

# $\gamma$ -hydroxybutyrate protects the liver from warm ischemia-reperfusion injury in rat

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**BACKGROUND:** Ischemia-reperfusion (I/R) syndrome remains an important clinical consideration in hepatic surgery, hemorrhagic shock, and liver transplantation.  $\gamma$ -hydroxybutyrate (GHB) has been reported to exert protective effects against ischemia-reperfusion injury to various organs. To investigate whether GHB protects the liver from warm ischemia-reperfusion injury, we performed this study in rats.

**METHODS:** Thirty male Wistar rats were randomly divided into a sham-operation group, a control group, and three I/R groups pretreated with GHB, GHB plus naloxone or naloxone. After 30 minutes of partial ischemia, followed by 60 minutes of reperfusion in the liver, histomorphological and enzymological changes, lipid peroxidation, apoptosis, and the plasma level of endothelin-1 were observed.

**RESULTS:** I/R increased the serum levels of alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase and the plasma level of endothelin-1 significantly ( $P < 0.01$ ), in addition to increase of apoptotic index (AI) from  $0.28\% \pm 0.25\%$  to  $17.68\% \pm 1.91\%$ . The levels of hepatic malondialdehyde were markedly increased, whereas the activities of superoxide dismutase were markedly decreased. GHB pretreatment prevented the liver from warm ischemia-reperfusion injury significantly, but naloxone partially blocked this effect.

**CONCLUSION:** GHB may significantly protect the liver from hepatic warm ischemia-reperfusion injury via several different mechanisms.

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**KEY WORDS:**  $\gamma$ -hydroxybutyrate; liver; ischemia-reperfusion injury; apoptosis

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## Introduction

Ischemia-reperfusion (I/R) is responsible for primary liver dysfunction and failure after transplantation, liver resection, and hemorrhagic shock. The characteristic features of I/R injury involve the activation of Kupffer cells and neutrophils which release reactive oxygen species into the vascular space and induce a network of inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>[1,2]</sup> and interleukin-1 (IL-1)<sup>[3]</sup> both of which participate in sinusoidal accumulation of granulocytes and microcirculatory failure. It has been proposed that the generation of oxygen free radicals can directly lead to oxidative damage of DNA, proteins and lipids, which contributes to cellular dysfunction.<sup>[4-6]</sup> Moreover, reactive oxygen species also activate redox-sensitive transcriptional factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B),<sup>[7]</sup> and then inducible NO synthase (iNOS), a determinant of hepatic I/R injury,<sup>[8]</sup> is activated and expressed by NF- $\kappa$ B in I/R injury. Many studies evaluating the therapeutic effect of free radical scavengers have suggested that reactive oxygen species, at least in part, likely play an important role in determining the fate of liver function after I/R.<sup>[9-12]</sup>

$\gamma$ -hydroxybutyrate (GHB) is a natural metabolite in many mammalian tissues,<sup>[13]</sup> and is widely distributed both in the central nervous system and in peripheral tissues.<sup>[14]</sup> Clinically, its synthesized compound is used as an intravenous anesthetic. Some studies<sup>[15-20]</sup> have shown that GHB can protect the central nervous system and peripheral tissues from I/R injury by scavenging oxygen radical species and by reducing cellular metabolism, thereby lowering tissue oxygen demand. Another possible mechanism of the protective effects of GHB may be the activation of opioid receptor by GHB. This effect on the central nervous system can be blocked by naloxone, an opiate receptor-antagonist.

In this study, a rat model of hepatic warm ischemia-reperfusion was used to investigate the potential effects of GHB on the liver.

## Methods

### Animal model

Thirty male healthy Wister rats weighing 200–250 g were purchased from the Hubei Academy of Medical Sciences, Wuhan, China. Before being used in the study, the rats were fasted for 12 hours and were allowed free access to water. Anesthetized by 3% sodium pentobarbital (30 mg/kg), the animal was subjected to a mid-line incision for the exposure of the vessels of the liver. The portal vein and liver artery, which drain blood to the middle and left lobes of the liver, were dissected and the blood flow was blocked with a pair of vessel clamps. After 30 minutes of ischemia, reperfusion for 60 minutes was allowed by releasing the clamps. All animals were killed at designated time to collect blood samples via the intrahepatic vena cava and the left lobe of the liver.

### Experimental design

The rats were divided into five groups (6 for each group): sham (A), I/R (B), I/R+GHB (C), I/R+GHB+naloxone (D), and I/R+naloxone (E). In rats of group A, the blood vessels of the liver were freed without occlusion. Group B animals were treated with I/R as described previously. Group C animals were pretreated via the portal vein with GHB (300 mg/kg) 10 minutes before ischemia. Group D animals received GHB in the same manner as those in group C and received naloxone via the portal vein (0.4 mg/kg) 10 minutes before injection of GHB. Group E was pretreated with naloxone in the same manner but without GHB.

### Hepatic enzyme

The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured by an auto-biochemistry analyzer (Hitachi 7060).

### Endothelin-1 plasma level

Endothelin-1 (ET-1) level was measured using 2 ml of blood samples, which was collected in a test tube containing 3  $\mu$ l of 10% disodium edentate and 40  $\mu$ l of aprotinin, and centrifuged at 3000 rpm for 10 minutes at 4 °C. The resulting plasma was stored at -20 °C until analysis with corresponding radioimmunoassay kit (Beijing Dongya-Kemei Corp., Beijing, China) according to the manufacturer's guide.

### Hepatic activities of superoxide dismutase and levels of malondialdehyde

Hepatic specimens (0.5 cm  $\times$  0.5 cm  $\times$  0.5 cm) were preserved in liquid nitrogen (-196 °C) until hepatic activities of superoxide dismutase (SOD) and levels of malondialdehyde (MDA) were assessed using corresponding SOD and MDA assay kits (Nanjing Jiancheng Corp., Nanjing, China) according to the manufacturer's guide respectively.

### Light microscopy

Liver specimens for light microscopy were fixed with 10% formalin and then embedded in paraffin. The sections of the specimens were stained with hematoxylin and eosin for histological examination.

### TUNEL reaction morphological analysis of apoptosis

Hepatic samples were dissected, fixed with 4% paraformaldehyde for 24 hours, and then embedded in paraffin. Subsequently they were cut into sections of 5  $\mu$ m and mounted on slides. In situ cell death detection kit, horseradish peroxidase (POD, Boehringer Mannheim, Germany) was used to detect apoptotic cells according to the manufacturer's instructions and other references. Positive cells were identified, counted and analyzed under a light microscope. Apoptotic cells were manifested by brownish staining in the nuclei. Ten images were randomly selected from each section for counting of at least 1000 cells as the percentage of TUNEL-positive cells. The apoptotic index (AI) was calculated by the following equation: AI = (number of apoptotic cells/total number)  $\times$  100%.

### Statistical analysis

The results are expressed as mean  $\pm$  standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) test. A *P* value less than 0.05 was considered statistically significant.

## Results

### Levels of AST, ALT and LDH in serum and ET-1 in plasma

More significantly increased levels of AST, ALT and LDH in serum and ET-1 in plasma were observed in group B subjected to ischemia and reperfusion than in group A. When GHB was given 10 minutes before ischemia, the increase of the levels of AST, ALT and LDH in serum and ET-1 in plasma was inhibited (*P* < 0.01). In contrast, injection of naloxone via the portal vein 10 minutes before GHB partially inhibited the effects of GHB, but the increase of the levels were still less significant than in group B (*P* < 0.05, Table 1).

### Hepatic activities of SOD and levels of MDA

Groups B and E had lower hepatic activities of SOD and higher levels of MDA in liver tissues than did other groups. GHB significantly increased the activities of SOD and decreased the levels of MDA in liver tissues (*P* < 0.01). Naloxone attenuated the effects of GHB but the differences between the two groups were not statistically significant (*P* > 0.05, Table 2).

### Cell apoptosis in different groups

**Table 1.** Levels of ALT, AST and LDH in serum (U/L) and ET-1 in plasma ( $\mu\text{g/L}$ )

Group	n	ALT <sup>a</sup>	AST <sup>b</sup>	LDH <sup>c</sup>	ET-1 <sup>d</sup>
A	6	49.04 $\pm$ 13.04	61.23 $\pm$ 14.95	785.53 $\pm$ 113.53	47.49 $\pm$ 11.58
B	6	369.49 $\pm$ 48.68	459.90 $\pm$ 48.26	2010.96 $\pm$ 140.19	137.69 $\pm$ 18.17
C	6	152.42 $\pm$ 29.81	154.36 $\pm$ 28.81	1466.47 $\pm$ 125.60	77.28 $\pm$ 12.32
D	6	224.50 $\pm$ 42.80	325.64 $\pm$ 31.34	1740.31 $\pm$ 131.76	106.00 $\pm$ 8.62
E	6	365.82 $\pm$ 32.04	416.61 $\pm$ 55.61	2069.72 $\pm$ 146.81	134.25 $\pm$ 14.79

a:  $P < 0.01$ , A vs B or E, C vs B or E, D vs B or E;  $P < 0.05$ , C vs D. b:  $P < 0.01$ , A vs B or E, C vs B or E, D vs B or E, C vs D. c:  $P < 0.01$ , A vs B or E, C vs B or E, D vs E;  $P < 0.05$ , D vs B, C vs D. d:  $P < 0.01$ , A vs B or E, C vs B or E, D vs B;  $P < 0.05$ , C vs D, D vs E.

**Table 2.** Hepatic activities of SOD (nmol/mg) and levels of MDA (U/mg)

Group	n	MDA <sup>a</sup>	SOD <sup>a</sup>
A	6	6.70 $\pm$ 2.51	35.65 $\pm$ 3.19
B	6	25.20 $\pm$ 2.93	19.03 $\pm$ 2.37
C	6	15.87 $\pm$ 1.44	27.70 $\pm$ 2.46
D	6	19.45 $\pm$ 1.83	25.38 $\pm$ 2.86
E	6	24.69 $\pm$ 2.08	14.43 $\pm$ 2.44

a:  $P < 0.01$ , A vs B or E, C vs B or E, D vs B or E;  $P > 0.05$ , C vs D, B vs E.

Apoptotic cells were found only occasionally in group A (AI = 0.28% $\pm$ 0.25%), but after 30 minutes of regional ischemia followed by 60 minutes of reperfusion, many apoptotic cells appeared. The AI of groups B (17.68% $\pm$ 1.91%) and E (17.79% $\pm$ 1.43%) was greatly higher than that of group A ( $P < 0.01$ ). In group C (6.94% $\pm$ 1.54%), GHB significantly decreased the number of apoptotic cells when compared with groups B and E ( $P < 0.01$ ). The AI of group D (12.29% $\pm$ 1.86%) was higher than that of group C ( $P < 0.01$ ) but still lower than that of groups B and E ( $P < 0.01$ ).

### Histopathologic evaluation

The liver tissue showed almost normal structure in group A under a light microscope. In groups B and E, shrunk hepatic sinus and swollen hepatic cells were observed with vesicular degeneration but no obvious necrosis. In groups C and D, however, these changes were attenuated markedly.

### Discussion

GHB is an intravenous anesthetic. Previous studies have shown that GHB can protect both the central nervous system<sup>[16,17]</sup> and peripheral tissues (heart,<sup>[15]</sup> lung<sup>[18]</sup> and intestine<sup>[19]</sup>) from I/R injury and has the potential to protect liver function after long-term hypothermic storage.<sup>[20]</sup> In the present study, the serum levels of ALT, AST and LDH elevated significantly ( $P < 0.01$ ) with the increase of apoptotic cells after 30 minutes of ischemia followed by 60 minutes of reperfusion. GHB

pretreatment attenuated all of these changes effectively ( $P < 0.01$ ). Our results indicated that GHB may significantly protect the liver from warm ischemia-reperfusion injury.

The mechanisms of the effects of GHB have not yet been elucidated except that the burst of reactive oxygen species generated after reperfusion may contribute to the initiation of postischemic liver injury.<sup>[21,22]</sup> During the hypoxic stage of I/R, hypoxanthine accumulates because of ATP depletion as total energy decreases. In a parallel process, hypoxia activates proteolytic enzymes which convert xanthine dehydrogenase (XOH) to xanthine oxidase (XO).<sup>[23]</sup> The increasing levels of XO then oxidize the accumulated hypoxanthine to urate after the oxygen supply is restored during the reperfusion phase. In this reaction molecular oxygen is converted to superoxide radicals which exceed the capacity of endogenous redox degrading systems, leading to direct cellular damage through protein oxidation and degradation, lipid peroxidation, and DNA damage.<sup>[3-5,24,25]</sup> Boyd et al<sup>[19]</sup> and Sherman et al<sup>[20]</sup> indicated that GHB protecting the intestine or liver from I/R injury may be correlated with its scavenging of oxygen radical species. This hypothesis is supported by the increased hepatic activities of SOD and the decreased hepatic levels of MDA, the metabolites of lipid peroxidation, after pretreatment of GHB in our study.

Primary stress in ischemia of the liver is oxygen-deficiency caused interruption of oxidative energy production, giving a rapid decrease of the level of intracellular adenosine triphosphate (ATP). Reduced metabolic activities like cooling of the organ or sustained ATP production such as adding glycogen<sup>[26]</sup> may help to prevent ischemic injury. GHB itself can serve as an energy substrate and may reduce cellular metabolism, thereby lowering tissue oxygen demand.<sup>[13]</sup> This may be correlated with its protective effects. The detailed mechanisms need further investigation.

ET-1 is one of the most potent and powerful vasoconstrictors, which are produced not only by vascular endothelial cells, but also a variety of non-endothelial cells and can induce microcirculatory disorders by mediating sinusoidal vasoconstriction, lowering perfusion rate,

and promoting leukocyte adhesion, all of which may play a role in the pathogenesis of I/R injury. In the early stage of hepatic reperfusion, the increased level of ET-1 in both plasma and hepatic parenchyma is correlated with the decrease of liver blood flow. On the other hand, low NO concentration is caused by the release of a large amount of arginase that breaks down L-arginine (the precursor required for NO synthesis).<sup>[4]</sup> Pretreatment with blockade of ET receptor<sup>[27]</sup> as well as with L-arginine<sup>[28]</sup> may attenuate liver injury. It has been suggested that I/R injury is a result of an imbalance between ET and NO levels during the reperfusion period.<sup>[4]</sup> Boyd et al<sup>[29]</sup> found that GHB could prevent the decrease of intestinal blood pressure during the ischemia phase, thereby ameliorates intestinal microcirculation, and that this effect was at least partly due to the hypertonicity of GHB solution. In our study the plasma level of ET-1 was increased significantly after pretreatment of GHB, indicating that GHB may reduce the activities of ET-1, while preventing hepatic microcirculation disorders during I/R. The mechanisms of this effect await further study.

GHB is known to increase the tissue concentration of dynorphin, an opiate peptide,<sup>[30]</sup> and can activate opioid receptors. It has been found that acute opioid can down-regulate the activities of Ca<sup>2+</sup> channel and depress the Ca<sup>2+</sup> intracellular flow<sup>[31]</sup> and therefore may prevent the I/R injury caused by intracellular Ca<sup>2+</sup> overload. Naloxone can block the action opioid-agonist on opioid-receptors in the central nervous system.<sup>[31]</sup> Boyd et al<sup>[19]</sup> found that naloxone partially blocked the protective effect of GHB on regional intestinal ischemia. In our study the effect of GHB on hepatic warm I/R was also partially blocked by naloxone, but GHB could still be effective when pretreated with naloxone ( $P < 0.05$ ). Furthermore, the differences between the increased hepatic level of MDA and the decreased hepatic activities of SOD were not significant when compared with those of the GHB group. It is suggested that the protective effect of GHB, at least the anti-lipid-peroxidation action of GHB, is not fully dependent on the opioid receptor system. Further studies are needed to clarify the relationship between the protective effect of GHB and the opioid system.

### Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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The persist on efforts without losing the aim will finally result in success.

— (Germany) Johann Wolfgang von Goethe