Hypertonic saline activates CD4\(^+\) and CD8\(^+\) T-lymphocytes in the small intestine to alleviate intestinal ischemia-reperfusion injury


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Introduction

Intestinal ischemia-reperfusion (I/R) injury is a common syndrome seen in clinic following abdominal aortic aneurysm surgery, intestinal transplantation, strangulated hernia, neonatal necrotizing enterocolitis, and shock.\(^1\)\(^-\)\(^3\) Intestinal I/R injury not only alters the absorptive function of intestine, but also causes increased mucosal permeability, bacterial translocation,\(^4\) and further mucosal hypoperfusion. Administration of resuscitation fluid is a common intervention for correction of the deranged hemodynamic status and management of I/R. However, mucosal injury may be exacerbated by perfusion with resuscitation fluids, such as crystalloid or artificial colloids (dextran and h espan) solutions.\(^5\)\(^,\)\(^6\) The initial response to this injury of gut is activation of local immune cells and generation of pro-inflammatory mediators, which contribute to both local and distant organ injuries, especially injuries to the lungs, ultimately leading to multiple organ failure and death.\(^9\)\(^,\)\(^10\)

Recently, it has been suggested that resuscitation with small volume of hypertonic saline (HS) (4 mL of 7.5% NaCl per kilogram of body weight) can effectively prevent inflammatory tissue damages\(^11\) and modulate post-injury immunologic disorders evoked by I/R.\(^2\)\(^,\)\(^3\)\(^,\) Rizoli et al.\(^14\) have demonstrated that HS could blunt neutrophils activation by down-regulating the expression of neutrophil adhesion molecules (CD11b and CD62L) that are activated after hemorrhage shock. In addition, HS could elicit monocyte redistribution, leading to increased ratio of CD14\(^+\) monocytes, but decreased percentage of pro-inflammatory CD14\(^-\)CD16\(^+\) subsets.

Abstract. – OBJECTIVE: Intestinal ischemia-reperfusion injury (I/R) is a common syndrome encountered in clinic following intestinal surgery, strangulated hernia, and shock. Hypertonic saline has been shown to prevent inflammatory tissue damages caused by I/R and regulate immunologic disorders in peripheral blood. However, the immunoregulatory effects of hypertonic saline on the small intestine response to intestinal I/R have not been reported.

MATERIALS AND METHODS: To investigate this, we created the intestinal I/R model by clamping the superior mesenteric artery in Sprague-Dawley rats. After 1 hour of ischemia, the vascular clamp was removed, and either normal saline (0.9% NaCl, NS group) or hypertonic saline (7.5% NaCl, HS group) was administered through the tail vein (6 ml/kg). The CD4\(^+\) and CD8\(^+\), primarily T-lymphocytes subpopulation yielded from the intestinal tissues, were determined by immunohistochemistry.

RESULTS: A pro-inflammatory cytokine, tumor necrosis factor (TNF)-\(\alpha\), and nuclear factor kappa B (NF-\(\kappa\)B), a critical transcription factor for the TNF-\(\alpha\) gene, were measured in the intestinal and lung tissues with ELISA. HS induced an increase in CD4\(^+\) and CD8\(^+\) T cells in the jejunum and ileum compared with the NS group. The levels of TNF-\(\alpha\) and NF-\(\kappa\)B in the intestinal and lung tissues were significantly decreased in the HS group compared with those of the NS group.

CONCLUSIONS: HS treatment may ameliorate the tissue damage induced by intestinal I/R. This protective effect is possible due to its ability to activate the CD4\(^+\) and CD8\(^+\) T-lymphocytes cells in the intestinal tissues and inhibit the intestinal I/R-induced expression of pro-inflammatory cytokines.

Key Words: Hypertonic saline resuscitation, Immune T cells, Intestinal ischemia reperfusion, Nuclear factor kappa B, Tumor necrosis factor.
Using a rat model of hemorrhagic shock, Lu et al. confirmed that HS could induce CD4+ markedly, but CD8+ T-lymphocytes to a certain extent, leading to high CD4+/CD8+ ratio in peripheral blood. The study carried out by Murao et al. indicated that HS could reduce gd T cells at 24 hours and increase apoptosis of CD4+CD25+ regulatory T cells at 48 hours in mice with hemorrhagic shock. However, these studies mainly focus on investigating the altered peripheral immunologic indexes of HS. To the best of our knowledge, whether HS also has regulatory effects on the immune cells of small intestine following the intestinal I/R remains not reported.

The present study was designed to investigate the effects of HS resuscitation on intestinal I/R-induced local responses (CD4+ and CD8+, primarily T-lymphocytes subpopulation in small intestine), systemic responses [Helper T-lymphocytes (CD3+CD4+), cytotoxic T-lymphocytes (CD3+CD8+), and nature killer (NK) cells in peripheral blood], and remote inflammatory responses [tumor necrosis factor (TNF)-α and nuclear factor kappa B (NF-κB) in lung tissues]. We hypothesize that HS treatment may have a beneficial effect on the intestinal I/R injury by inhibiting the intestinal I/R-induced expression of pro-inflammatory cytokines and modulating the balance of immune T cells.

Materials and Methods

Animals

Twenty-four male Sprague-Dawley rats weighing 200-220 g, provided by the Experimental Animal Center of Second Military Medical University, were used for this study. Rats were housed with free access to food and water under a natural day/night cycle, and acclimated for 7 days before any experimental procedures. All rats received humane care according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The protocol study was approved by the ethics committee for animal research at the Second Military Medical University, China.

Experimental Design

Rats were randomly divided into three groups: sham-operated group (SHAM group, n = 8); intestinal ischemia + normal saline group (NS group, n = 8) and intestinal ischemia + hypertonic saline group (HS group, n = 8). All animals were anesthetized with 10% chloral hydrate (4 mL/kg, i.p.) and placed in a supine position. After an incision was made on the midline of the abdomen, the intestinal ischemia model was created by occlusion of the superior mesenteric artery and vein with atraumatic vascular clamps. The ischemia was confirmed when the mesenteric pulsations ceased and the intestines became pale. After 1 hour of ischemia, a vascular clamp was removed, and either 6 ml/kg normal saline (0.9% NaCl, NS group) or 6 ml/kg hypertonic saline (7.5% NaCl, HS group) was administered to rats through the tail vein. Then the abdomen was closed and intestinal reperfusion was induced for 6 hours. The SHAM group served as a control in which rats were subjected to the same surgical manipulations as NS group without undergoing superior mesenteric artery and vein clamping.

Peripheral Blood and Tissue Sampling

Animals were sacrificed for peripheral blood and tissue sampling at the end of 6-hour reperfusion. Peripheral blood samples of the three groups were collected from the inferior vena cava for the measurement of blood routine, CD3+CD4+ T cells, CD3+CD8+ T cells, and NK cells. Subsequent to the blood sampling, the small intestine from the Treitz ligament to the ileal-cecal valve was stripped from the mesentry. About 1 cm of jejunum (1 cm distal to Treitz ligament) and ileum (1 cm proximal to ileal-cecal valve) were harvested for hematoxylin-eosin (HE) staining and immunohistochemical assessment of CD4+ and CD8+ T-lymphocytes in the intestinal intraepithelial spaces and lamina propria. The middle small intestine and left lung were harvested and frozen in liquid nitrogen for the measurement of TNF-α and NF-κB.

HE Staining of the Small Intestine Tissues

Small intestine sections were embedded in paraffin after fixation with 10% neutral buffered formalin for 48 hours and, then, sectioned and stained with HE. Two samples from each segment were examined under a light microscope. The histological evaluation of re-perfused intestinal tissues was performed independently and blindly by two reviewers according to the method previously described. The intestinal injury was graded on a scale ranging from 0 to 5: Grade 0, normal structure; Grade 1, sloughing of surface epithelium, mild mucosal damage; Grade 2,
loss of one-third of mucosal crypts, moderate damage; Grade 3, loss of two-thirds of mucosal crypts, extensive damage; Grade 4, mural infarct, mucosal and submucosal necrosis were present; and Grade 5, transmural infarct, necrosis in areas throughout the thickness of the intestinal wall. The overall injury score in each group was calculated as the mean of the grade for the eight rats.

**Immunohistochemistry**

The distribution of CD4+ and CD8+ T-lymphocytes in the intestinal intraepithelial spaces and lamina propria was detected by immunohistochemical staining. Briefly, these specimens were dewaxed and incubated with 3% H2O2 in methanol at 37°C for 10 minutes to quench endogenous peroxidase activity. After being blocked at room temperature for 30 minutes, the sections were incubated with rabbit polyclonal antibody against rat CD4 and CD8 (Groundwork Biotechnology Diagnosticate Ltd, San Diego, CA, USA) overnight at 4°C and then with goat polyclonal antibody against rabbit EnVision-HRP (Dako Company, Giostrup, Denmark) for 40 minutes at room temperature. The specimens were counterstained by immersion in hematoxylin. The positive-staining cells were enumerated in 20 randomly selected fields under a light microscope (magnification, 400×).

**Flow Cytometry Analysis**

CD3+CD4+ T cells, CD3+CD8+ T cells and NK cells in peripheral blood were analyzed by two-color or single-color flow cytometry (Becton Dickinson FACScan, San Jose, CA, USA) as previously described19. Briefly, 0.5 ml of peripheral blood was collected in a microfuge tube, in which one drop of heparin sodium was previously added. Phosphate buffered saline (PBS) (0.5 ml) was added to each microfuge tube. After thorough mixing, the suspension was layered over 1 ml of lymphocytes separation medium (LSM) and centrifuged at 1500 rpm for 20 minutes at 23°C. The buffy layer was aspirated from the PBS/LSM interface and washed with PBS. Lymphocytes were stained with the appropriate antibodies for 20 minutes at 23°C. Fluorescein isothiocyanate (FITC)-conjugated anti-rat CD3 (clone G4.18, MultiSciences Biotech Co. Ltd., Shanghai, China) and allophycocyanin (APC)-conjugated anti-rat CD4 (clone OX-35, MultiSciences Biotech Co. Ltd., Shanghai, China) were utilized for staining of CD3+CD4+ T cells. FITC-conjugated anti-rat CD3 and phycoerythrin (PE)-labeled anti-rat CD8a (clone G28, MultiSciences Biotech Co. Ltd., Shanghai, China) were used for staining of CD3+CD8+ T cells. PE-labeled anti-Rat natural killer cell receptor (NKR)-P1A (clone 10/78, Invitrogen, Carlsbad, CA, USA) was utilized for NK cells identification. Samples were collected on a Becton Dickinson FACScanlibur and analyzed with Cell QuestTM acquisition/analysis software.

**Determination of TNF-α and NF-κB Levels in the Small Intestine and Lung Tissues**

Small intestine and lung tissues samples were homogenized at 4°C for 30 seconds in polypropylene tubes containing lysis buffer (including 2 mM phenylmethyl-sulfonyl fluoride, 2 mg/ml of pepstatin A, aprotinin, antipain, and leupeptin (all purchased from Sigma Chemical Co., St. Louis, MO, USA) at a ratio of 10 ml buffer/g of wet tissues (WT). Homogenates were centrifuged at 100,000 g for 1 hour at 4°C. Levels of TNF-α and NF-κB were determined by using ELISA kits (Blue Gene, Shanghai, China). The absorbance was read on a microplate reader and the concentrations were calculated according to the standard curve.

**Statistical Analysis**

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Significant differences between the three groups were analyzed using one-way ANOVA followed by a post-hoc Tukey’s test. The Kruskal-Wallis and then Mann-Whitney U tests were performed to assess the pathological score values. p < 0.05 was considered statistically significant.

**Results**

**Histopathology of Small Intestine Tissues**

In SHAM group, the structure of the small intestinal mucosa was intact, and normal intestinal mucosa was observed (Figure 1). In NS group, glands of small intestine were apparently damaged, showing severe small intestine interstitial edema, sloughing of surface epithelium (vacularization, prominent neutrophil infiltration), loss of mucosal crypts, and necrosis in the mucosa and submucosa (Figure 1). However, in the HS group, the above damages were alleviat-
ed (Figure 1). In fact, the intestinal injury score of NS and HS groups was significantly higher than that of SHAM group \((p < 0.01)\), but the injury score of HS group was significantly decreased compared with NS group \((p < 0.05)\) (Table I).

**CD4+ and CD8+ T-lymphocytes Distribution in Small Intestine Tissues**

The CD4+ and CD8+ T-lymphocytes of HS and NS groups markedly increased, with significant differences compared with SHAM group \((p < 0.01)\), Table II). In addition, the CD4+ and CD8+ T-lymphocytes in the jejunum and ileum were also significantly increased in HS group compared with the NS group \((p < 0.01)\).

**Changes in White Blood Cells, Lymphocytes and CD3+CD4+ T Cells, CD3+CD8+ T Cells, and NK Cells in Peripheral Blood**

As shown in Table IV, there was no significant difference in the number of white blood cells, the ratios of lymphocytes and CD3+CD4+ T cells in peripheral blood among SHAM group, NS group and HS group (Table III). However, when compared with SHAM group where the animals did not experience intestinal I/R injury, the percentage of CD3+ T cells was reduced markedly, and the percentage of NK cells increased significantly in NS group where the animals experienced intestinal I/R injury \((p < 0.05)\). No significant differences in the ratios of CD3+ T cells between NS group and HS group were observed, but both the ratios of CD3+CD8+ T cells and NK cells were significantly higher in NS group than those in HS group \((p < 0.05)\).

**Cytokines Profiles in the Intestinal and Lung Tissues**

Compared with the SHAM group, levels of TNF-α and NF-κB in the intestinal and lung tissues were significantly increased in the NS and HS groups \((p < 0.01)\). However, the expressions of TNF-α and NF-kB in the intestinal and lung tissues were significantly lower in the HS group compared with the NS group \((p < 0.05)\).
Discussion

A number of studies have indicated that intestinal mucosa is the first-affected and most rapid change site when shock or other low-flow conditions occur. Because of containing a large number of T cells, the intestinal mucosa is known as a gut-associated lymphoid tissue. Sensitized lymphocytes from Peyer patches move to mesenteric lymph nodes and return to the systemic circulation via the thoracic duct, then home to intestinal (lamina propria and intraepithelial spaces) and extra-intestinal mucosal systems. Lamina propria lymphocytes (LPLs) are primarily conventional CD4+TCRαβ+ or CD8αβ+TCRαβ+ T cells, and intestinal intraepithelial lymphocytes (IELs) are primarily CD8α+ T cells. In a 60-min intestinal I/R model, lymphocytes yielded from the IELs and LPLs are shown to be decreased to only 20%-30% of those from SHAM animals, which may be an important mechanism underlying the dysfunction of gut barrier. To alter the above changes may be a potential strategy to relieve intestinal I/R. Our results showed that HS treatment could significantly increase the numbers of CD4+ T cells and CD8+ T cells in the IELs and LPLs of jejunum and ileum compared with the SHAM group, indi-

### Table II. CD4+ or CD8+ T cell number in different small intestine epithelium and laminae propria.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Group</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cell</td>
<td>SHAM</td>
<td>10.9 ± 3.8</td>
<td>11.9 ± 3.0</td>
<td>19.8 ± 6.6</td>
<td>35.7 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>17.3 ± 3.6*</td>
<td>16.9 ± 3.3*</td>
<td>36.1 ± 2.8*</td>
<td>39.5 ± 6.0</td>
</tr>
<tr>
<td>CD8+ T cell</td>
<td>SHAM</td>
<td>12.4 ± 3.6</td>
<td>19.7 ± 6.5</td>
<td>32.5 ± 6.6</td>
<td>46.2 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>21.5 ± 4.7*</td>
<td>29.9 ± 5.5*</td>
<td>36.7 ± 5.6</td>
<td>48.9 ± 14.3</td>
</tr>
</tbody>
</table>

The positive-staining cells were expressed as mean number of positive cells/20 randomly selected fields. The data are shown as mean ± SD. *p < 0.01 compared with SHAM group; †p < 0.01 compared with NS group. Groups are defined in the Table I. IELs, intestinal intraepithelial lymphocytes; LPLs, lamina propria lymphocytes.

### Table III. The number and percentage of CD3+CD4+ T cells, CD3+CD8+ T cells, and NK cells in peripheral blood.

<table>
<thead>
<tr>
<th>Group</th>
<th>White blood cells (×10^9/L)</th>
<th>Lymphocytes (%)</th>
<th>CD3+ cells (%)</th>
<th>CD3+CD4+ T cells (%)</th>
<th>CD3+CD8+ T cells (%)</th>
<th>NK cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>6.6 ± 1.6</td>
<td>88.5 ± 11.5</td>
<td>41.2 ± 6.9</td>
<td>28.8 ± 5.8</td>
<td>10.0 ± 1.6</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>NS</td>
<td>7.4 ± 2.2</td>
<td>84.6 ± 18.3</td>
<td>32.5 ± 4.32*</td>
<td>24.4 ± 7.4</td>
<td>9.7 ± 1.1</td>
<td>11.0 ± 3.9*</td>
</tr>
<tr>
<td>HS</td>
<td>6.1 ± 1.4</td>
<td>85.2 ± 12.9</td>
<td>35.4 ± 2.9</td>
<td>27.3 ± 3.9</td>
<td>7.6 ± 1.7*†</td>
<td>7.3 ± 1.7†</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SD. *p < 0.05 compared with SHAM group; †p < 0.05 compared with NS group.

### Table IV. Levels of TNF-α and NF-κB in the intestinal and lung tissues.

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α in small intestine (pg/ml)</th>
<th>TNF-α in lung (pg/ml)</th>
<th>NF-κB in small intestine (ng/ml)</th>
<th>NF-κB in lung (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>246.9 ± 7.4</td>
<td>248.2 ± 9.1</td>
<td>89.7 ± 5.4</td>
<td>87.8 ± 7.9</td>
</tr>
<tr>
<td>NS</td>
<td>307.2 ± 20.8*</td>
<td>317.2 ± 15.1*</td>
<td>120.0 ± 6.7*</td>
<td>114.9 ± 7.1*</td>
</tr>
<tr>
<td>HS</td>
<td>282.7 ± 14.4*†</td>
<td>296.2 ± 15.6*†</td>
<td>108.5 ± 10.1*†</td>
<td>104.4 ± 7.9*†</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SD. *p < 0.01 compared with SHAM group; †p < 0.05 compared with NS group.
cating HS resuscitation has immunomodulatory effects for the small intestine, which was not reported previously.

The CD4⁺ T-lymphocytes can be differentiated into the mature Th1 or Th2 cells to participate in cell-mediated or antibody-mediated immunity²⁴. The CD8⁺ T-lymphocytes can be divided into suppressor (TS) or cytotoxic T (TC) cells. TS cells can suppress inflammatory responses and TC cells can effectively kill the target through release of cytotoxic granules²⁵. The cooperation of CD4⁺ and CD8⁺ subpopulations ultimately inhibits bacterial translocation from the gastrointestinal tract²⁶,²⁷. The tightly regulated local response reduces a systemic immune reaction. Our study indicated that after HS resuscitation, both the ratios of CD3⁺CD8⁺ T cells and NK cells in peripheral blood were significantly decreased, which was firstly investigated although several studies have demonstrated that HS resuscitation can down-regulate the following immune cells in peripheral blood, including neutrophils, CD14⁺CD16⁺ monocytes¹⁴, and CD4⁺CD25⁺ regulatory T cells¹⁵.

NF-κB is a critical nuclear transcription factor known to induce several pro-inflammatory genes, including TNF-α, interleukin (IL)-1β, and IL-6²⁸. Funaki et al²⁹ have reported that attenuation of NF-κB activation with subsequent reduction in TNF-α mRNA expression may be an important protective mechanism of ischemic preconditioning against hepatic I/R injury. NF-κB has been demonstrated to be related with local intestine and remote lung injuries in rat intestinal I/R models⁵⁰. Thus, we investigated the expression levels of NF-κB and TNF-α in the intestinal and lung tissues. Our findings suggested that the expression of NF-κB and TNF-α in the intestinal and lung tissues was significantly increased after intestinal I/R, and HS treatment could significantly reduce their expression levels compared with the NS group, which is consistent with previous studies⁵¹,²⁵,³¹. However, Soares et al²⁵ recently report that TNF-α is not associated with intestinal I/R-induced lung inflammation. Therefore, further investigations are still needed to confirm our conclusions.

Conclusions

HS treatment significantly alleviates the severity of intestinal I/R injury via inhibiting the expression of TNF-α and NF-κB and activating CD4⁺ T cells and CD8⁺ T cells in the small intestine. This may provide a promising method to treat acute intestinal I/R injury that occurs in intestinal transplantation or small intestinal surgery clinically.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

Hypertonic saline induces CD4+/CD8+ T cells in IIR injury


31) Shihr HC, Huang MS, Lee CH. Magnolol attenuates the lung injury in hypertonic saline treatment from mesenteric ischemia reperfusion through diminishing iNOS. J Surg Res 2012; 175: 305-311.