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Protective effects of exogenous β-hydroxybutyrate on paraquat toxicity in rat kidney

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ABSTRACT

In this study, we demonstrated the protective effects of β-hydroxybutyrate (β-HB) against paraquat (PQ)-induced kidney injury and elucidated the underlying molecular mechanisms. By histological examination and renal dysfunction specific markers (serum BUN and creatinine) assay, β-HB could protect the PQ-induced kidney injury in rat. PQ-induced kidney injury is associated with oxidative stress, which was measured by increased lipid peroxidation (MDA) and decreased intracellular anti-oxidative abilities (SOD, CAT and GSH). β-HB pretreatment significantly attenuated that. Caspase-mediated apoptosis pathway contributed importantly to PQ toxicity, as revealed by the activation of caspase-9/-3, cleavage of PARP, and regulation of Bcl-2 and Bax, which were also effectively blocked by β-HB. Moreover, treatment of PQ strongly decreased the nuclear Nrf2 levels. However, pre-treatment with β-HB effectively suppressed this action of PQ. This may imply the important role of β-HB on Nrf2 pathway. Taken together, this study provides a novel finding that β-HB has a renoprotective ability against paraquat-induced kidney injury.

1. Introduction

Paraquat (1,1-dimethyl-4-4-bipiridinium dichloride, PQ) is a potent, widely used herbicide, especially in developing countries [1]. Unfortunately, thousands of accidental as well as intentional paraquat poisonings of human beings have been reported, and most cases are end with death because of lack of effective drugs [2]. Paraquat causes severe multiple organ failure in mammal. Regardless of its administration route, paraquat is rapidly distributed in most tissues, with the highest concentration found in the kidneys, where it produces early and severe nephrotoxicity [3]. In addition, as it is mainly excreted unchanged via the kidneys, the consequent impaired renal function increases the plasma concentrations of paraquat by up to fivefold, which contributes to paraquat toxicity in other organs, especially the lungs [4,5]. Ultimately, respiratory failure, in the presence of nephrotoxic acute renal failure, is responsible for most deaths caused by paraquat [5–7]. Therefore, maintaining renal function in patients suffering from paraquat poisoning remains a therapeutically important treatment strategy [8].

Paraquat is a potent free radical producer. Once entering cells, PQ is reduced primarily by nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase to form PQ monocation free radical. The electron transferred to PQ rapidly moves to oxygen with subsequent production of superoxide. Consequently, excessive oxidative damage occurs from reactive oxygen species [9]. Since PQ toxicity is mainly mediated via oxidative stress-induced mechanisms, researchers and clinicians used to focus on the use of antioxidants as a treatment. Most of the antioxidants used in treating PQ-exposed humans and animals have failed to modify the toxicity of the herbicide largely attributed to their inability to cross cell membrane barriers and/or their rapid clearance from cells [10].

β-HB is the predominant member of ketone bodies. β-HB rapidly diffuses through peripheral tissues and readily penetrates membranes to enter cells, making it superior to conventional antioxidant drug [11]. Many reports have showed that exogenous β-HB has therapeutic benefits against stress conditions, such as hemorrhagic shock [12,13], extensive burns [14], and cerebral hypoxia, anoxia, and ischemia [15]. In these cases, β-HB attenuates tissue damage, protein catabolism, and metabolic dysfunction. However, the role of β-HB on PQ-induced kidney injury has not yet been investigated. Therefore, in this study we aimed to assess the protective effect and potential mechanisms of β-HB in PQ-induced kidney injury.

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2. Materials and methods

2.1. Ethics statement

Procedures were conducted according to the US NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Welfare and Research Ethics Committee at Jilin University (Approval ID: 20111210-3).

2.2. Materials

β-HB and PQ (1,1′-dimethyl-4,4′-bipyridiniumdichlo-ride) were obtained form Sigma Aldrich (USA). Cleaved PARP antibody was obtained from Cell Signaling Technology (USA). Nrf2 antibody was obtained from Santa Cruz Biotechnology (USA). Cleaved caspase-9, -3, Bax, Bcl-2 antibodies were obtained from Boster Biotechnology (China). β-actin antibody was obtained from Sungene Biotech (China). MDA assay kit, GSH assay Kit, SOD assay kit and CAT assay kit were obtained from Beyotime Biotechnology (China). BUN assay kit and creatinine assay kit were obtained from Jiancheng Bioengineering Institute (China). All the other reagents, unless otherwise stated, were obtained from Beyotime Biotechnology (China).

2.3. Animals and treatment

Twenty-four adult male Wistar rats weighting 200–250 g were used in these experiments, provided by the Center of Experimental Animals of Baiqiuen Medical College, Jilin University, China. The rats were divided randomly to four groups of six each. The control group was given the vehicle. The PQ group was intravenously injected by PQ without β-HB pretreatment. The β-HB + PQ group was intravenously injected by PQ with β-HB pretreatment. The β-HB group was injected by the vehicle with β-HB pretreatment. The β-HB pretreatment (2 mmol/kg, body weight) was given intravenously at 24 h intervals five days prior to PQ injection (30 mg/kg, body weight). 12 h after paraquat injection, rats were sacrificed and kidney tissues were rapidly excised.

2.4. Determination of renal dysfunction specific markers

Blood samples collected from puncturing rat hearts were collected and then centrifuged for serum. The blood urea nitrogen (BUN) and serum creatinine were determined by using commercially available kits according to the manufacturer's recommended protocol (Jiancheng Bioengineering Institute, China).

2.5. Histological studies

Kidney tissues from the experimental animals were fixed in 10% buffered formalin and were processed for paraffin sectioning. The paraffin-embedded kidney was cut into serial sections (5 μm). Sections were stained with haematoxylin and eosin to evaluate under light microscope (Olympus, Japan).

2.6. Assay of oxidative biochemical parameters

Kidney tissues were homogenized in RIPA lysis buffer. The mixture was centrifuged at 12,000g for the supernatant. The activities of SOD and CAT, the levels of GSH and MDA were determined by using commercially available kits according to the manufacturer's recommended protocol (Beyotime Biotechnology, China).

2.7. Western blot analysis

Total proteins or nuclear proteins were extracted by using commercially available kits according to the manufacturer's recommended protocol (Beyotime Biotechnology, China). Samples with equal amounts of protein (30 μg) were fractionated on 12% SDS–PAGE, transferred to a polyvinylidene fluoride membrane, and blocked in 3% BSA for 40 min at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies (anti-PARP, 1:1000 dilution; anti-Nrf2, 1:500 dilution; anti-caspase-9, anti-caspase-3, anti-Bax, anti-Bcl-2, 1:250 dilution). After rinsed in TBST, the blots were incubated with appropriate secondary antibodies (1:5000 dilution) conjugated to horseradish peroxidase for 1 h at room temperature. Protein expression was detected with an enhanced chemiluminescence detection system, and the membrane was exposed to an X-ray film. The β-actin signal was used as a loading control.

2.8. Data analysis

All data are represented as the mean ± S.E.M. (Standard Error Mean). Comparison between groups was made by one-way analysis of variance (ANOVA) followed by an appropriate post hoc test to analyze the difference. A value of $p < 0.05$ was considered as being significant.

3. Results

3.1. Changes in serum biochemical parameters

To investigate the kidney functions, we investigated BUN and creatinine, two serum biochemical parameters. As shown in Table 1, in PQ-treated group, there was a significant increased level of BUN and creatinine compared to the control group. However, pretreatment with β-HB could prevent PQ-induced increased levels of these serum biochemical parameters close to the control. Meanwhile, β-HB treatment alone showed no effect on the levels of BUN and creatinine.

3.2. Changes in histological morphology

Fig. 1 shows the histopathological changes from kidney segments of the normal and experimental groups. We observed the degeneration of renal tubules and atrophy of glomerulus in PQ-treated group. However, pretreatment with β-HB ameliorated PQ-induced alterations and kept the pathological lesions close to normal range. Meanwhile, β-HB treatment alone showed no effect on the integrity of tubules and glomerulus.

3.3. Changes in lipid peroxidation

MDA is a degradation product of polyunsaturated lipid, which is known as the major bioactive marker of lipid peroxidation. It was measured and represented in Table 2. Compared with the control

Table 1
Changes of kidney injury indicators in different groups.

Experimental group	BUN (mmol/L)	Creatinine (μmol/L)
Control group	4.15 ± 0.23	23.24 ± 0.93
PQ group	6.15 ± 0.47*	28.41 ± 2.99*
β-HB + PQ group	5.00 ± 0.36#	25.33 ± 1.81#
β-HB group	4.24 ± 0.18	23.51 ± 1.46

Values are expressed as mean ± SD, for 6 animals in each group.
* $p < 0.05$, compared with control group.
# $p < 0.05$, compared with PQ treatment group.

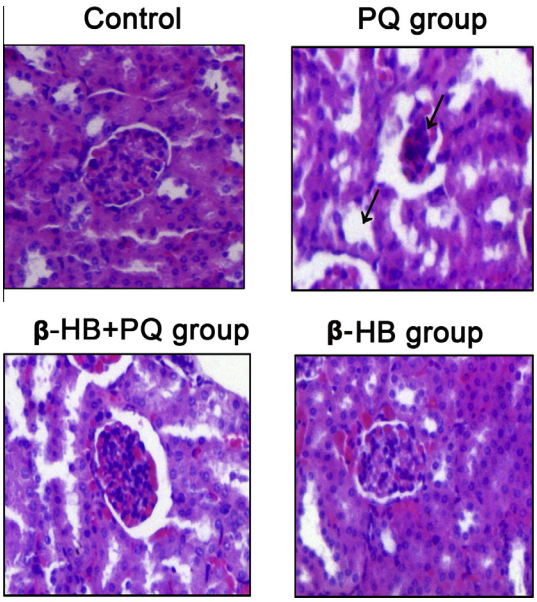

Fig. 1. Effects of β-HB on PQ-induced histological changes in kidney section. Histological examination was performed by H&E staining, loss of integrity of glomerulus and tubules is marked with arrow.

Table 2
Changes of oxidative stress related biomarkers in different groups.

Experimental group	MDA (nmol/mg pro)	SOD (U/mg pro)	CAT (U/mg protein)	GSH (μg/g pro)
Control group	2.33 ± 1.53	146.84 ± 15.54	87.36 ± 25.73	7.87 ± 1.76
PQ group	3.75 ± 1.67*	115.08 ± 23.91*	67.72 ± 17.53*	5.53 ± 1.68*
β-HB + PQ group	2.08 ± 1.37#	141.58 ± 11.26#	81.39 ± 20.53#	8.03 ± 2.37#
β-HB group	2.10 ± 0.96	165.43 ± 14.31*	100.36 ± 14.53*	8.21 ± 2.38

Values are expressed as mean ± SD, for 6 animals in each group.
* $p < 0.05$, compared with control group.
# $p < 0.05$, compared with PQ treatment group.

group (2.33 ± 1.53 nmol/mg protein), the MDA content in the PQ group (3.75 ± 1.67 nmol/mg protein) was significantly higher. The kidney MDA content was significantly reduced in rats pre-treated with β-HB (2.08 ± 1.37 nmol/mg protein). These results indicated that β-HB inhibits lipid peroxidation on PQ-induced kidney injury.

3.4. Changes in the activities of SOD and CAT

Alterations in activities of SOD and CAT, two antioxidant enzymes were represented in Table 2. PQ treatment significantly reduced the activities of SOD and CAT compared to the control, however, β-HB pretreatment could maintain their activities almost close to normal against PQ toxicity. Interestingly, β-HB treatment alone increased the activities of these two enzymes, compared to the control.

3.5. Changes in GSH content

GSH is the major endogenous antioxidant. The levels of GSH were measured and represented in Table 2. Compared with the control group (7.87 ± 1.76 μg/g protein), the GSH content in the PQ group (5.53 ± 1.68 μg/g protein) was significantly higher. The kidney GSH content was significantly reduced in rats pre-treated with β-HB (8.03 ± 2.37 μg/g protein). These results indicated that β-HB effectively maintained GSH content against PQ toxicity.

3.6. Changes in caspase-9, caspase-3 and PARP activation

To investigate possible involvement of caspase-mediated apoptosis pathway, the expression of cleaved caspase-9, -3 and PARP in total proteins were detected by Western blot. As shown in Fig. 2, PQ treatment significantly increased the expression of cleaved caspase-9, -3 and PARP, namely, their activation, indicating the involvement of the caspase-mediated apoptosis pathway. However, β-HB pretreatment was found to be effective to prevent PQ-induced activation of the three apoptotic proteins.

3.7. Changes in Bcl-2 and Bax protein expression

The Bcl-2 family are important apoptotic regulators. Two members, Bcl-2 and Bax in total proteins, were detected by Western blot. As shown in Fig. 3, PQ treatment significantly up-regulated pro-apoptotic Bax protein and down-regulated anti-apoptotic Bcl-2 protein compared to the control. However, β-HB pretreatment induced a marked reduction in amount of Bax and increased the level of Bcl-2 protein against PQ toxicity.

3.8. Changes in nuclear content of Nrf2

Nrf2 has been recognized as a key transcription factor against oxidative damage.

It translocates into the nucleus to regulate a battery of endogenous cytoprotective genes, including those encoding for both antioxidant- and anti-inflammatory proteins, such as HO-1 and NQO-1. The nuclear content of Nrf2 in nuclear proteins was detected by Western blot. As shown in Fig. 4, PQ treatment significantly decreased nuclear expression of Nrf2 compared to the control. However, β-HB pretreatment strongly suppressed the inhibitory effects of PQ on Nrf2.

4. Discussion

Before the onset of acute renal failure, renal clearance of PQ exceeds that of the glomerular filtration rate, indicating that PQ is actively secreted by the proximal renal tubules [16,17]. PQ is also nephrotoxic in the proximal convoluted tubules as a result of high concentrations [18]. Many studies have reported that PQ-induced nephrotoxicity associated with direct tubular toxicity, inflammation, oxidative stress and apoptosis [19,20]. In the present study, we found that β-HB played a potent role in PQ-induced kidney injury in rat. β-HB pretreatment effectively protected against PQ-induced loss of integrity in structure and reduction in renal function, which credited to attenuation of lipid peroxidation through recovering intracellular antioxidant abilities and inhibition of apoptosis.

High doses of PQ caused acute renal failure, which is characterized by loss of integrity in structure and reduction in renal function [21]. In this study, first of all, serum BUN and creatinine levels, two important biomarkers for PQ poisoning, were detected. BUN is produced as a waste product of the digestion of protein by the liver in the urea cycle, and creatinine is a breakdown product of creatine phosphate produced at a fairly constant rate. Normally they are excreted unchanged by the kidneys and kept at constant level in blood. However, in cases of severe renal dysfunction, such as PQ poisoning, their levels elevate because kidney is inefficient to excrete them [22,23]. From our study, we observed that the administration of PQ caused marked elevation of BUN accompanied by accumulation of creatinine, indicating that PQ exposure impaired renal function. Moreover, histological examination was also performed to examine the extent of kidney injury. Our results show that paraquat induced many histological alterations in

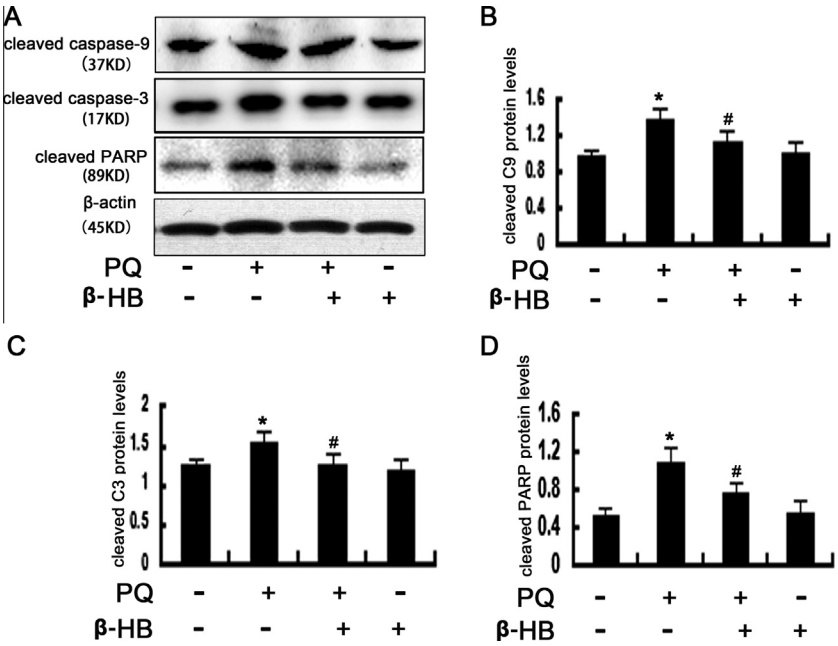

Fig. 2. Effects of β-HB on PQ-induced protein expression of cleaved caspase-9, -3 and PARP. Total proteins were assayed. (A) and (B) cleaved caspase-9, (A) and (C) cleaved caspase-3, (A) and (D) cleaved PARP were measured by Western blot, and the ratio to β-actin was calculated, respectively. The mean of three independent experiments is shown. $^{*}p < 0.05$, versus control group. $^{\#}p < 0.05$, versus PQ treatment group.

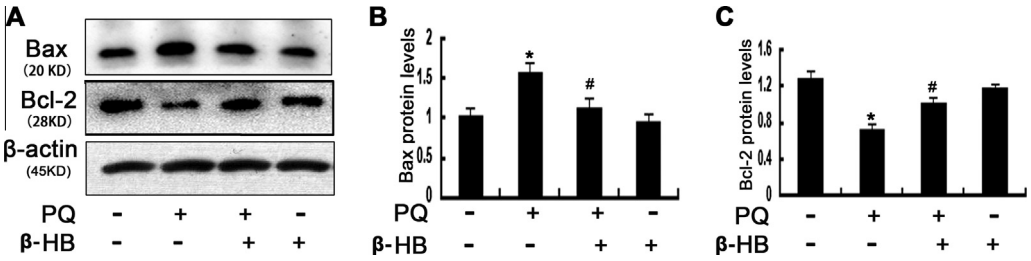

Fig. 3. Effects of β-HB on PQ-induced protein expression of Bax, Bcl-2. Total proteins were assayed. (A) and (B) Bax, (A) and (C) Bcl-2 were measured by Western blot, and the ratio to β-actin was calculated, respectively. The mean of three independent experiments is shown. $^{*}p < 0.05$, versus control group. $^{\#}p < 0.05$, versus PQ treatment group.

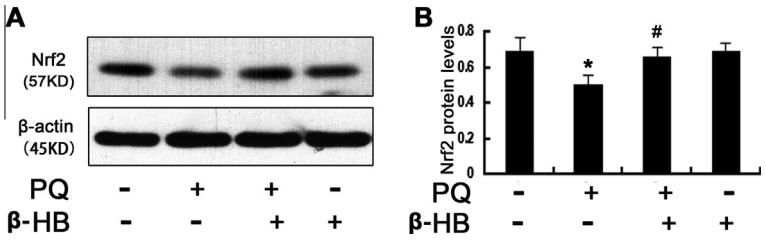

Fig. 4. Effects of β-HB on PQ-induced nuclear content of Nrf2. Nuclear proteins were assayed. (A) and (B) Nrf2 was measured by Western blot, and the ratio to β-actin was calculated. The mean of three independent experiments is shown. $^{*}p < 0.05$, versus control group. $^{\#}p < 0.05$, versus PQ treatment group.

kidneys of rats including degeneration of renal tubules and atrophy of glomerulus. These observations are in harmony with previous reports [21–23]. Although exposure with high doses of PQ, β-HB pretreatment effectively recovered renal function and prevented kidney injury. These results indicated that β-HB had a protective effect on kidney tissue, a novel finding.

Despite the specific mechanisms of the pathogenesis of PQ-induced kidney injury have not been fully elucidated, it is commonly recognized that oxidants exceeding antioxidant defenses is involved. As PQ easily undergoes redox cycling by NADPH-cytochrome c reductase, it results in generation of active oxygens and oxidative damage to membrane lipids. Lipid peroxidation by these active oxygens is generally believed to be a biochemical event in its toxicity, as described in many clinical reports and experimental researches [24–26]. In this study, the levels of MDA, a degradation product of polyunsaturated lipid and accumulates in response to oxidative stress, were detected as the marker of lipid peroxidation. Our results showed that elevated MDA levels were observed in PQ-treated group, indicating oxidative stress in kidney tissues. SOD, CAT and GSH are essential antioxidant molecules in cell. SOD plays a vital role in the balance between oxidation and antioxidation. SOD catalyzes the dismutation of superoxide anions into H_2O_2 and eliminates the cytotoxic effects of the superoxide anion [27]. CAT is a ubiquitous enzyme

that prevents cell oxidative damage by degrading H_2O_2 to H_2O and O_2 with high efficiency [28]. GSH is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms [29]. From our study, significant decrease in activities of SOD and CAT, and levels of GSH were observed in PQ-treated group, implying that an impaired ability to detoxify ROS via antioxidase results in excessive lipid peroxidation. However, β-HB showed ability to elevate the activities of SOD and CAT, which is also seen β-HB alone group, thus recovering intracellular antioxidatant system against PQ toxicity.

PQ toxicity is also characterized by cell apoptosis. Previous in vitro study has showed that PQ-induced apoptosis of HK-2 cells (human kidney proximal tubular cell) by activation of multiple caspase-mediated mechanisms, including both of the extrinsic and intrinsic apoptosis pathway, and the mitochondria-mediated intrinsic pathway was found to play a more important role during this process [30]. Our results showed that apoptosis was involved in PQ-induced nephrotoxicity. Caspase-9 is an important initiator caspase. It could be stimulated by mitochondria death signal and is able to activate downstream caspases like caspase-3. Cleaved caspase-3, a well-known effector of apoptosis, which able to cleave PARP that serves as a biochemical hallmark of apoptosis and acts as a substrate of caspase activation, was also triggered by PQ [31]. Their activations were both prevented by β-HB pretreatment, suggesting that β-HB had anti-apoptotic effects against PQ through suppression of caspase-mediated apoptosis in rat kidney. The Bcl-2 family proteins play a crucial role for regulating cell apoptosis [32]. Bcl-2 is known as an anti-apoptotic protein which protects the cells from apoptosis, whereas the pro-apoptotic proteins such as Bax promote the programmed cell death. In this study, we observed that the administration of PQ up-regulated Bax protein and down-regulated Bcl-2 protein, however they were both recovered by β-HB pretreatment. This suggested that regulation of Bcl-2 and Bax by β-HB is also involved in its anti-apoptotic ability.

NF E2-related factor 2 (Nrf2) is a member of the cap 'n' collar family of basic leucine zipper proteins. It is known as the primary transcription factor responsible for initiating the response to oxidative stress [33]. Normally, Nrf2 translocates into the nucleus where it binds DNA at a consensus sequence known as the antioxidant or electrophilic response element. In this way, Nrf2 regulates transcription of a large group of detoxification genes, among which are the subunits of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH biosynthesis. Under stress conditions, such as PQ poisoning, Nrf2 pathway was inhibited thus makes cell susceptible to chemical toxicity [30]. From our study, PQ significantly inhibited Nrf2 translocation, as revealed by the decrease in the nuclear Nrf2 levels, however, pre-treatment with β-HB with PQ, strongly suppressed the inhibitory effects of PQ on Nrf2. It seems that β-HB protects kidney cells against oxidative stress via induction of the Nrf2 pathway.

The kidney is an important organ for reabsorbing and excreting ketone bodies. The concentration of β-HB in the early proximal tubule almost equals that in plasma [34]. The concentration of β-HB in kidney is controllable. Fasting [35], ketogenic diet [36] or exogenous administration [37] could elevate its level. We tested the effects of 1–7 days pretreatment with β-HB on renal function specific markers in the preliminary experiment (data not shown). 5–7 days pretreatment showed similar protection effects on paraquat toxicity but significantly better than 1–4 days pretreatment, so 5 days pretreatment was chosen for the present study. Our results support that β-HB had a protective effect against PQ-induced toxicity in rat kidney via suppression of lipid peroxidation, regulation of Bcl-2 and Bax, and inhibition of caspase activation. What's more, induction of Nrf2 pathway by β-HB might play an important role involved. This study describes the renoprotective effect of β-HB. It provides novel clinical application of ketone bodies. Future studies should provide more information for the metabolic mechanism of exogenous β-HB and its effects on other organs.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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