Original Article
Butyrate protects rats from hepatic ischemia/reperfusion injury

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Abstract: Background: Hepatic ischemia/reperfusion (HI/R) injury is a common pathologic process caused by many clinical settings, such as liver resection, liver transplantation, hypovolemic shock and trauma. The use of butyrate, which acts as a four-carbon fatty acid, normally produced by bacterial fermentation of fiber in mammalian intestines, provides anti-oxidant and anti-apoptotic effects. Methods: Male Sprague-Dawley (SD) rats model of HI/R were subjected to a partial (70%) hepatic ischemia for 45 minutes (min) after pretreatment with either saline or butyrate, followed reperfusion. 30 rats were randomly allocated to three main experimental groups (n = 10 each): (1) The sham-operated group underwent laparotomy without hepatic ischemia. (2) Butyrate was injected into the tail vein in the butyrate group 30 min before HI/R. (3) The control group underwent the same procedure as the butyrate group but with administration of physiological saline. Rats from each group were randomly euthanized to collect blood and liver samples. Results: Butyrate treatment markedly improved hepatic function and histology, as indicated by reduced transaminase levels and ameliorated tissue pathologic changes. SD rats that received butyrate displayed reduced HI/R injury compared with controls. Use of butyrate reduced the histologic injury and significantly decreased serum Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. In addition, butyrate decreased myeloperoxidase (MPO) activity and malondialdehyde (MDA) tissue contents. Apoptotic cells in I/R rats were also significantly reduced after butyrate treatment. Furthermore, butyrate also decreased the mean number of apoptotic cells (positively stained for TUNEL) and increased the mean number of proliferating cells (positively stained for Ki-67). The expression levels of TNF-α and IL-6 were attenuated after butyrate treatment. Conclusions: Our results suggest that butyrate attenuated I/R-induced liver injury through upregulation of intracellular anti-oxidant stress and anti-apoptotic signaling pathways.

Keywords: Butyrate, ischemia reperfusion, liver, apoptosis

Introduction

Liver transplantation is the most effective mean for the treatment of various types of end-stage liver disease. However, its utilization has been severely limited by a critical shortage of donors. Donation after cardiac death (DCD) is the most likely source of donation. However, primary graft non-function and biliary complications remain significant risks for recipients of DCD livers, comparing with brain death and living organ donations [2]. The main reason for thus risks is the prolonged ischemic insult as a result of liver retrieval, with preservation and engraftment leading to more serious reperfusion injury [2, 3]. Hence, reducing ischemia/reperfusion (I/R) injury, and improving DCD transplantation outcomes are of great clinical significance. The molecular mechanisms underlying I/R, however, have not been fully clarified, despite a recent resurgence of interests in this area.

Ischemia/reperfusion (I/R) injury occurs by the interrupted blood flow for a short period in the tissues and consequently aggravates the tissue injury after a period of ischemia. HI/R injury is an important clinical problem complicating liver surgery and transplantation [1]. The injury may also occur during hypovolemic shock or after severe trauma, may often lead to liver dysfunction, and even acute and chronic rejection after transplantation, especially when grafts from non-heart-beating donors are used [2, 3], which results in a high morbidity and mortality.

Therefore, HI/R injury has been an obstacle to the development of liver surgery.

Although the nature of I/R has been widely studied, the mechanisms by which organ damage occurs are unclear. The initial HI/R injury is known to be triggered by reactive oxygen species (ROS), with inflammation involving chemokines and cytokines, followed by neutrophil-mediated hepatic injury occurring in the late period of reperfusion [4]. Recent evidence has shown that ROS can also induce apoptosis, which is one reason for cell death following reperfusion of the ischemia liver [5].

Butyrate, a four-carbon short-chain fatty acid, normally produced by the colonic bacterial anaerobic fermentation of undigested carbohydrates and fiber polysaccharides, has received considerable attention as a potential therapeutic agent for cancers due to its histone deacetylase (HDAC) inhibition [6]. In addition to their anticancer activity, recent data has demonstrated that short-chain fatty acids have potent anti-inflammatory or immunomodulatory effects, at non-cytotoxic dosing levels [7]. Kim et al [8] have demonstrated that HDAC inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat ischemic model of stroke. However, it remains unclear whether pretreatment with butyrate can protect the liver from I/R injury.

The present study examined the dose-related anti-oxidative and anti-apoptosis actions of butyrate. Specifically, we examined the protective effects of butyrate against I/R-induced hepatic injury, particularly on the oxidative stress and apoptosis.

Materials and methods

Animals

Male SD rats (200-250 g) were purchased from the Department of Laboratory Animal Science at Fudan University and housed in a laminar flow, specific pathogen-free atmosphere. Animal protocols were approved by the Fudan University Animal Care Committee, and the experiments were performed in adherence to the guidelines provided by the National Institutes of Health for the use of animals in laboratory experiments.

Warm hepatic I/R animal model experiments

Partial (70%) warm ischemia was performed as previously described [8]. In brief, after a midline laparotomy, a microvascular clamp was used to occlude the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes for 45 min to introduce partial warm ischemia. This method of partial hepatic ischemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Sham controls underwent the same procedure without vascular occlusion. Reperfusion was initiated by removal of the clamp. The rectal temperature was maintained at 37°C throughout the surgery by a warming pad. For the pretreatment experiments, some rats were injected intravenously with 100 mg/kg and 300 mg/kg sodium butyrate (Sigma), as previously described [8], or vehicle (normal saline solution) at 30 min prior to ischemia. At the indicated time points (3 h, 6 h, 12 h, 24 h) after the operation, 10 rats from each group were randomly euthanized, blood was collected via inferior vena cava puncture, and the liver were harvested. Blood samples were centrifuged at 4500 × g for 10 min to collect serum, which were stored at -80°C. Each liver was divided into two halves: one half was fixed with 4% buffered paraformaldehyde and the other half was kept at -80°C.

Liver damage assessment

To assess hepatic function and cellular injury following liver ischemia, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured in blood samples obtained at predetermined time points (6 h, 24 h) after reperfusion with a standard automatic analyzer (type 7150, Hitachi).

Histopathology

Liver tissues were fixed by immersion in 4% buffered paraformaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin-eosin (HE) and assessed for inflammation and tissue damage.

Myeloperoxidase (MPO) activity and malondialdehyde (MDA) contents in liver tissues

Tissue-associated MPO activity, an indicator of neutrophil infiltration, was determined as previ-
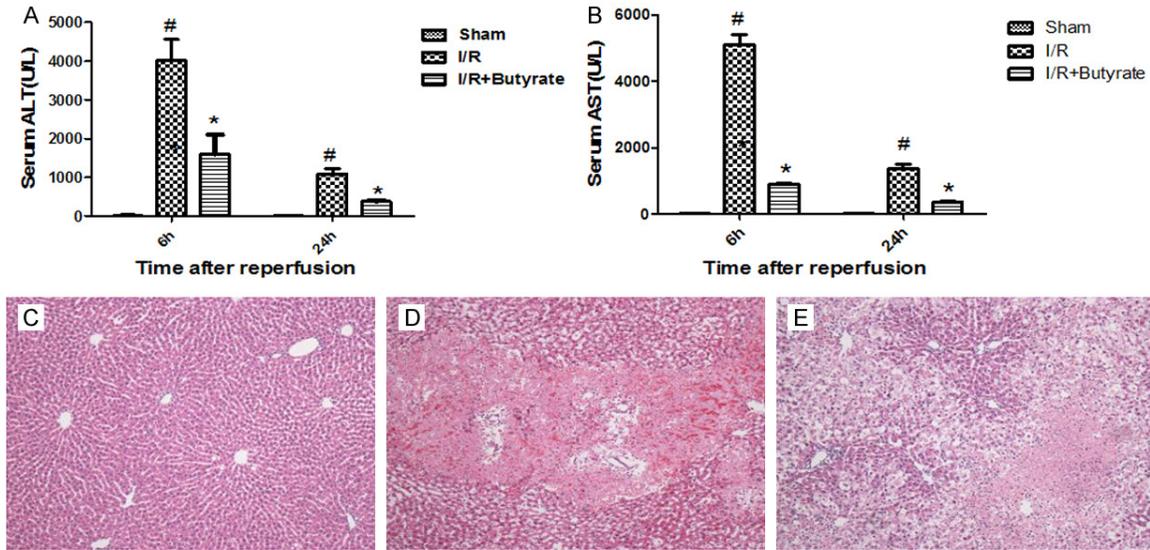
Figure 1. Effect of Butyrate on liver tissue injury after hepatic I/R. (A, B) Serum samples were collected 3 h, 6 h, 12 h, 24 h after reperfusion from the sham, vehicle, and Butyrate pretreatment groups for measuring ALT (A) and AST (B). Data presented as means ± SE (n = 4-6/group) and compared by one-way ANOVA; *P < 0.05 vs. the sham group, #P < 0.05 vs. the vehicle group. (C-E) Hematoxylin-eosin stained liver sections from the sham (C), vehicle (D), and butyrate (E) groups at 6 h after reperfusion (× 200). Large hemorrhages and massive necrosis with neutrophil infiltration were obvious in the vehicle group, whereas pretreatment with butyrate ameliorated these pathologic changes, only a few areas of hepatocyte swelling and necrosis were observed, with less neutrophil infiltration.

ously described [9]. The contents of MDA in liver tissues were measured to assess lipid peroxidation. Samples of liver tissues were homogenized with ice-cold 150 mM potassium chloride, and the MDA levels were measured spectrophotometrically. The results were expressed as nmol of MDA per gram tissue [10].

Immunostaining of TUNEL and Ki67

To detect apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed on paraffin sections using the apoptosis detection commercial kit (Roche, Shanghai, China), the assay was conducted according to the instructions of manufacturer. Then the sections were counterstained with hematoxylin. Finally, total hepatocytes and TUNEL-positive cells were observed under light microscopy. Additionally, to determine the proliferative status of hepatocytes in our study, we detected liver tissues for Ki67, which is a nuclear protein strictly present in proliferating cells and absent from resting/G_0 cells. The primary antibody was a mouse monoclonal anti-human Ki67 antigen (MIB-1 clone). Immunohistochemistry on paraffin-embedded sections with Ki67 was performed using a fully automated system (Ventana Benchmark) as

previously described. Briefly: The heat retrieval was standard for 60 min. Sections were incubated with Ki67 (DAKO, Glostrup, Denmark M7240), diluted 1:100 in Zymed antibody diluent (Zymed, California, USA003118).

Real-time reverse-transcriptase polymerase chain reaction

Total RNA was extracted from the liver using TRIzol® reagent (Life Technologies, Carlbad, USA) according to the manufacturer's instructions. The mRNA for tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6), and glyceraldehyde 3-phosphate dehydrogenase(GAPDH) was quantified in duplicate by SYBR green two-step, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) with an ABI-Prism 7500 Sequence Detector (Applied Biosystems, Foster City, USA) using the primers previously described [9]. GAPDH mRNA levels were used as the invariant control for each sample.

Statistical analysis

Group sizes are indicated in the figure legends. Data are presented as means ± SD, unless oth-
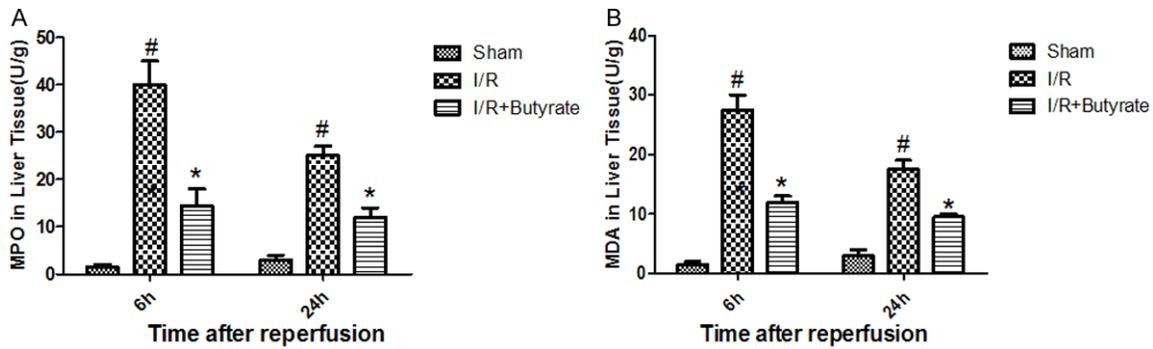
Figure 2. Effect of Butyrate on Myeloperoxidase (MPO) activity and Malondialdehyde (MDA) content after hepatic I/R. Male SD rats were subjected to partial warm hepatic I/R injury or sham operation and pretreated with butyrate or vehicle. MPO activity and MDA content in the liver were measured at 6 h and 24 h after reperfusion. MPO activity and MDA content in liver tissues. Liver tissues in **Figure 2** were used to measure the levels of MPO (A) and MDA (B) as described in Materials and Methods. The results are expressed as means ± SD, N = 4-6 rats per group. P < 0.05 vs. the sham group, P < 0.05 vs. the vehicle group.

erwise noted. Statistical analysis was performed by either an ANOVA or Kaplan-Meier test. A difference of P < 0.05 was considered statistically significant.

Results

Butyrate pretreatment ameliorates hepatic I/R injury

To assess the effects of butyrate on hepatic I/R injury, the levels of ALT and AST in sera acquired from each group of rats were measured. Rats pretreated with vehicle or sodium butyrate were subjected to 30 min of partial liver warm ischemia. The serum ALT and AST levels significantly increased in the vehicle group at 3 h, 6 h, 12 h, 24 h after reperfusion (**Figure 1A** and **1B**). In contrast, pretreatment with a dose of 300 mg/kg butyrate protected against I/R-induced injury, as evidenced by the significantly decreased the serum levels of ALT and AST at the observation points (**Figure 1A** and **1B**). The protection was also confirmed by liver pathology after reperfusion (**Figure 1C-E**). Severe lobular distortion with massive necrosis, increased swelling, cytoplasmic vacuolization, hemorrhage, and neutrophil infiltration were present in the portal area of liver tissue in the vehicle group. In contrast, pretreatment with butyrate markedly reduced above pathologic changes, hepatocyte swelling and necrosis were observed only in a few areas of the liver sample from the butyrate group, with less neutrophil infiltration.

Butyrate reduces the levels of neutrophil infiltration and lipid peroxidation

Neutrophil infiltration after reperfusion in the vehicle group, analyzed by MPO activity (U/g tissue), increased significantly. Compared to the vehicle groups at 6 h, 24 h after reperfusion, butyrate pretreatment significantly reduced MPO activity at both time points after the operation (both P < 0.05) (**Figure 2A**), suggesting that butyrate may inhibit neutrophil infiltration. Additionally, the MDA content in liver tissues, marker of lipid peroxidation, was significantly increased in sham-operated groups 6 h and 24 h after the operation as compared with control group (P < 0.001). However, after administration of butyrate (300 mg/kg), the MDA content significantly decreased (P < 0.05) (**Figure 2B**).

Butyrate suppresses hepatocyte apoptosis after hepatic I/R

To examine hepatocyte apoptosis, we performed the TUNEL assay. As shown in (**Figure 3A-D**), the vehicle-treated rats showed a significant increase in the number of TUNEL-positive cells compared to the sham controls, the TUNEL positive cells were sparsely observed in the sham livers, whereas there were more positive cells in the vehicle-treated animals (**Figure 3A** and **3B**). Additionally, few TUNEL positive hepatocytes were observed in the livers of rats treated with butyrate (**Figure 3C**). These results were confirmed with microscopic TUNEL-positive cell counting.
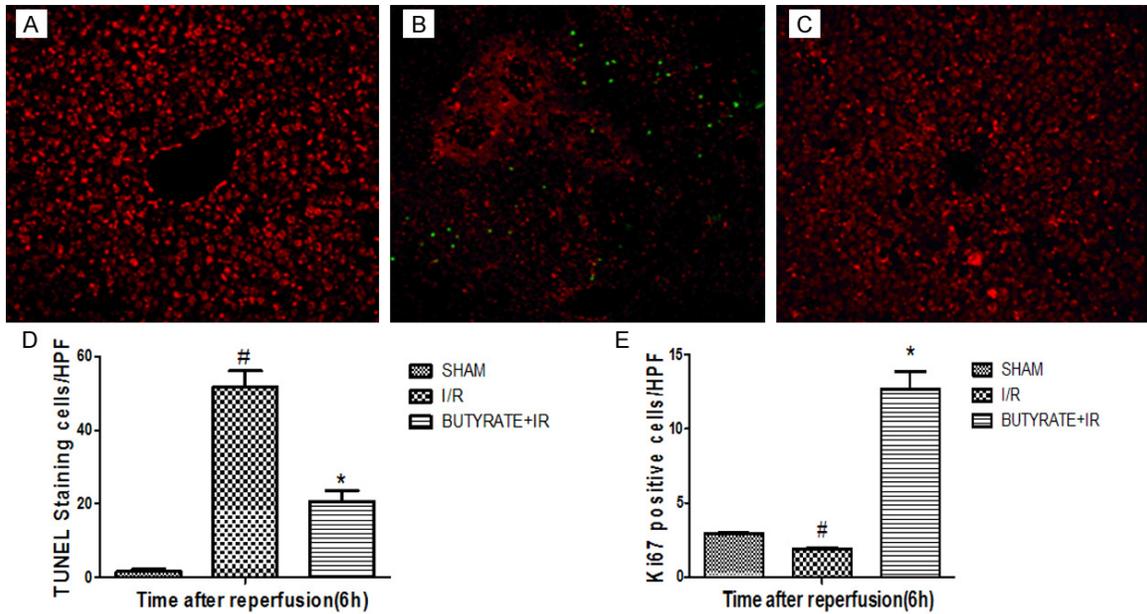
Figure 3. Effect of Butyrate on hepatocyte apoptosis and proliferation after hepatic I/R. Male SD rats were subjected to partial warm hepatic I/R injury or sham operation and pretreated with butyrate or vehicle. Hepatocyte apoptosis and proliferation in the liver was measured at 6 h after reperfusion. Liver tissues were harvested 6 h after reperfusion. (A-C) Liver sections were subjected to TUNEL assay to detect positive hepatocytes. Representative images of Sham- (A), Vehicle (B), and Butyrate pretreatment (C) groups. A large number of Tunel positive hepatocytes were detected in vehicle group. Only a few Tunel positive cells were detected in Butyrate pretreatment group. (D) A graphical representation of TUNEL positive staining cells averaged over 10 microscopic fields/animal. (E) A graphical representation of Ki67 positive staining cells averaged over 10 microscopic fields/animal. The results are expressed as means ± SD, N = 4-6 rats per group. P < 0.05 vs. the sham group, P < 0.05 vs. the vehicle group.

Butyrate promotes hepatocyte proliferation after hepatic I/R

In order to determine the effect of butyrate administration on hepatocyte proliferation after I/R injury, we performed immunohistochemical staining against Ki67. Minimal immunostaining of Ki67 was seen in liver tissue sections of either the sham or vehicle groups, but was markedly increased after hepatic I/R with butyrate treatment. With quantification by counting, I/R with vehicle administration resulted in a 58% reduction in Ki67 positive staining cells, compared to sham-operated animals. However, the number of Ki67 positive staining cells was increased 3.5-fold when butyrate was given (**Figure 3E**). These results indicate that butyrate was able to promote cellular proliferation despite the presence of physiologic insult.

Butyrate decreases hepatic I/R-induced inflammatory cytokine production

The injury occurring as a result of hepatic I/R has also been demonstrated to be associated with various inflammatory cytokines such as TNF-α and IL-6 [11]. We analyzed the rats' mRNA expression patterns in the liver following I/R using RT-PCR. As shown in (**Figure 4A** and **4B**), butyrate significantly reduced the intrahepatic expression of mRNA coding for TNF-α and IL-6 at 6 h after reperfusion in comparison with the vehicle group. Furthermore, the serum levels of TNF-α and IL-6 assessed by ELISA were consistent with the mRNA results (**Figure 4C** and **4D**).

Discussion

HI/R injury is the main cause of liver dysfunction, and even acute and chronic rejection after transplantation, especially using grafts from non-heart-beating donors [1, 2]. Serracino-Inglott et al. have demonstrated that the morbidity associated with liver transplantation and major hepatic resection is closely associated with I/R injury [12]. Hence, minimizing the I/R-induced injury should improve not only liver transplantation outcomes, but also expand liver donor pool. As a result, HI/R injury has
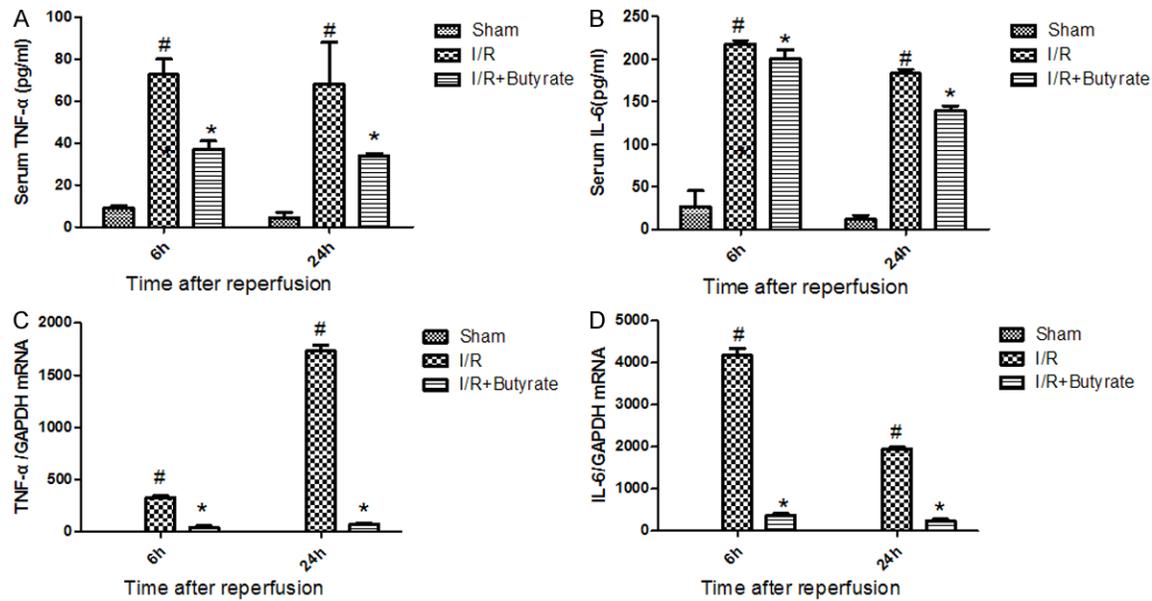
Figure 4. Effect of Butyrate on inflammatory cytokine production. Male SD rats were subjected to partial warm liver I/R injury or sham operation and pretreated with butyrate or vehicle. Liver TNF-α, IL-6 mRNA expression (A and B) and protein levels (C and D) assessed by real-time PCR and Elisa at 6 h and 24 h after reperfusion. (A and B) Liver TNF-α and IL-6 mRNA expression was measured by RT-PCR at 6 h and 24 h after reperfusion. The expression of TNF-α and IL-6 mRNA was significantly increased in the vehicle group, which can be markedly reduced by butyrate administration. Data represent means ± SD, N = 4-6 rats per grou. *P < 0.05 vs. the sham grou, #P < 0.05 vs. the vehicle group. (C and D) Serum TNF-α and IL-6 levels were assessed by Elisa at 6 h after reperfusion. Serum TNF-α and IL-6 levels were significantly increased in the vehicle group, which can be markedly reduced by butyrate administration. Data represent means ± SD, N = 4-6 rats per grou. *P < 0.05 vs. the sham grou, #P < 0.05 vs. the vehicle group.

already attracted the attention of scientists worldwide. The exploration of more effective drugs and instruments is urgently required.

HDAC inhibitors, butyrate, a short-chain fatty acid, is emerging as a novel approach to treat a variety of diseases. Butyrate has also recently emerged as a potent anti-inflammatory drug that reduces the production of pro-inflammatory cytokines, both in vivo and in vitro [13-15]. HDAC inhibitors have previously shown robust neuroprotective effects in a focal cerebral ischemia model of rats [16], protecting the heart against ischemic injury [17]. Here, we have shown that butyrate plays a pivotal role in attenuating HI/R injury and that histopathological changes caused by I/R, evidenced by decreased serum aminotransferase levels(ALT, AST), and improved tissue pathology, which is manifested in alleviated hepatocyte apoptosis and neutrophil infiltration, and promote hepatocyte proliferation.

Butyrate has displayed anti-inflammation and anti-oxidant properties that protect livers [18].

Accordingly, the present study has demonstrated that butyrate reduced the production of MPO in livers and several inflammatory factors in the sera. Myeloperoxidase, an enzyme present in neutrophils, is a widely used marker of neutrophil infiltration [19]. By producing oxidative stress, neutrophils activate Kupffer cells, and contribute to microvascular dysfunction and edema [20]. Hepatic ischemia/reperfusion could increase the MPO activity in livers in accordance with our previous report [18]. We observed that hepatic MPO activity significantly increased as early as 3 h after reperfusion then peaked at 6 h. Pretreatment with butyrate (300 mg/kg) in livers significantly reduced MPO activity. The present results showing that butyrate decreased MPO activity may indicate that butyrate ameliorates inflammation-induced liver injury by inhibiting oxidative stress.

The liver tissue MDA content has been identified as an indicator for such oxidative damage [21]. In our study, we have herein shown that the content of MDA in liver tissues was dramatically is increased during I/R, while the MDA

content significantly lower after applying butyrate. Therefore, the present results indicated that the actions of butyrate to alleviate HI/R injury might be associated with its antioxidant activity.

Hepatocyte apoptosis plays a significant role in the damage induced by HI/R. Our study showed that I/R increased remarkably the number of TUNEL-positive cells compared with the butyrate pretreatment group. Butyrate alleviated HI/R injury by suppressing apoptosis. In the present study, we have observed a striking phenomenon is that despite the severe damage caused by 45 min of warm HI/R, animals treated with butyrate had an abundance of hepatocytes staining with Ki67, indicating that these cells were proliferating instead of suffering from apoptosis or necrosis. Although the liver is unique amongst visceral organs in its ability to rapidly regenerate almost all of its mass after partial hepatectomy or metabolic stress [22], we did not observe this in vehicle-treated animals following I/R, which was likely due to the severity of our model. The presence of proliferating hepatocytes in our butyrate treatment group was consistent with other studies. Many factors contribute to I/R injury with ROS accumulation, massive granulocyte infiltration, and hepatocyte apoptosis. ROS play significant roles during HI/R injury [23, 24]. Enhanced hepatic anti-oxidant ability can reduce damage induced by I/R.

Cytokines play an important role in the responses after surgical stress [25]. TNF-α and IL-6, played as important pro-inflammatory cytokines, contributing to the recruitment of neutrophils in the liver, are ultimately responsible for the later phases of injury. The increased mRNA levels of TNF-α and IL-6 were also inhibited by butyrate administration, consistent with the Elisa results. A similar effect of butyrate has been previously confirmed in cell culture in vitro, in which butyrate could reduce the production of pro-inflammatory cytokines including TNF-α and IL-6, whereas enhance the production of anti-inflammatory cytokine IL-10 [26]. Whether butyrate can increase the IL-10 expression in liver after I/R needs further study. Therefore, the hepatoprotection of butyrate might be attributed to its inhibition of inflammatory factors. The present study showed that butyrate reduced the serum level of TNF-α, IL-6 in liver tissues elevated by HI/R.

In conclusion, we demonstrated that butyrate plays a significant role in hepatoprotection against I/R injury by reducing oxidative stress, apoptotic cell death and regulating cell proliferation. These findings are of clinical importance as many relevant clinical conditions in the field of hepatic surgery are associated with warm I/R injury. In this regard, and considering the low hazardous potentials of butyrate, it can be viewed as an attractive modality for tackling liver I/R injury in clinical liver surgery. However, its potential therapeutic usefulness warrants the investigation in large animal models and clinical trials.

Disclosure of conflict of interest

None.

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