

Temporal profile of intestinal tissue expression of intestinal fatty acid-binding protein in a rat model of necrotizing enterocolitis

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OBJECTIVES: Necrotizing enterocolitis is a severe multifactorial intestinal disorder that primarily affects preterm newborns, causing 20-40% mortality and morbidity. Intestinal fatty acid-binding protein has been reported to be a biomarker for the detection of intestinal injuries. Our aim was to assess intestinal tissue injury and the molecular expression of intestinal fatty acid-binding protein over time in a necrotizing enterocolitis model.

METHODS: A total of 144 Newborn rats were divided into two groups: 1) Control, which received breastfeeding (n=72) and 2) Necrotizing Enterocolitis, which received formula feeding and underwent hypoxia and hypothermia (n=72). A total of six time points of ischemia (2 times a day for 3 days; 12 pups for each time point) were examined. Samples were collected for analysis of body weight, morphological and histological characteristics, intestinal weight, intestinal weight/body weight ratio, injury grade, and intestinal fatty acid-binding protein levels.

RESULTS: Body and intestinal weights were lower in the Necrotizing Enterocolitis group than in the Control group ($p < 0.005$ and $p < 0.0005$, respectively). The intestinal weight/body weight ratio was higher in the Necrotizing Enterocolitis group than in the Control group ($p < 0.005$) only at the sixth ischemia time point. The Necrotizing Enterocolitis group displayed higher expression of intestinal fatty acid-binding protein ($p < 0.0005$) and showed greater tissue damage than the Control group.

CONCLUSION: Intestinal fatty acid-binding protein was an efficient marker of ischemic injury to the intestine and a good correlation was demonstrated between the time of ischemic injury and the grade of intestinal injury.

KEYWORDS: Necrotizing Enterocolitis; Rats; Ischemia; Intestinal Injury; I-FABP.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe intestinal inflammatory disease of multifactorial origin that attacks the intestines of premature and full-term neonates. NEC is considered to be a major cause of morbidity and mortality (20-40%) among neonates receiving care in intensive care units (1,2). The factors leading to the stimulation of the

inflammatory cascade that results in NEC include prematurity; intestinal hypoxia/ischemia; early enteral feeding, especially with formula; and bacterial colonization (3,4).

Hypoxia/ischemia affects both the integrity and the homeostasis of the intestinal epithelial barrier, inducing epithelial injury (5,6). In addition, not only the immaturity of gastrointestinal motility but also the composition of formula milk increases the likelihood of intraluminal bacterial fermentation of malabsorbed lactose, increasing the risk of NEC development in premature neonates (7).

The discovery of a more specific diagnostic marker of NEC has been a challenge for neonatologists and surgeons aiming to reduce the high morbidity and mortality rates of this disease. Intestinal fatty acid-binding protein (I-FABP) has been used as a marker for the detection of intestinal injuries and has been investigated as a candidate marker for the

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detection of enterocyte death or rupture. Although several studies have correlated the presence of I-FABP in urine and in the blood circulation with NEC, its direct function and tissue expression have been little described (8). Given that NEC is a progressive inflammatory process, our objective was to evaluate the expression of I-FABP as an indicator of intestinal tissue injury over time in an experimental NEC model using newborn rats.

■ MATERIAL AND METHODS

Evaluation by the Ethics Committee on Animal Experimentation

All procedures involving animals were approved by the Ethics Committee on Animal Experimentation of the Ribeirão Preto Medical School (CETEA – FMRP-USP), protocol number 040/2011.

Experimental NEC Model

Experimental NEC was induced according to the protocol described by Jilling et al. and adapted by Gonçalves et al. (9,10). Newborn rats were fed 0.1 mL of Esbilac[®] artificial milk (PetAg, Hampshire, IL, USA) every 3 hours beginning at 6:00 am for 24 hours using an adapted, peripherally inserted central catheter (PICC, 1.9 Fr 26 gauge, 1 lumen, BD[®], Sandy, UT, USA). Newborns in the NEC group were positioned laterally on a heated mattress for placement of the orogastric probe for every procedure. Before each procedure, the newborns were evaluated for the presence of abdominal distension and bloating; if either clinical sign was detected, the diet was suspended for that feeding time. Hypoxia was induced by exposure of the animals to 35 L/min nitrogen (N₂) in an acrylic anoxic chamber for 70 seconds, followed by hypothermia (4°C) in a refrigerator for 10 minutes; this procedure was performed twice a day, at 8:00 AM and 8:00 PM, for three days (11).

Experimental groups

On the 22nd day of gestation, the neonates were weighed at birth and randomly allotted to two groups of 144 Sprague-Dawley rat pups: Control (C) group: newborn pups were exclusively fed maternal milk (n=72); NEC group: the pups were separated from the dams immediately after birth, placed in a compartmented box maintained under thermal control at a temperature of 36-38°C, fed artificial Esbilac[®] milk (PetAg, Hampshire, IL, USA ~200 kcal/kg/day) 8 times a day, and subjected to hypoxia and hypothermia for three days (n=72). The two groups were divided into six subgroups. Every 12 hours (8:00 AM and 8:00PM) over three days, we performed ischemia on the NEC group (from the 1st to the 6th procedure), and each subgroup was compared with the subgroup of animals in the C group (breastfeeding) sacrificed at the same time point.

Sample processing

Euthanasia was performed on a subset of animals in each group according to the timing of the experimental procedures, i.e., two hours after the ischemic episode and hypothermia. The pups were weighed and euthanized by decapitation. The intestines from each subgroup were removed and divided into two fragments for immunohistochemistry and histological analysis. The first fragment, 1 cm of jejunum, was obtained at 6 cm from the pylorus, and the second fragment included the 2 cm preceding the

ileocecal valve. The fragments were fixed in 4% paraformaldehyde and embedded in histological paraffin, respectively.

Body weight and morphological evaluation of the groups

Body weight (BW), intestinal weight (IW) and the IW/BW ratio were used to analyze the differences between the groups. The change in weights from birth to the day of tissue collection (day 3) were determined and the intestinal loops were examined macroscopically.

Processing for histological analysis

Crosswise 5- μ m-thick histological sections of the intestinal segments were obtained with a Leica Model RM 2145 microtome (Leica, Nussloch, Germany). The sections were stained with Masson's trichrome for histological identification and mounted on Permount[®] (Fisher Scientific, Pittsburgh, PA, USA).

Histological grading of ileal injury

Four intestines per subgroup were randomly selected for study. During microscopic evaluation, six histological parameters were observed in the intestinal segments: flattening of the villi, separation of the layers from the mucosa to the longitudinal muscular layer, edema of the layer, edema of the villi, desquamation of the villi, and loss of villi of mild, moderate and severe intensity. Based on the studies by Dvorak *et al.* and Granger *et al.*, a modified protocol was designed for more precise evaluation of the levels of tissue damage (12,13). A score was recorded for each injury observed and a summed score was calculated at the end of each investigation. The score ranged from 0 to 25 points, with zero representing no signs of injury to the animals and 25 representing maximum injury, such as loss of villi. The score was calculated as follows: A, no signs of injury: 0 - zero; B, flattening of villi: mild - 0.5, moderate - 1.0, or severe - 1.5; C, layer of separation: mild - 2.0, moderate - 2.5, or severe - 3.0; D, edema layer: mild - 3.5, moderate - 4.0, or severe - 4.5; E, edema of villi: mild - 5.0, moderate - 5.5, or severe - 6.0; F, desquamation of the villi: mild - 6.5, moderate - 7.0, or severe - 7.5; and G, loss of villi: severe - 25. NEC was considered present in animals with histological scores ≥ 12.5 . The scores were recorded separately by three examiners and assigned arbitrary values (au) according to the signs of injury. Then, a mean score was calculated for each histological section. The score of injury was classified in mild, moderate and severe, respectively 33%, 66% and 100% according to the damaged of intestinal wall.

Immunohistochemical analysis

Immunohistochemistry was used to detect proteins in 3- μ m tissue sections mounted on slides. The slides were deparaffinized with xylene and hydrated in an ethanol series. The sections were first treated with 3% H₂O₂ (30%) in methanol for 10 minutes at room temperature and then with 50 mM Tris-HCl buffer, pH 9.5, containing 5% urea for antigen recovery under steam. Subsequently, the sections were washed in phosphate-buffered saline (PBS: 20 mM Na₂HPO₄ and 0.45 M NaCl, pH 7.4). The slides were incubated in 10% normal rabbit serum blocking solution for 30 minutes to block nonspecific binding sites. Then, the samples were incubated overnight with a goat anti-I-FABP antibody (sc-16063, Santa Cruz Biotechnology, Santa Cruz,



CA, USA) diluted 1:200 in bovine serum albumin (BSA) at 4°C. After washing, the slides were incubated with a biotin-conjugated rabbit anti-goat antibody (sc-2768, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in BSA for two hours. The primary antibody was omitted as a negative control. The histochemical reaction was visualized using the Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) and 3,3'-diaminobenzidino-tetra-hydrochloride (Sigma, St Louis, MO, USA). Finally, the slides were counterstained with Harris hematoxylin, dehydrated in a graded alcohol series and xylene, and covered with coverslips mounted with Permount[®] (Fisher Scientific, Pittsburgh, PA, USA).

Semiquantitative immunohistochemical analysis

The slides were photographed with a photomicroscope (NIKON Eclipse E200 80i, Tokyo, Japan) at 200X magnification for three sections per slide, using 4 slides from different animals per subgroup. The images were obtained using NSI-Elements F 3.2 software (NIKON, Tokyo, Japan). Staining intensity was scored as follows: 0=negative, 1=weak, 2=moderate, 3=strong, and 4=very strong. In addition, intermediate scores of 0.5, 1.5, 2.5 and 3.5 were used for more precise scoring of tissue injury based on immunolabeling. Three examiners independently assigned arbitrary scores (au) according to the immunostaining intensity. Then, a mean score was calculated for each section.

Western blotting

Three samples from the C group and four samples from the NEC group were used for molecular analysis. The intestines were homogenized in extraction buffer, consisting of 100 mM Tris buffer (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg aprotinin, and 1% Triton X-100, at 4°C using a TE-103 small sample homogenizer (Tecnal, Paulínia, SP, Brazil) at 30,000 rpm for 30 seconds. The samples were then centrifuged (Hettich Mikro 200R, Münster, Germany) at 12,000 rpm at 4°C for 30 minutes and the protein concentrations in each sample were measured using the Bradford method standardized by the laboratory (14). Aliquots of 20 µg protein were boiled for 5 minutes, rapidly centrifuged at 8,000 rpm for 10 seconds and applied to 15% polyacrylamide gel containing 0.1% SDS and running buffer (SDS-PAGE). The proteins were separated by electrophoresis at a constant current intensity of 100 V for approximately 2 hours. The protein bands were then electrotransferred to a nitrocellulose membrane in transfer buffer at 120 V for 90 minutes at 4°C,

followed by blocking with Molico[®] skim milk (Nestlé, São Paulo, SP, Brazil) with constant shaking for 30 minutes. Next, the membranes were incubated overnight at 4°C with a primary anti-I-FABP antibody (sc-16063, Santa Cruz Biotechnology) diluted 1:100 in 3% PBS/BSA. On the subsequent day, the membranes were washed with 0.01 M PBS buffer, pH 7.4, and incubated with peroxidase-conjugated secondary anti-goat antibodies (sc-2768, Santa Cruz Biotechnology) diluted 1:2,000 in 3% PBS/BSA for two hours. The membranes were washed again, and a set of chemiluminescence reagents (Pierce, Rockville, IL, USA) was applied for 5 minutes. The material was developed and photographed using a ChemiDoc XRS+ imager equipped with Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are reported as means ± standard deviation. The body weight and the morphological data were analyzed using two-way ANOVA followed by the Tukey post-test; molecular biology data were analyzed using two-way ANOVA followed by the Sidak post-test; and immunohistochemistry and lesion score data were analyzed using the Kruskal-Wallis test followed by the Dunn post-test, with the level of significance set at $p < 0.05$. All analyses were conducted using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

A total of 146 newborn Sprague-Dawley rats obtained from 13 dams were studied; 72 animals were assigned to the C group and 74 were assigned to the NEC group. The mortality rate after the NEC protocol was 2/146 pups (1%). Two animals (3%) in the NEC group were excluded from the protocol due to possible perforation of the esophagus; as a result, 144 pups were examined in this study.

The body weight and the morphological results are summarized in Figure 1. The change in BW from birth to the day of euthanasia demonstrated increased weight of the C pups receiving maternal milk compared to the NEC pups ($p < 0.005$ or $p < 0.0005$) on the first, fourth, fifth and sixth ischemia time points. The C group pups also showed increased IW compared to the NEC group pups ($p < 0.005$ or $p < 0.0005$) from the third to the sixth ischemia time point. The IW/BW ratio increased over time in the NEC group compared to the C group ($p < 0.005$) only at the sixth ischemia time point.

The classification of injury grade is presented in Figure 2. Grading of ileal injury showed that group C had a normal

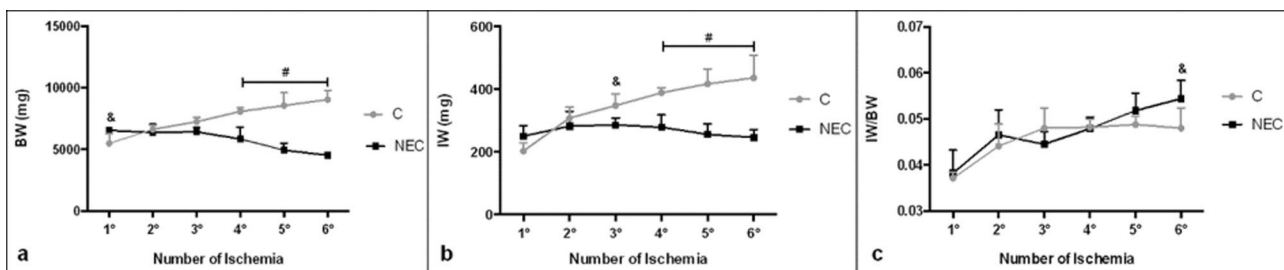


Figure 1 - Body weight and the morphological results. **a)** Body weight (mg); **b)** Intestinal weight (mg); **c)** Intestinal weight / Body weight ratio. Groups: control (C) and necrotizing enterocolitis (NEC). The samples were collected at the 1st-6th time points. & $p < 0.005$; # $p < 0.0005$.

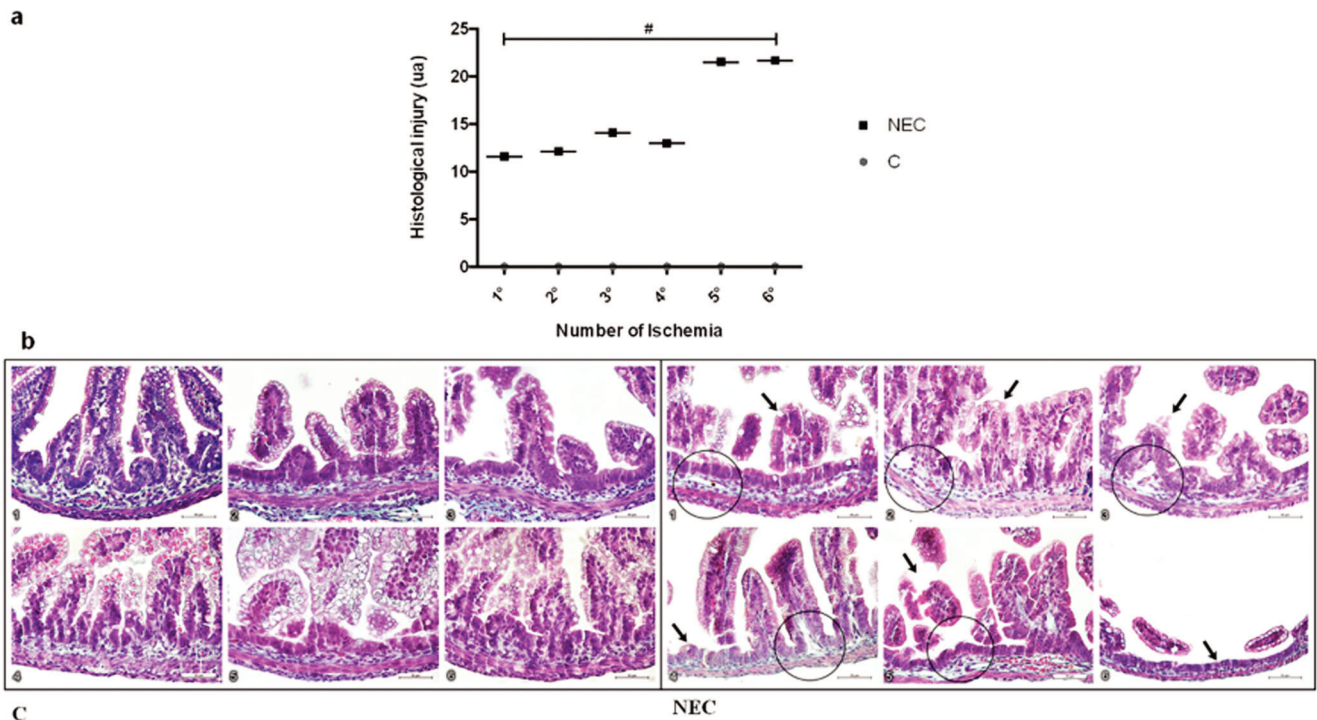


Figure 2 - Lesion scores expressed in arbitrary units (au). **a**) The differences in the mean injury severity score based on histological analysis were compared at six time points between the Control and NEC groups. The samples were collected at the 1st-6th time points. Graphs of the lesion scores are presented. Note that the Control group exhibited no lesion (score of zero). **b**) Photomicrographs of histological sections from the C group at the six experimental time points. The samples were collected at the 1st-6th time points. Preserved layers and villi were observed (scale bar=50 μm). Photomicrographs of histological sections of the NEC group at the six experimental time points. 1: first collection time point; edema, separation of the mucosa and submucosa layers, and villus injury were observed. 2: second collection time point; edema, separation of the submucosal layer; peeling and villus injury were observed. 3: third collection time point: edema, separation of the submucosal layer and the lamina propria were observed. 4: fourth collection time point; villus scaling, submucosal layer separation, and decreased thickness of the submucosal and circular muscle layers were observed. 5: fifth collection time point; edema in the mucosal layer, separation of the submucosal layer, and decreased thickness of the circular muscle layer were observed. 6: sixth collection time point; loss of villi (arrows and circles) was observed (scale bar=50 μm). # *p* < 0.0005.

intestine with intact villi and layers. Beginning from the first ischemia time point, the NEC group exhibited high scores of tissue injury. Maximal involvement of the intestinal wall was observed as moderate separation of the submucosal and mucosal layers, severe edema, desquamation with loss of villi, and atrophy from the mucosal to the longitudinal muscle layer. The NEC animals subjected to ischemia at the third time point (2nd day) were assigned a score > 12.5. The NEC group exhibited significantly different injury severity compared to group C at all experimental time points (*p* < 0.0005).

The immunohistochemistry results are shown in Figures 3, 4 and 5. The expression of I-FABP in the jejunum based on immunohistochemistry showed that the neonates in group C expressed increased I-FABP levels on the second, fourth and sixth time points. Alternatively, the animals in the NEC group demonstrated increased I-FABP expression at the third and sixth ischemia time points and I-FABP expression was decreased between the NEC and C groups at the second and fifth experimental time points (*p* < 0.005 and *p* < 0.0005, respectively).

I-FABP expression in the ileum of the C group showed increased immunostaining compared to the NEC group at the third ischemia time point. I-FABP expression subsequently decreased and stabilized by the end of the study period. I-FABP expression was increased between the NEC

and C groups from the fourth through the sixth ischemia time points (*p* < 0.0005).

The Western blotting results (Figure 6) showed that the C group displayed higher I-FABP expression at the first two time points than at the other time points examined. Reduced I-FABP expression at the fourth ischemia time point was observed compared to the other time points, including the initial time point. The NEC group showed higher expression at the first, fifth and sixth ischemia time points compared with the second, third and fourth ischemia time points. Notably, the NEC group showed lower expression of I-FABP at the first ischemia time point and greater expression at the sixth ischemia time point (*p* < 0.0005) than the C group at the second and sixth ischemia time points.

DISCUSSION

Formula feeding, ischemia and hypothermia are considered to be risk factors for the development of NEC, which has a mortality rate ranging from 10 to 50% among neonates treated in intensive care units (15,16). In this study, the mortality rate after NEC induction was low (approximately 7%) compared to the 38% mortality rate observed by other authors (11). The reduced mortality of the present protocol may be attributed to the more precise intubation technique and the discontinuation of the diet when the pups showed

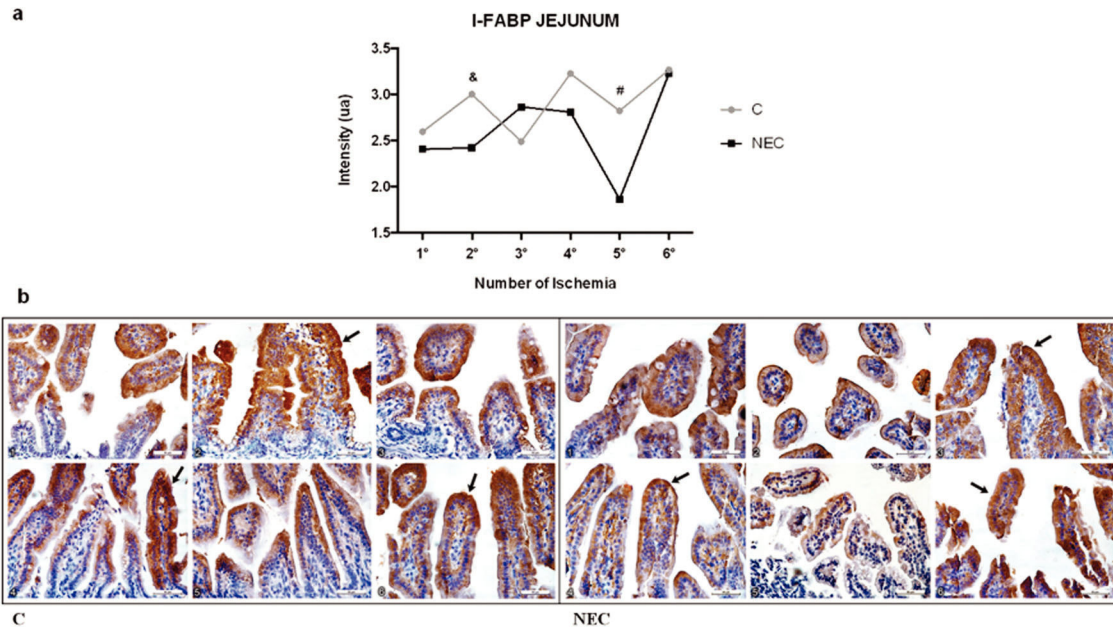


Figure 3 - Graphs of I-FABP expression in the jejunum. **a)** The means and standard deviation of the arbitrary scores of I-FABP immunolabeling intensity (au) in the jejunum over three days were compared between the two groups. **b)** Immunohistochemistry for I-FABP in the jejunum relative to the C group. I-FABP immunolabeling intensity increased from the first to the sixth experimental time point (scale bar=50 μm). & # $p < 0.005$; # $p < 0.0005$.

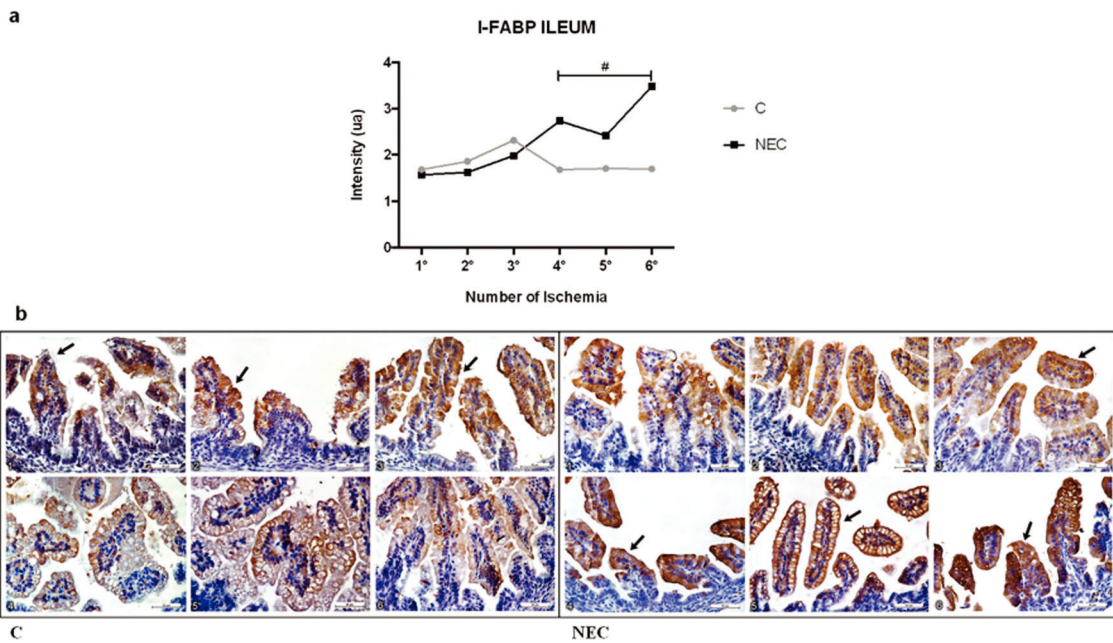


Figure 4 - Graphs of I-FABP expression in the ileum. **a)** The means and standard deviation of the arbitrary scores of I-FABP immunolabeling intensity (au) in the ileum over three days were compared between the two groups. **b)** Immunohistochemistry for I-FABP in the ileum relative to the C group. I-FABP immunolabeling intensity increased from the first to the sixth experimental time point (scale bar=50 μm). # $p < 0.0005$.

gastric distention or respiratory distress (16). We observed that the BW of the NEC group was significantly reduced compared to the C group (breastfed). Other authors obtained similar results, showing weight loss among formula-fed animals (15,17-19). One exception is the study by Dvorak *et al.* (20), who detected increased BW in their NEC group.

The IW/BW ratio was higher in the NEC group than in the C group. This finding could be explained by the nutritional or inflammatory intestinal processes occurring as a result of NEC (10).

Regarding the degree of histological injury, NEC induced microscopic changes such as mild to moderate necrosis of the

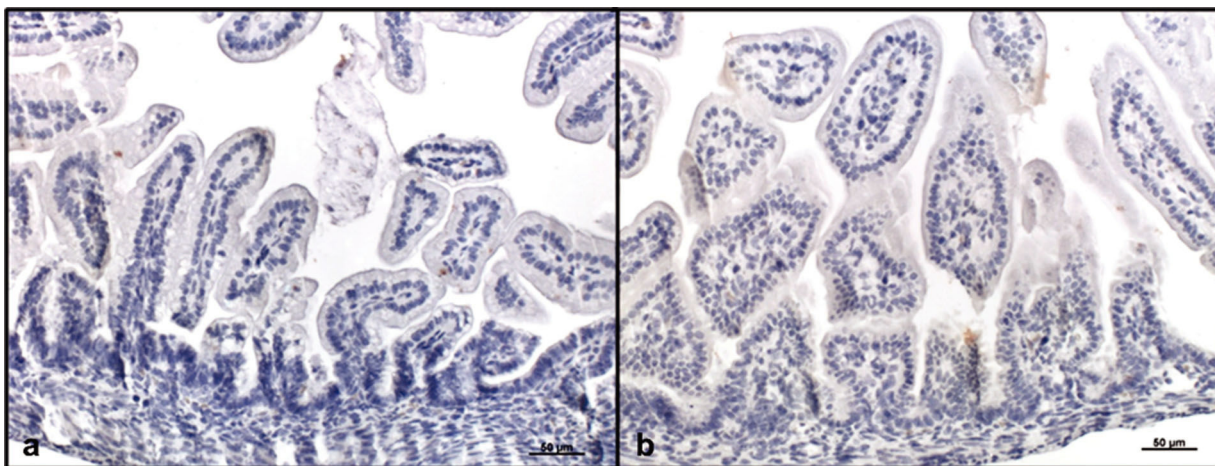


Figure 5 - Negative control. Photomicrographs show the negative controls for immunohistochemistry. **a)** Ileum. **b)** Jejunum (scale bar=50 µm).

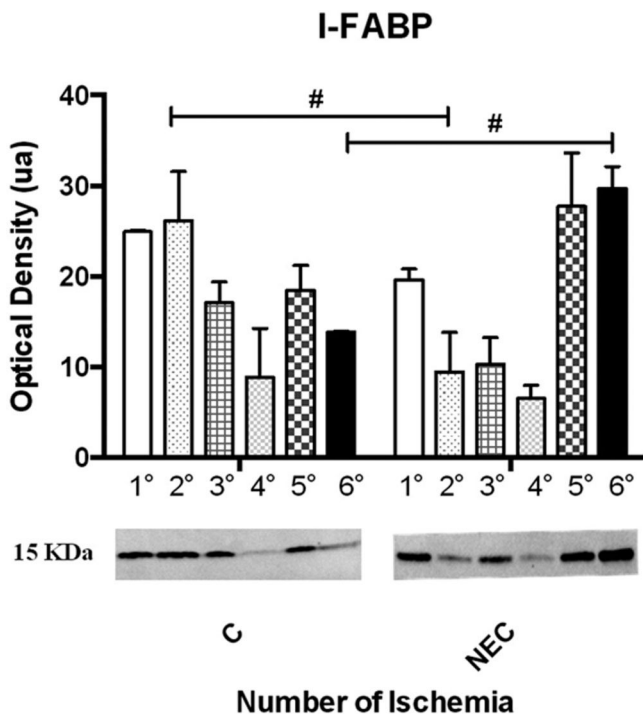


Figure 6 - Western blotting. The mean optical density values of the Western blotting bands for I-FABP (au) at the six experimental time points (numbers correspond to the time points of ischemia) in the C and NEC groups. The molecular weight of I-FABP is indicated. # $p < 0.0005$.

villi and crypt and submucosal layer injury; these results were similar to those reported in another experimental study of NEC in rats (17,20). Although maternal milk has a protective effect against NEC, exclusive breastfeeding may not completely eradicate the disease (15). Pitt et al. demonstrated that freezing and thawing maternal milk completely eliminated its protective effect against NEC (21,22).

I-FABP has been indicated as a possible biomarker of NEC because it is an abundant protein that represents 2-3% of the cytoplasmic protein content of mature enterocytes and is normally undetectable in the peripheral circulation (1,16,23).

After the death of enterocytes, their cytoplasmic content is released into the circulation, and an increase in the circulating concentration of I-FABP has been demonstrated in both experimental models of NEC (1,23) and clinical samples from neonates with NEC (16,24). Biochemical evaluation of I-FABP in rats demonstrated that its content can be 1.5 times higher in the jejunum than in the ileum, as also observed in humans (25). During the progressive 3-day hypoxia/ischemia process, the pups in the C group showed stable I-FABP levels, but the pups in the NEC showed a progressive increase in I-FABP levels, especially in the ileum, compared to those in the C group.



Although we did not perform other measurements to determine the level of oxidative stress, various signaling mechanisms that promote the induction and the progression of ischemic injury, such as increased synthesis of adhesion molecules combined with neutrophil infiltration, increased production of hyper-reactive peroxides, increased lipid peroxidation and increased production of inflammatory mediators such as cytokines, may have been activated in the NEC group (26). We suggest that in group NEC, lipid peroxide formation and oxidative stress may have been induced by feeding with Esbilac[®] milk, which contains 44% lipids.

Lipid peroxide formation was previously observed in a clinical study of extremely low birth weight infants treated with parenteral lipid emulsion. In that study, increased urinary L-FABP levels were detected, and this result suggested that the fat emulsion produced oxidative stress in the renal tubules (27).

High serum and urinary I-FABP concentrations have been observed in patients with intestinal ischemia, with systemic inflammatory response syndrome or with NEC (16). During cardiopulmonary bypass surgery, patients showed increased urinary levels of I-FABP; this result suggested that gastrointestinal perfusion changed during the surgery, leading to an increased risk of intestinal ischemia and mesenteric infarction (28,29). In neonates with NEC, the urinary concentrations of I-FABP were associated with the severity of clinical signs and symptoms, and to treat NEC in these patients (30). Serial evaluation of the I-FABP levels may be a useful marker for early diagnosis of NEC and for the prediction of disease severity (1,2,3), as I-FABP is rapidly eliminated from the circulation through the kidneys (25,26,31).

In conclusion, we demonstrated that I-FABP was highly expressed and that its expression correlated with the degree of tissue injury during hypoxia/ischemia in an experimental rat model of NEC. These data support the hypothesis that I-FABP may serve as a diagnostic marker of NEC, although further studies are needed to assess the correlation between the serum and urinary levels of I-FABP to characterize the evolution of NEC.

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AUTHOR CONTRIBUTIONS

Simões AL performed the research, including the immunohistochemistry and Western blotting experiments, and wrote the manuscript. Figueira RL performed the mating of the rats and feeding of the newborns and helped with Western blotting. Gonçalves FL performed the mating of the rats and feeding of the newborns and helped in immunohistochemical cell counting. Mitidiero LF performed the mating of the rats and feeding of the newborns. Castro e Silva O helped to determine the appropriate I-FABP dosage in his laboratory. Peiró JL helped to write the Discussion section and to translate the report. Sbragia L served as the mentor and tutor of the project and revised the manuscript.

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