Effects of maternal ischemic preconditioning in the colon of newborn rats submitted to hypoxia-reoxygenation insult[1]

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ABSTRACT

PURPOSE: To evaluate the effects of maternal remote ischemic preconditioning (IPCr) in the colonic mucosa of newborn rats subjected to hypoxia and reoxygenation.

METHODS: Newborn Wistar rats were divided into three groups. Control Group (CG), Hypoxia and Reoxygenation Group (HRG) and Remote Ischemic Preconditioning Group (IPCrG). Hypoxia and reoxygenation was performed 2x per day, with an interval of 6 hours, on the 1st, 2nd and 3rd days of life, with 10 minutes of CO_2 at 100%, followed by 10 minutes O_2 at 100%(HRG/IPCrG). The maternal IPCr was performed 24 hours before delivery by applying a rubber band tourniquet to the left hind limb (IPCrG). Segments of the colon underwent histological (HE) and immunohistochemical analysis for caspase-3 and COX - 2.

RESULTS: The histological findings showed no intestinal mucosal damage in the CG group and severe lesions in HRG that was attenuated in the IPCrG ($p<0.05$). The expression of the apoptotic cells was lower in the HRG group than in the CG and IPCrG. The COX-2 expression was intense in HRG and attenuated in the IPCrG ($p<0.05$).

CONCLUSIONS: Maternal IPCr protected the colonic mucosa of newborn rats subjected to hypoxia and reoxygenation, reducing the morphological alterations and inflammatory response. It ameliorates the occurrence of apoptosis, keeping the physiological process of renewal and regeneration in the epithelial lining of the colonic mucosa.

Key words: Enterocolitis, Necrotizing. Colon. Apoptosis. Rats.

Introduction

Necrotizing Enterocolitis (NEC) is the most common and severe surgical emergency in newborns. It affects mostly premature infants with low weight at birth, remaining the most important cause of mortality and morbidity in this group of patients. Several pathophysiological mechanisms have been proposed in an attempt to clarify the phenomena that are involved in the origin and evolution of NEC, but its etiology remains uncertain and their pathophysiology is not completely understood[1,2]. Moreover, there is not an established prophylactic and treatment protocols with proven efficacy[3]. Etiopathological findings of NEC are closely related with ischemic events, and the fact that NEC most often affects the distal ileum and proximal colon, suggests the existence of a derangement in the local circulatory system. The preterm infants are more susceptible to intestinal ischemia and hypoxia due to the immaturity of the mechanisms of regulation of vascular resistance[2-4].

Apoptosis plays an important role in the architecture of the intestinal epithelium and responds to the stress of intestinal epithelial cells, which begins through distinct pathways, and can be seen in the epithelium depending on the position of the cell along the crypt, the axis of the villous, the level of differentiation and the type of stimulation was initiated[5].

Another remarkable aspect of these patients is the inappropriate production of enzymatic products of prostanoids related to cyclooxygenase 1 e 2 (COX-1 and COX-2). Prostanoids regulate cellular proliferation, migration and cell apoptosis, as well as gastrointestinal secretion, contraction and relaxation of smooth muscle, the body temperature, and are flags in inflammatory cascade[6,7]. The COX-1 and 2 have an important role in the maintenance of the intestinal epithelium (COX-1) and the triggering of apoptotic phenomena induced by stress (COX-2)[8].

Ischemic preconditioning (IPC) is a technique that increases the tolerance to ischemia in either a local or distant organ[9,10]. Ischemic Remote Preconditioning (IPCr) is a strategy that also has demonstrated promising results in local and systemic tolerance against hypoxia and reoxygenation. The IPCr protects the organs exposed to lethal ischemia and the magnitude of protection is equivalent to the IPC, through the release of biochemical messengers in the circulation or by activation of neuronal pathways, or a combination of both[11,12].

Considering the intolerance to ischemic events in the pathophysiology of the necrotizing enterocolitis, as well as the role of IPCr as a promising alternative to prevent deleterious mechanisms of ischemic and reperfusion injury in the intestinal mucosa, it was decided to study the potential benefits of IPCr applied on the pregnant to inhibit colonic mucosa injury of newborn rats subjected to hypoxia and reoxygenation.

Methods

The project was approved by the Research Bioethics Committee of the School of Medicine, UNIFESP, under registration No. 0468/11.

It was used three pregnant rats and their 31 newborn, Wistar OUT B EPM-1 (*Rattus norvegicus* albinos, Rodentia mammalia) from the Institute of Pharmacology, Paulista School of Medicine-UNIFESP. The weight of the newborn rats ranged from 5.3 to 6.7 grams.

The three pregnant rats were allocated randomly forming three groups with their newborns: Control Group (CG) (n=10): Newborn rats that had not undergone any intervention; Hypoxia-Reoxygenation Group (HRG) (n=9): Newborn rats were subjected to hypoxia and reoxygenation; Remote Ischemic Preconditioning Group (IPCrG) (n=12): The pregnant mother was subject to IPCr, 24 hours before delivery, and the newborn were exposed to hypoxia and reoxygenation, according to the following protocols.

Remote ischemic preconditioning protocol

The pregnant rat was submitted to ischemic preconditioning 24 hours before delivery by applying a rubber band tourniquet[11], by using an elastic band in the proximal region of the left hind paw. It was induced 10 minutes of ischemia, after what the tourniquet was removed allowing reperfusion. The parameter used to monitor the ischemia was to compare the color in the plantar region of the left paw, which was cyanotic, to the contralateral paw, which remained with a pink pattern showing adequate perfusion.

Hypoxia and reoxygenation protocol

The newborn rats from HR and IPCr groups were submitted hypoxia and reoxygenation by using the model described by Ozkan *et al.*[13]. The rats were placed on an special acrylic chamber for controlled inhalation of gases, which measured 32 cm high, 34 cm wide and 50 cm long (model SB CO2 G - mark Beira-Mar - Brazil®) and underwent hypoxia through the exclusive supply of carbon dioxide (CO_2) at a concentration of 100% over a period of 10 minutes. After this period of hypoxia, the animals were resuscitated by means of oxygen (O_2) at 100% over a period of 10 minutes. This procedure was held twice a day, an interval of

six hours for three consecutive days (1st, 2nd and 3rd days of life).

The animals were euthanized by lethal anesthesia dose on the fourth day of life. It was collected a segment from the proximal colon for histological and immunohistochemical assays.

Histological and immunohistochemical assays

The colonic segments were fixed in 10% formalin for 16 hours, and transferred to 70% alcohol. Four-micrometer sections of the paraffin tissue blocks were stained with hematoxylin-eosin (HE). The degree of colonic damage was evaluated according to Chiu *et al.*[14].

In order to study apoptosis, it was determined the expression of cleaved caspase-3 and inflammatory process was evaluated by the COX-2 expression. For that, it was used four micrometers thick paraffin-embedded tissue sections at 60°C degree glass-house, subjected to xylene, rehydrated in absolute alcohol, performing antigen retrieval by Steamer, with citric acid at a pH 6.0 for 60 minutes, incubated for primary cleaved caspase-3 antibody and anti-COX-2 at a dilution of 1:30 (Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb antibody and anti-COX-2 rabbit antibody- Cell Signaling Technology®). The samples were incubated in secondary antibody with a polymer detection system. These markers received, as a counter staining, Mayer's hematoxylin.

A specific scale was used in order to quantify the number of positive cells for cleaved caspase-3 as proposed by Le Mandat Schultz *et al.*[15]: scale (0), 0 to 2 positive cells; scale (1), 2 to 5 positive cells; scale (2), 5 to 20 positive cells, scale (3), 20 to 50 positive cells, and scale (4), more than 50 positive cells The counting was performed analyzing 5-10 microscopic fields in each slide at x200 magnification.

For COX-2, it was used a calculated immunohistochemical score as proposed by Perrone *et al.*[16] by multiplying the quantity and staining intensity scores. The raw data were obtained from an estimate percentage of immunoreactive cells (quantity score), ranging from zero to four; and an estimate of the color intensity (staining intensity score), which was assessed on a scale from zero to three. The immunohistochemical score could range from 0 to 12; where a score of 9–12 was considered strong immunoreactivity, 5–8 was considered moderate, 1–4 was considered weak, and 0 was scored as negative.

The statistical analyzes were done using ANOVA test (two-way), in order to identify differences in histological alterations, and for cleaved Caspase-3 and COX-2, it was used the Kruskal-Wallis test. The significance level was 5%.

Results

The normal cellular architecture can be observed at control group (Figure 1A). The hypoxia and reoxygenation induced atrophy and cellular necrosis in the colon (HR group; Figure 1B). However, the maternal ischemic preconditioning promoted cell regeneration in the colon of the newborn rat submitted to HR (IPCr group; Figure 1C).

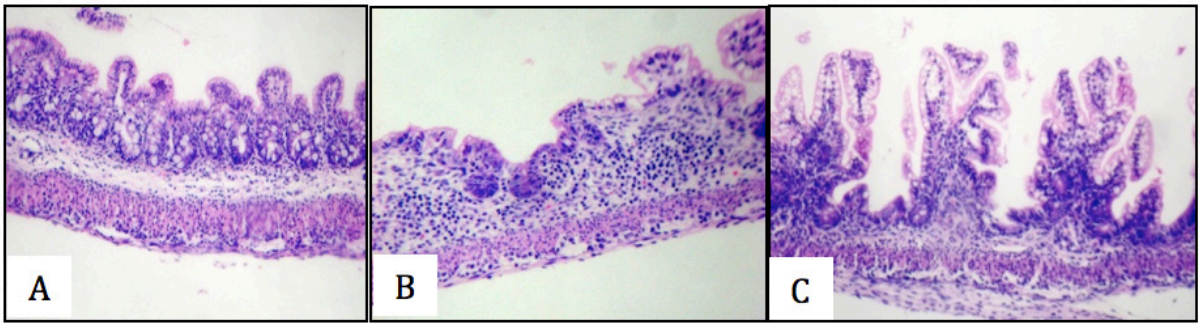

FIGURE 1 – Photomicrographs of the colonic segments from studied groups (HE-x100). Normal architecture (CG; **A**), atrophic architecture with necrosis (HRG; **B**) and regenerated architecture (IPCrG; **C**).

The basal occurrence of apoptosis observed in the animals of the Control group was modified in the HRG. The samples from HR group had a low occurrence of apoptosis (p=0.013), but a high degree of destruction of the colonic epithelium architecture when compared to the other groups (p=0.026). Maternal ischemic preconditioning ameliorates the occurrence of apoptosis, similarly to the Control group (p=0.30), demonstrating the preservation of the physiological process of renewal and regeneration in the epithelial lining of the colonic mucosa (Figures 2 and 3).

The degree of inflammation, analyzing the immunohistochemical score of COX-2 (Table 1 and Figure 4), showed significant difference among the groups (p<0.05).

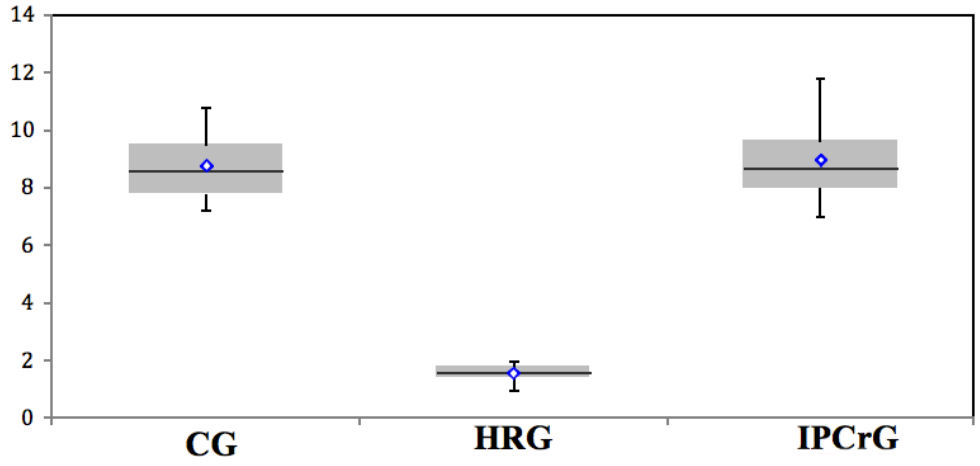

FIGURE 2 - Apoptotic corpuscles count among the studied groups. Kruskal-Wallis test: HRG> IPCrG=CG (p<0.05)

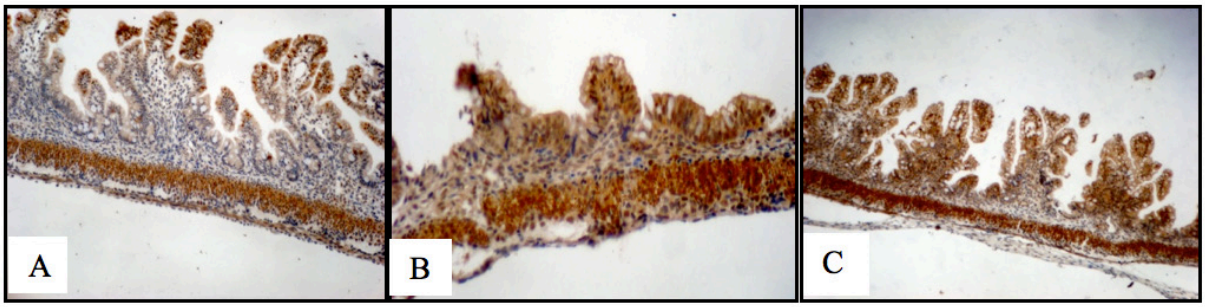

FIGURE 3 - Photomicrograph showing numerous apoptotic corpuscles at the tip of colonic villi tips in Group CG (left, arrows) (x200). Absence of apoptotic corpuscles in consequence of cell damage at the tips of colonic villi in group HRG - (middle) (x 200). Apoptotic Corpuscles at the tip of colonic villi in group IPCrG (Right, black arrows. (x200)

TABLE 1 - Immunohistochemical score calculated for inflammatory evaluation based on COX-2 data.

	Groups		
	Control	**HR**	**IPCr**
+ Cells (Quantity score)	2.0	3.5	1.5
Staining intensity score	1.0	3.0	1.0
Immunohistochemical score	**2.0**	**10.5**	**1.5**

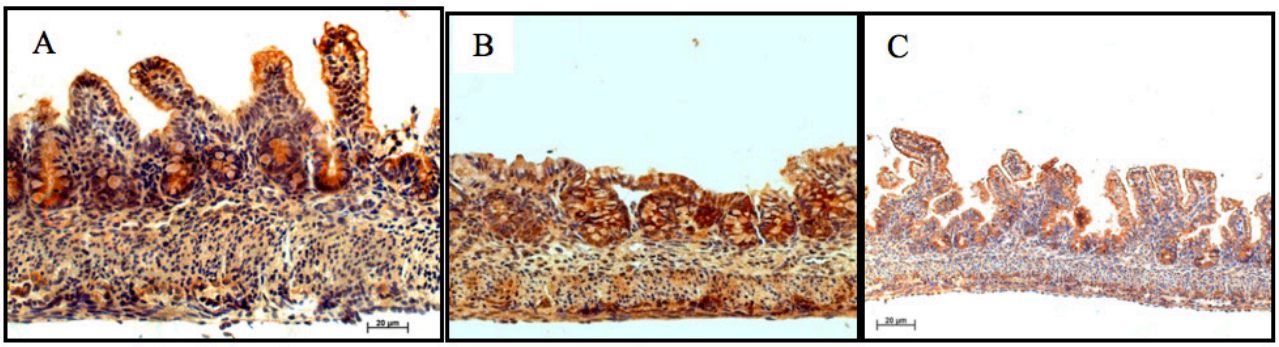

FIGURE 4 - Photomicrographs showing the expression of COX-2: (**A**) Group CG - Grade 1 (x200); (**B**) Group HRG - Grade 3 (x200); (**C**) Group IPCrG - Grade 1 (x200).

Discussion

This is the first study that was performed aiming to respond whether applying remote ischemic preconditioning yet in the pregnant rat, therefore before the delivery, would be able to provide protective effects to their newborn rats which would be later subjected to hypoxia and reoxygenation. The promising results confirming the unequivocal benefits of the maternal remote ischemic preconditioning, was demonstrated by a statistically significant attenuation of the inflammatory response due to severe acute hypoxia and reoxygenation, measured by decreasing the expression of COX-2, a specific well-known pro-inflammatory mediator.

Despite of these physiopathological benefits, we found another important mechanism of protection through maintaining the physiological capability of epithelial renewal, by normal apoptosis occurrence.

This present research is a continuation of a series initiated by Cintra *et al*.[17], Meyer *et al*.[18], using the model published by Ozkan *et al*.[13] causing damage in the ileum from newborn rat subjecting them to 10 minutes hypoxia and reoxygenation for 10 minutes.

Okur *et al*.[19] demonstrated that there was mucosal colonic damage in rats exposed to 5 minutes of hypoxia with CO2 at 100%, followed by reoxygenation for five minutes with O2 at 100%. Based on this same model, other authors have succeeded in producing ischemic bowel injuries. The frequency and duration of hypoxia and reoxygenation was relatively short, nevertheless causing marked intestinal lesions, concurring with results of other authors[13].

Several strategies have being used in experimental studies in attempt to reduce or prevent injuries caused by intestinal ischemia and reperfusion, among them the IPCr which was able to attenuate the intestinal lesions in the ileum. It was decided to evaluate this experimental model in the colon of newborn rats because this is, together with the small intestine, one of the segments most involved in the NEC[2].

It is uncertain the optimal number of cycles and timing of ischemia and reperfusion when performing IPC and IPCr in these experimental models. Reviewing the literature for similar IPC studies, it was found ischemic periods ranging from five to 20 minutes, with reperfusion varying from five to 15 minutes[20]. With regard to the models of remote ischemic preconditioning, a number of cycles varying from two to four, as well as reperfusion time between five and ten minutes could be found[12].

Studies assessing the intracellular mechanisms by which IPC protects tissues from damage due to IR are controversial. In those studies, ischemia and reperfusion promote increasing occurrence of apoptosis in different tissues analyzed[21,22,23].

This is a study in which IPCr applied in pregnant rats brought benefit to the newborn rats subjected to ischemia and reperfusion protocols, assessed by histological and immunohistochemical analysis. The mucosa of the intestinal epithelium of the colon provides an inappropriate production of prostanoid, enzymatic products of COX-1 and COX-2. Prostanoids regulate cell proliferation, migration and apoptosis, being directly involved in processes that regulate gastrointestinal secretion, also acting as triggers in inflammatory cascades[7]. COX-1 and 2 have an important role in the maintenance of the intestinal epithelium (COX-1) and for triggering inflammatory processes which culminate in apoptotic phenomena induced by stress (COX-2)[8].

Cyclooxygenase is an enzyme that limits the rate of conversion of arachidonic acid in to prostaglandins[24,25]. This enzyme has three isoforms, COX-1, COX-2 and COX-3. COX-1 and COX-3 are expressed constitutively in most tissues, and controls normal physiological processes. COX-2 is expressed only after being stimulated by growth factors, cytokines, mitogens, interleukins, tumor necrosis factor, and prostanoids. The COX-2 is expressed in normal gastrointestinal tract in undetectable levels, but their expression is evident in situations of hemodynamic tissue stress

like syndrome of hypoxia and reoxygenation, resulting significantly expression in the mucosa of the intestinal epithelium[24,26].

Hypoxia and reoxygenation triggers a series of deleterious events culminating by irreversibly altering the cellular and tissue architecture leading to intestinal perforation, with coagulative necrosis of the colonic wall. When this occurs, the presence of serious injury due to hypoxia and reoxygenation does not permit the occurrence of the sequence of events involved in programmed cell death[23]. In our study, we observed a higher frequency of apoptosis in groups R and IPCr, and a lower occurrence of apoptosis in group H/R, confirming a protective effect by reducing the deleterious effects due to hypoxia and reoxygenation injury, which were much more severe in group H/R leading to further destruction of the colonic mucosa with necrosis, whereas in the groups R and IPCr presented with apoptotic corpuscles on the tip of colonic villi, where epithelium desquamation takes place into the intestinal lumen as part of the process of cell renewal[23].

The present study demonstrated that IPCr interferes positively in the process of hypoxia and reoxygenation by mitigating the histopathological manifestations as well as modulating the expression of immunohistochemical markers closely related to inflammatory and apoptotic events in the colonic mucosa, increasing the number of goblet cells and by decreasing morphologic structural changes in the mucosa of the colon, with reduced inflammatory response as evidenced by a lower expression of COX-2 and by maintaining the apoptotic capacity evidenced by the expression of caspase-3.

There are remarkable anatomical, microcirculatory and functional differences between the ileum and colon[26,27], highlighting a higher concentration of bacteria in the colon, which are closely related to the initial cascade of inflammatory phenomena found in hypoxia and reoxygenation syndrome, resulting in increased expression of COX-2[28,29] and a decrease in physiological apoptotic function, represented by a decreased expression of caspase-3[30,31].

To our best knowledge, there are no studies using this experimental protocol of maternal IPCr evaluating inflammatory response due to hypoxia and reoxygenation through COX-2 expression in the colonic epithelium. By demonstrating that the expression of COX-2 in rats from group IPCr was similar to the Control group, the study strongly suggests a cytoprotective effect of maternal remote ischemic preconditioning in the syndrome of hypoxia and reoxygenation applied to its progeny.

Future studies should be conducted to clarify the mechanisms of protection against ischemia and reperfusion by using different immunohistochemical markers and their application in clinical practice.

Conclusions

Maternal remote ischemic preconditioning attenuates the morphological alterations and inflammatory response induced by hypoxia and reoxygenation in the colon of its newborn rats. Furthermore, it ameliorates the occurrence of apoptosis, keeping the physiological process of renewal and regeneration in the epithelial lining of the colonic mucosa.

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