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Lung Histological and Immunohistochemical Evaluation after Mesenteric Ischemia /Reperfusion in Rats

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Abstract

Purpose: The present study aimed to evaluate pulmonary inflammatory and apoptotic processes following intestinal ischemia/reperfusion injury and the modulation by pentoxifylline and hypertonic saline solution.

Methods: Thirty male Wistar rats were allocated into 5 groups (n=6; 200 to 250 g) and, except SHAM, were subjected to intestinal ischemia for 40 min and reperfusion for 80 min: prior to reperfusion. The Control group did not receive any treatment; the HS group received hypertonic saline (4mL/kg-IV); the PTX group received pentoxifylline (30mg/kg-IV); and the HSPTX group received hypertonic saline and pentoxifylline. All animals were heparinized (100U/kg). At the end of the reperfusions, right lung fragments were removed and stained for morphological evaluation (hematoxylin-eosin) and histochemical studies (COX-2, Bcl-2 and cleaved caspase-3).

Results: Immunohistochemical stainings revealed reduced expressions of COX-2 (p=0.0015) and Bcl-2 (p=0.0012) in the HSPTX group compared to the others. Reduced expression of cleaved caspase-3 was observed in the lungs of the IR group (p=0.0090 for the comparison of the PTX and IR groups).

Conclusion: The combination of hypertonic saline and pentoxifylline yields better results than using them separately in these same doses in terms of lung tissue morphology and attenuation of inflammation and apoptosis after I/Rm in this rat model.

Keywords: Reperfusion; Apoptosis; Inflammation; Pentoxifylline

Abbreviations: PTX: Pentoxifylline; HSPTX: Hypertonic Saline + Pentoxifylline; I/R m: Mesenteric Ischemia and Reperfusion; HE: Hematoxylin and Eosin; ROS: Reactive Oxygen Substances; RL: Ringer's Lactate; ATP: Adenosine Triphosphate

Introduction

The outbreak of inflammatory and apoptosis cascades in the acute lung injury that occurs after mesenteric ischemia and reperfusion (I/R m) in lung tissue which is targeted by cytokines and reactive substances oxygen produced in the mesenteric circulation are responsible for the endothelial barrier breakdown and the evolution of the process until the acute respiratory distress syndrome is installed [1-3]. The type of intervention to reverse the I/Rm process may affect the tissue response in target organs such as the lung. In hemorrhagic shock, pentoxifylline associated with hypertonic saline (7.5%), proved to be able to inhibit the activation of neutrophils and attenuate the production of TNF and interleukins and thus decrease the histological damage to the mesenteric territory and to target organs [3-6]. In this study, the aim was to assess the performance of the aforementioned substances in lung tissue in animals subjected to I/Rm by temporary occlusion of mesenteric vessels.

Methods

The Ethics Committee of the Medical School of the University of São Paulo (120/2010) approved this research project. Thirty male Wistar rats (Rattus norvegicus) weighing from 200 to 250g were used. The animals were anesthetized with a mixture of

ketamine and xylazine intraperitoneally. They were then fixed in a heated table (Insight) ™ so that the body temperature was maintained at 37°C during the experiment. After acrural incision, the right femoral vein received a 24 fr polyethylene catheter for the infusion of substances. The animals were separated into five groups: S = Sham, C = Control, HS = 7.5% hypertonic saline, PTX = pentoxifylline and HSPTX = 7.5% hypertonic saline + pentoxifylline. Through a midline incision, the mesenteric vessels were dissected and the ileum was exposed.

The animals of the S group did not receive any intervention from that point and were kept under observation. The group C had the superior mesenteric vessels clamped for 40 minutes and they were observed for further 80 minutes reperfusion. The terminal ileum vessels that connect with the colonic arch were ligated to prevent blood reflux. Animals from groups HS, PTX and HSPTX underwent the same procedure of group C animals, but one minute before reperfusion, they received infusions of 7.5% hypertonic saline (4ml/kg), pentoxifylline (30mg/kg diluted in 4ml/kg of 0.9% saline solution) and Pentoxifylline diluted in the 7.5% hypertonic saline, respectively. At the end of 80 minutes of reperfusion the animals were given a lethal dose of 19.1% KCI and then the right lung was collected to be subjected to histological and immunohistochemical staining.

Tissue samples were dehydrated embedded in paraffin and sliced 4µm thick. They were subjected to four staining methods: hematoxylin and eosin (HE) staining, immunohistochemistry for COX-2, cleaved caspase-3 and Bcl-2 (Cox-2 (m-19) sc-1747, Santa Cruz, G2205, monoclonal goat, AP-1027 Anti cleaved caspase 3 (Asp 175), Calbiochem, D29363, Rabbit pAb; Bcl-2 (C-2), sc-7382, Santa Cruz, J0203, Monoclonal mouse). Two pathologists, blinded to the groups, performed histological examinations. In HE staining, pulmonary and vascular congestion as well as neutrophil infiltration in the interstitium were evaluated and each slide was given a graduation ranging from zero (no change) to three (severe damage).

Immunohistochemical appraisal system combined a semi-quantitative parameter (percentage of immunoreactive cells) and qualitative (tissue staining intensity) whose results were ranked from one (minor changes) to four (major changes) as previously described [7]. The results are presented as mean and standard deviation. Statistical analysis was performed by Kruskal-Wallis test followed by the Student-Newman-Keuls post hoc test. The level of significance was set at $p \leq 0.05$.

Results

The degree of lung injury in Staining with HE was lower in group S (0.67 ± 0.82), HS (0:33 ± 0:52), PTX (01.17 ± 0.69) and HSPTX (0.67 ± 0.82). The tissue injury score assigned to group C (2:50 ± 0.84) was higher and a significantly different compared to S groups (p = 0.0421), HS (p = 0.0421), PTX (p = 0.0003) and HS + PTX (p = 0.0010). In immunohistochemical staining for COX-2, the C group (2.80 ± 00:30) had the highest value and significant differences compared to groups that received treatment (HS = 1.80 ± 0:45, p = 0.0455; PTX = 1.40 ± 1, p = 0.0143; HSPTX = 1.60 ± 0:55, p = 0.0455). Two groups, C (3:50 ± 0.84) and HS (3.17 ± 0.75), showed the highest values of cleaved caspase-3 expression in immunohistochemical and differed significantly from the groups S (2:17 ± 0:41) and PTX (2:17 ± 0:41) (C: p = 0.0388, p = 0.0105; HS: p = 0.0388 and p = 0.0105, respectively). The HSPTX group (2:50 ± 0:55) showed intermediate values and did not differ statistically from the others. The cytoplasmic expression of Bcl-2 was mild in lung tissue on group C (1.67 ± 0.52) and differed from the expression found in HS (3.33 ± 1.03; p = 0.0087), PTX (3.50 ± 0.55; p = 0.0035) and HSPTX (3:50 ± 0.84; p = 0.0037) groups (Figures 1 & 2).

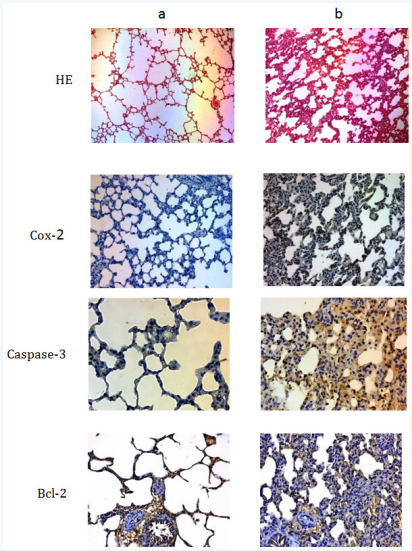

Figure 1: Pulmonary tissue photomicrographs (200x): A: Minor Tissue Injury; B: Major Tissue Injury.

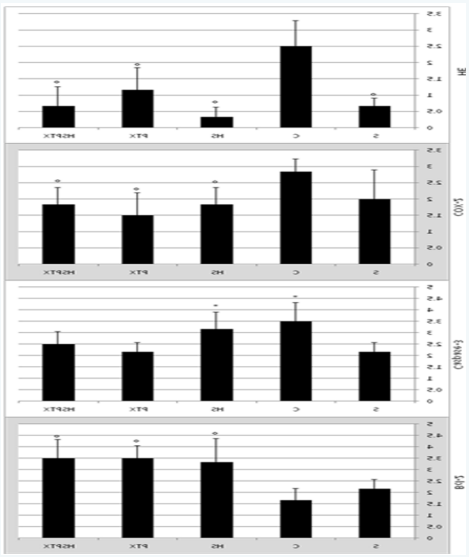

Figure 2: Morphological and immunohistochemical scores. *Statistically significant vs group C, *Statistically significant vs groups S and PTX (p<0, 05).

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Discussion

The lower degree of lung injury in the overall assessment, occurred in the group treated with the association of hypertonic saline and pentoxifylline, in this animal model. Hypertonic saline solution has the property of restoring the mesenteric blood flow resulting in reduced production of reactive oxygen substances (ROS) and other deleterious substances that, by blood circulation, reach the lung. Compared to Ringer's lactate (RL) in an experimental model of hemorrhagic shock, hypertonic saline promoted a lower degree of acute pulmomary injury [8]. The use of anti-TNF substances protects the alveolar architecture in animals subjected to I/Rm [9]. Pentoxifylline, a nonspecific phosphodiesterase inhibitor, showed similar results for the prevention of acute lung injury when was previously applied to animals subjected to I/Rm.

The bronchic aspiration of the treated animals showed a lower protein concentration and a lower concentration of TNF [10,11]. The evaluation of the degree of lung injury in HE presented in this study is comparable to the results found in the literature. Both substances, 7.5% pentoxifylline and hypertonic saline, are capable of inhibiting the inflammatory cascade and production of COX-2 from arachidonic acid [8,12]. The active pentoxifylline adeniciclase and increases the intracellular level of cyclic adenosine monophosphate, which, in turn, reduces the amount of arachidonic acid suffering peroxidation. The overall effect is a reduction in systemic and local inflammatory agents such as cyclooxygenase. Hypertonic saline besides restoring the mesenteric blood flow improves hepatic metabolism in a way that many of the harmful substances produced in the mesenteric region are metabolized by the liver before reaching the lungs. Thus, one may expect that an animal treated with both substances present lower level of cytoplasmic expression of COX-2 as shown [8,10].

The expression of cleaved caspase-3 pro-apoptotic protein was different among the treated groups, with higher cytoplasmic expression in the SH group, lower in the PTX group and intermediate in HSPTX group. The expression of this protein is sometimes misinterpreted once severe tissue damage can lead to cell death by necrosis and thus there is less apoptosis identification [13]. In addition, apoptosis is a cell death mechanism dependent on energy (ATP) and energy depletion leads to subexpression of pro-apoptotic proteins. Moreover, the recovery of energy substrates in animals receiving treatment promotes tissue regeneration and thus apoptosis, which is a mechanism of regeneration, may be overestimated [6].

The mechanisms mentioned above promote increased cytoplasmic expression of Bcl-2 anti-apoptotic protein that prevents or delays the release of cytochrome c by stabilizing mitochondrial channels, and blocking the occurrence of apoptosis that could exacerbate tissue damage [6,14]. This process ensures a milder tissue injury such as the one found in the evaluation in HE in this study. In a recent paper, the authors demonstrated that the over expression of Bcl-2 in transgenic mice is able to prevent the damage to the intestinal mucosa of animals subjected to sepsis [15]. The combination of hypertonic saline and pentoxifylline yields better results than using them separately in these same doses in terms of lung tissue morphology and attenuation of inflammation and apoptosis after I/Rm.

Conclusion

Despite the common use of such substances in hospitals and the investigations of the role of these substances in ischemia and reperfusion, the action and interaction mechanisms as well as the combination strategies with other substances still deserve to be studied. Prevention of target organ damage is still a challenge to be overcome.

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