

Use of Sildenafil and L-arginine in Preventing Neonatal Necrotizing Enterocolitis: An Experimental Model in Rats

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Abstract

Necrotizing enterocolitis (NEC) has a 45% mortality in neonatal intensive care units. This paper aimed to evaluate the isolated and combined effects of sildenafil and l-arginine in intestinal wall and survival rates. Neonatal rats were fed formula milk and submitted to hypoxia under a 100% N₂ atmosphere for 70 seconds. Then, animals were subjected to hypothermia (4°C for 10 minutes), twice a day for three days. Forty neonatal rats were divided into five groups: negative control – not submitted to the protocol (n = 5), sildenafil group - NEC protocol (n = 9), l-arginine group - NEC protocol (n = 9), l-arginine and sildenafil group - NEC protocol (n = 9) and positive control - NEC protocol and intraperitoneal saline solution (n = 8). Jejunum and terminal ileus were removed for histopathologic and immunohistochemical Ki-67 analysis. Kruskal-Wallis test was used to analyze mortality, survival, body weight, intestinal injury score and Ki-67 proliferation index. All animals submitted to the protocol developed enterocolitis. Mortality rate was higher in group that received only l-arginine (p = 0.0293). The Ki-67 analysis showed a higher proliferative index in groups that received interventional drugs (p = 0.017). In conclusion, sildenafil and l-arginine were not effective to reduce intestinal injury.

Introduction

Neonatal necrotizing enterocolitis (NEC) is an intestinal inflammatory disorder. It affects approximately 5–7% of premature newborns and it is one of the major causes of mortality in neonatal intensive care units (45%)¹. Prematurity is the main risk factor for the disease, and more than 90% of newborns affected by NEC are born at less than 37 weeks of gestational age².

Multiple factors are involved in the pathophysiology of the disease, including the immaturity of the immune system to the complex changing composition of the intestinal microbiota. In this regard, perinatal hypoxia, early feeding with formula milk, and bacterial colonization play a fundamental role in the activation of the inflammatory cascade of NEC. These factors induce intestinal damage, which may progress to coagulative necrosis of the gastrointestinal tract^{1, 3}.

When harmful bacteria reach the circulation, they inhibit the expression of the Endothelial nitric-oxide synthase (eNOS). Consequently, this process reduces the availability of nitric oxide (NO), an important modulator of homeostatic and inflammatory conditions¹. The reduction in plasma NO levels promotes intense vasoconstriction, changes intestinal perfusion, and generates hypoxia, evolving to the characteristic necrosis of NEC^{4,5}.

In this context, the administration of sildenafil, a substance that increases eNOS signaling, emerges as a possible preventive strategy for the development of NEC. Sildenafil is a robust selective inhibitor of Phosphodiesterase-5 (PDE5) which is responsible for the degradation of cyclic guanosine monophosphate (cGMP). The accumulation of cGMP leads to the activation of the NO/cGMP system, favoring the production of NO and increasing intestinal perfusion. Thus, it may prevent the appearance of necrosis¹.

To enhance the activation effect of eNOS, we included an l-arginine supplementation. This amino acid, the precursor of NO, provides a greater amount of substrate for enzyme activity^{4, 5, 6, 7}.

It has been observed that, although the NO released in the endothelium is essential in the modulation of peripheral vascular tone, l-arginine plays an important role in endothelium-dependent relaxation, a decisive issue in the prevention of tissue damage ^{4, 5,6,7} .

In a previous study, intraperitoneal administration of l-arginine in an experimental model of NEC showed an increase of NO in the intestinal tissue, which resulted in a lower degree of injuries produced by hypoxia and hypothermia events⁸.

Given that NEC is an aggressive inflammatory process, we set out to study the effects of sildenafil and l-arginine – both isolated and combined – on intestinal injuries in an experimental model of necrotizing enterocolitis.

Results

After the NEC protocol, the mortality rate was 14/40 (35%). Group C, which only received l-arginine, obtained the highest mortality rate among the groups (66.67%), as shown in Fig. 1 (Kruskal-Wallis, $p = 0.0293$). Contrarily, group B, which only received sildenafil, obtained the lowest mortality rate among the groups submitted to the NEC protocol (14%).

Among newborn rats that died, group E had the highest survival rate (with a mean of 63.83 hours of life since the beginning of the experiment) as shown in Fig. 1. Group C, on the other hand, demonstrated the lowest survival among groups (with a mean of 37.3 hours of life) (Kruskal-Wallis, $p = 0.0293$). Among the groups that received the studied substances (sildenafil and/or l-arginine), group D had the highest survival rate (with a mean of 62.25 hours of life). One animal in group E was excluded from the protocol due to esophageal perforation (2.5%). As a result, a total of 25 animals were examined in this study.

The bodyweight of the newborn rats is summarized in Fig. 2. During the experiment, groups submitted to the NEC protocol showed decreased body weight gain. The final bodyweight of the rats in group A obtained a mean of 12.66 g. This weight was significantly higher than all NEC groups, whose means were of 5.31 g in group B, 5.87 g in group C, 6.23 g in group D, and 5.92 g in group E (Kruskal-Wallis, $p = 0.0088$).

The histopathological injury score is shown in Fig. 3. According to histopathological analysis, all animals submitted to the protocol developed enterocolitis (score > 18). Group A presented a normal intestine with intact villi and layers. There was a significant difference in injury severity among NEC protocol groups and group A (Kruskal-Wallis, $p = 0.0288$). Group D had the lowest mean score for intestinal injury (26.66).

The amount of metabolically active cells, measured by Ki-67, is shown in Fig. 4. A mean of 95% cell activity rate was observed in rats in group A. Group E exhibited the lowest proliferative index (a mean of 38%) (Kruskal-Wallis, ($p = 0.017$)). Among the groups submitted to the protocol that received interventional

drugs, group D (administration of sildenafil and l-arginine) had the highest rate of cellular activity (with a mean of 68.33%).

Discussion

Formula feeding, hypoxia and hypothermia are considered risk factors for the development of NEC. The mortality rate of the disorder varies from 10 to 50% among newborns treated in neonatal intensive care units 9,10. In this study, the mortality rate after NEC induction in neonatal rats was 35%, compared with a 38% mortality rate observed by other authors who used the same experimental model¹¹.

Contrary to expectations, group C, which received only l-arginine, obtained the highest mortality rate and the lowest survival rate in relation to the other groups. It is difficult to determine which mechanisms are responsible for the harmful effect of l-arginine. We wondered if the substrate supply for NO synthesis, with its consequent increased production, would be the aggressor factor of tissue injury.

Group B, which only received sildenafil, had the lowest mortality rate. On the other hand, group D had the highest survival rate among NEC groups, which concomitantly received sildenafil and l-arginine. This difference could be due, above all, to the increase in cGMP intracellular levels, which is responsible for stimulating NO production.

We observed that the final body weight of the groups submitted to the NEC protocol was significantly lower in comparison to the negative control group, which received maternal milk. Other authors have obtained similar results, showing weight loss among animals fed formula milk^{9,11}. An exception is a study by Dvorak et al.⁹, that detected an increase in bodyweight in the groups submitted to the NEC protocol.

Regarding the degree of histological injury, the results were similar to those reported in other experimental studies. The NEC induced microscopic changes were such as villous flattening, reduction in the number of villi and thinning of the intestinal wall^{12,13}. As shown in Fig. 5, our study did not find villous edema, edema in layers, separation of layers, and peeling of the villi as other studies^{9,14}. The probable explanation for this fact would be the immediate fixation of the jejunum and the terminal ileum in 10% buffered formaldehyde, which we performed shortly after the removal of the material. This fixation could have avoided slide artifacts, which could have been considered as injury findings in other studies.

We observed that the intestinal injuries presented high intensity and structural damage in the animals of group E, which were submitted exclusively to the conditions of hypoxia, formula milk, and hypothermia. Hence, the accuracy of the protocol in inducing NEC was confirmed.

The experimental model also demonstrated a higher proliferative index in the groups that received the interventional drugs compared with the positive control group. However, the use of sildenafil and l-arginine has not been able to prevent the development of NEC. One hypothesis is that the intestine of newborns rats would be unable or immature to synthesize NO. Another rationale is that there might not be

enough eNOS available nor able to synthesize NO when the supply was high. Sukhotnik and Cols found that exposure of rats to l-arginine does not prevent the intestine from ischemic damage, but reasonably accelerates the repair of the damaged intestinal mucosa^{15,16,17}. Another study showed that l-arginine is not able to inhibit the occurrence of intestinal injuries produced by hypoxia/hypothermia. It has rather shown a higher level of NO and a lower degree of morphological changes in the intestinal wall of animals⁸.

The present study also observed whether there would be any additive effect when concomitantly administering sildenafil and l-arginine to neonatal rats. As verified through the results of group D, we can affirm that there was a reduction in intestinal injury (with a mean of 26.66) and a higher proliferative rate (68.33%) compared with the other groups submitted to the protocol. However, such a reduction was not statistically significant. Therefore, it would not justify the association of substances.

In conclusion, results from the present study demonstrate that the administration of sildenafil and l-arginine is not able to prevent the occurrence of intestinal injuries produced by hypoxia, formula feeding, and hypothermia. Nevertheless, we have evidenced a reduction in the morphological changes in the intestinal wall of animals that received both substances concomitantly. The intervention drugs likely play a decisive role in the number of metabolically active cells, considering evidence from the groups that received the drugs in isolation or combination.

Our study is relevant both for its innovative character of testing sildenafil as a preventive strategy for NEC, as well as for adding new data on l-arginine literature. We were also able to prove the reproducibility of the NEC protocol and its effectiveness in inducing the disease. However, further studies are needed to better understand the highest mortality and lowest survival rate in the group with isolated use of l-arginine.

Methods

Evaluation by Ethics Committee on animal experimentation

All procedures were approved by the Animal Use Ethics Committee of the Federal University of Paraná (CEUA – UFPR), under number 1249 and verification code 1728778. All methods were performed in accordance with the relevant guidelines and regulations. This study follows the recommendation in the ARRIVE guidelines.

Experimental model

The experimental NEC model was performed based on the protocol described by Jilling et al. and further adapted by Gonçalves et al.^{18,19}. Neonatal rats were fed animal formula milk (Esbilac®, PetAg, Hampshire, IL, USA) at 0,1mL every four hours for 24 h. Feedings were instituted employing an orogastric tube, adapted from a peripherally inserted central catheter (PICC, 1. 9Fr 26G, 1 lumen, BDs, Sandy, UT, USA). The newborns were placed on a heated surface for the introduction of the orogastric tube at each

feeding. Hypoxia was accomplished by stressing the animals with a nitrogen atmosphere (100% N₂) for 70 seconds in an anoxic acrylic chamber. Then, neonatal rats were subjected to hypothermia (4°C for 10 minutes). This procedure was performed twice a day, at 9 pm and 9 am, for three days²⁰.

Experimental design

Forty newborn rats (*Rattus norvegicus*) from the Wistar strain were used. They were divided into five groups:

Group A - negative control group – 5 rats, maternal milk-fed (without NEC);

Group B – 9 rats, fed 0.1 mL of Esbilac® formula milk every 4 hours plus 0.05 mL of intraperitoneal sildenafil every 12 hours;

Group C – 9 rats, fed 0.1 mL of Esbilac® formula milk every 4 hours plus 0.1 mL of intraperitoneal L-arginine (10%) every 12 hours;

Group D – 9 rats, fed 0.1 mL of Esbilac® formula milk every 4 hours plus 0.1 mL of intraperitoneal L-arginine (10%) every 12 hours plus 0.05 mL of intraperitoneal sildenafil every 12 hours;

Group E - positive control group – 8 rats, fed 0.1 mL of Esbilac® formula milk every 4 hours (with NEC) plus 0.1 mL of intraperitoneal saline solution every 12 hours.

The neonatal rats in groups B, C, D, and E were removed from maternal contact at 24 hours of life. They were placed in a compartmented box under thermal control of 36–38°C, fed Esbilac® formula milk every 4 hours (beginning at 4 pm), and submitted to hypoxia and hypothermia twice a day (9 pm and 9 am) for three days. The groups submitted to the NEC protocol (B, C, D, and E) were weighed on all experiment days at 4 pm and immediately before euthanasia. Group A was weighed only once, immediately before euthanasia. The animals of all groups were euthanized at the same time on the fourth day of the experiment, 6 hours after the last hypoxia/hypothermia event.

Sample processing

The experiment was terminated on the fourth day, and animals were euthanized by decapitation. The jejunum was obtained 6 cm from the pylorus, and the terminal ileum was obtained 2 cm from the ileocecal valve. Both organs were removed from each group, then fixed in 10% buffered formaldehyde for histopathological and immunohistochemical analysis.

Histopathological scoring of slides

Each slide was examined by a blinded observer under an optical binocular microscope. A standard analysis of the slides was performed to assess tissue damage levels, according to the protocol by Dvorak et al. and modified by Granger et al.^{21,22}. During the microscopic evaluation, three histological parameters were measured: villous flattening, reduction in the number of villi and thinning of the intestinal wall, of mild, moderate, and severe intensity. A score was assigned for each injury, and an

added score was calculated at the end of each investigation. The score ranged from 0 to 36 points. Score 0 represented intact villi, while 36 represented maximum villi injury. The score was calculated as follows: A: no signs of injury: 0 - zero; B, villous flattening: mild - 2, moderate - 4 or severe - 6; C, reduction in the number of villi: mild - 8, moderate - 10 or severe - 12; D, thinning of the intestinal wall: mild - 14, moderate - 16 or severe - 18. NEC was considered present in animals with histological scores > 18. Scores were recorded separately, and arbitrary values were assigned according to the signs of injury. Then, an average score was calculated for each group²⁰.

Immunohistochemistry analysis of Ki-67

5- μ m-thick section intestinal samples were dewaxed in xylol and rehydrated with alcohol and running water solutions. Antigenic recovery was performed in citrate buffer at 90°C for 15 minutes. Endogenous peroxidase was blocked with two baths of hydrogen peroxide for 15 minutes. Nonspecific protein sites were also blocked according to technical specifications. The primary antibody was the anti-Ki-67 monoclonal antibody, dilution 1: 100, MIB1 clone, refrigerated overnight. Subsequently, the sections were washed and incubated with Polyvalent biotinylated antibody (LSAB kit) and streptavidin- peroxidase (LSAB kit). Washing happened for 30 minutes at room temperature. After washing, samples were stained with diaminobenzidine (DAB) and counterstained with hematoxylin solution (Mayer's Protocol). Finally, slides were mounted using an Erv-Mount solution. Positive control was used. Negative control was obtained by replacing the primary antibody, incubating slides in a phosphate-saline buffer. Only active cells with brownish nuclear staining were regarded as positive. The results were evaluated considering the percentage of cells stained in the nuclei in relation to the total number of cells in the proliferative zone of the intestinal mucosa, which is illustrated in Fig. 6. This zone consists of the interface between villi and crypts²³.

Statistical analysis

Quantitative variables were expressed as means and standard deviations. Kruskal-Wallis test was used to analyze mortality, survival, body weight, intestinal injury score and Ki-67 proliferation index for independent samples.

Declarations

Author contributions statement

G.A.M, C.G.F. and A.I.B.S.D. conceived the experiment; G.A.M, S.M.S.C.C., M.L.C.F. and S.O.I. conducted the experiment; G.A.M, C.G.F. and A.I.B.S.D analysed the data; G.A.M and C.G.F conducted the critical analysis, and G.A.M wrote the manuscript. All authors reviewed and approved the manuscript.

Additional information

The authors declare no competing interests.

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Figures

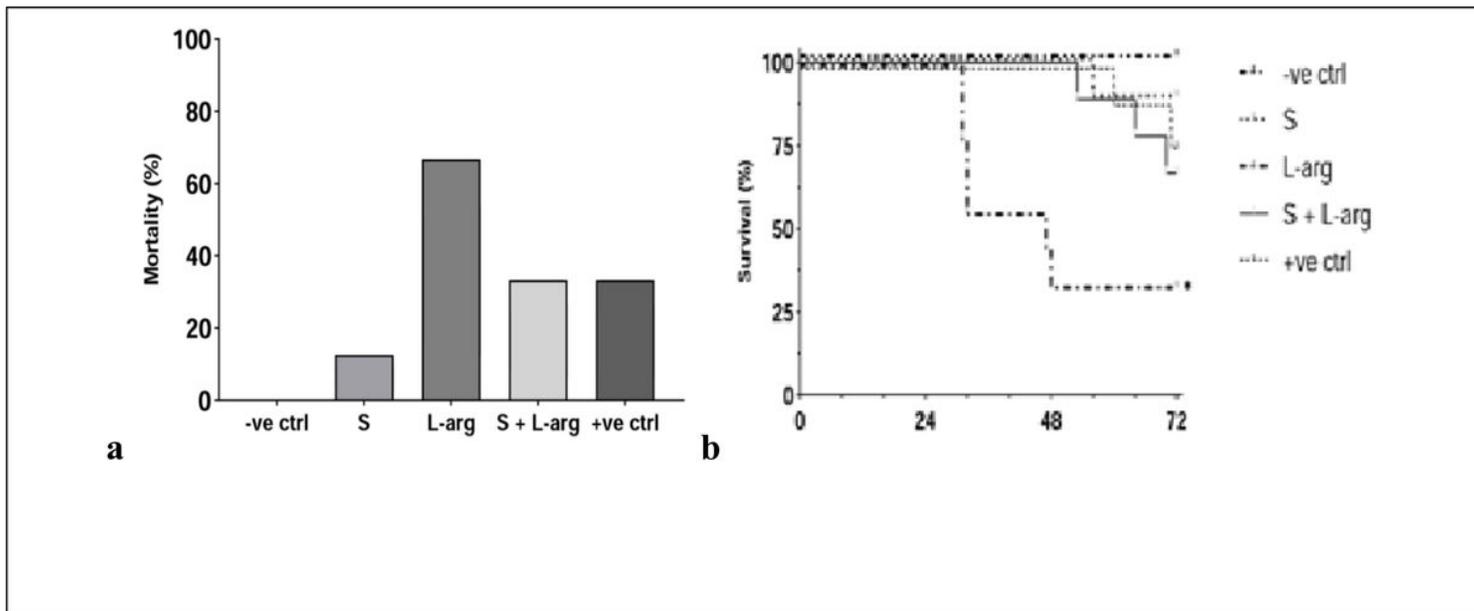


Figure 1

Mortality and Survival a) Percentage of deaths per group over total deaths. b) Kaplan Meier curves with the mean lifespan per group (hours), from the beginning to the end of the experiment. Groups: A - negative control (-ve ctrl), B - sildenafil (S), C - l-arginine (L-arg), D - sildenafil and l-arginine (S + L-arg) and E - positive control (+ve ctrl) (Kruskal- Wallis, $p=0.0293$).

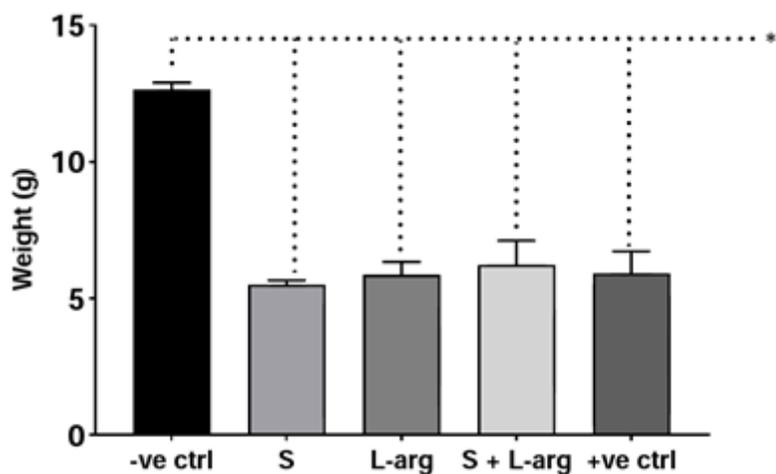


Figure 2

Mean body weight of the groups at the end of the experiment Groups: A - negative control (-ve ctrl), B - sildenafil (S), C - l-arginine (L-arg), D - sildenafil and l-arginine (S + L-arg) and E - positive control (+ve ctrl) (Kruskal- Wallis, $p=0.0088$).

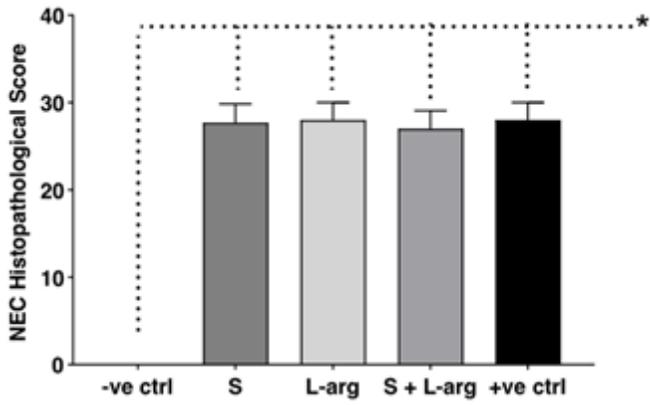


Figure 3

Mean histopathological injury score of the groups Groups: A - negative control (- ve ctrl), B - sildenafil (S), C - l-arginine (L-arg), D - sildenafil and l-arginine (S + L-arg) and E - positive control (+ ve ctrl) (Kruskal-Wallis, $p=0.0288$).

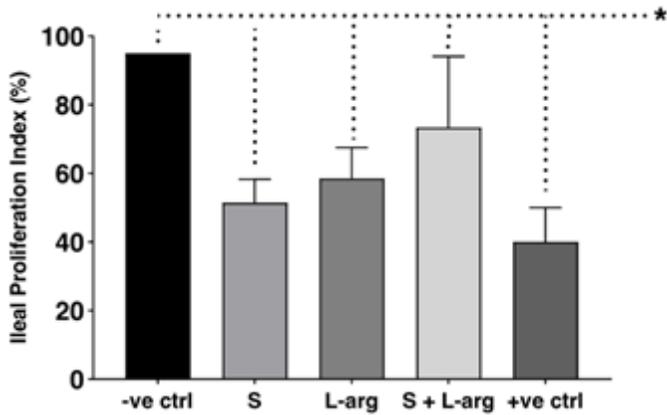


Figure 4

Mean Proliferative Index of Groups Groups: A - negative control (- ve ctrl), B - sildenafil (S), C - l-arginine (L-arg), D - sildenafil and l-arginine (S + L-arg) and E - positive control (+ ve ctrl) (Kruskal-Wallis, $p=0.0017$).

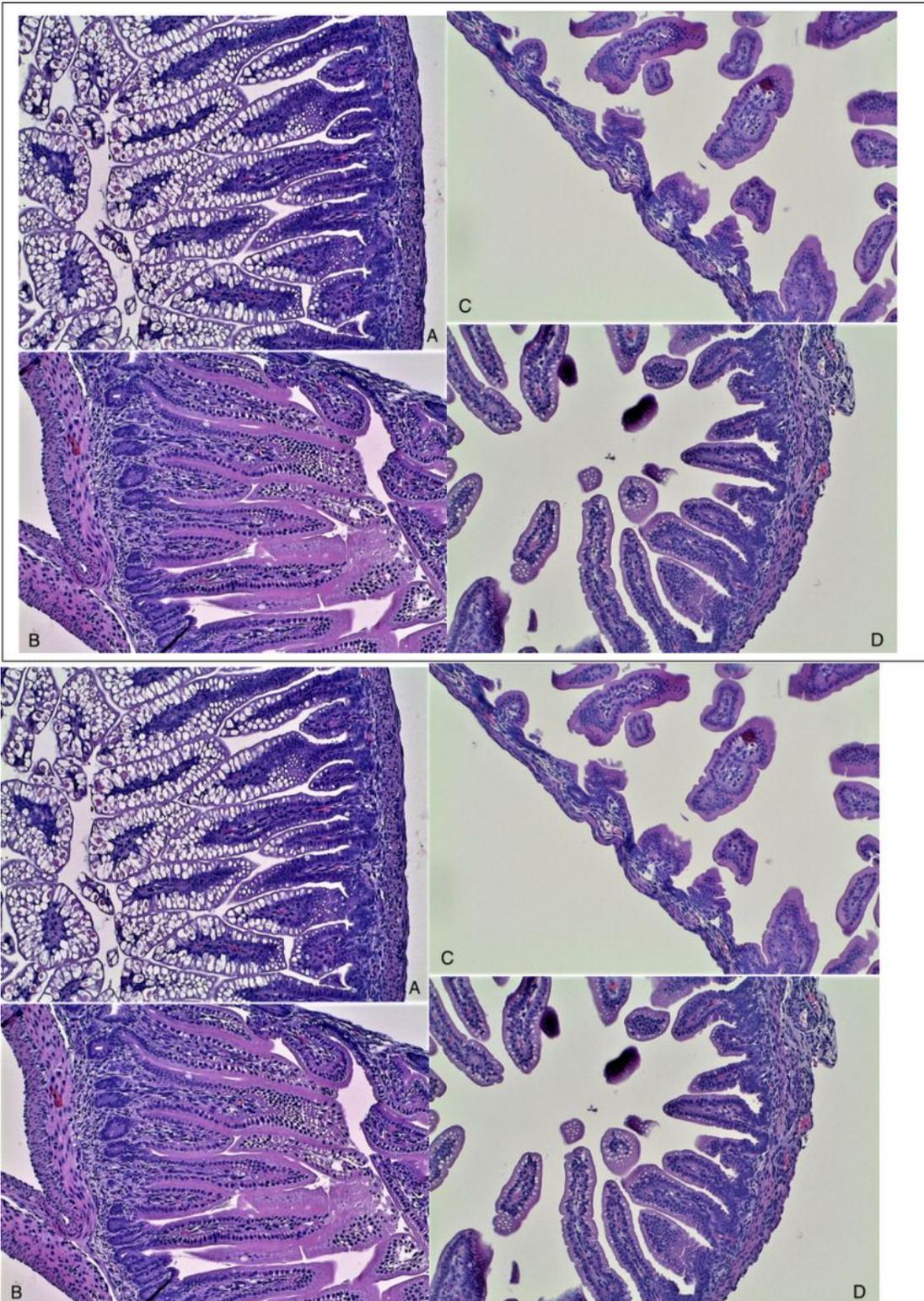
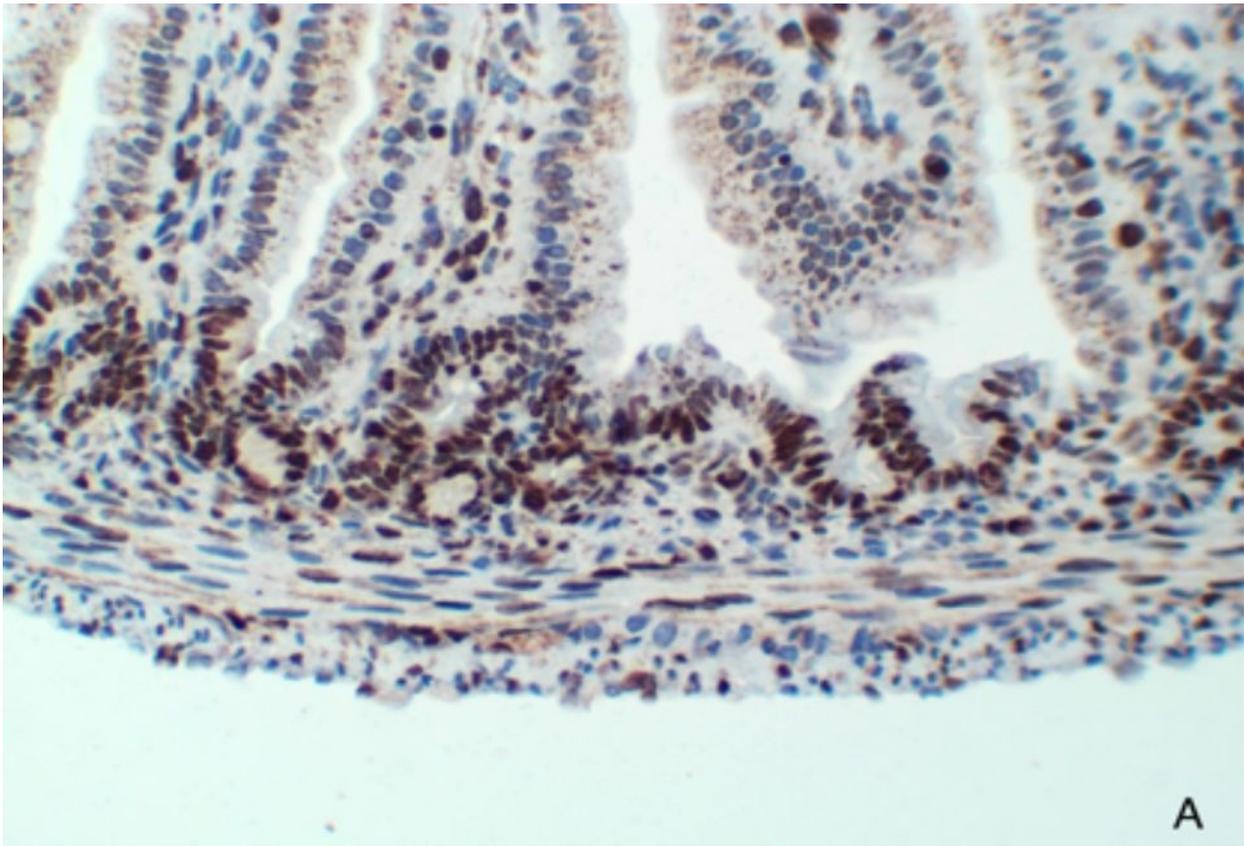
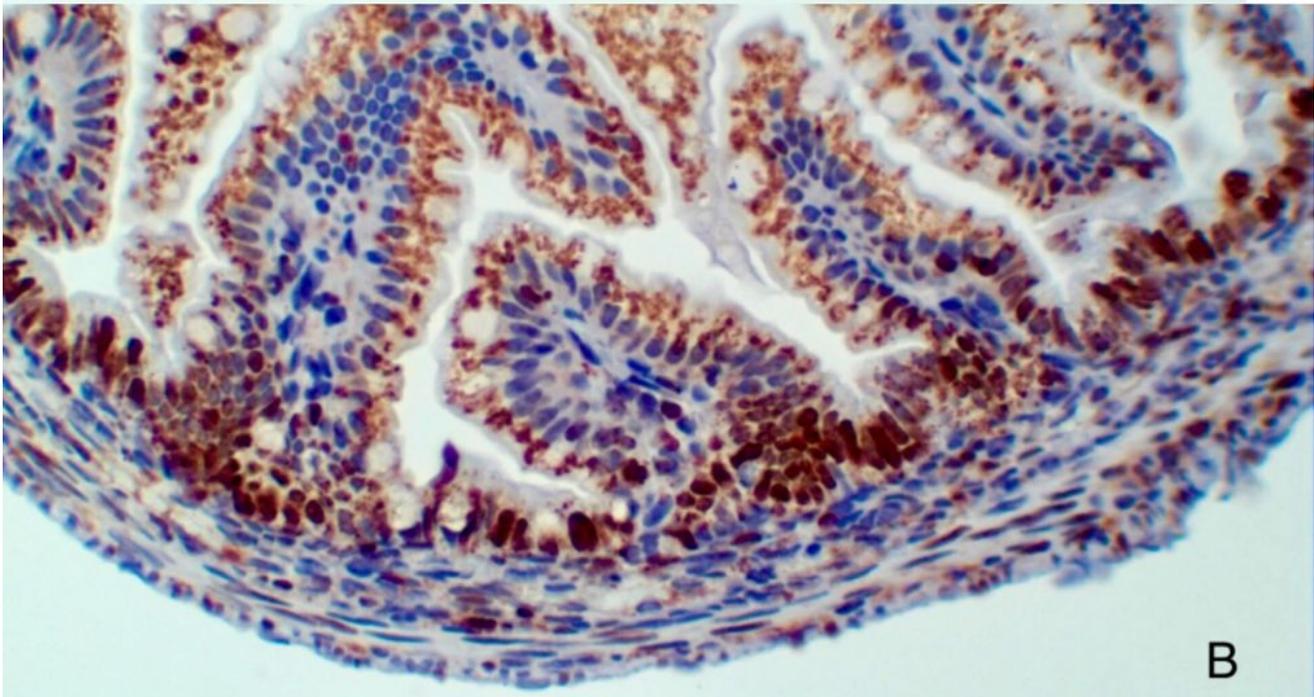


Figure 5

Histopathological analysis of NEC intestinal injuries A) Negative control group - normal villi: normal length and appearance. B) Negative control group - Intact and preserved intestinal wall. C) Group submitted to the NEC protocol - villous flattening, reduction in number of villi and thinning of the intestinal wall. D) Group submitted to the NEC protocol – villous flattening and reduction in number of villi.



A



B

Figure 6

Immunohistochemical analysis of Ki-67 A) Group submitted to the NEC protocol - Reduction of the percentage of brown nuclei cells (Ki-67 stained) in relation to the total number of cells in the proliferative zone of the intestinal mucosa. B) Negative control group - Higher percentage of brown nuclei cells (Ki-67 stained) in relation to the total number of cells in the proliferative zone of the intestinal mucosa.