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*Original Article*

# Protection of berberine on intestinal mucosa following autologous orthotropic liver transplantation and its correlationship with p-AMPK activation and p-mTOR inactivation

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**Abstract:** Background: Ischemia-reperfusion injury occurs during the process of liver transplantation and remains a formidable challenge clinically. Current study aimed to demonstrate whether berberine (BBR) protects against intestinal damage caused by autologous orthotropic liver transplantation (OLAT). Methods: The present study was performed by using rat model of orthotropic liver autotransplantation (AT). Adult male Sprague-Dawley rats were randomly separated into 3 different groups including sham (S), AT and BBR administrated AT groups. After being anesthetized, blood vessels and ligaments of rats in S group were simply freed, and ringer lactate solution was injected for liver reperfusion as an inflow tract with 30 minutes of anhepatic phase. In AT group, rats underwent OLAT, of which entire anhepatic phase lasted for 30 minutes as well. In BBR group, BBR was administered intragastrically at the dosage of 200 $mg \cdot kg^{-1} \cdot d^{-1}$ for 7 days prior to AT conduction at 8 hours following surgery; inferior vena cava (IVA) blood was obtained to detect the levels of D-lactate and endotoxin. Rats were sacrificed at 8 and 24 hours after reperfusion. Then, immediately, the intestines were harvested to determine histologic injury and wet/dry (W/D) ratio, intestine homogenate was adopted to detect the levels of superoxide dismutase (SOD) and malondialdehyde (MDA). Western blot was applied to assess the protein expression levels of Bax, Bcl-2, phosphorylated AMP-activated protein kinase (p-AMPK) and phosphorylated mammalian target of rapamycin (p-mTOR). The cell apoptosis of intestine was evaluated by using Terminal-deoxynucleoitidyl transferase mediated nick end labeling. Results: It is shown that the tissue damage of rats in BBR group was significantly reduced compared with AT group, while no evident injury was observed in rats of S group. D-lactate, endotoxin, W/D ratio, MDA, Bax, p-AMPK, p-mTOR and cell apoptosis in rats of AT group and BBR group were significantly higher than that of S group. In AT group, these markers were significantly higher than in BBR group except AMPK protein level ($P<0.05$). In AT group and BBR group, the expression levels of SOD and Bcl-2 were significantly lower than that in S group, moreover, in BBR group, they were dramatically higher than AT group ($P<0.05$). Conclusion: Our data indicates a protective role of BBR against I/R injury during AT in rats, and the underlying mechanism might attribute to p-AMPK activation and p-mTOR in-activation.

**Keywords:** Berberine, reperfusion injury, liver, intestines

## Introduction

The underlying mechanisms of Ischemia-reperfusion (I/R) injury have not been fully elucidated. Mounting clinical and experimental studies suggested that oxidative stress, neutrophils, endothelial factors, cytokines and apoptosis are tightly associated with I/R injury [1], which frequently generates fatal impacts including sepsis and multiple organ failure to patients. Additionally, small intestine is the most sensitive and vulnerable viscus to I/R injury [2], which manifests congestion and hypoxia in anhepatic phase when the portal vein is blocked.

Berberine (BBR), a traditional herbal medicine, has been used to treat gut infection and diar-

rhea for thousands of years in China. In the early 20th century, it was founded to possess multiple pharmacological effects such as anti-inflammatory [3], anti-oxidant [4], anti-tumor [5], and cholesterol-lowering [6] effects. Recently, BBR was reported to exhibit protective effects on radiation-induced intestinal injury [7]. Moreover, BBR administration was demonstrated to attenuate intestinal mucosa injury induced by I/R, suppress intestinal permeability up-regulation and protect tight junction (TJ) architecture [8]. In addition, Sheng and his colleagues [9] discovered that in human renal proximal tubular cells, BBR reduced hypoxia/re-oxygenation-induced injury via suppressing mitochondria/ER stress.

AMPK, a heterotrimer composed of α, β, and γ subunits, functions as a serine-threonine kinase, acting as an emerging crucial modulator of diverse cellular pathways in the setting of energetic stress [10, 11]. It can be activated by depletion of cellular ATP and elevation of AMP induced by glucose deprivation, hypoxia, and ischemia. As a well-conserved protein, AMPK is widely expressed in endothelial cells with diverse origins. Its activation depends on phosphorylation of Thr172 in the activation loop, which is mediated by at least two kinases including Peutz-Jeghers syndrome kinase (LKB1) and $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK). BBR was reported to activate AMPK signaling pathway via inhibiting respiratory complex I in mitochondria [12, 13], which plays a pivotal role in energy metabolism and balance.

In this study, we aimed to investigate the effects of BBR on intestinal I/R injury via detection of plasma D-lactate and intestinal cell apoptosis.

## Materials and methods

### *Animals*

Twenty-four male SD rats (Laboratory Animal Center of Military Academy of Medical Sciences, Beijing, China) weighing 200-250 g were fed under controlled temperature and humidity, fasted 12 hours before surgery but free to water. All studies were approved by China Laboratory Animal Management Committee.

### *Groups*

Rats were randomly separated into three different groups: sham (S), orthotropic liver autotransplantation (AT) and BBR administrated AT groups (n=8/group). In S group, blood vessels and ligaments were simply freed. Rats in AT group underwent orthotropic liver autotransplantation. In BBR group, rats were administered intragastrically with 200 mg/kg BBR daily for 7 days prior to AT.

### *Surgical procedure*

AT model was conducted as previously described by Jin and his colleagues [14]. Briefly, rats were anesthetized by intraperitoneal injection of 2.5% pentobarbital (50 mg/kg). Then, the abdomen of rat was incised, and the falciform ligament of liver was resected. Blood vessels along esophagus were removed. The liver was severed until suprahepatic vena cava (SVC) was totally liberated. A homemade retractor was utilized to guide the SVC for blockage. The liver was replaced orthotopically with IVC above the right renal vein. Portal vein (PV) was dissected from the convergence point of inferior mesentery and splenic veins.

Thereafter, hepatic artery and biliary tract were liberated together in virtue of their anatomic relationshiop. Vascular clamps were adopted at the convergence point of the inferior mesentery, splenic veins, hepatic artery, SVC and IVC. PV was punctured with a No. 4 transfixion pin for reperfusion followed by fixation with a vascular clamp. Ringer lactate solution was injected at the speed of 2.5 ml/min for reperfusion; a 1-mm incision was performed in IVC as an outflow tract. The liver would gradually turn yellow when the reperfusion was successfully performed. Eventually, the transfixion pin was pulled out, IVC incisions were repaired utilizing 8-0 sutures, PV, SVC, IVC and hepatic artery were unclamped. Normal anatomical position was maintained to avoid uneven perfusion. The entire anhepatic phase lasted for 30 minutes. 0.5ml pre-warmed (37°C) saline was instilled into peritoneal cavity before closure by suturing. In BBR group, BBR was administrated intragastrically at the dose of 200 $mg \cdot kg^{-1}$ $day^{-1}$ for 7 days prior to AT. There was no significant difference in survival rate between AT group and BBR group. The rats of all groups were sacrificed at 8 and 24 hours after reperfusion. Experiments were repeated at least for three times. The blood samples of rats were used for detection of SOD, MDA, D-lactate and endotox-

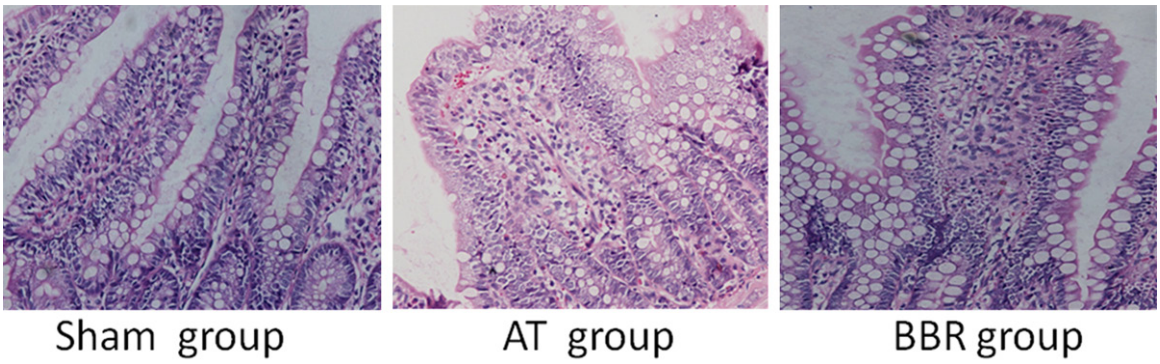


**Figure 1.** Intestine microstructure in sham, AT and BBR groups (×400). The Intestines in BBR group exhibits significantly less swollen compare to AT group.

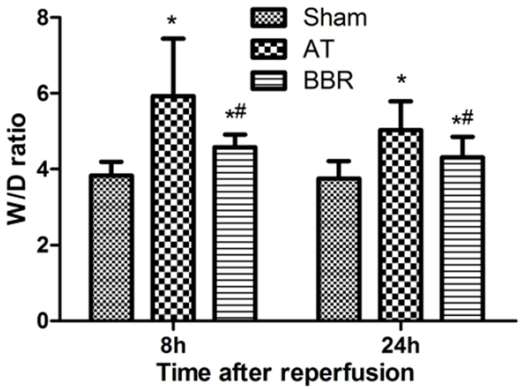


**Figure 2.** W/D ratio in the AT group was the highest among the three groups; BBR pretreatment can obviously attenuate intestinal mucosa edema. *$P$<0.05, compared with sham group, #$P$<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation.

in. The small intestines, beginning from jejunum and ending with terminal ileum, were excised to assess the alteration of W/D ratio, histological damage, the expression levels of SOD, MDA, D-lactate and endotoxin, apoptosis and western blot.

*Histological examination*

Intestinal samples were immersed in 10% formalin for at least 24 hours followed by dehydration in ethanol and embedding in paraffin. Sections were gathered for hematoxylin-eosin (H&E) staining.

*Blood homogenization*

At the end of the experiment, serum D-lactic acid and endotoxin levels were analyzed. Briefly, IVC blood samples were collected in polypropylene tubes, centrifuged at the speed of 3000 rpm/min for 15 minutes, then serum samples were separated and stored at -80°C until the assessment of D-lactic acid and endotoxin levels using enzyme-linked immunosorbent assay (ELISA) (Groundwork Biotechnology Diagnosticate Ltd., USA).

*Detection of lipid peroxidation and superoxide dismutase activity in intestinal tissues*

Intestines were homogenized with a homogenizer in saline, which was performed on ice. The homogenates were centrifuged at 4000g for 10 min at 4°C. The levels of lipid peroxidation products, i.e. MDA level and SOD activity from supernatants were obtained and assayed in accordance with the manufacturer's instructions (Jian cheng Bioengineering Institute, Nanjing, China). Each measurement was performed in duplicate. The amount of MDA was expressed as nmol/mg, SOD activity was expressed as U/mg.

*TUNEL assay*

TUNEL staining was operated according to manufacturer's instruction (Roche, USA). Briefly, deparaffinating of paraffin-embedded sections was carried out with xylene followed by 100%, 95%, 85%, 70% and 50% ethanol. Sections were rinsed with phosphate-buffered saline (PBS) for 5 min and administrated with protease K (20 μg/ml) for 20 min at room temperature. After washing with PBS twice, sections were incubated with 100 μl TUNEL reaction solution in humidified environment at 37°C for 60 min, followed with PBS washing three

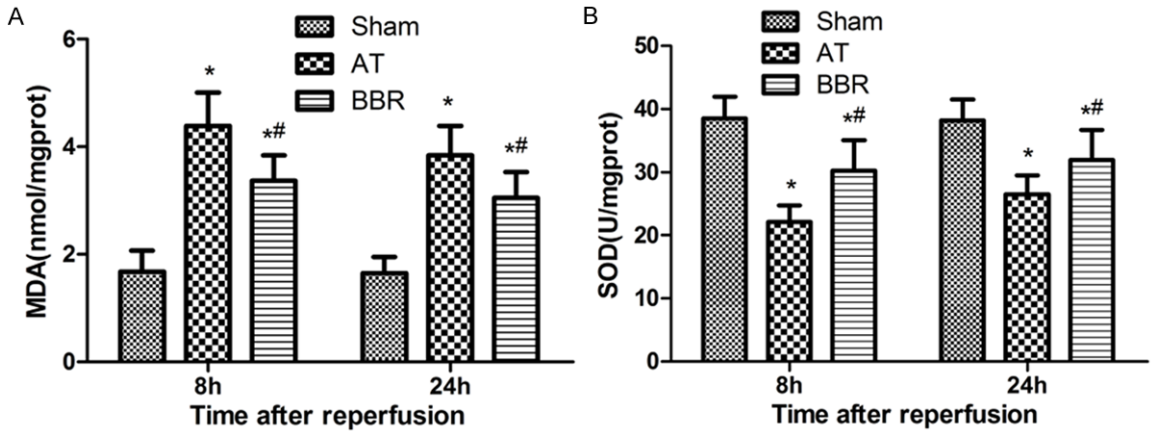


**Figure 3.** A. Effect of BBR on MDA levels after reperfusion. The level of MDA was significantly increased in the AT group compared with the sham group. BBR administration can significantly reduce the MDA level. Data were the mean ± SD values of rats. **P*<0.05, compared with sham group, #*P*<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation. B. Effect of BBR on the activity of SOD after reperfusion. The activity of SOD was significantly decreased in the AT group compared with the sham group. BBR pretreatment could significantly increase the SOD level. Data were the mean ± SD values of rats. **P*<0.05, compared with sham group, #*P*<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation.

times. Sections were treated with 100 μl streptavidin HRP at room temperature at 37°C for 30 min. Then sections were rinsed with PBS twice followed by incubation with 100 μl substrate at room temperature for 6 min. Mounting was performed after washing with deionized water for several times. The number of TUNEL positive cells was counted under a microscope (400×), and the quantity of apoptotic cells were presented by the average of the numbers of 7 independent fields.

*Western blot analysis*

Proteins were extracted from intestines, followed by homogenization through sonication and centrifugation at 14,000 rpm/min for 10 min at 4°C. Twenty-five micrograms of protein was separated on 10% SDS polyacrylamide gel and transferred to 0.2-mm nitrocellulose membrane which was blocked with TBST (5% skim milk, 10 mM Tris-HCl, pH 7.5,150 mM NaCl, and 0.1% Tween 20) for 1 h. After rinse with TBST for three times, the membranes were incubated with primary antibodies including Bcl-2, Bax, β-actin, p-AMPK, AMPK, p-mTOR and mTOR (1:1000, Cell Signaling Technology, USA) over night at 4°C, followed by incubation with their corresponding secondary antibody for 30 min at 37°C. After rinsed with TBST for 3 times, the positive protein bands were developed via a chemiluminescent system and scanned.

*Statistical analysis*

Continuous variables were summarized using mean ± standard deviations (SD). The mean values of W/D ratios, MDA and SOD contents in intestines, serium D-lactic acid and endotoxin, Bax, Bcl-2, p-AMPK and p-mTOR expressions in the three groups were compared using analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. *P* values <0.05 were considered as statistically significant. All analyses were performed using SPSS (version 17.0).

## Results

*Morphological examination of intestine*

No apparent pathologic changes were observed in the intestines of S group (**Figure 1**). However, the intestines in AT group presented severe inflammatory cell infiltration and edema. In BBR group, BBR pre-treatment remarkably attenuated AT induced edema as well as inflammatory cell infiltration.

*W/D ratio*

Our data revealed that BBR suppressed mucosal edema at both 8 h and 24 h after AT. As shown in **Figure 2**, W/D ratios were significantly elevated in both AT and BBR groups compared with S group, whereas BBR pretreatment dramatically attenuated the increase.

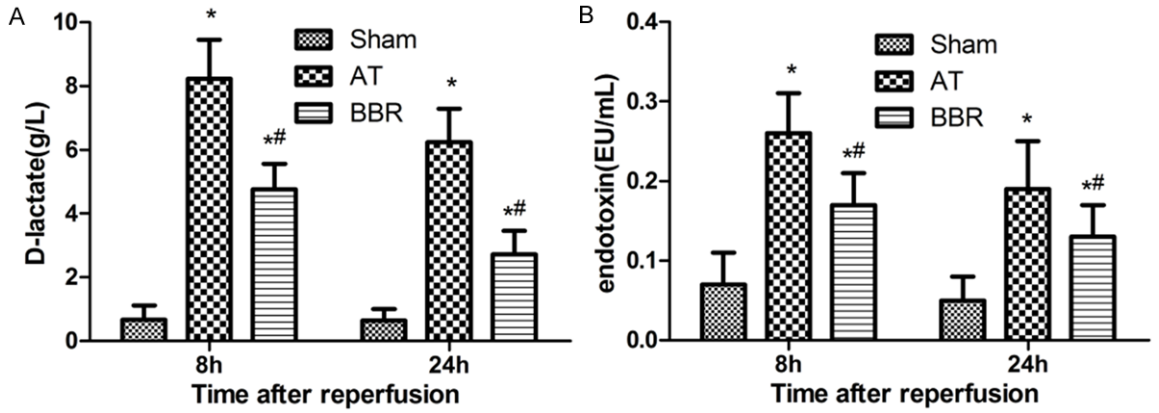


**Figure 4.** A. Effect of BBR on serum D-lactate concentration after reperfusion. The concentration concentration of serum D-lactate was significantly increased in the AT group compared with the sham group. BBR pretreatment could significantly decrease the serum D-lactate concentration. Data were the mean ± SD values of rats. **P*<0.05, compared with sham group, #*P*<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation. B. Effect of BBR on serum endotoxin concentration after reperfusion. The concentration of serum endotoxin was significantly increased in the AT group compared with the sham group. BBR pretreatment could significantly decrease the serum endotoxin concentration. Data were the mean ± SD values of rats. **P*<0.05, compared with sham group, #*P*<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation.

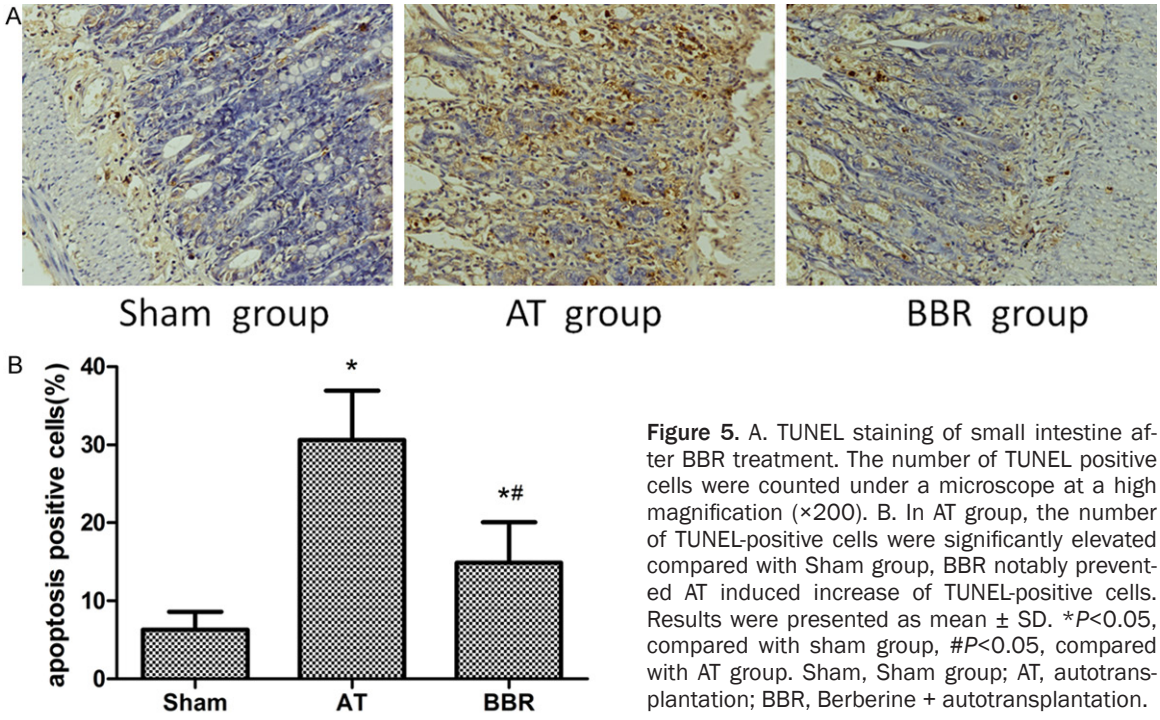


**Figure 5.** A. TUNEL staining of small intestine after BBR treatment. The number of TUNEL positive cells were counted under a microscope at a high magnification (×200). B. In AT group, the number of TUNEL-positive cells were significantly elevated compared with Sham group, BBR notably prevented AT induced increase of TUNEL-positive cells. Results were presented as mean ± SD. **P*<0.05, compared with sham group, #*P*<0.05, compared with AT group. Sham, Sham group; AT, autotransplantation; BBR, Berberine + autotransplantation.

*MDA and SOD contents in the intestinal tissue*

MDA levels were dramatically increased in both AT and BBR groups than sham group (*P*<0.05). In AT group, MDA level was still remarkably higher than that of BBR group (*P*<0.05). SOD was predominantly lower in AT and BBR groups compared with S group (*P*<0.05), meanwhile, was significantly lower in AT group than BBR group (*P*<0.05) (**Figure 3**).

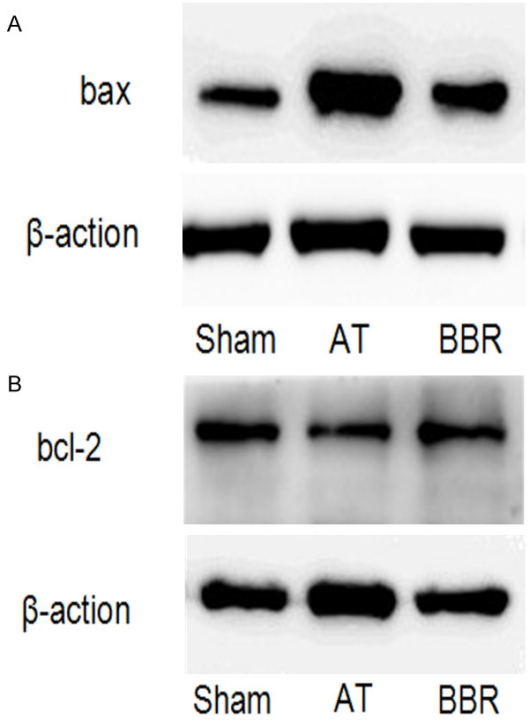


**Figure 6.** (A) Bax protein level. (B) Bcl-2 protein level. Effects of BBR on Bax and Bcl-2. Bax translational level was predominantly lower in BBR group compared with AT group (A). Bcl-2 translational level was significantly lower in both AT group and BBR group than in sham group, and was still greatly higher in BBR group than in AT group ($P$<0.05) (B).

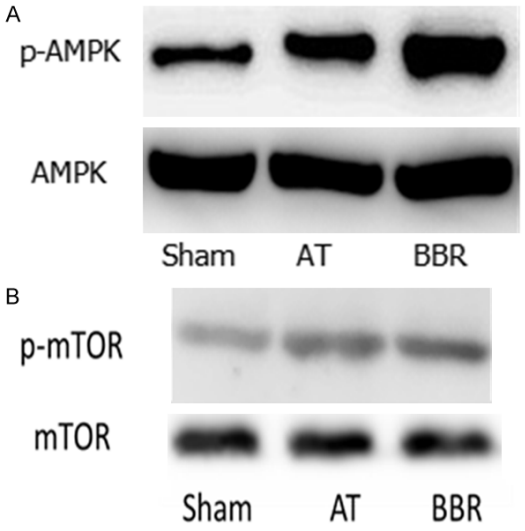


**Figure 7.** A. p-AMPK protein level. B. p-mTOR protein level.

*D-lactic acid and endotoxin in the serum*

Eight hours after I/R, serum D-lactate and endotoxin levels in AT and BBR groups were memorably up-regulated compared with S group, at the same time, their levels in BBR group remained lower than AT group (**Figure 4A**, **4B**). The result indicated that BBR notably reduced AT evoked elevated serum D-lactate and endotoxin levels.

*BBR affects the apoptosis of intestinal tissue*

The data demonstrated that the highest amount of TUNEL-positive cells was in AT group when compared with S group ($P$<0.05), and BBR noticeably restrained AT induced TUNEL-positive cell up-regulation ($P$<0.05) (**Figure 5**).

*BBR down-regulated pro-apoptotic protein Bax and up-regulated anti-apoptotic protein Bcl-2*

To explore the mechanism of pro-/anti-apoptotic effects of BBR, we detected Bax and Bcl-2 levels in intestines. In AT group, Bax expression level was significantly higher and Bcl-2 expression level was remarkably lower in comparison with BBR group (**Figure 6**).

*Western blot analysis of p-AMPK and p-mTOR*

We utilized western blot to investigate the effects of I/R and BBR. p-AMPK expression level in AT and BBR groups was increased compared with S group, BBR pretreatment activated p-AMPK protein activity ($P$<0.05) (**Figure 7A**). p-mTOR protein level was obviously higher in AT group than in BBR group and S group (**Figure 7B**).

## Discussion

Cessation of hepatic blood supply is a frequent maneuver adopted to reduce intra-operative blood loss during liver resection and transplantation. The blockage of liver PV is one of the effective steps that contribute to dramatic changes in hemodynamics during OLAT. In current study, the histological features in rats of AT group were characterized by edema, inflammatory cell infiltration and hemorrhage into the intestinal wall, which was consistent with previous studies [15, 16].

Multiple studies have demonstrated that reactive oxygen species (ROS) are produced during I/R injury. Oxidative stress induced by free radicals is considered to be the primary cause of tissue injury [17]. Exposure to O2- damaged bio-membranes and enzymes, generated cell apoptosis, and promoted leukocyte-endothelial

cell adherence. Oxidative stress and lipid peroxidation are crucial during intestinal IR, which brings about injury to small intestines [18], whereas SOD attenuates the production of oxygen radical [19]. MDA is the product of polyunsaturated fatty acid peroxidation, which functions as the marker of lipid peroxidation. Regarding attenuation of the damage caused by oxidative stress, a large number of agents have been employed to prevent I/R injury via diverse approaches [20-22]. The imbalance between oxidant and antioxidant play a major role in intestinal damage induced by ischemia-reperfusion, which was validated in current study, particularly in the early phase. Interestingly, BBR plays a protective role in improving antioxidant defense and lipid peroxidation level.

The intestinal mucosa possesses four kinds of barriers including mechanical, chemical, biological, and immune barriers to protect organisms from infection. During OLAT surgery, intestinal tract undergos I/R injury, which lead to the releases of large amounts of inflammatory mediators and cytokines, further induces the increase of intestinal permeability and translocation of bacteria and endotoxins. D-lactic acid is a major metabolic end product released from resident bacteria of gastrointestinal tract and poorly appeared in mammalian organisms. When the intestinal mucosa defense is impaired, the concentration of D-lactate in the plasma obviously increased. D-lactic is therefore considered as a sensitive index to assess intestinal mucosa permeability. In this study, we found that plasma D-lactic acid and endotoxin levels in AT group were notably elevated in comparison with S group.

Our study illuminated that OLAT caused serious injury to intestinal pathological structure. BBR pretreatment exerted protective effects on preventing intestine from apoptosis and injury. Meanwhile, western blot results demonstrated that expression of Bax greatly degraded in BBR group compared with AT group. It is possible that BBR protects against the injury through down-regulation of Bax and subsequent bax/bcl-2 ratio.

The pro-apoptotic Bax gene and anti-apoptotic Bcl-2 gene regulate the release of apoptosis inducing factor and further alter the mitochondrial membrane permeability [23]. The Bcl-2/Bax ratio is a parameter to imply the apoptotic state. Bcl-2 mainly promotes cell survival and Bax mediats cell apoptosis. When Bcl-2/Bax ratio increases, Bcl-2/Bax forms heterodimer. The mitochondrial permeability transition pores is closed, and apoptosis is inhibited. Thus, the alteration of Bcl-2/Bax ratio is of great importance in regulating apoptosis [24-26]. TUNEL staining was widely applied as a marker of apoptosis. We demonstrated that OLAT caused a significant elevation in the number of TUNEL-positive cells, and intestinal apoptosis was accompanied by dramatic increase of histologic injury. Previous studies have verified that apoptosis is a major mode of cell death in intestine damage induced by I/R [27, 28].

AMPK, a heterotrimeric protein which plays a crucial role in the regulation of whole-body energy homeostasis, is one attractive drug target. BBR significantly increased AMPK activity via ROS production, and knockdown of AMPKα1 abolished the effect of BBR [29]. The activation of AMPK negatively regulated metabolism, cell growth, proliferation and autophagy [30, 31], moreover, down-regulated mTOR signaling [32], which is a major positive stimulus to cellular stress-regulated protein synthesis, cell growth/size. AMPK improved ventricular function after cardiac I/R injury in diabetic rats [33]. I/R injury was evidenced to up-regulate p-AMPK level and down-regulate p-mTOR level in renal tubular cells [34]. Chen [35] reported that quercetin activated AMPK-regulated autophagy signaling pathway and inhibited phosphorylation of mTOR, which might offere a protective effect in renal I/R injury.

Taken together, current data suggested a protective effect of BBR on intestine apoptosis in intestinal I/R injury caused by OLAT in rats, which might be associated with activated AMPK in intestinal mucosa. These findings might shed light on the potential of BBR as a therapy for pathological conditions associated with intestinal I/R injury. Further study will be performed in the near future to explore the underlying mechanisms.

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## Disclosure of conflict of interest

None.

## Authors' contribution

Conceived and designed the experiments: HD YW WL. Performed the experiments: YW WL WY YIW QW. Analyzed the data: MS. Contributed to reagents/materials/analysis tools: ZS. Wrote the manuscript: YW WL. All authors read and approved the final manuscript.

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