Journal of Pediatric Surgery 57 (2022) 183–191

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg.org

Comparative effects of fresh and sterile fecal microbiota transplantation in an experimental animal model of necrotizing enterocolitis

Christian Prado, Mariane Rocha Abatti, Monique Michels, Emily Córneo*, Luana Cucker, Heloisa Borges, Rodrigo Dias, Luana Bezerra Rocha, Felipe Dal-Pizzol, Cristiane Ritter

Laboratory of Experimental Pathophysiology, Graduate Program in Health Sciences, University of Southern Santa Catarina (UNESC), Criciúma, SC, 88806-000, Brazil

ARTICLE INFO

Article history:
Received 19 July 2021
Revised 1 December 2021
Accepted 8 December 2021

Keywords:
Necrotizing enterocolitis
Fecal microbiota transplant
Sterile
Probiotic

ABSTRACT

Introduction: Necrotizing Enterocolitis (NEC) is a serious intestinal disease that affects premature neonates, causing high mortality, despite the technological development in neonatal intensive care, with antibiotics, parenteral nutrition, surgery, and advanced life support. The correction of dysbiosis with fecal microbiome transplantation (FMT) has shown beneficial effects in experimental models of the disease. The different forms of administration and conservation of FMT and mixed results depending on several factors lead to questions about the mechanism of action of FMT. This study aimed to compare the effectiveness of fresh, sterile FMT and probiotic treatment under parameters of inflammation, oxidative stress, and tissue damage in a neonatal model of NEC.

Methods: One-day-old *Wistar* rats were used to induce NEC model. Animals were divided in five groups: Control + saline; NEC + saline; NEC + fresh FMT; NEC + sterile FMT and NEC+ probiotics. Parameters of inflammatory response and oxidative damage were measured in the gut, brain, and serum. It was also determined gut histopathological alterations.

Results: Proinflammatory cytokines were increased in the NEC group, and IL-10 levels decreased in the gut, brain, and serum. Fresh and sterile FMT decreased inflammation when compared to the use of probiotics. Oxidative and histological damage to the intestine was apparent in the NEC group, and both FMT treatments had a protective effect.

Conclusion: Fresh and sterile FMT effectively reduced the inflammatory response, oxidative damage, and histological alterations in the gut and brain compared to an experimental NEC model.

1. Introduction

Necrotizing Enterocolitis (NEC) is a multifactorial disease that affects the gastrointestinal tract of newborns, causing partial or complete necrosis [1,2]. It is a pathology mainly of the premature, reaching 10% of newborns weighing less than 1500 gs, from which 20 to 30% do not survive [3–6]. The overall incidence of NEC is approximately 1 in 1000 live births, and its incidence is inversely related to birth weight and gestational age. It affects up to 10% of infants weighing less than 1500 g [7]. Additionally, at school age, the motor functions and intelligence of many children with NEC were borderline or abnormal. Specifically, attention and visual perception were impaired, suggesting that the gut and the brain could be indirectly affected [8].

Dysbiosis is related to NEC development [9–12]. Other factors can contribute to it: episodes of ischemia-reperfusion due to hemodynamic instability of the newborn, bacterial colonization, and exposure to artificial formulas [1]. Inflammatory mediators play a critical role in the development of NEC, increasing the permeability of the intestinal membrane, allowing the translocation of bacteria and toxins, leading to the collapse of the integrity of the intestinal mucosa [13]. The rupture of the epithelial barrier results in apoptosis, increased production of cytokines, toxins, and bacterial products [4].

The microbiota has the function of producing metabolites that positively affect the host, including anti-inflammatory and antioxidant activity, regulation of intestinal barrier function, and participating in the development and maintenance of the gut's immune, sensory, and motor functions [14]. The normal microbiota confers health benefits, and a disruption of this balance (unbalanced diet, infection, illness, and use of antibiotics) can cause dysbiosis that confers susceptibility to diseases [15].

* Corresponding author at: UNESC – Universidade do Extremo Sul Catarinense, Av. Universitária, 1105 – Bairro Universitário, Criciúma - SC, CEP: 88806-000.
E-mail address: emilycorneo@hotmail.com (E. Córneo).

https://doi.org/10.1016/j.jpedsurg.2021.12.013
0022-3468/

Fecal microbiota transplantation (FMT), a strategy in which feces are transferred from healthy patients to patients with dysbiosis to balance the intestinal flora, has been used to treat recurrent pseudomembranous colitis [9]. Furthermore, FMT has been studied in multiple diseases, from inflammatory diseases or intestinal motility [16], obesity [17], non-alcoholic liver steatosis [18], type 2 diabetes [19], and NEC [20, 21]. The changes in the microbiome proposed by the transplant are not permanent if no interactions with the host perpetuate them. In this context, the FMT may have an adjuvant role in the therapeutic proposals understudy for these pathologies [22].

Although already recognized as the second line of treatment for pseudomembranous colitis, FMT still lacks standardization in performing the technique [9,23]. Some studies show that FMT has more beneficial effects than probiotics already used in the clinic. They decrease the inflammatory response in necrotizing enterocolitis, both locally and at the systemic level [18,22]. Data in the literature suggest that the transplantation mechanism is related to other products of bacterial metabolism and substances present in feces, such as short-chain fatty acids, and not exclusively by the microbiome's interaction with the intestinal mucosa [24].

Therefore, based on the importance of this therapeutic strategy, a simple methodology for FMT sterilization by ultraviolet radiation was proposed to ensure safer administration but preserving its protective effects in an experimental model of NEC. We hypothesized that FMT sterilization was not inferior to fresh FMT protecting different aspects of NEC pathology in an animal model.

2. Methods

The experimental procedures involving animals were performed following the National Institutes of Health (Bethesda, MD, USA) Guide for Care and Use of Laboratory Animals and with the approval of our institutional ethics committee (protocol number:68/2020).

2.1. FMT sterilization method standard

Feces from five healthy 2-month-old male rats were removed from the cecum and used for FMT standardization. Using adult feces aims to provide a mature microbiota, differently from the obtained from newborn animals. For standardization, 1 g of fecal material was homogenized in 10 ml of sterile PBS, filtered, and centrifuged for 30 s at 3000 r.p.m. An equivalent of 3×10^8 cells (Optical density of 0,5) in 100μL solution was used for the procedure described previously [24].

2.1.1. Standardization of the sterilization process

Feces homogenized in sterile PBS (1 g/10 ml) were placed in different Petri dishes and subjected to varying times of ultraviolet light (U.V.) 245 nm - 280 nm: 30 min, 1, 1.5, 2, 3, and 4 h. At the end of the U.V. exposition, fecal content was seeded in Mueller Hinton (aerobic) (KASVI - Brazil) or Macconkey (anaerobic) medium, and bacterial growth was evaluated for up to 48 h under these conditions. Samples were incubated in duplicate with $n = 5$.

Bacterial multiplication reaches its peak of proliferation in 48 h, and the accumulation of bacteria in a single locus of the plaque makes the colony visible. Colony counting was performed, and results were expressed in colony-forming units (CFU)/plate. After a calculation was based on the formula below and the result was expressed in CFU/ml.

$$\text{Mean (duplicate) x } \frac{1}{\textit{dilution level}} \times \frac{1}{\textit{volume aliquot}}$$

2.2. Animal model

Ninety newborns (one-day-old) Wistar rats from our breeding colony were used. Of the 90 newborn Wistar rats, 80 were separated from their progenitors on the first day of life and submitted to NEC (see below). Another ten animals were breastfed naturally and were used as a control group.

2.3. NEC induction

NEC induction was described by Barlow et al. [25] and Besner et al. [2] and included different stimuli (feeding with artificial hyperosmolar formula, hypoxia, hypothermia, and endotoxin exposition). First, LPS was administered (a single 2 mg/kg dose by gavage) 24 h after birth. The animals were then submitted twice a day to hypoxia and hypothermia. Briefly, animals were exposed to hypoxia in a plexiglass chamber infused with 100% nitrogen for 60 s, followed immediately to the exposition to 4 °C for 10 min. During the protocol, animals were fed exclusively with artificial formula (Similac 15 g diluted in 75 ml of Max Milk from Total Food), with 200 Kcal/kg/day divided into six doses/day. The rats were fed with 0.4 ml of the formula every 4 h until the third day of life. After NEC induction, animals were killed and organs isolated for subsequent analyses.

2.4. Fecal microbiota transplant (FMT)

For the FMT, 1 g of fecal material from the cecum of a healthy adult rat was homogenized in 10 ml of sterile PBS, filtered, and centrifuged for 30 s at 3000 r.p.m. An equivalent of 3×10^8 cells (Optical density of 0.5) in a 100μL solution was given to each neonatal as a single dose by gavage on the first day. Li et al. and Seekatz et al. [23,26] developed this method adapted to be used in the NEC model in our laboratory [24]. Sterile FMT used feces exposed to UV for 4 h.

2.5. Experimental procedure

Neonates were grouped as follows:

1) Control group (breastfed and maintained with progenitor);
2) NEC + saline;
3) NEC + fresh FMT
4) NEC + sterile FMT
5) NEC + probiotics *Lactobacillus acidophilus* (THT SA- Belgium) + *Bifidobacterium bifidum* (THT SA - Belgium) 10^9 UFC each [30].

All animals received their respective treatments on the first day of life and were fed every 4 h with the formula previously described. On day 2, NEC was induced. Seventy-two hours after it was collected serum, brain, and terminal ileum for analysis of local and peripheral inflammation, in addition to oxidative, nitrosative damage, and tissue damage. It was used $n = 5$ for each of the above-described analyses and was all performed in duplicate.

2.6. Analyses

2.6.1. Survival rate

Animals were followed for up to three days to determine mortality after NEC induction

2.6.2. Histology

Samples of the gut were washed with saline solution and immediately immersed in 4% paraformaldehyde, where they remained for 48 h. After that period, the tissues were removed, placed in 70% ethanol, and stored for further histological analysis.

Tissues were stained with hematoxylin-eosin for subsequent evaluation of tissue damage. The lesion was graded by a score developed by Caplan et al. [27]. In this system, Grade 0 is considered normal, without injury; Grade 1 when there is separation or elevation of the mucosa; Grade 2 when there is edema and lesion of the mucosa up to the level of half of the intestinal villus; Grade 3 when there is necrosis of all intestinal villi and Grade 4 when there is transmural necrosis. All analyses were performed by one of the authors (C.P.) blinded to the group.

2.6.3. Oxidative damage

The formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction was measured in the brain and gut as an oxidative stress index as described previously [28]. The samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% (Sigma-Aldrich) and then heated in a boiling water bath for 15 min. Malondialdehyde (MDA) equivalents were determined by measuring the absorbance at 535 nm in SpectraMax Molecular Devices M2 (San José, Califórnia, EUA) using 1,1,3,3- tetramethoxypropane (Sigma-Aldrich) as an external standard. Results were expressed as MDA equivalents per mg of protein [28].

2.6.4. Myeloperoxidase activity

The tissue was homogenized (50 mg/ml) in 0.5% of hexadecyltrimethylammonium bromide (Sigma-Aldrich) and centrifuged (8765 x g) for 10 min. The suspension was sonicated, and an aliquot of the supernatant was mixed with a solution of 1.6 mmol/L 3,3′,5,5′-tetramethylbenzidine (TMB) and one mmol/L H2O2. The MPO activity was measured spectrophotometrically at 650 nm at 37 °C. The results were expressed as mU/mg protein [29].

2.6.5. Levels of cytokines

Concentrations of IL-1β, IL-10, and IL-6 were determined in serum, brain, and gut by enzyme-linked immunosorbent assay (ELISA) on a microplate reader (Molecular Devices SpectraMAX M2, San José, Califórnia, EUA) using commercial kits (R & D System, Mineápolis, Minnesota, EUA). Briefly, 96-well plates were sensitized with a specific monoclonal antibody incubated overnight. The plates were blocked with 1% albumin, and samples were added to the plate. Specific detection antibodies were added and incubated for two hours. Then, streptavidin peroxidase was added to the plate, and tetramethylbenzidine (TMB) substrate solution was added. The reaction was stopped with the addition of 2 N hydrochloric acid solution (stop solution). At each stage, the plates were washed with a wash buffer. Average detection (pg/ml): IL-1 (0.62 - 4.000); IL-10 (0.62 - 4.000), and IL-6 (1,25–14.000).

2.7. Statistical analysis

The Shapiro Wilk test evaluated the normality of data. Kaplan Meier curve was constructed, and a log-rank test was performed for survival analysis. Data collected were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey post-hoc method and expressed as mean ± standard deviation in Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 21. Graphs were obtained using GraphPad Prism (San Diego, California, USA) version 7. For all comparisons, $p < 0.05$ indicates statistical significance.

3. Results

3.1. Stool sterilization protocol

Fig. 1 shows representative images of stool sterilization in U.V. light for 30 min (A); 1 h (B); 1,5 h (C); 2 h (D); 3 h (E) and 4 h (F).

Table 1
Colony forming unit counting.

	Aerobic conditions		Anaerobic conditions	
U.V Exposure time	Number	CFU/ml	Number	CFU/ml
30 min	>200	2×10^6	>200	2×10^6
1 h	>200	2×10^6	>200	2×10^6
1.5 h	>200	2×10^6	>200	2×10^6
2 h	12 ± 2	1.2×10^7	>200	2×10^6
3 h	5 ± 1	0.5×10^7	4 ± 1	0.4×10^7
4 h	0	0	0	0

Bacterial growth in aerobic conditions remained observable until 1.5 h of exposure to U.V (Table 1). From two to three hours, there was a major decrease in CFU counting, but only after four hours there was no more CFU in growth plates (Table 1)

Fig. 2 shows representative images of FMT after U.V. radiation cultivated in anaerobic conditions. Bacterial growth remained observable until two hours of exposure to U.V (Table 1). After three hours, there was a decrease, but only after four hours, there was no more colony growth (Table 1).

3.2. NEC doesn't change the survival rate of animals

The survival rate was 60% in the NEC group, which was lower when compared to the control group ($p = 0.014$). There is no difference between the other groups that received the treatments (Fig. 3).

3.3. Inflammatory response in serum and tissue from animals submitted to NEC

Systemic inflammation was measured in the serum of neonates submitted to NEC and treated with fresh or sterile FMT or probiotics (Fig. 4). Pro-inflammatory cytokines are increased in the NEC + saline group (IL1:2.17±0.72 pg/ml; IL6:13.4 ± 3.28 pg/ml) and in the group treated with fresh FMT (Fig. 4A and 4B) (IL1: 2.16±0.69 pg/ml [$p = 0.006$]; IL6:10.5 ± 2.66 pg/ml [$p<0.0001$]). In the sterile ECN + FMT group, the increase in IL-6 was also significant (9.01±1.43 pg/ml). There was a decrease in IL-10 levels in the NEC group (0.51±0.16 pg/ml). On the other hand, there is a considerable increase in all treated groups (Fig. 3C) (fresh FMT:1.9 ± 0.86 pg/ml; sterile FMT: 0.99±0.52 pg/ml; and probiotic: 1.16±0.41 pg/ml [$p = 0.0013$]).

MPO activity was measured in the brain of neonates submitted to NEC and treated with fresh or sterile FMT or probiotics. NEC induction increased MPO activity in the brain tissue (0.009±0.004 mU/mg protein) and all treatments reduced it (Fig. 5A) (fresh FMT 0.003±0,002 mU/mg protein; sterile FMT 0.004±0.001 mU/mg protein; and probiotic 0.003±0.001 mU/mg protein [$p<0.0001$]). Furthermore, pro (IL-1 and IL-6) and anti-inflammatory (IL-10) cytokines were measured. Fig. 5B and 45C demonstrated a significant increase in IL-1 (3.84±1.2 pg/ml) and IL-6 (13.25 ± 2.29 pg/ml) in neonates submitted to NEC. IL-1 levels were decreased by both fresh FMT (2.47±1.19 pg/ml) and probiotics (2.55±0.71 pg/ml [$p = 0.0001$]). Furthermore, IL-6 levels decreased after both fresh (7.05±2.5 pg/ml) and sterile FMT (8.38±2.54 pg/ml [$p<0.0001$]). IL-10 was decreased in NEC group (1.00±0.41), and an increase was observed only in NEC + sterile FMT group (2.26±0.49 pg/ml [$p = 0.0013$]).

In the gut, MPO activity was increased in NEC group (0.009±0.003 mU/mg protein), and this was only reversed by fresh FMT (Fig. 6A) (0.005±0.002 mU/mg protein [$p = 0.004$]). IL1 (7.74±1.47 pg/ml) and IL6 (32.69±4.23 pg/ml) increased in NEC group. However, both fresh and sterile FMT were able to decrease IL-1 (fresh FMT: 4.68±1.05 pg/ml; sterile FMT:5.46±0.72 pg/ml

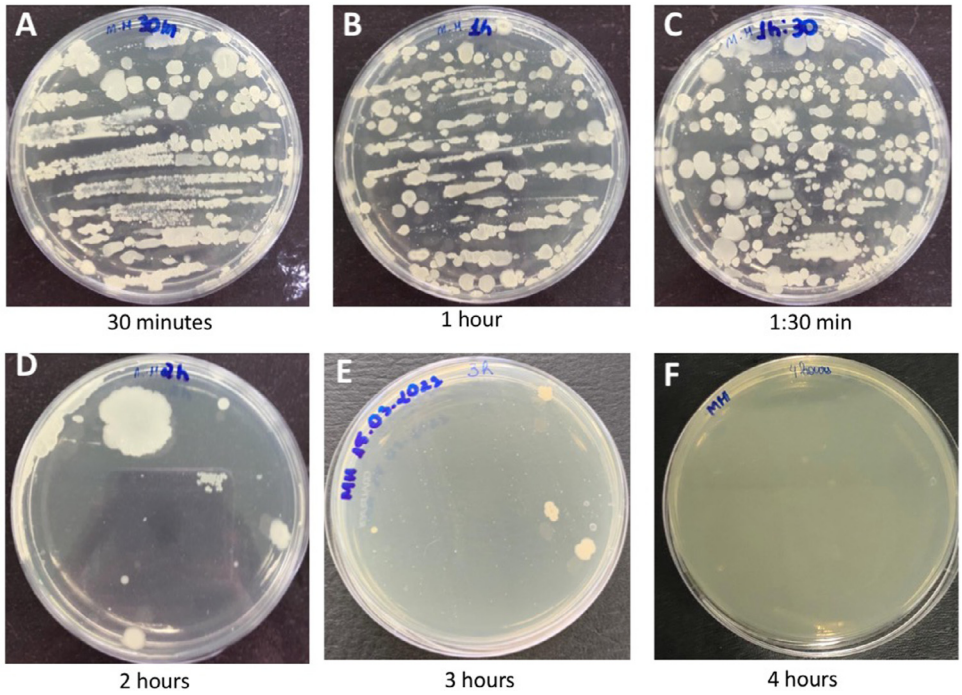

Fig. 1. Representative images of FMT in aerobic conditions. Fecal contents of the gut were removed from a healthy adult rat, the feces were homogenized in sterile PBS. One ml of supernatant was placed in petri dishes and subjected to different times under U.V. At the end of the incubation each sample was plated in Mueller Hinton medium and bacterial growth was evaluated up to 48 h after. 30 min (A); 1 hour (B); 1: 30 min (C); 2 h (D), 3 h (E) and 4 h (F). Samples were incubated in duplicate with $n = 5$.

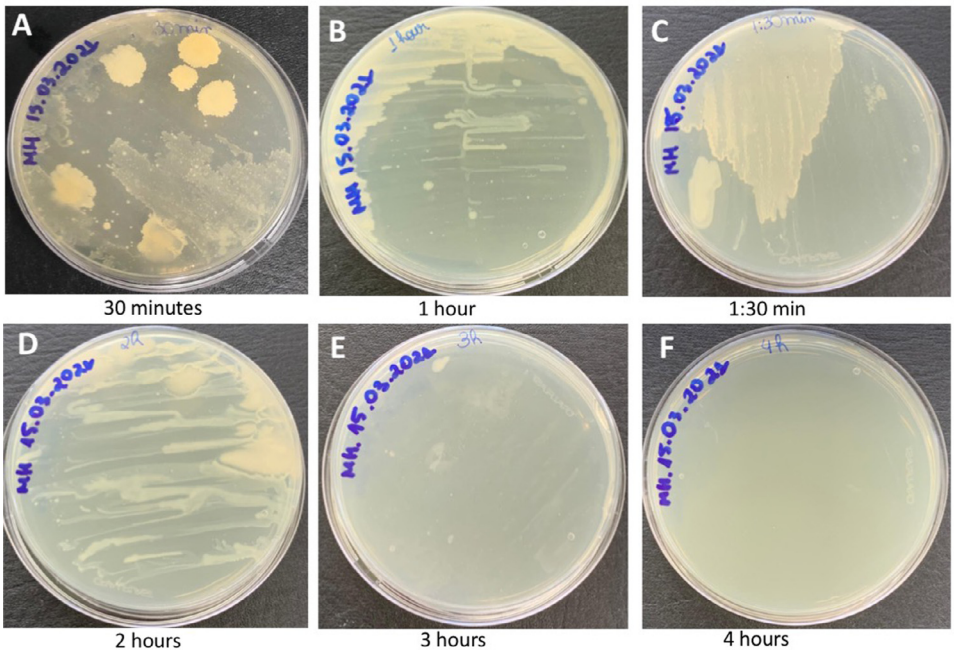

Fig. 2. Representative images of FMT in anaerobiosis conditions. Fecal contents of the gut were removed from a healthy adult rat, the feces were homogenized in sterile PBS. One ml of supernatant was placed in petri dishes and subjected to different times under U.V. At the end of the incubation each sample was plated in Mueller Hinton medium and bacterial growth was evaluated up to 48 h after. 30 min (A); 1 hour (B); 1: 30 min (C); 2 h (D), 3 h (E) and 4 h (F). Samples were incubated in duplicate with $n = 5$.

[$p<0.0001$]) and IL-6 (fresh FMT: 18±2.27 pg/ml; sterile FMT: 24.17±3.98 pg/ml [$p<0.0001$] ±) gut levels (Fig. 6B and 6C). IL-10 levels didn't change in any group in the gut (Fig. 6D) ($p = 0.064$).

3.4. Oxidative stress in tissue from animals submitted to NEC

Oxidative damage and inflammation are simultaneous events, so brain and gut levels of TBARS were measured. There was no change in TBARS levels in the brain after NEC induction of FMT (Fig. 7A) ($p = 0.67$). However, in the gut (Fig. 7B), there was an increase in TBARS levels in NEC group (0.071±0.017 nmol/mg protein), and only in NEC + fresh FMT group (0.034±0.008 nmol/mg protein [$p<0.0001$]) was observed protection against oxidative damage.

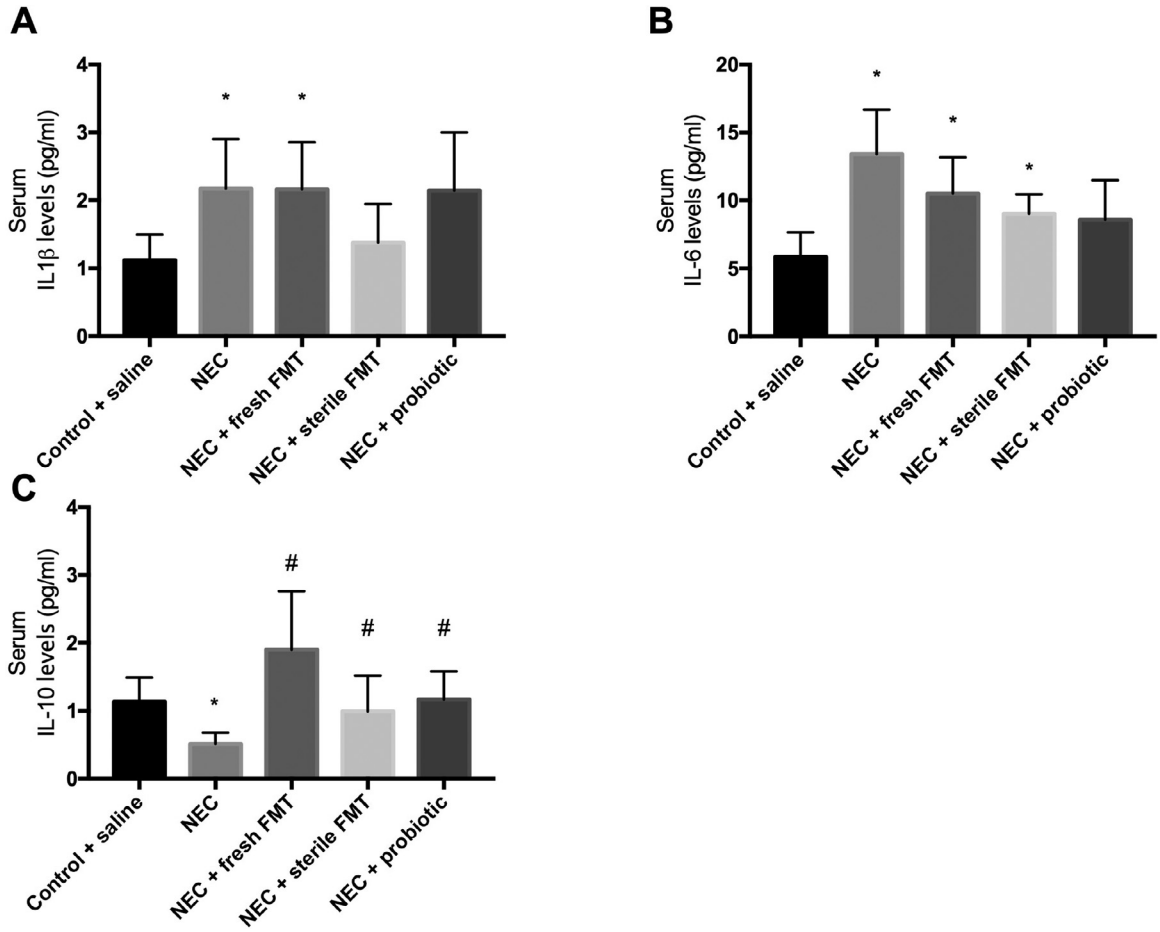

Fig. 3. Interleukin levels IL-1 (A); IL-6 (B) and IL-10 (C) in serum of neonates submitted to NEC and treated with fresh or sterile FMT or probiotics. Samples were analyzed by ELISA and quantified as pg/ml. Data are presented as mean ± SD. $n = 12$ per group. * different from control; # different from NEC. p-value <0.05.

Table 2
Score developed by Caplan et al. (1994).

Lesion Grade				
Control	NEC	NEC + fresh FMT	NEC + sterile FMT	NEC + probiotic
0	3	2	0	2
0	2	1	1	2
0	3	0	0	2
0	3	0	0	1
0	3	0	0	1

3.5. Histological analysis in tissue from animals submitted to NEC

Fig. 8A exhibits representative histological images of the gut from the animals. In the control group, immature and preserved villi were noted, while the villi were shortened and damaged after NEC. Fig. 8B and Table 2 show that the grade of the gut lesion and fresh FMT and sterile FMT effectively reduced histological damage.

4. Discussion

NEC is an inflammatory disease of the gastrointestinal tract characterized by ischemic necrosis of the intestinal mucosa, primarily affecting premature neonates. It is the most common life-threatening emergency involving the gastrointestinal tract of infants in the neonatal intensive care unit. Microbiota transplant is becoming a popular process to restore "healthy" gut microbiota, but its safety and efficacy are not well known in this context. In a recent review, Liu et al., (2020)[30] suggest that the current approaches of microbiota transplant can introduce significant health risk factors to the recipients, and the newborns are especially susceptible. Thus, we demonstrate that sterile FMT was as effective as fresh FMT in an animal model of NEC.

Ultraviolet radiation is a physical process that can be used for the sterilization of different materials. Ultraviolet radiation treatment has a long and efficient history in microbiological control [31]. If used with sufficient intensity and exposure time, ultraviolet irradiation has a microbiocidal effect, finding diverse applications such as air sterilization, equipment surfaces, packaging, and materials. U.V. decontamination exploits different wavelength bands where UVC (200–280 nm) is superior to UVB (280–300 nm) and UVA (320–400 nm). Optimum irreversible molecular damage occurs around the 260 nm wavelength. Wavelengths less than 200 nm are inefficient for sterilization, as the waves are rapidly absorbed by oxygen and water. Ultraviolet irradiations in the 240 to 330 nm range are more efficient as germicide because they are absorbed by proteins and nucleic acids, causing chromosome disruption, genetic mutations, enzyme inactivation, and, consequently, cell death [32]. Theoretically, if sterile FMT did not lose effectiveness, it could be safer for a more widespread clinical application.

FMT is a current therapy that has shown promising results. Studies with FMT are mainly focused on specific gastrointestinal diseases. Few studies have been published on the effect of FMT on NEC, most are still experimental, and recently our group demonstrated the impact of fresh FMT on gut inflammation and oxidative stress [24]. Thus, we tried to determine if these protective ef-

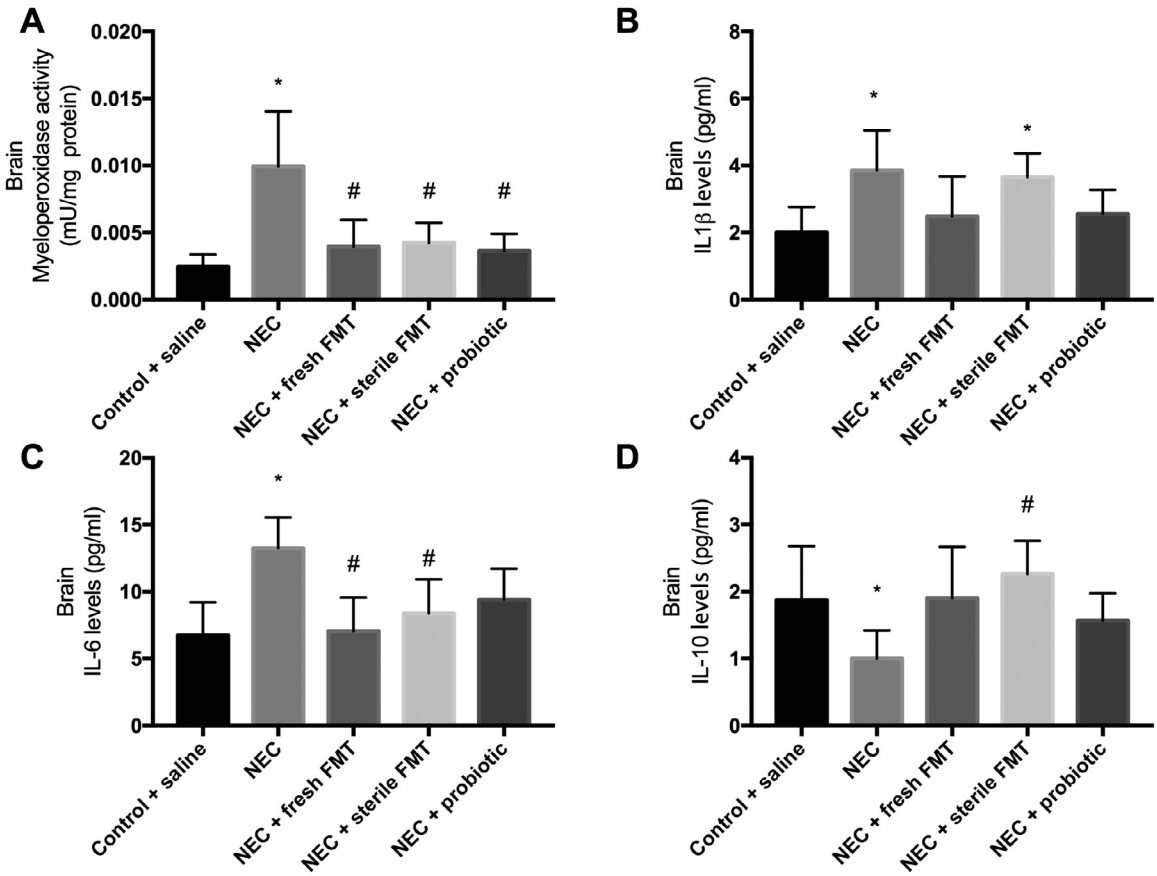

Fig. 4. MPO activity (A) and interleukin levels IL-1 (B); IL-6 (C) and IL-10 (D) in brain of newborns submitted to NEC and tretated with fresh or sterile FMT or probiotics. Samples were analyzed by ELISA and quantified as pg/ml. Data are presented as mean ± SD. $n = 12$ per group. * different from control; # different from NEC. p-value <0.05.

fects were not lost if feces were sterilized by U. V. light. The results presented here suggest that both fresh and sterile FMT have similar protective effects in an animal model of NEC. Inflammation and oxidative stress were measured in the serum, gut, and brain to evaluate the impact of FMT on the gut-brain axis. It is known that pathological alteration of the intestinal microbiota and impaired function of the intestinal barrier, which occurs in NEC, can influence immunity and initiate an inflammatory response and, therefore, reflect on the health and behavior of the host [33]. Both treatments decreased gut and brain inflammatory response and were superior to probiotics, a different approach to modulate microbiota [34]. These results open the perspective to further explore FMT on the treatment or prevention of NEC and other diseases of the neonatal period (for example, neonatal sepsis) and serve as a basis to the proposal of clinical studies in the field.

NEC directly impacts the gut structure, and this is consistent with our results. Histological lesions were detected in the small intestine of neonates with NEC. Although we have not reported here, the number and diversity of the microbiota are also altered in NEC [20], which can trigger a severe inflammatory condition. It has been described those changes in microbiota colonization have a strong relationship with inflammatory bowel [35]. Studies have also shown that the proinflammatory response in the intestinal mucosa is associated with the production of oxidative stress, followed by mucosal permeability and, consequently, bacterial translocation [36,37]. An inflammatory condition generates free radicals since inflammatory cells produce nitric oxide (NO) and hypochlorous acid (HOCL) that can interact and form the hydroxyl radical (OH). Free radicals produced in the tissue generate lipoperoxidation, the formation of aldehydes, and other highly cytotoxic products, which induces an increase in the permeability of cell membrane, leading to tissue damage in the gut and brain. In addition to lipid peroxidation, free radicals can oxidize amino acids, resulting in the formation of oxidized thiol groups, among other changes that alter the function and standard structure of the protein [38]. According to the results, the levels of proinflammatory cytokines are elevated in the context of NEC, suggesting that dysbiosis alter inflammatory response in neonates.

At the phylum level, changes in the microbiota in neonates with NEC are characterized by decreased *Firmicutes* and *Bacteroidetes* and increased *Proteobacteria*, and at the gender level by decreased *Lactobacillus* and *Bifidobacterium* and increased *Gammaproteobacteria* [39,40]. Correcting intestinal dysbiosis can be a strategy to contribute to the recovery of intestinal damage in NEC. The proposed methods of using the FMT promote the restoration of the intestinal microbiota and microbial diversity [18]. Although probiotics of *Lactobacillus* strains protect from infection by preventing colonization and virulence of pathogens [35], the non-consistent role was observed for common probiotic species of *Lactobacillus* and *Bifidobacterium* in NEC [37]. In our results, probiotics had a protective effect on some of the inflammatory parameters measured but had no impact on gut histopathology. However, FMT (fresh and sterile) was generally more protective, at least in the evaluated parameters. Li et al. [20] showed that the levels of *Lactobacillus* and *Bifidobacterium* decreased significantly in mice submitted to the NEC experimental model and only recovered after treatment with FMT. This can be justified because this group of probiotic bacteria is sensitive to environmental changes in the inflammatory phase, but it recovers after FMT administration. In addition, it has been reported that

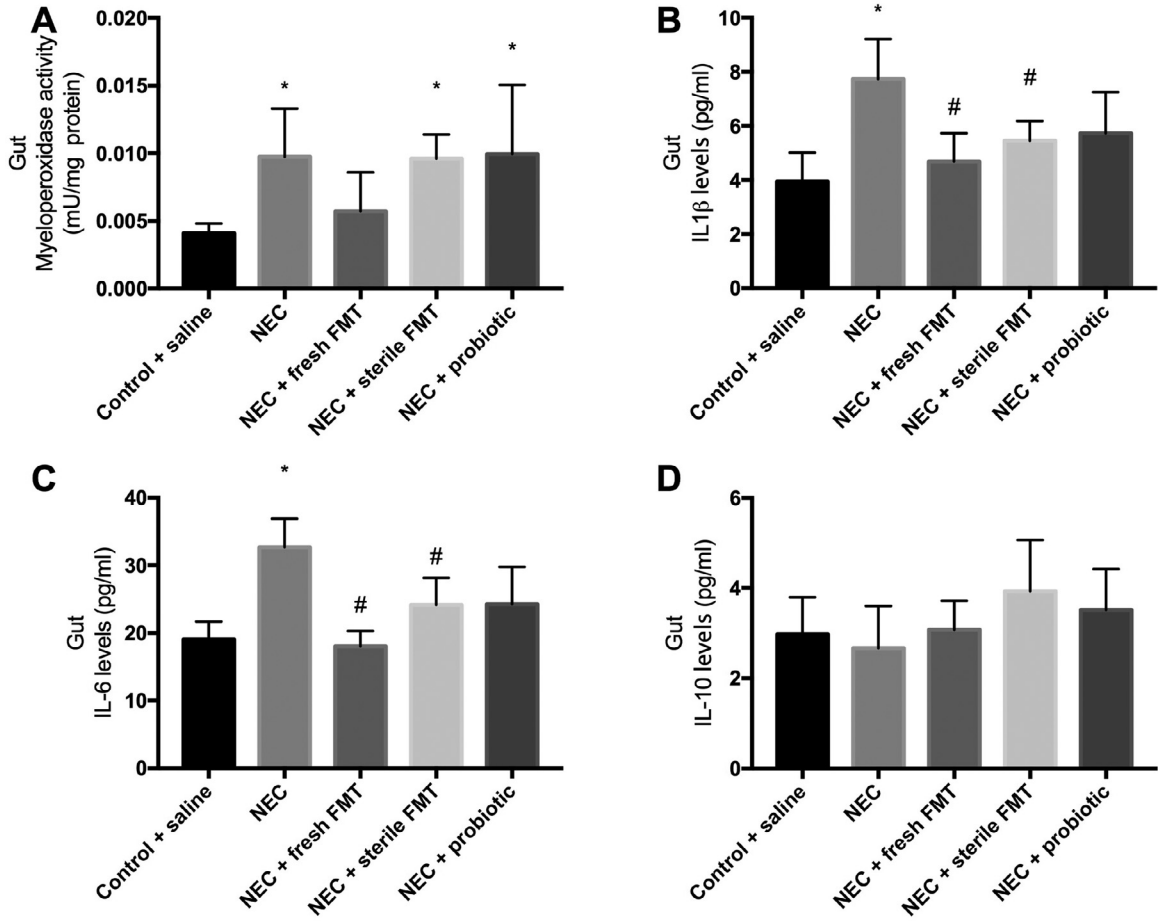

Fig. 5. MPO activity (A) and interleukin levels IL-1 (B); IL-6 (C) and IL-10 (D) in gut of newborns submitted to NEC and tretated with fresh or sterile FMT or probiotics. Samples were analyzed by ELISA and quantified as pg/ml. Data are presented as mean ± SD. $n = 12$ per group. * different from control; # different from NEC. p-value <0.05.

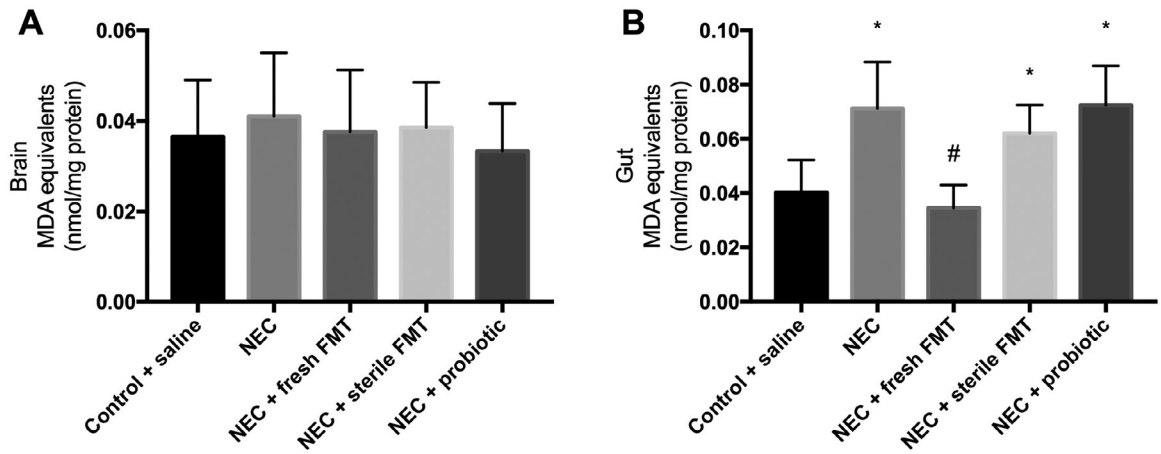

Fig. 6. Oxidative damage to lipids in brain (A) and gut (B) of newborns submitted to NEC and treated with fresh or sterile FMT or probiotics. Samples were analyzed by ELISA and quantified as pg/ml. Data are presented as mean ± SD. $n = 12$ per group. * different from control; # different from NEC. p-value <0.05.

the level of *E. coli* increases after NEC but decreases significantly after treatment with FMT [23]. In addition, FMT has been shown to suppress intestinal apoptosis and bacterial translocation across the intestinal barrier [20,40]. Recently Liu et al. [40] demonstrated that treatment with FMT significantly decreases mRNA expression of IL-6 and TNF-cytokines. In contrast, claudin-7 expression is increased, indicating that FMT can alleviate the severity of NEC by reducing intestinal inflammation and improving the function of the intestinal barrier [40].

Another objective was to evaluate the effect of FMT on brain dysfunction. First, it could have a brain-gut-microbiota axis that plays an essential role in homeostasis and, consequently, in health and disease [41,42]. Second, brain damage could occur during NEC development, which would impact the long-term performance of survivors [8]. Recently, it was demonstrated that cognitive impairments caused by NEC are secondary to activation of Toll-like receptors on brain microglia [43]. This study shows the beneficial effects of fresh and sterile FMT on brain inflammation, which opens

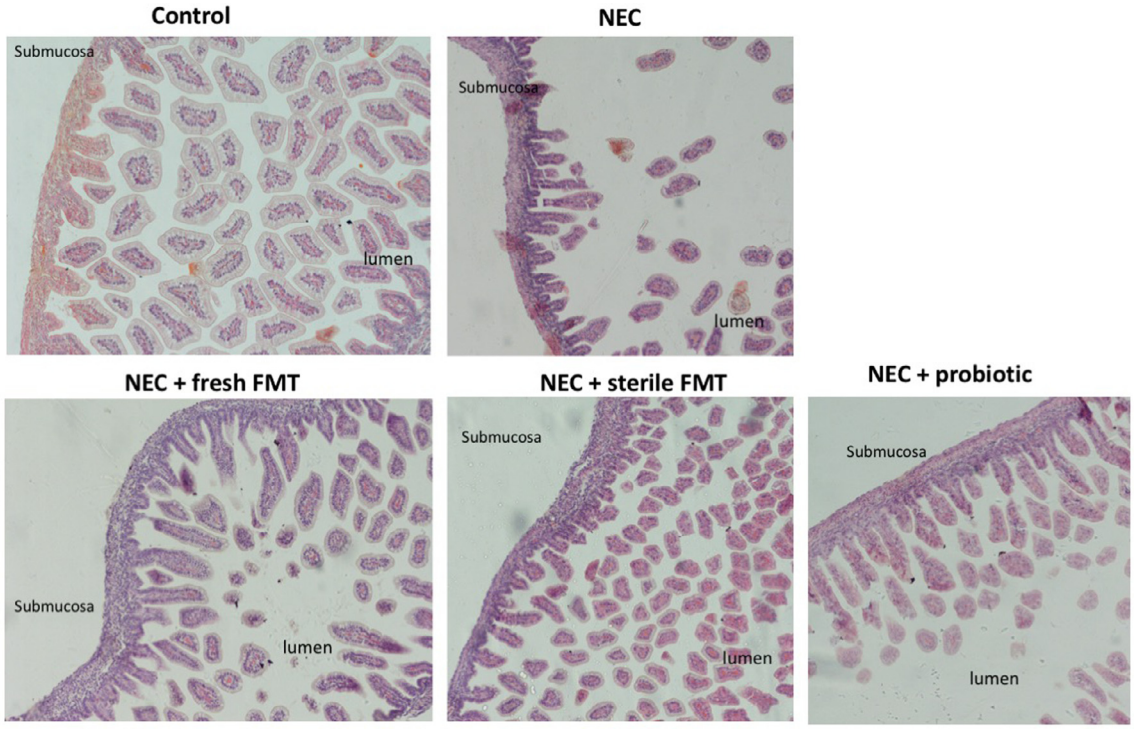

Fig. 7. Representative images of gut of neonates submitted to NEC and treated with fresh or sterile FMT or probiotics. Magnitude of 40x show submucosa, lumen and gut villi. Control group (A); NEC (B); NEC + fresh FMT (C); NEC + sterile FMT (D); NEC + probiotic (E).

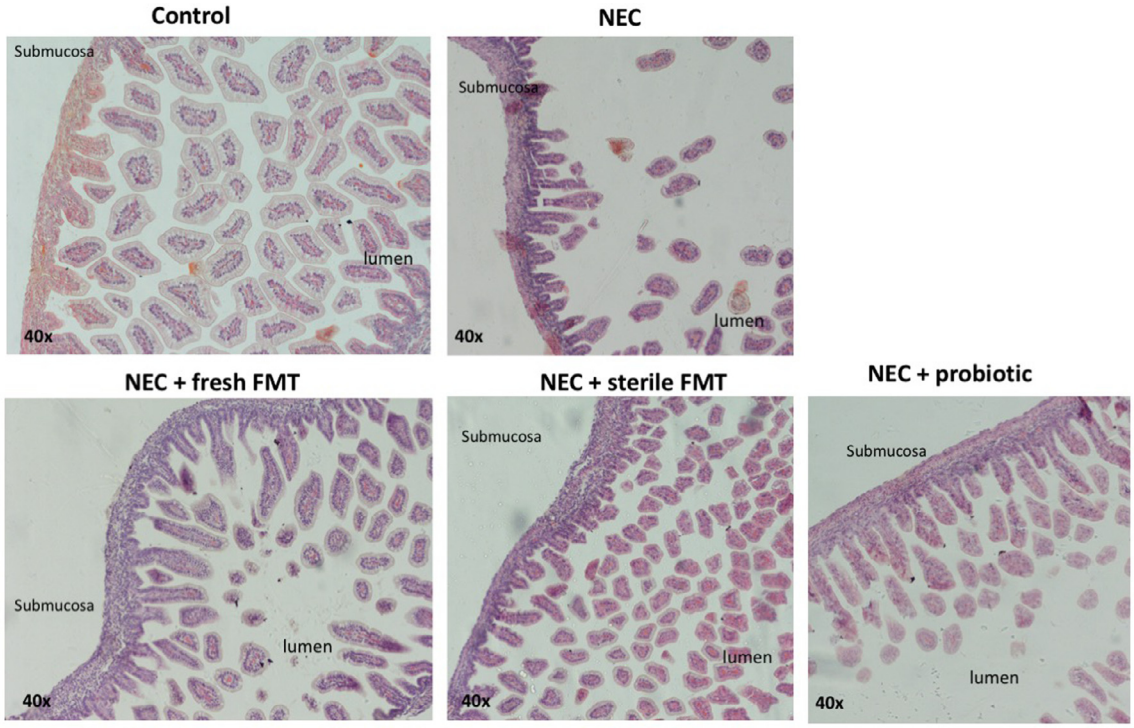

Fig. 8. Histological analysis of tissues from animals submitted to NEC. Representative histological images of the animals' intestine at 40x magnitude. Control group (A); NEC (B); NEC + fresh FMT (C); NEC + sterile FMT (D); NEC + probiotic (E).

the perspective to study FMT both to decrease mortality and morbidity associated with NEC.

Finally, it is worth mentioning that FMT is under investigation by the FDA (*United States Food and Drug Administration*) due to the complexity of the procedure and the insecurity it causes to the patient. However, safety and tolerance of FMT have been reported in adults and children [44,45], including in a recent randomized clinical trial [46]. The regulation remains controversial, but two studies showed that patients reported being willing to receive FMT if it was a medical indication [47,48]. Thus, sterilization of the FMT can be an exciting strategy to approach the patient who

needs use, bringing safety and maintaining the effectiveness of the treatment.

Some limitations should be taken into account when interpreting our results. First, from a mechanistic point of view, it would be essential to determine the impact of FMT on NEC microbiome (for example, using 16 s rRNA sequencing) or measure some metabolites (such as butyric acid) trying to understand better the effects of fresh and sterile FMT on NEC. This should be addressed in further studies. Second, our experimental design allowed us to suggest the use of FMT as a prophylactic measure, and further studies should be performed treating animals with FMT after NEC induction.

Funding

CAPES, CNPQ.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jpedsurg.2021.12.013.

References

[1] De Souza JCK. Cirurgia pediátrica – teoria e prática: enterocolite Necrosante. São Paulo: Editora Roca 2008:385–97.
[2] Besner GE. A pain in the NEC: research challenges and opportunities. J Pediatr Surg 2015;50:23–9.
[3] Högberg N, Stenbäck A, Carlsson PO, et al. Genes regulating tight junctions and cell adhesion are altered in early experimental necrotizing enterocolitis. J Pediatr Surg 2013;48:2308–12.
[4] Bergholz R, Zschiegner M, Eschenburg G, et al. Mucosal loss with increased expression of IL-6, IL-8, and COX-2 in a formula-feeding only neonatal rat model of necrotizing enterocolitis. J Pediatr Surg. 2013;48:2301–7.
[5] Karadag A, Ozdemir R, Kurt A, et al. Protective effects of dexpanthenol in an experimental model of necrotizing enterocolitis. J Pediatr Surg 2015;50:1119–24.
[6] Yu Y, Klemann C, Feng X, et al. Increased inflammatory reaction to intestinal ischemia-reperfusion in neonatal versus adult mice. Eur J Pediatr Surg 2015;25:46–50.
[7] Dominguez KM, Moss RL. Necrotizing enterocolitis. Clin Perinatol 2012;39:387–401.
[8] Roze E, Ta B, van der Ree M, et al. Functional impairments at school age of children with necrotizing enterocolitis or spontaneous intestinal perforation. Pediatr Res 2011;70:619–62.
[9] Rentea RM, Liedel JL, Fredrich K, et al. Enteral intestinal alkaline phosphatase administration in newborns decreases iNOS expression in a neonatal necrotizing enterocolitis rat model. J Pediatr Surg 2013;48:124–8.
[10] Coran AG. Pediatric surgery - seventh edition: necrotizing enterocolitis. Philadelphia: Elsevier; 2012. p. 1187–207.
[11] Burke KE, Lamont JT. Fecal transplantation for recurrent clostridium difficile infection in older adults: a review. J Am Geriatr Soc 2013;61:1394–8.
[12] Wang Y, Hoenig JD, Malin KJ, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3:944–54.
[13] Claud EC. Neonatal necrotizing enterocolitis -inflammation and intestinal immaturity. Antiinflamm Antiallergy Agents Med Chem 2009;8(3):248–59.
[14] Berrington JE, Stewart CJ, Cummings SP, et al. The neonatal bowel microbiome in health and infection. Curr Opin Infect Dis 2014;27:236–43.
[15] Patole S, Keil AD, Chang A, et al. Effect of Bifidobacterium breve m-16v supplementation on fecal bifidobacteria in preterm neonates - a randomised double-blind placebo controlled trial. PLoS ONE 2014;9:e89511.
[16] Jenke AC, Postberg J, Mariel B, et al. S100A12 and hBD2 correlate with the composition of the fecal microflora in elbw infants and expansion of *E. coli* is associated with NEC. Biomed Res Int 2013:150372.
[17] Di Mauro A, Neu J, Riezzo G, et al. Gastrointestinal function development and microbiota. Ital J Pediatr 2013;24 39-15.
[18] Berg D, Clemente JC, Colombel JF. Can inflammatory bowel disease be permanently treated with short-term interventions on the microbiome? Expert Rev Gastroenterol Hepatol 2015;9:781–95.
[19] Jayasinghe TN, Chiavaroli V, Holland DJ, et al. The new era of treatment for obesity and metabolic disorders: evidence and expectations for gut microbiome transplantation. Front Cell Infect Microbiol 2016;19:6–15.
[20] Konturek PC, Haziri D, Brzozowski T, et al. Emerging role of fecal microbiota therapy in the treatment of gastrointestinal and extra-gastrointestinal diseases. J Physiol Pharmacol 2015;66:483–91.
[21] Esteve E, Ricart W, Fernández-Real JM. Gut microbiota interactions with obesity, insulin resistance and type 2 diabetes: did gut microbiote co-evolve with insulin resistance? Curr Opin Clin Nutr Metab Care 2011;14:483–90.
[22] Li X, Li X, Shang Q, et al. Fecal microbiota transplantation (FMT) could reverse the severity of experimental necrotizing enterocolitis (NEC) via oxidative stress modulation. Free Radic Biol Med 2017;108:32–43.
[24] Prado C, Michels M, Ávila P, et al. The protective effects of fecal microbiota transplantation in an experimental model of necrotizing enterocolitis. J Pediatr Surg. 2019;54:1578–83.
[23] He Canxia, Shan Yujuan, Song Wei. Targeting gut microbiota as a possible therapy for diabetes. Nutr Res 2015;35:361–7.
[25] Berg D, Clemente JC, Colombel JF. Can inflammatory bowel disease be permanently treated with short-term interventions on the microbiome? Expert Rev Gastroenterol Hepatol 2015;9:781–95.
[26] Zhou H, Sun J, Yu B, et al. Gut microbiota absence and transplantation affect growth and intestinal functions: an investigation in a germ-free pig model. Anim Nutr 2021;7:295–304.
[27] Barlow B, Santulli TV, Heird WC, et al. An experimental study of acute neonatal enterocolitis - the importance of breast milk. J Pediatr Surg 1974;9:587–95.
[28] Seekatz AM, Theriot CM, Molloy CT, et al. Fecal microbiota transplantation eliminates clostridium difficile in a murine model of relapsing disease. Infect Immun 2015;83:3838–46.
[29] Carlisle EM, Poroyko V, Caplan MS, et al. Gram negative bacteria are associated with the early stages of necrotizing enterocolitis. PLoS ONE 2011;6:e18084.
[30] Zhu X, Yang Y, Gao W, et al. Capparis spinosa alleviates DSS-induced ulcerative colitis via regulation of the gut microbiota and oxidative stress. Evid Based Complement Alternat Med 2021;15:1227876.
[31] Niu W, Dong Y, Fu Z, et al. Effects of molecular weight of chitosan on anti-inflammatory activity and modulation of intestinal microflora in an ulcerative colitis model. Int J Biol Macromol 2021;15:1927–36.
[32] Liu H, Wang HH. Impact of microbiota transplant on resistome of gut microbiota in gnotobiotic piglets and human subjects. Front Microbiol 2020;19:932.
[33] Stannard CJ, Abiss JS, Wood JM. Combined treatment with hydrogen peroxide and ultra-violet irradiation to reduce microbial contamination levels in preformed food packaging cartons. J Food Prot 1983;46:1060–4.
[34] Bayliss CE, Waites WM. The combined effect of hydrogen peroxide and ultraviolet irradiation on bacterial spores. J Appl Bacteriol 2020;47:236–69.
[35] Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nat Neurosci 2017;20:145–55.
[36] Ávila PRM, Michels M, Vuolo F, et al. Protective effects of fecal microbiota transplantation in sepsis are independent of the modulation of the intestinal flora. Nutrition 2020;73:1107–27.
[37] Ganguli K, Meng D, Rautava S, et al. Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation. Am J Physiol Gastrointest Liver Physiol 2013;304:132–41.
[38] Good M, Siggers RH, Sodhi CP, et al. Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. Proc Natl Acad Sci USA. 2012;109:11330–5.
[39] Yazji Ibrahim, Chhinder P, Elizabeth SK, et al. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS–NO–nitrite signaling. Proc Natl Acad Sci USA 2013;110:9451–6.
[40] Barichello T, Machado RA, Constantino L, et al. Antioxidant treatment prevented late memory impairment in an animal model of sepsis. Crit Care Med 2007;35:2186–90.
[41] Liu J, Miyake H, Zhu H, et al. Fecal microbiota transplantation by enema reduces intestinal injury in experimental necrotizing enterocolitis. J Pediatr Surg 2020;55:1094–8.
[42] Repa A, Thanhaeuser M, Endress D, et al. Probiotics (Lactobacillus acidophilus and Bifidobacterium infantis) prevent NEC in VLBW infants fed breast milk but not formula [corrected]. Pediatr Res 2015;77:381–8.
[43] Mu C, Yang Y, Zhu W. Gut microbiota: the brain peacekeeper. Front Microbiol 2016;7:345.
[44] Rea K, Dinan TG, Cryan JF. The microbiome: a key regulator of stress and neuroinflammation. Neurobiol Stress 2016;4:23–33.
[45] Niño DF, et al. Cognitive impairments induced by necrotizing enterocolitis can be prevented by inhibiting microglial activation in mouse brain. Sci Transl Med 2018:10.
[46] Gurram B, Sue PK. Fecal microbiota transplantation in children: current concepts. Curr Opin Pediatr 2019;31:623–9.
[47] Nowak A, Hedenstierna M, Ursing J, et al. Efficacy of routine fecal microbiota transplantation for treatment of recurrent Clostridium difficile infection: a retrospective cohort study. Int J Microbiol 2019;7 395-127.
[48] Kahn SA, Gorawara-Bhat R, Rubin DT. Fecal bacteriotherapy for Ulcerative colitis: patients are ready, are we? Inflamm Bowel Dis 2012;18:676–84.