in 40 newborn Sprague-Dawley rats by asphyxia and hypothermia applied in 3 consecutive days. Rats were subdivided into 4 subgroups as NEC, NEC + BA, NEC + 2-APB, and controls. BA and 2-APB were applied daily before the procedure. Serum total antioxidant status, superoxide dismutase (SOD), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and erythrocyte glutathione (GSH) levels were measured. Pathological changes for NEC in intestinal architecture were evaluated by a grading system.

Results: Pretreatment with BA and 2-APB resulted in a decrease in NEC incidence. In all of the NEC groups, decreased serum levels of GSH and SOD were measured. Boron limited GSH consumption but had no effect on SOD levels. Total antioxidant status levels were not statistically different among groups. In our experimental NEC model, BA, but not 2-APB, prevented the increase of TNF-α. Pretreatment with BA and 2-APB downregulated the activity levels of IL-6 in NEC.

Conclusions: In the experimental NEC model, BA and 2-APB partly prevent NEC formation, modulate the oxidative stress parameters, bring a significant decrease in GSH consumption, and enhance the antioxidant defense mechanism, but have no effect on total antioxidant status. BA inhibits the hypoxia and hypothermia-induced increase in both IL-6 and TNF-a, but 2-APB only in IL-6. Boron may be beneficial in preventing NEC.

Key Words: Boron, inflammation, necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is the most common life-threatening condition of the gastrointestinal tract in newborns (1). NEC is a multifactorial disease, and the risk factors for NEC are controversial. The triad of intestinal ischemia, enteral nutrition, and bacterial translocation has been linked to NEC. In most cases of NEC, no pathogen has been identified. Early and/or aggressive enteral feeding may predispose infants to the development of NEC. The incidence of NEC is low in breast-fed infants. Loss of mucosal integrity because of a variety of factors (ischemia, infection, and inflammation) and the host response to injury (circulatory, immunologic, and inflammatory) are the leading causes of necrosis of the affected area (1). Oxidative stress is implicated in the pathogenesis of NEC, and oxidative damage could be enhanced by a relative deficiency in the oxidant/antioxidant balance (2).

Glutathione (GSH) is the major intracellular antioxidant that protects against free radical–mediated damage (3). As in other tissues of the body, the GSH antioxidant detoxifying system has been proven to be of great importance in the gut barrier response to toxic oxidants (4). At birth, smaller preterm infants have significantly lower levels of GSH than do larger preterm infants, and full-term small-for-gestational-age (SGA) infants have significantly lower levels of GSH than do full-term appropriate-for-gestational-age (AGA) infants (5). The GSH concentrations in the cord blood are higher in preterm infants than in term infants; however, GSH levels fall rapidly in preterm infants within a few days of birth, possibly as a consequence of increased oxidative stress (6).

Superoxide dismutases (SODs) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. High levels of SODs of the immature and newborn intestine are well known to exert a protective effect against intestinal ischemia and reperfusion (I/R) injury (7–9).

Interleukin (IL)-6 and tumor necrosis factor (TNF)-α are produced locally, but have significant effects on the induction and regulation of systemic infection and are well known to be involved in NEC pathogenesis (10–12). In particular, IL-6 is a more reliable indicator of NEC than TNF-α (13).

Mucins are produced and secreted by epithelial goblet cells as a component of the immune system and possess a barrier function for bacterial invasion (14). Alternations in goblet cell morphology and mucin production are a component of NEC pathogenesis (15). TNF-α causes a loss of goblet cells only in immature mice, which may contribute to NEC formation, and induces Muc2 and Muc3 mRNA upregulation only in the mature ileum, which may be protective (14).

Boron is involved in a substantial number of metabolic processes in humans. Therefore, boron deficiency and supplementation have many effects. By means of an enzyme or hormone system, boron can affect aspects of vitamin D_3 metabolism, can affect bone and cartilage mineralization and growth, and can influence the metabolisms of some minerals (lithium, calcium, phosphorus, and especially magnesium) (16–18). Boron is also important for the replication and development of animal cells (19). Boron has some beneficial effects on preventing lipid peroxidation and DNA damage, strengthening tissue antioxidant defenses (20–22), and reducing the genotoxic effects of heavy metals (23). Boron plays an important role in anti-inflammatory processes (22,24–26). Antimicrobial activity has also been reported (27). Boron deficiency and supplementation have various biological effects in human tissues (in plasma lipid profiles, brain function,

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From the *Department of Pediatrics, Medical Faculty, the †Department of Biochemistry, Faculty of Veterinary Medicine, the ‡Department of Pharmachology, the §Department of Histology, Balıkesir University, Medical Faculty, Balıkesir, and the ||Department of Pediatrics, Ministry of Health, Dr Behçet Uz Pediatric Hospital, İzmir, Turkey.

Address correspondence and reprint requests to Selçuk Yazıcı, MD, Medical Faculty, Department of Pediatrics, Balıkesir University, 10145 Balıkesir, Turkey (e-mail: selcuk.yaz@hotmail.com).

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osteoporosis, and arthritis) (28). Boron may also affect insulin and energy metabolism, and the metabolic aspects of boron may be attributable to its overall effect on energy substrate use (16,29). Animal studies using high doses of boron reported that boron is toxic to reproduction and development; however, recent human studies performed on workers exposed to high levels of boron did not conclude that boron is toxic to reproduction (30–32). The biological functions of boron may act via the cell signaling molecules capable of complexing with boron (33). There is growing evidence of the essentiality of boron in humans and animals, but the mechanism of action underlying the benefits of boron is not well defined.

The antioxidant role of boron compounds has been previously reported in several studies (20,21,34). The use of natural or synthetic free radical scavengers could be a potential chemoprotective strategy for NEC. In the present study, the efficacy of 2 boron compounds (boric acid [BA] and 2-aminoethoxydiphenyl borate [2-APB]) in protecting rodents from NEC was examined. This relation has not been previously studied. We examined the hypothesis that rats with NEC would exhibit lower levels of GSH and total antioxidant status (TAS) and higher levels of SOD, TNF-α, and IL-6 compared with controls. Furthermore, we predicted that pretreatment with boron compounds would prevent the decrease in GSH and TAS as well as prevent the increase in SOD, TNF-α, and IL-6.

METHODS

Experimental Design

The study was conducted at the Balıkesir University Veterinary Faculty Animal Research Laboratory. The study was undertaken after obtaining approval from the experimental animal ethics committee at Balıkesir University. A total of 40 term newborn Sprague-Dawley rats (15 days old, 20–35 g) were enrolled in the study. All of the study subjects were healthy. The animals were kept in an environmentally controlled room at room temperature (24°C ± 0.5°C) in a 12-hour light and 12-hour dark cycle. Rats were given 3 days to adapt to the animal room conditions. The newborn rats were kept in identical cages (1 per group with 10 pups) with their mothers. A standard rodent diet and tap water were used for feeding the mothers. Diet and water for the mothers were provided ad libitum. During the study period, newborn rats were fed freely on breast milk by their mothers.

Rats were subdivided into 4 subgroups: NEC (group I—subjected to the NEC procedure), NEC + BA (group II—pretreated with BA and subjected to the NEC procedure), NEC + 2-APB (group III—pretreated with 2-APB and subjected to the NEC procedure), and controls (group IV—breast-fed freely by their mothers and not subjected to the NEC procedure). Each subgroup included 10 newborn rats.

NEC Procedure

Experimental NEC was induced by asphyxia (breathing 100% nitrogen gas for 120 seconds) and hypothermia (4°C for 10 minutes) twice daily for 3 consecutive days (35,36). The NEC procedure was not performed on the control group (group IV). All of the animals were weighed daily, and weight gain or loss was recorded. On the fourth day, the animals were sacrificed via decapitation, and the biochemical estimations were completed on the same day.

BA and 2-APB Application

BA and 2-APB were applied by intraperitoneal injection once daily to avoid absorption differences of the gastrointestinal tract. BA ($H_3(BO)_3$, 4% solution; Sigma Aldrich, CAS no. 10043–35–3) and 2-APB ($(C_6H_5)_2BOCH_2CH_2NH_2$, Sigma Aldrich, CAS no. 524958) was obtained from Sigma Chemical Co (St Louis, MO).

The dose for BA was 4 mg/kg, the dose mentioned to be the most protective by Pawa and Ali (37). The dose for 2-APB was 2 mg/kg, which was reported to be effective for I/R injury (38).

Biochemical Analysis

A total of 2 mL of blood was obtained from each subject via decapitation. Two tubes were used for blood collection. One tube contained sodium citrate and was used to measure the level of GSH.

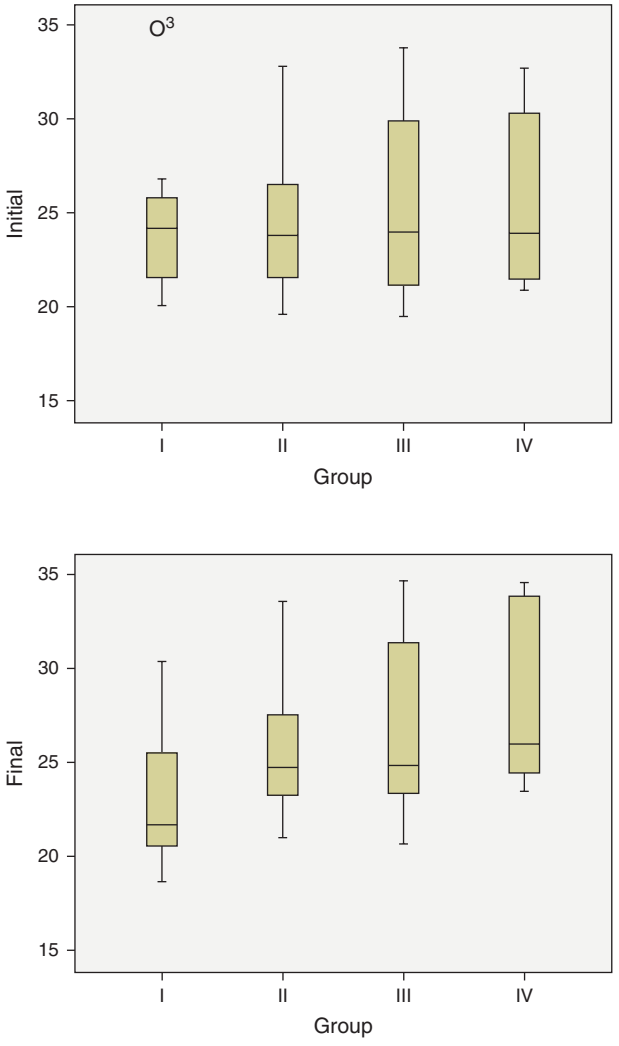

FIGURE 1. Box plots demonstrating the initial and final weights in grams for each group (group I—subjected to the necrotizing enterocolitis [NEC] procedure; group II—pretreated with boric acid and subjected to the NEC procedure; group III—pretreated with 2-APB and subjected to the NEC procedure; group IV—breast-fed freely by their mothers and not subjected to the NEC procedure). There was no difference in the initial weights among the 4 groups ($P = 0.09$). The final weights in all groups did not differ significantly different ($P = 0.17$); however, when the initial and final weights were compared, there was a statistically significant weight loss in group I ($P < 0.01$) and a significant weight gain in groups II, III, and IV (group II, $P < 0.01$; group III, $P = 0.03$; and group IV, $P < 0.01$).

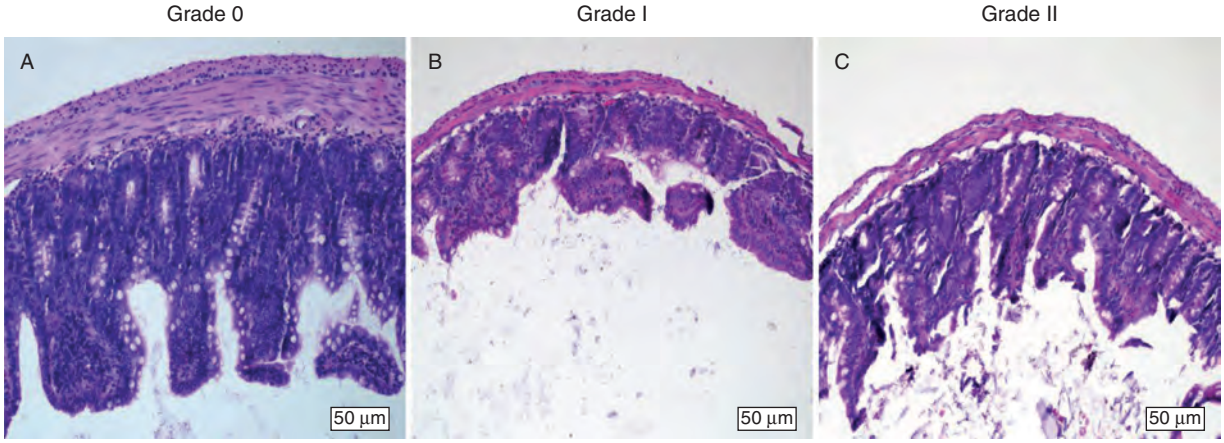

FIGURE 2. Representative histological injury scores of the terminal ileum damage in hematoxylin & eosin–stained sections. A, This figure belongs to a sample from the control group. Normal intestinal histology seen in the figure is graded as grade 0. B, This figure belongs to a sample from necrotizing enterocolitis (NEC) + boric acid group. Slight submucosal and/or lamina propria separation seen in the figure is graded as grade 1. C, This figure belongs to a sample from the NEC group. Moderate separation of submucosa and/or lamina propria and/or edema in the submucosal and muscular layers seen in the figure is graded as grade 2. The bars at the bottom of the figures represent 50 μm.

The other tube included no chemicals and was used to measure levels of TAS, SOD, TNF-α, and IL-6. The blood samples were centrifuged at 3000*g* at 4°C for 10 minutes. The samples were analyzed at the Balıkesir University veterinary faculty.

Commercially available kits were used to measure the total antioxidant status (Total Antioxidant Status Assay Kit, Rel Assay Diagnostics, RL0017, Gaziantep, Turkey), SOD (OxiSelect Superoxide Dismutase Activity Assay, STA-340, Cell Biolabs, San Diego, CA), TNF-α (Rat TNF-α platinum ELISA, BMS622, eBioscience, San Diego, CA), and IL-6 (Rat IL-6 platinum ELISA, eBioscience) in the serum. GSH was measured in whole blood by the method described by Fairbanks and Klee (39).

Histological Injury Grading and NEC Evaluation

Upon sacrifice, the intestines from the rats in all of the groups were resected. A 2-cm piece of distal ileum was removed and formalin-fixed, paraffin-embedded, sectioned at 5-μm thickness and stained with hematoxylin and eosin for histological evaluation. Pathological changes in intestinal architecture were evaluated by the use of the NEC histologic injury scoring system described by Dvorak et al (40). The grading system was as follows: 0 (normal), no damage; 1 (mild), slight submucosal and/or lamina propria separation; 2 (moderate), moderate separation of the submucosa and/or lamina propria and/or edema in submucosal and muscular layers; 3 (severe), severe separation of the submucosa and/or lamina propria, severe edema in submucosa and muscular layers and/or region villous sloughing; and 4 (necrosis), loss of villi and necrosis.

TABLE 1. Histological NEC scoring in each group

	Grade 0	Grade 1	Grade 2	Any injury	NEC +
Control, %	90	10	0	10	0
NEC, %	0	40	60	100	60*
Boric acid, %	50	40	10	50	10
2-APB, %	60	30	10	40	10

2-APB = 2-aminoethoxydiphenyl borate; NEC = necrotizing enterocolitis.
* $P < 0.05$ vs control.

Tissues were graded by the same histologist, who was blinded to the experiment. Animals with histologic scores <2 were considered negative for NEC, and animals with histological scores ≥2 were considered positive for NEC.

TUNEL Staining for the Detection of Apoptotic Cells

Apoptotic cells in the ileum sections were detected with transferase-mediated dUTP nick end labeling (TUNEL) assay by an observer who was blinded to the group assignment. The TUNEL staining was conducted using a TUNEL assay kit according to the manufacturer's instructions (ApopTaq Peroxidase In Situ Apoptosis Detection Kit; S7101-KIT, Merck Millipore, Billerica, MA).

Statistical Analysis

The statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL). The Mann-Whitney *U* and Kruskal-Wallis tests were used for comparisons between groups. The Wilcoxon test was used for interpreting the data in each group. The χ^2 test and Fisher exact test were also used where appropriate. Nonparametric tests were used, and the descriptive statistics are expressed as the median (minimum–maximum). A P value <0.05 was considered statistically significant.

RESULTS

All of the newborn rats survived until the end of the study. On the third day, hypotonia, hypoactivity, lack of appetite, and partial hair loss were observed only in the NEC group (group I). The other groups exhibited none of those changes.

There was no difference in the initial weights among the 4 groups ($P = 0.09$). During the procedure, only the NEC group lost weight; the other groups gained weight. At the end of the procedure, the final weights in all of the groups did not significantly differ ($P = 0.17$); however, when compared with the initial weights, there was a statistically significant weight loss in group I ($P < 0.01$) and a weight gain in the other groups (group II, $P < 0.01$; group III, $P = 0.03$; and group IV, $P < 0.01$) (Fig. 1).

Effect of BA and 2-APB on the Incidence of NEC

In this study, the severity of the histological changes of the ileal segments and the incidence of NEC in all of the groups were determined by using a scoring system from 0 to 4 (Fig. 2). Ileal damage in group II (NEC + BA) and group III (NEC + 2-APB) was reduced to a median histological NEC score of 0.5 and 0, respectively, compared with a median score of 2 in group I (NEC group). The incidences of NEC were 60% (6/10) in group I and 10% (1/10) in group II and group III. NEC did not develop in the control group. The incidence of NEC was significantly higher in group I when compared with the control group ($P < 0.05$), but there were no significant differences among the NEC incidences of group II, group III, and the control group (Table 1).

TUNEL Staining

The ileum sections from all of the groups were stained by TUNEL staining to investigate apoptosis. There were no differences among the groups (Fig. 3).

Effect of BA and 2-APB on Oxidative Stress

A significant decrease in the serum levels of GSH was observed in groups I, II, and III when compared with the controls, implicating oxidative stress ($P < 0.01$). Both group II (NEC + BA) and group III (NEC + 2-APB) exhibited significantly lower GSH levels than did the controls (group II–IV; $P < 0.01$ and group III–IV; $P < 0.01$), and significantly higher GSH levels than did the NEC group (group I–II; $P < 0.01$ and group I–III; $P < 0.01$). Interestingly, GSH levels were not significantly different between groups II and III ($P = 0.07$) (Fig. 4).

There was no significant difference in the total antioxidant status of the groups subjected to the NEC procedure (groups I, II, and III) compared with the controls ($P = 0.906$).

A significant decrease was present in the SOD levels in groups subjected to the NEC procedure (groups I, II, and III) when compared with the controls ($P < 0.01$). In the control group, SOD levels were significantly higher than in group I ($P < 0.01$), group II ($P < 0.01$), and group III ($P < 0.01$). The differences between groups I and II ($P = 0.063$), between groups I and III ($P = 0.057$), and between groups II and III ($P = 0.21$) were not statistically significant (Fig. 4).

Effect of BA and 2-APB on the Activity Levels of TNF-α and IL-6

TNF-α levels in the NEC group were significantly higher than in the controls ($P < 0.01$). TNF-α levels in group II (NEC + BA) were significantly lower than in the NEC group ($P < 0.01$) and group III (NEC + 2-APB) ($P < 0.01$), but did not differ from the controls ($P = 0.57$). The levels in group III were higher than in groups II ($P > 0.01$) and IV ($P = 0.01$). The NEC and NEC + 2-APB groups exhibited no difference in their TNF-α levels ($P = 0.57$) (Fig. 4).

IL-6 levels in group I were significantly higher than in group II (group I–II; $P < 0.01$), group III (group I–III; $P < 0.01$), and group IV (group I–IV; $P < 0.01$). IL-6 levels in the groups pretreated with BA and 2-APB were similar to those of the controls (group II–IV; $P = 0.97$ and group III–IV; $P = 0.06$). The IL-6 levels did not differ between groups II and III ($P = 0.21$) (Fig. 4).

The present study demonstrated that the NEC procedure increased TNF-α and IL-6 levels, preadministered BA prevented this increase, 2-APB was effective in normalizing IL-6 levels but not TNF-α levels, and the ILs involved in the inflammation process appear to be normalized by pretreatment with boron compounds.

DISCUSSION

Several studies have used hypoxia and hypothermia to produce a model of NEC in rats (35,36,41). To date, many

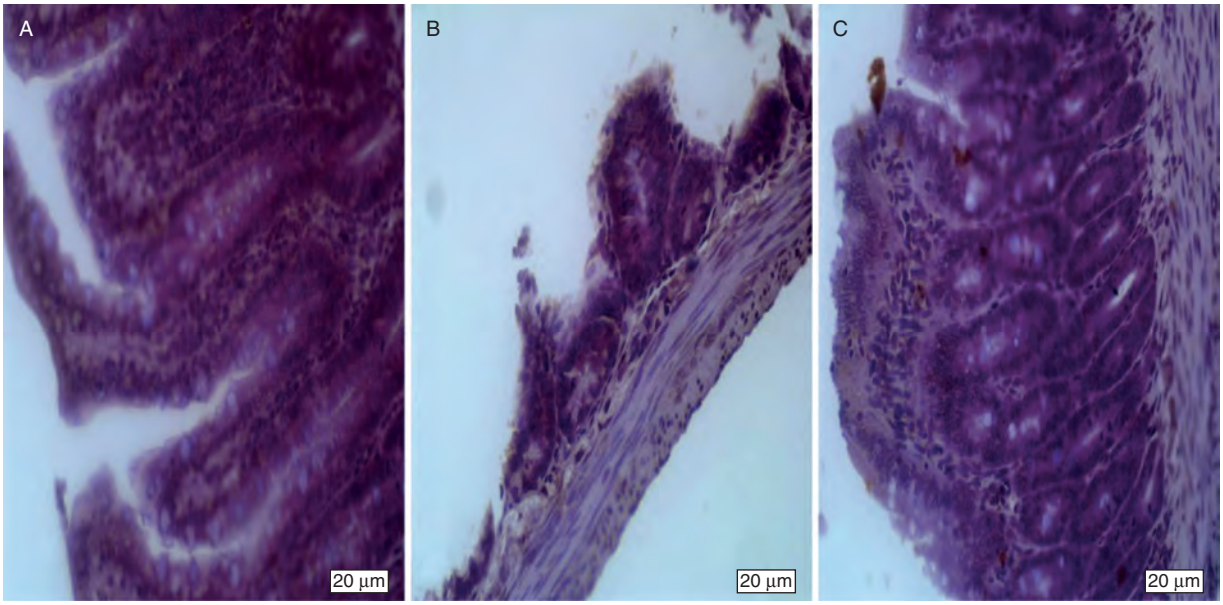

FIGURE 3. Representative transferase-mediated dUTP nick end labeling (TUNEL) staining sections of the terminal ileum from the experimental groups: A, Control group; B, NEC group; and C, necrotizing enterocolitis (NEC) + boric acid group. Although some histological findings, the slight lamina propria separation, are seen in the NEC group, there is no difference between the numbers of TUNEL-positive apoptotic cells among the 3 groups. The bars at the bottom of the figures represent 20 μm.

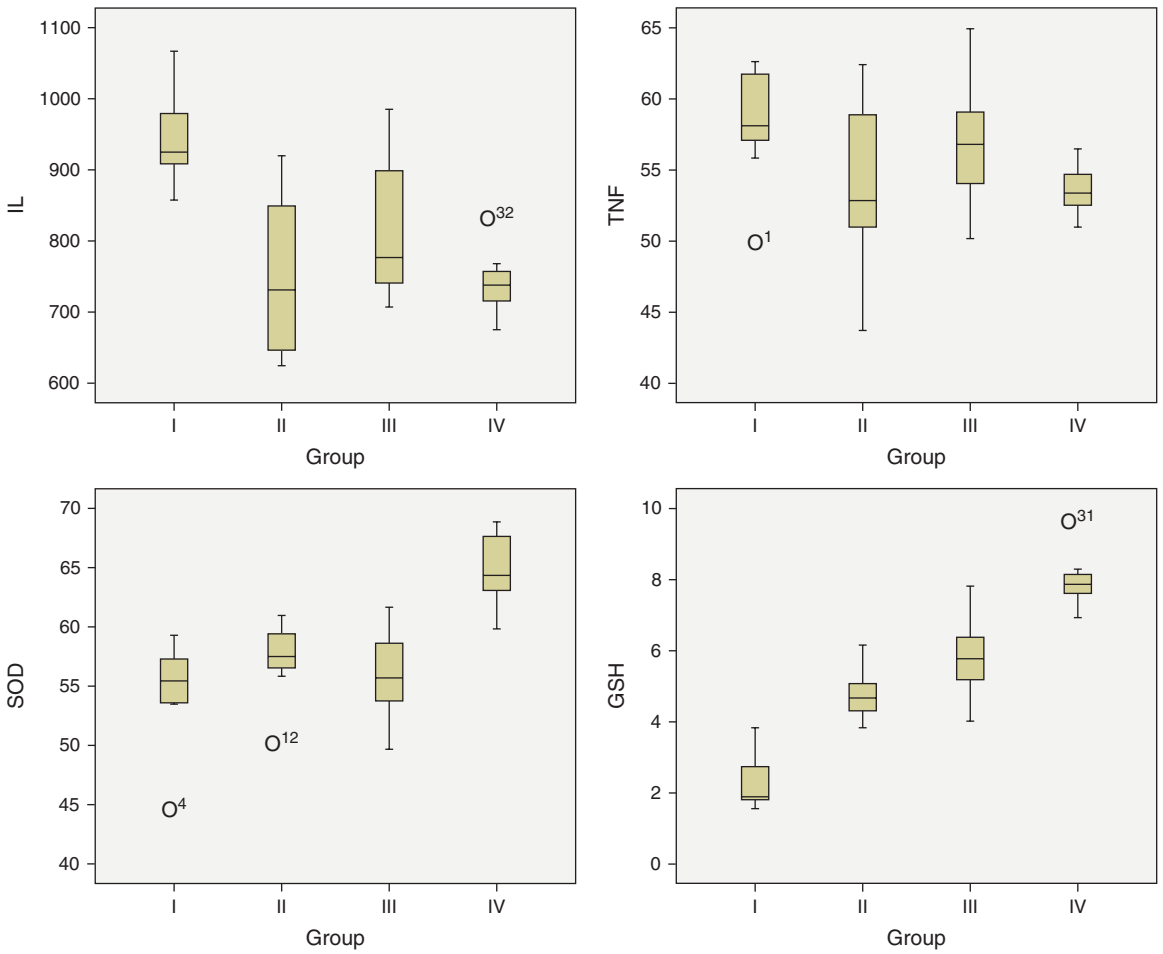

FIGURE 4. Box plots for the glutathione (GSH) (mg/dL), superoxide dismutase (SOD) (İnhibition %), tumor necrosis factor (TNF)-α (pg/mL), and interleukin (IL)-6 (pg/mL) levels in each group (group I—subjected to the NEC procedure; group II—pretreated with boric acid and subjected to the NEC procedure; group III—pretreated with 2-aminoethoxydiphenyl borate and subjected to the NEC procedure; group IV—breast-fed freely by their mothers and not subjected to the NEC procedure). GSH levels were lower in groups I, II and III than they were in the controls ($P < 0.01$). GSH levels in group II and group III did not significantly differ ($P = 0.07$) but were lower than in the controls (group II–IV; $P < 0.01$ and group III–IV; $P < 0.01$) and higher than in the NEC group (group I–II; $P < 0.01$ and group I–III; $P < 0.01$). SOD levels in groups I, II, and III were lower than controls ($P < 0.01$) but were not from each other (group I–II, $P = 0.063$; group I–III, $P = 0.057$; and group II–III, $P = 0.21$). TNF-α levels in group I were significantly higher than those of the controls ($P < 0.01$). TNF-α levels in group II were significantly lower than those in group I ($P < 0.01$) and group III ($P < 0.01$) but did not differ from levels in the controls ($P = 0.57$). TNF-α levels in group III were higher than in groups II ($P > 0.01$) and IV ($P = 0.01$) and did not differ from those of group I ($P = 0.57$). IL-6 levels in group I were significantly higher than those in group II (group I–II; $P < 0.01$), group III (group I–III; $P < 0.01$), and group IV (group I–IV; $P < 0.01$). The IL-6 levels did not differ among groups II, III, and IV (group II–III, $P = 0.21$; group II–IV, $P = 0.97$; and group III–IV, $P = 0.06$).

compounds have been tested, but no drug has been approved to prevent NEC. In the English-language literature, no study has been published on the effects of boron compounds on NEC. We used a rodent model to examine the probable protective, antioxidant, and anti-inflammatory effects of 2 different boron compounds.

In our rodent model, the NEC incidence was lower in the boron-pretreated groups, suggesting that boron exerts protective effects. Histologic scoring revealed that the protective effects of both boron compounds on NEC development are certain. The mechanism remains unclear. TUNEL staining to investigate apoptosis revealed no difference between the groups. Lipid peroxidation, an important potential mechanism of boron protection against cell damage, was not studied in the present work.

In the present study, there was a strong correlation between boron pretreatment and a reduction in oxidative stress. The total antioxidant status in experimental NEC models has been reported as increased (2), decreased (42), or unchanged (21). A study performed on peripheral human blood cultures involving 4 boron compounds revealed an increase in the levels of GSH, SOD, and TAS up to a dose of 20 mg/kg (22). The effects were dose related and higher doses contributed to oxidative stress and a decrease in GSH, SOD, and TAS levels. Our doses of 4-mg/kg BA and 2-mg/kg 2-APB resulted in an increase in GSH levels and a decrease in SOD levels. Interestingly, the total antioxidant status did not change. The results of the present study revealed that boron compounds appear to normalize the antioxidant defense in our experimental NEC

model and the effects may be dose dependent. Further studies with various doses are needed.

Previous reports have suggested that NEC pathogenesis is related to a decrease in GSH levels, as expected in oxidative stress, and that GSH supplementation may be protective in animal models of intestinal ischemia (4). Data from our NEC model are consistent with the study of Kelly et al (4). Interestingly, Hall et al (3) reported a trend toward lower GSH levels in infants with more extensive NEC stages, but the decrease was not significant.

SOD levels in our model were significantly lower in all NEC groups than in controls. The results are consistent with similar studies in the English-language literature (43,44). In previous reports, the stimulatory effect of BA on SOD in human blood has been reported (34). Unexpectedly, boron pretreatment had no effect on SOD levels in our NEC model.

Several studies investigated the relation of boron and inflammation (24,25,45,46). Cao et al (24) found that BA had no significant effect on TNF-α formation or intracellular GSH contents in THP-1 cells; however, BA administration led to a decrease in lipopolysaccharide (LPS)-induced TNF-α formation. The study of Benderdour et al (45) reported that BA acts as a stimulus for the release of TNF-α from cultured human fibroblasts and chick embryo cartilage. Armstrong et al (46) reported that pigs that consumed a boron-supplemented diet exhibited a decreased local inflammatory response to an intradermal injection of phytohemagglutinin. Following stress in pigs, Armstrong and Spears (25) reported an increase of TNF-α after dietary boron intake, implicating an increase in the systemic inflammatory response. Finally, Armstrong et al concluded that models of localized tissue inflammation are most likely not equivalent to whole-body inflammatory disease models. Conversely, our study demonstrated a limitation of systemic inflammation. The results revealed that in our experimental NEC model, pretreatment with BA prevented the increase of TNF-α, but pretreatment with 2-APB did not result in the same effect. The study clearly revealed that pretreatment with BA or 2-APB downregulated the activity levels of IL-6 in NEC. This result confirms the finding that IL-6 is more specific than TNF-α in NEC pathogenesis (11,13). Our route of administration was intraperitoneal, and we did not assess local tissue inflammation.

The data in our rodent model suggest that boron pretreatment could temper the decrease in GSH and inhibit the increase in the proinflammatory cytokines TNF-α and IL-6. Unfortunately, there is no similar experimental NEC study in the English-language literature to which our results can be compared.

CONCLUSIONS

Our results provide the first data on the effects of boron compounds (BA and 2-APB) on NEC. The data clearly demonstrate that BA and 2-APB modulate oxidative stress parameters, enhance the antioxidant defense mechanism, and partly prevent NEC formation. In rats with NEC, pretreatment with BA or 2-APB resulted in a significant decrease in GSH consumption but had no effect on total antioxidant status. The inhibition of hypoxia- and hypothermia-induced increases in serum proinflammatory cytokines and the enhancement of antioxidant defense suggested that boron could help prevent systemic inflammation and maintain the oxidant/antioxidant balance of the affected tissue. Although limited conclusions can be drawn from animal studies, boron may be beneficial for protection against NEC. Further characterization of the cell-signaling molecules capable of complexing with boron should provide insights into the effects of boron. The exact mechanisms need to be further elucidated.

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