The impact of hypertonic and normal saline in gut reperfusion after ischemia in rats

O impacto das soluções hipertônica e salina fisiológica na reperfusão do trato gastrintestinal pós-isquemia em ratos

INTRODUCTION

Gut ischemia can cause local and systemic harmful events.\(^1,2\) Arterial occlusion, inflammation, trauma, and all types of shock states have been associated with intestinal ischemia.\(^3\) The extent of the intestinal system involved in the ischemic/reperfusion injury determines the severity of damage to distant organs. Rupture of the blood-intestinal barrier occurs precociously after ischemic/reperfusion injury, and it takes substantial time for the intestinal mucosa to be completely repaired.\(^4\) During this time, endotoxins and intestinal bacteria can reach the systemic circulation and amplify the inflammatory response.\(^5,6\)
Gut reperfusion leads to extensive oxidative stress in the previously ischemic intestinal tissue.\textsuperscript{(12,7)} Under these conditions, the deleterious effects of reactive oxygen species (ROS) on cells are numerous. Adverse effects of ROS include peroxidation of the cellular membranes (both plasmatic cell membrane and intracellular organelles), DNA breakdown, and cell death.\textsuperscript{(9)}

The reperfusion of a previously ischemic organ is an intricate process, in which different regions of full blood flow restoration coexist with other regions of partial or no arterial blood perfusion.\textsuperscript{(9)} In situations of severe hypoperfusion, excessive ROS production can have a cumulative effect and can impair organ perfusion as a result of the formation of cellular plugs (aggregation of erythrocytes or leukocytes) at the microcirculatory level.\textsuperscript{(10)} Reperfusion of the intestine causes redistribution of the total intravascular volume, which leads to a transient period of systemic hypovolemia.\textsuperscript{(4)}

The pioneering study by Velasco et al. showed that small amounts of 7.5% saline solution restored vital parameters and decreased mortality in dogs subjected to severe hemorrhagic shock.\textsuperscript{(11)} Since this discovery, the 7.5% hypertonic saline solution has been extensively studied in experimental and clinical settings.\textsuperscript{(12-16)} After hemorrhagic shock, a small volume of 7.5% hypertonic saline solution improves the cardiac ejection fraction and the perfusion pressure, also with improvements in the cardiac index.\textsuperscript{(17)} Moreover, in addition to its hemodynamic properties, 7.5% hypertonic saline solution appears to have a significant action as an anti-inflammatory drug.\textsuperscript{(18,19)}

Compared with the conventional treatment of hemorrhagic shock with a high volume of normal saline solution, the use of a small volume of 7.5% hypertonic saline solution minimizes tissue edema formation.\textsuperscript{(20)} The concentration of 7.5% sodium chloride (NaCl) in colloid solutions (dextran 70 or hydroxyethyl starch) increases the pharmacological properties of these hypertonic solutions compared with the use of a standard 7.5% saline solution.\textsuperscript{(21)}

We compared the effects of a small volume of 7.5% NaCl hypertonic saline solution to those of a 0.9% NaCl isotonic saline solution in the treatment of gut ischemia and reperfusion. We analyzed the oxidative stress and its correlation with the systemic inflammatory response in an experimental model of transient intestinal ischemia in rats. The hypothesis was that compared with an isotonic saline solution, a hypertonic saline solution has more potential benefits in the treatment of intestinal ischemia.

METHODS

The experimental protocol was approved by Research Ethics Committee’s of Hospital das Clínicas of Faculdade de Medicina de Universidade de São Paulo and followed the Guide for the Care and Use of Laboratory Animals (USA National Academy of Science). This study followed the National Institutes of Health (NIH) animal care guidelines. Animals that exhibited poor clinical recovery or that showed signs of severe distress after surgery were euthanized and counted as a death in their experimental groups.

Animal procedure

Adult male Wistar rats with body weight of 250 to 300g (n=101) were fasted overnight from food but had free access to water prior to the experiment. Under general anesthesia (2% xylazine hydrochloride by intraperitoneal injection - ip - in the dose of 5mg/kg of body weight prior to pentobarbital ip 5g/kg of body weight), the rats underwent tracheal intubation (polyethylene cannula, 1.7mm inner diameter) and were maintained with spontaneous ventilation. Polyethylene catheters (P10) were inserted in the right common carotid artery and the right external jugular vein. The catheters were exteriorized through the dorsal region.

A midline abdominal wall incision was performed, the abdominal viscera were gently manipulated to visualize the abdominal aorta, and the origin of the superior mesenteric artery (SMA) was identified. In the ischemia groups, the SMA was occluded with a micro vascular clamp immediately next to the abdominal aorta. The animals were maintained under general anesthesia during the SMA occlusion. Animals that exhibited poor clinical recovery or signs of distress were sacrificed by humane euthanasia methods.

Experimental design

The animals were randomized to four groups: (Sham Group, animals with no occlusion of the SMA and no treatment (n=20); 7.5% HS Group, with animals subjected to SMA occlusion and treated with 7.5% hypertonic saline solution (n=30); NS Group, with animals subjected to SMA occlusion and treated with 0.9% isotonic saline solution (n=30); and NT Group, with animals subjected to SMA occlusion, but not treated with a solution (n=21). The animals received the solutions through the right external jugular vein immediately prior to releasing the SMA clamp. The duration of SMA occlusion was 45 minutes.
The dose of hypertonic solution was 4mL/kg, which has previously been demonstrated to be efficacious in the literature. The normal saline dose was of 32mL/kg; this volume of normal saline has the same amount of sodium, which is the element that determines the extracellular expansion.

Sequential blood samples were collected immediately after SMA reperfusion and at 2 and 4 hours post SMA. A blood sample (0.5mL) was collected from the animals in each group. The blood samples were centrifuged (Centrifuge 5804 R®, Eppendorf, Hamburg, Germany), and the plasma was immediately frozen and stored in a freezer (-80°C) for posterior cytokine analysis.

Tissue samples from the lungs (median portions), liver (right lobe), and small intestine (10cm proximal to the ileocecal valve) were collected, snap frozen (-80°C) in liquid nitrogen and stored for posterior analysis. The animals were euthanized at 2, 4, or 6 hours after intestinal reperfusion.

**Myeloperoxidase assay**

For the myeloperoxidase (MPO) assay, we utilized a procedure previously described. The tissues were homogenized (50mg/mL) in 0.5% hexadecyltrimethylammonium bromide in 10mM 3-N-morpholinopropanesulfonic acid (MOPS) and centrifuged at 15,000g for 40 minutos. An aliquot of supernatant was mixed with a solution of 1.6mM tetramethylbenzidine and 1mM hydrogen peroxide. The activity was measured spectrophotometrically as the change in absorbance at 650nm at 37°C using a Spectramax™ microplate reader (Molecular Devices, LLC, Sunnyvale, California, United States). The results are expressed as the milliunits of MPO activity per milligram of protein, which were determined with the Bradford assay.

**Malondialdehyde assay**

Thiobarbituric acid-reactive formation was used to quantify lipid peroxidation in tissues; the thiobarbituric acid-reactive substances were measured as previously described. The tissues were homogenized (100mg/mL) in 1.15% potassium chloride (KCl) buffer and 100mL of the homogenates was then added to a reaction mixture that consisted of 750mL of 0.8% thiobarbituric acid, 100mL of 1.15% KCl, 750mL of 20% acetic acid (pH 3.5), and 300mL of distilled water. The mixture was then heated to 90°C for 60 minutes. After cooling at 4°C, the samples were cleared by centrifugation (10,000g for 10 minutes), and their absorbance was measured at 532nm, using 1,1,3,3-tetramethoxypropane as the external standard. The level of lipid peroxides was expressed as the mmol malondialdehyde/mg of protein.

**Interleukin 6 and interleukin 10**

The concentration of interleukin 10 (IL-10) and IL-6 were measured in lung tissues using an enzyme-linked immunosorbent assay (ELISA) with a DuoSet kit (R&D Systems™, Minneapolis, MN, USA). Briefly, the samples that contained 100mg of tissue (lungs, liver, or small intestine) were homogenized with a solution of 100μL 1.15% KCl. Aliquots of the supernatant were used for the measurement of IL-6 and IL-10 by the ELISA method. The samples were read by spectrophotometry at 450nm using a GENios Plus™ microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). The tissue sample data are presented as pg/mg of protein (Bradford assay), and the plasma data are presented as pg/mL.

**Statistical analysis**

Statistical analyses were performed using SigmaStat™ 3.1 software (Systat Software Inc, San Jose, California, USA). All data are presented as the means±standard error deviation (±SD). The group differences were evaluated by two-way analysis of variance (two-way ANOVA) with the Holm-Šídák method as a post-hoc test. The correlation between IL-6 and IL-10 was tested by Spearman’s rank method. A p value≤0.05 was considered significant.

**RESULTS**

**Survival rate**

Twenty animals were euthanatized. The total mortality rate for the individual groups was 12.12% for the Sham Group, 20% for the 7.5% HS Group, 12% for the NS Group, and 27% for the NT Group. The mortality occurred within the initial 4 hours after reperfusion, and most cases occurred between 2 and 4 hours after reperfusion.

**Lipid peroxidation**

There were significantly lower values of malondialdehyde (MDA) (Figure 1) in the treated groups (NS or 7.5% HS Group) compared with the non-
treated animals at each time point studied (2, 4, or 6 hours after reperfusion). Furthermore, it is noteworthy that the treated ischemic animals had similar MDA levels compared with the Sham Group.

**Neutrophil infiltration**

Similar to the results for lipid peroxidation, the reperfused groups showed basal MPO values (Figure 2) despite the increase present in the not treated animals. MPO activity was used as an index of neutrophil infiltration.

**Cytokines**

Two hours after reperfusion, there were no group differences in the IL-6 tissue concentrations. After 4 hours, the IL-6 concentrations were significantly higher in the liver and intestine of the NT and NS Groups compared with the Sham and 7.5% HS Groups. After 6 hours, there were no group differences except for the intestine, in which the IL-6 concentrations were higher in the NT Group (Figure 3).

Two hours after reperfusion, there were no group differences in the IL-10 tissue concentrations. Four hours after reperfusion, the IL-10 concentrations were significantly higher in the liver and intestine in the non-treated animals and in the animals treated with normal saline solution compared with the sham and the animals treated with 7.5% hypertonic saline solution (similar to the IL-6 data). Six hours after reperfusion, the IL-10 levels in the liver were significantly higher in the saline treated group and in the non-treated animals compared with the Sham and 7.5% HS Groups. The IL-10 concentrations measured in the intestines of the non-treated animals were significantly higher compared with the Sham and 7.5% HS Groups 6 hours after reperfusion. Six hours after reperfusion, the IL-10 levels present in the lungs were significantly higher in the animals treated with 7.5% hypertonic saline solution compared with the ones treated with normal saline solution (Figure 4).

The plasma concentrations of IL-6 and 10 were studied at three intermediate time points (immediately after reperfusion and 2 and 4 hours after reperfusion) and compared among groups (Figure 5). Following the initial period of 2 hours, the plasma IL-6 concentrations were significantly higher in the animals treated with 7.5% hypertonic saline solution. There was also a trend towards lower IL-6 levels in tissues 4 hours after reperfusion in the animals treated with 7.5% hypertonic saline solution compared with the other groups.

After 2 and 4 hours of reperfusion, the plasma IL-10 concentrations had a trend towards higher values in the animals treated with hypertonic saline solution compared with the other groups. These differences were significant compared with the Sham Group at each time point studied. There was a strong correlation between the IL-6 and IL-10 concentrations in tissues (R value in lungs=0.858; R value in liver=0.732; and R value in intestine=0.813) and a moderate correlation in the plasma cytokine levels (R value=0.432) using Spearman’s method (Figure 6).

**DISCUSSION**

Animals that underwent an SMA occlusion received intravascular fluid replacement with physiological or hypertonic saline solutions immediately prior to intestinal reperfusion. The effects of both solutions were compared with the results in the sham and non-treated animals. Our results demonstrated that crystalloid fluid treatment of ischemic animals caused significant decreases in oxidative stress markers and the inflammatory response. A hypertonic solution produced a delayed increase in gut and liver cytokines. There was an important increase in IL-10 in the lungs and plasma, as well as IL-6 in the plasma.

The arterial occlusion of large vascular territories triggers systemic mechanisms that alter the physiological condition. It has been hypothesized that the infusion of crystalloid solutions immediately prior to SMA reperfusion can protect against hemodynamic alterations after the intestinal blood flow reestablishment. The pre-treatment with crystalloid solutions produces a rapid and efficient bowel reperfusion.

Oxidative stress, inflammation, and anti-inflammatory events that occur immediately after reperfusion contribute to the outcome following SMA ischemia. Hence, we chose the period of 6 hours to study oxidative stress and inflammation after transient intestinal ischemia. Both crystalloid solutions decreased the oxidative stress and the inflammatory response studied in the splanchnic territory (bowel and liver) and lungs.

In this model, severe hypoperfusion occurs at the splanchnic territory, which leads to intestinal ischemia. Treatment with 7.5% hypertonic saline solution yields hemodynamic results comparable with traditional therapeutics that use large volumes of normal saline solution. In addition, it has been demonstrated that inflammatory responses are better attenuated with 7.5% hypertonic saline solution compared with a conventional normal saline solution infusion, and our data showed a similar pattern for gut ischemia and reperfusion. Both
Figure 1 - Tissue concentrations of malondialdehyde (µmol/mg) in the lungs (A), liver (B) and intestine (C) in the sham group, group of animals treated with 7.5% hypertonic saline, group with normal saline solution, and not treated group. Graphics show the malondialdehyde activity at 2 hours (black bar), 4 hours (light gray bar), and 6 hours (dark gray bar) after reperfusion. Gut ischemia and reperfusion increased malondialdehyde in the lung, liver and gut; volume infusion (normal saline solution and hypertonic saline) protected tissues against oxidative stress. MDA - malondialdehyde; HS - hypertonic saline; NS - normal saline solution; NT - not treated. *p<0.05; results as mean±standard deviation.

Figure 2 - Myeloperoxidase activity (U/mg) in the lungs (A), liver (B) and Intestine (C) in the sham group, group of animals treated with 7.5% hypertonic saline, group with normal saline solution, and not treated group. Graphics show the myeloperoxidase activity at 2 hours (black bar), 4 hours (light gray bar), and 6 hours (dark gray bar) after reperfusion. Gut ischemia and reperfusion increased neutrophil infiltration in the lung, liver and gut; volume infusion (normal saline solution and hypertonic saline) protected tissues by reducing inflammatory cell migration. MPO – myeloperoxidase; HS - hypertonic saline; NS - normal saline solution; NT - not treated. *p<0.05; results as mean±standard deviation.

Figure 3 - Tissue concentrations of interleukin 6 (pg/mg) in the lungs (A), liver (B) and intestine (C) in the sham group, group of animals treated with 7.5% hypertonic saline, group with normal saline solution, and not treated group. Graphics show the IL-6 concentrations at 2 hours (black bar), 4 hours (light gray bar), and 6 hours (dark gray bar) after reperfusion. Gut ischemia and reperfusion increased interleukin 6 in the lung, liver and gut; volume infusion (normal saline solution and hypertonic saline) protected tissues by reducing inflammation. The hypertonic solution induced better protection in the liver and gut compared with normal saline. IL-6 - interleukin 6; HS - hypertonic saline; NS - normal saline solution; NT - not treated. *p<0.05; results as mean±standard deviation.
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Gut ischemia and reperfusion increased the interleukin 10 concentrations in the lung, liver and gut; volume infusion (normal saline solution and hypertonic saline) reduced the amount of interleukin 10 inflammation. The normal saline group presented higher interleukin 10 in the liver and gut, and the hypertonic saline presented higher levels in the lung. IL-10 - interleukin 10; HS - hypertonic saline; NS - normal saline solution; NT - not treated. *p<0.05; results as mean±standard deviation.

Gut ischemia and reperfusion did not increase interleukin 6 or interleukin 10 concentrations in the plasma. The hypertonic solution presented higher plasma levels of interleukin 6 and interleukin 10 compared with the not treated and normal saline. IL-6 - interleukin 6; IL-10 - interleukin 10; HS - hypertonic saline; NS - normal saline solution; NT - not treated. *p<0.05; results as mean±standard deviation.

The osmotic and hyperoncotic characteristics of 7.5% hypertonic saline solution have been associated with this particular effect in this experimental model. Gonzalez et al. studied the therapeutic effects of small volumes of hypertonic saline solutions (4mL/kg of body weight) with different concentrations of sodium chloride in the SMA occlusion model in rats. The authors showed that hypertonic saline solutions (2%, 5%, 7.5%, or 10% hypertonic saline solution) decreased intestinal injury and the neutrophil infiltration in the intestinal mucosa. The
Figure 6 - Correlation between the levels of interleukin 6 and interleukin 10 in the lungs $R=0.858$ (A), liver $R=0.732$ (B), intestine $R=0.813$ (C) and plasma $R=0.432$ (D) in all groups by Spearman’s Method. IL-6 - interleukin 6; IL-10 - interleukin 10. $p<0.05$.

Results obtained with these different hypertonic solutions were similar compared with the results achieved with higher volumes of normal saline solution (calculated as 33mL/kg of body weight). However, the authors attained the best results with 7.5% hypertonic saline solution.\(^{(28)}\)

In our study, a small volume of 7.5% hypertonic saline solution and a high volume of normal saline solution had similar effects in the restoration of physiological parameters. In addition, both solutions efficiently attenuated oxidative stress and the inflammatory response.
It is important to highlight that the hypertonic solution delayed the inflammatory response, which is an effect that can be protective. The results obtained with crystalloid solutions were comparable with the results obtained for sham-operated animals. It is important to consider that in our study, the sham animals were subjected to moderate surgical trauma (abdominal incision, intestinal viscera manipulation, and superior mesenteric artery handling). This moderate surgical trauma was sufficient to cause oxidative stress and the inflammatory response observed in our results.\(^{(29,30)}\)

Reperfusion injury has been related to an overproduction of ROS.\(^{(2)}\) MDA was used as an oxidative stress marker in this study. Our results showed that the production of MDA in different organs was similar in the sham and treated groups. Oxidative stress measured by MDA formation in tissues was significantly more intense in the non-treated animals at each time point studied. Moreover, MDA concentrations decreased over time in all experimental groups. These data showed that oxidative stress was reduced by the adequate replacement of the total circulatory volume.

MPO activity was analyzed in different organs from the abdominal and extra-abdominal cavities. MPO activity has been widely used as an index of neutrophil infiltration in tissues.\(^{(22)}\) In this study, the MPO activity in the 7.5% HS and NS-treated Groups was similar compared with the sham animals. Significantly lower levels of MPO activity were identified in the treated animals compared with the non-treated animals. Leukocyte infiltration in tissues has been associated with the local synthesis of chemokines and cytokines.\(^{(31)}\) The different interleukins outperform both inflammatory and anti-inflammatory actions.\(^{(32)}\)

The concentrations of IL-6 (pro-inflammatory) and IL-10 (anti-inflammatory) were analyzed in different organs, and a temporal profile of these plasmatic interleukin concentrations was studied. Many studies have proposed that the synthesis of IL-10 could be regulated by the production of IL-6, and, furthermore, their ratio could predict the clinical outcome.\(^{(32-36)}\) The concentrations of IL-6 and IL-10 in tissues were very similar between the 7.5% hypertonic saline solution and sham animals throughout the time course studied. The analysis of IL-6 and IL-10 levels in tissues in the animals treated with normal saline solution peaked in the liver and small intestine in at least one measured time point compared with the Sham and 7.5% HS Groups. Four hours after reperfusion, there was a trend towards higher IL-6 and IL-10 tissue concentrations in the NT Group. Despite the overall results for IL in tissues, the plasmatic concentrations of IL-6 and IL-10 were higher in the animals treated with 7.5% hypertonic saline solution. There are some data that demonstrated that the hypertonic solution had an anti-inflammatory action, which increased IL-10. In addition, the increased IL-10 in the plasma and lungs can explain the reduced neutrophil infiltration and oxidative stress in lungs.

We analyzed two important mechanisms of injury that occurred after transient intestinal ischemia in rats. The results of the hemodynamic parameters and biochemistry analyses were similar after treatment with crystalloid infusion. However, the clinical outcome for the animals treated with 7.5% hypertonic saline solution had a trend towards lower survival compared with the animals treated with normal saline solution, but it was not significant.

This study had some limitations. We could not analyze a more chronic period post-ischemia because of the difficulties in maintaining animals alive and for ethical reasons. In addition, mesenteric ischemia requires a volume infusion on a daily basis, and repeated hypertonic solution infusions present as a complication of hypernatremia. A point of strength was to compare the advantages of experimental studies in reproducing the identical scenario to ensure the therapeutic actions of a compound.

**CONCLUSION**

In conclusion, utilizing a rat model of transient intestinal ischemia, we determined that treatment with a small volume of 7.5% hypertonic saline attenuates reactive oxygen species formation and inflammatory responses in different organs. A hypertonic solution resulted in higher levels of interleukin 10 in the plasma and lung tissue, which tipped the balance in the anti-inflammatory profile. The hypertonic saline effects were similar compared with the effects obtained with the infusion of higher volumes of normal saline solution. The advantage of hypertonic saline solution compared with the normal is the production of less edema.
Investigar o papel de duas diferentes soluções salinas nos mecanismos de lesão após isquemia intestinal: estresse oxidativo e respostas inflamatórias.

Métodos: Ratos Wistar foram submetidos a oclusão transitoria da artéria mesentérica superior e estudados durante as 6 horas seguintes à reperfusão. Após randomização, os animais foram divididos em quatro grupos: Falso; Solução Hipertônica, os quais receberam infusão de solução salina a 7,5% (4mL/kg de peso corpóreo); Solução Fisiológica, os quais receberam infusão de solução salina a 0,9% (33mL/kg); e Sem Tratamento. A infusão foi realizada imediatamente antes da reperfusão. Foram realizadas dosagens sequenciais de interleucina 6 e interleucina 10 nas concentrações plasmáticas de interleucina 6 e interleucina 10 no plasma. Foram coletadas amostras de tecidos (pulmão, fígado e intestino) para medir malondialdeído, mieloperoxidase e estrufia oxidativa.

Conclusão: Neste modelo de isquemia intestinal transitória, a manutenção adequada de volume intravascular diminuiu o estresse oxidativo e a síntese de marcadores de inflamação. Tanto a solução hipertônica quanto a fisiológica atenuaram os efeitos deletérios observados após isquemia intestinal.

Autores’ contributions

Wilson Kohama Chimabucurro was responsible for all analyses and the content of the article. Bomfim Alves da Silva Junior contributed to the article elaboration. Ana Iochabel Soares Moretti contributed to the biochemistry analysis. Ester Correia Sarmento Rios contributed to article adjustment. Irineu Tadeu Velasco and Francisco Garcia Soriano are responsible for the idea and the project.
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