Protective effect of salvianolic acid B against intestinal ischemia reperfusion-induced injury in a rat model

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Abstract

Purpose: To evaluate the gastro-protective efficacy of salvianolic acid B (SAB) against intestinal ischemic-reperfusion injury (IIRI) in a rat model.

Methods: Forty-eight healthy male rats were randomly chosen and divided into 4 groups of 12 rats each. Control group rats underwent laparotomy without occlusion; IIRI group rats underwent laparotomy with occlusion for 60 min, followed by 24 h of reperfusion; SAB + IIRI group received 7 days of pretreatment with 40 mg/kg of SAB + IIRI; while the fourth group received only SAB. The antioxidant, inflammatory markers, intestinal permeability marker, as well as intestinal histopathological changes were assessed.

Results: The activities of antioxidants including reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were significantly ameliorated (p < 0.01) in SAB-supplemented group (SAB + IIRI). The concentration of inflammatory markers, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and nuclear factor p65 (NF-p65) as well as small intestinal permeability marker (FITC-Dextran), were significantly reduced (p < 0.01) following administration of SAB for 7 days. In addition, pretreatment with SAB reverted intestinal (ileum) histopathological changes to almost normal architecture with significant reduction in Chiu score.

Conclusion: The results of this study demonstrate that SAB may protect the intestine by attenuating oxidative stress and inflammatory response and hence, may be potentially for treating IIRI.

Keywords: Salvianolic acid B, Intestinal Ischemia-reperfusion, Antioxidants, Inflammation, Intestinal permeability

Introduction

Intestinal ischemia-reperfusion injury (IIRI) is a serious clinical condition occurred during septic shock, cardiopulmonary bypass, small bowel transplantation, strangulated hernias due to gastric barrier dysfunction and bacterial translocation [1]. During intestinal ischemia, rapid restoration of blood flow is vital, nevertheless reperfusion might collapse the systemic circulation that triggers systemic inflammatory response and mucosal injury and ultimately ends up in multi-organ failure (MOF) and thus entails with increased mortality and morbidity rate [2]. Several researchers have indicated that inflammatory response and oxidative stress are the two crucial events that act as vicious cycle and elicit mucosal damage and ultimately results in IIRI [3,4]. Previously, studies have highlighted that phytocompounds with antioxidant and anti-
inflammatory properties abolish IIRI in various animal models [5,6].

Salvianolic acid B (SAB), a natural water-soluble phenolic acid extracted from *Salvia miltiorrhiza* (Danshen), is prescribed for improving blood circulation related to menstrual and dermal disorders [7]. SAB also exert various pharmacological properties including antioxidant, anti-cancer, anti-inflammatory, and immunomodulatory [8,9]. Previously, *Salvia miltiorrhiza* has shown to protect gastric mucosa owing to the presence of salvianolic acid A/B [10]. Moreover, SAB has a positive effect on various experimental IR model in different organs [7,11,12]. Hence, it is probable that SAB may also alleviate IR-induced injury in rat model. Therefore, this pre-clinical study aimed to explore the gastroprotective property of SAB by evaluating various inflammatory markers (cytokines), antioxidant status and ileum histopathology analysis in IIRI a rat model.

**EXPERIMENTAL**

**Experimental rats**

Male healthy Sprague-Dawley rats (n = 48) weighing 280 ± 10 g were procured from Animal center of Nanchang University, China. All the animals were maintained at 22 ± 2 °C with 12 h day light/ night dark cycle with full access to food and water (*ad libitum*) in a stainless-steel cage. The Institutional Ethical Committee Board have approved this experimental study/protocol (NCU no. 8305-16) and was performed at the Animal Center of Nanchang University following the guidelines of the United States National Institutes of Health (NIH) [13].

**Surgical procedure/IRII insult**

All the rats were fasted for 12 h and anesthetized using pentabarbital sodium at the dose of 50 mg/kg via intraperitoneal (i.p) injection. Rats were placed on a warm pad to maintain at 37 °C throughout the surgical protocol. The intestinal IR injury was induced by performing midline laparotomy followed by isolation of the superior mesenteric artery (SMA) and followed by clamping by atraumatic arterial clamp for 60 min to block splanchnic circulation. Ischemia was confirmed by the absence of pulsation as well as pale color appearance. Then, reperfusion was performed by removing the clamp, and the reperfusion was confirmed by the recurrence of the pulse as well as the pinkish color appearance of the intestine. The surgical wound was closed using nylon sutures and reverted to respective cage. All the experimental rats underwent midline laparotomy followed by occlusion and reperfusion except the control rats which were not clamped.

**Animal groups**

Forty-eight healthy male SD rats were divided into 4 groups with 12 rats in each. Group I rats underwent laparotomy without occlusion or clamping and received only saline and served as control group. Group II rats underwent laparotomy with occlusion for 60 min and followed by 24 h of reperfusion (as indicated above) and received only saline and served as IIRI group. Group II rats were pretreated with 40 mg/kg of SAB (based on preliminary studies) via i.p. by dissolving with saline for 6 days prior IIR induction and 1 h prior IIRI induction and served as SAB + IIRI group. Group IV rats underwent laparotomy without occlusion received only SAB for 7 days and served as SAB alone group.

**Sample preparation**

Rats were sacrificed by cervical decapitation under pentobarbital sodium at the dose of 50 mg/kg via i.p after IIRI induction. Blood samples were collected in a heparinized tube to separated plasma. The small intestine-ileal tissues (20 cm) were excised immediately and a portion of ileal tissue (5 cm) was fixed in 10 % formalin for histological analysis and the remaining ilial tissues are homogenized (10 %) using phosphate buffered saline (PBS) solution to yield ileal tissue lysate (supernatant), were used for various biochemical analysis.

**Determination of lipid peroxidation products and antioxidant activity**

Lipid peroxidation product like melondialdehyde (MDA), as well as antioxidant activities of reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were assessed in ileal lysate using kit from Nanjing Bioengineering Institute (Nanjing, China). One Unit of SOD activity is equivalent to the amount required to inhibit 50 % of auto-oxidation. One Unit (U) of CAT One unit was defined as mg of tissue protein that consumed 1 µmol H₂O₂ at 405 nm in 1 s.

**Evaluation of myeloperoxidase (MPO) activity and proinflammatory cytokines**

Neutrophil infiltration was determined in the form of myeloperoxidase (MPO) activity in ileal lysate by MPO assay kit from Nanjing Bioengineering Institute (Nanjing, China). One Unit (U) of MPO...
activity was equal to the amount required for degrading 1 mmol peroxidase/min at 25 °C is expressed as unit per g wet weight (U/g). Moreover, nuclear factor kappa B (NF-kB) subunit (NF-p65) in the ileal tissue lysate were evaluated using nuclear fraction (extracted by NR-PER nuclear/cytoplasmic extract reagent from Thermo Fisher Scientific, MA, USA) and evaluated by NF-kB p65 transcription factor assay kit (Thermo Fisher Scientific, MA, USA). Furthermore, the levels of various pro-inflammatory cytokines such as interleukins-1β (IL-1β), interleukins-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were measured using commercial ELISA kit (USCN Business Co., Ltd. Wuhan, China).

### Intestinal permeability assay

To assess the intestinal permeability (epithelial barrier) of ileal tissue, fluorescein isothiocyanate (FITC)-conjugated dextran assay procedure was used by following the methods of Chang and his coworkers with slight modification [14]. Shortly, 1 h before the sacrifice, rats were anaesthetized under pentobarbital sodium at the dose of 50 mg/kg via i.p and ileum region (10 cm) were clamped again and administered the solution of FD4 dissolved in PBS (0.5 mg/mL). After 1 h all the rats were euthanized, and a blood sample was collected via cardiac puncture and the fluorescence concentration (FD4) of the plasma was measured by microplate reader with an excitation/emission wavelength 485/528 nm with FITC standards. The concentration of FITC-dextran was calculated using a standard curve and expressed as µg/mL.

### Histopathological studies

Freshly isolated ileum tissue (5 cm) were cut down to small pieces and fixed in formaldehyde (10 %) for 10 h and embedded in paraffin wax to form a tissue block. Those blocks were sliced into 4 µm sections and transferred to a glass slide and followed by staining with H & E stain. The stained slide was covered with cover slip and viewed under a light microscope (Olympus-BX53 Tokyo, Japan) at 400 x for any histopathological changes. The degree of histopathological changes of the ileal section was graded according to Chiu scoring system based on Chiu method [15].

### Data analysis

For data statistical analysis SPSS software (version 21, IBM Inc, NY, USA). Values are expressed as the mean ± standard deviation (SD). Chiu score was analyzed with Kruskal-Wallis test. For other parameters, the significance difference between the control group and SAB group were analyzed using one-way ANOVA followed by LSD post-hoc test. were determined by one-way ANOVA followed by least significant difference (LSD) for multiple comparison. $P < 0.05$ was considered statistically significant.

### RESULTS

#### Effect of SAB on the activities of ileal antioxidants and lipid peroxidation products

The activities of ileal antioxidants and lipid peroxidation products in experimental rats were represented in Table 1. The activities of GSH, CAT and SOD were significantly abolished (p < 0.01) with increased lipid peroxidation in IIR induced rats compared with control rats. Whereas, pretreatment with SAB for 7 days in IR-induced rats could significantly improve (p < 0.01) the antioxidants activity with decreased lipid peroxidation product.

#### Effect of SAB on ileal MPO levels

Figure 1 illustrate the effect of SAB on ileal MPO levels in experimental rats. Rats in the IIRI group showed increase (p < 0.01) levels of MPO than control rats. Administration of 40 mg of SAB in IIRI induced rats could considerably decreased (p < 0.01) the MPO levels than IIRI rats.

### Table 1: Effect of SAB on the activities of ileal antioxidants and lipid peroxidation products in experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>CAT (U/mg Protein)</th>
<th>SOD (U/mg Protein)</th>
<th>GSH (U/mg Protein)</th>
<th>MDA (nmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40.31±5.10</td>
<td>5.03±0.44</td>
<td>1.15±0.20</td>
<td>1.79±0.12</td>
</tr>
<tr>
<td>IIRI</td>
<td>28.33±4.00 a**</td>
<td>3.78±0.27 a**</td>
<td>0.71±0.12 a**</td>
<td>2.34±0.10 a**</td>
</tr>
<tr>
<td>SAB + IIRI</td>
<td>36.67±4.09b**</td>
<td>4.51±0.55b**</td>
<td>0.95±0.09b**</td>
<td>1.95±0.09b**</td>
</tr>
<tr>
<td>SAB alone</td>
<td>39.34±6.23</td>
<td>4.88±0.62</td>
<td>1.08±0.11</td>
<td>1.82±0.07</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation. **p < 0.05 (a) IIRI group compared with control rats, (b) SAB + IIRI compared with IIRI group

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Effect of SAB on ileal MPO levels of experimental rats. Values are expressed as the mean ± standard deviation; **p < 0.05 (a) IIRI group compared with control rats; (b) SAB + IIRI compared with IIRI group

Effect of SAB on ileal inflammatory markers

Table 2 epitomized the effect of SAB on the concentration of ileal inflammatory markers in experimental rats. The concentration of NF-p65 subunit, IL-1β, IL-6, and TNF-α in the ileal tissue of IIR induced rats were markedly increased (p < 0.01). SAB supplementation significantly decreased (p < 0.01) the concentration of NF-p65 subunit, IL-1β, IL-6, and TNF-α as compared to IIR group.

Effect of SAB on small intestine permeability

The effect of SAB on small intestinal permeability (ileum) in experimental rats are showed in Figure 2. A pronounced increase (p < 0.01) in the levels of FITC-Dextran (FD4) was observed in the intestinal IR-induced group. Treatment with SAB for 7 subsequent days prior to IIR induction, could significantly decreased (p < 0.01) the levels of FITC-dextran in comparison with IIR rats.

Table 2: Effect of SAB on the concentration of ileal inflammatory markers in experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α (ng/g protein)</th>
<th>IL-1β (ng/g protein)</th>
<th>IL-6 (pg/g protein)</th>
<th>NF-p65 (ng/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>72.66±7.21</td>
<td>41.10±8.80</td>
<td>46.05±4.67</td>
<td>65.23±5.12</td>
</tr>
<tr>
<td>IIRI</td>
<td>165.03±12.57 a**</td>
<td>78.44±10.16 a**</td>
<td>92.70±10.21 a**</td>
<td>121.47±13.39 a**</td>
</tr>
<tr>
<td>SAB + IIRI</td>
<td>126.56±15.20 b**</td>
<td>52.26±7.31 b**</td>
<td>60.82±11.60 b**</td>
<td>81.05±9.27 b**</td>
</tr>
<tr>
<td>SAB alone</td>
<td>93.72±10.11</td>
<td>44.91±10.22</td>
<td>48.40±10.10</td>
<td>69.11±11.22</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation; **p < 0.05 (a) IIRI group compared with control rats, (b) SAB + IIRI compared with IIRI group

Figure 1: The effect of SAB on ileal MPO levels of experimental rats. Values are expressed as the mean ± standard deviation; **p < 0.05 (a) IIRI group compared with control rats; (b) SAB + IIRI compared with IIRI group

Figure 2: The effect of SAB on small intestinal permeability (ileum) in experimental rats. Values are expressed as the mean ± standard deviation; **p < 0.05 (a) IIRI group compared with control rats, (b) SAB + IIRI compared with IIRI group
Figure 3: Effect of SAB on the ileal section with H & E staining in experimental rats as well as Chiu score. The slide of the ileal tissue of control rats (nil Chiu score) showed the normal morphology/architecture of villus (Figure 3A). The slide of IIRI induced rats (highest Chiu score) represents the presence of denuded villi and lamina propria with massive infiltration of neutrophils and extensive necrosis (arrow mark) accompanied by disrupted brush border and edema (Figure 3B). Slide of SAB pretreated with IIRI induced rats (mild Chiu score) revealed a mild villi denudation with lesser edema and necrosis (arrow mark) (Figure 3C). Whereas, the slide of SAB + IIRI rats (nil Chiu score) displayed normal morphology of ileum with prominent villi and looked similar to control rats (Figure 3D); Values are expressed as the mean ± standard deviation; **p < 0.05 (a) IIRI group compared with control rats, (b) SAB + IIRI compared with IIRI group

Effect of SAB on histological changes in ileal tissue

The histological changes in ileal tissue were evaluated using Chiu scores (Figure 3). The control rats ileal section showed normal mucosa with nil chiu score (Figure 3A). The ileal section of IIRI induced rats characterized by the presence of denuded villi and lamina propria with massive infiltration of neutrophils and extensive necrosis with highest (p < 0.01) Chiu score (Figure 3B). SAB pretreated with IIRI induced rats revealed a mild villi denudation with lesser edema and necrosis with lesser (p < 0.01) Chiu score (Figure 3C). SAB alone treated rats showed normal morphology of ileum with zero Chiu score (Figure 3D).

DISCUSSION

The present study highlighted that IIRI induced rats pretreated (preconditioned) with SAB for 7 days could significantly enhance the antioxidant status, with reduced inflammatory markers, intestinal permeability and histopathological changes and thus showcasing its gastrointestinal protective activity. Intestine and intestinal mucosa are highly susceptible to ROS (reactive oxygen species) after ischemia-reperfusion (IR) injury, as they act as intestinal barrier (prevent bacterial translocation) and directly involved in maintaining immune response as well as maintaining the nutritional homeostasis (exogenous antioxidants) by protecting enterocytes of villi [16].
In ischemia, lack of oxygen supply, would provoke the excessive production of ROS. Upon reperfusion, the ROS generation were enhanced and ultimately end in tissue or cell damage via lipid peroxidation [17] and hence the levels of MDA were increased with decreased antioxidant activities. Pretreatment with SAB significantly lowered the excessive production of free radicals in IR-induced rats. The results are in agreement with the results of Liu et al [18], who also noticed that pretreatment with salvianolic acid B could significantly increased the activities of GSH, CAT, and SOD in the PC12 cell line model.

Tongqiang et al [19] demonstrated that salvianolic acid B could up-regulate Nrf2 (Nuclear factor erythroid-2-related factor) protein expression and thereby enhance the production of various endogenous antioxidants like SOD and GSH. Several researchers have indicated that two free hydroxyl groups of salvianolic acid B could contribute to its free radical quenching ability and thus concomitantly lowered the lipid peroxidation products production [20,21].

Rats in the IIR group showed increased levels of MPO than control rats due to exuberant ROS production via activation of polymorphonuclear neutrophils (PNMs) and ultimately increases the neutrophil infiltration in the intestine and results in MOF. SAB administration in IIR induced rats significantly lowered the MPO levels by inhibiting neutrophil infiltration to the small intestine and thus protect the ileum from further damage. Previously, salvianolic acid B has been shown to alleviate neutrophil infiltration (activation) in ischemic reperfusion rabbit model owing to its anti-inflammatory property [22].

The concentration of NF-p65 subunit, IL-1β, IL-6, and TNF-α in the ileal tissue of IIR induced rats were significantly increased. As mentioned earlier, during IIR condition excessive activation of PNMs might upregulate the proinflammatory cytokines like IL-1β, IL-6, and TNF-α via NF-κB signaling pathway [4]. SAB intervention significantly brought back those inflammatory markers concentration to near normal level due to anti-inflammatory activity. Similar to our results, Wang and his colleague also documented that SAB could substantially restrain the levels of IL-1β, IL-6 and TNF-α by down-regulating NF-κB via inhibiting a TLR4 signaling pathway in cerebral ischemia/reperfusion injury model [23].

A marked increase in the levels of FITC-dextran (FD4) was observed in IIRI group due to the disruption of the tight junction (loss of adhesion molecules) [24]. Treatment with SAB might have decreased the levels of FITC-dextran owing to its antioxidant and anti-inflammatory properties. Deng et al indicated that salvianolic acid B with ginsenoside (Rg1) downregulates cytokine expression such as TNF-α, IL-1β and thus, improves the microvascular permeability in cardiac IR injury model [25].

Histological findings on ileal section stained with H & E of a control rats illustrate a normal mucosa with prominent villus with increased oxidative stress, an inflammatory response which results in increased intestinal permeability and eventually end in extensive necrosis and edema (intestinal damage). The above results are in accord with the results of Yan et al [26]. The ileal section of SAB-pretreated rats revealed mild villi denudation with lesser neutrophil infiltration, edema and necrosis with lower Chiu score, thus indicating its gastroprotective activity. The ileal section of SAB control rats displayed normal morphology with prominent villi and appeare similar to control rats with nil Chiu score.

CONCLUSION

The finding of this study demonstrate that SAB protects the rat small intestine (ileum) by alleviating oxidative stress, inflammatory response, neutrophil infiltration, intestinal permeability, and histopathological changes. Hence, SAB has a potential for treating ischemic/reperfusion condition in combination with standard drugs. Nevertheless, further experiments are needed to evaluate the underlying mechanisms of its gastro-protective activity.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

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Contribution of Authors

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