



Protective effects against and potential mechanisms underlying the effect of magnesium isoglycyrrhizinate in hypoxia-reoxygenation injury in rat liver cells

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ABSTRACT. We examined the protective effects of magnesium isoglycyrrhizinate (MgIG) on hypoxia-reoxygenation injury in rat liver cells. Rat liver cells in the logarithmic growth phase were divided into the hypoxia-reoxygenation injury model group and MgIG pretreatment group (0.01, 0.1, 1, 10, 100 mg/mL). After 24-h pretreatment, we detected the effects of MgIG on liver cell viability using the methyl thiazolyl tetrazolium (MTT) assay at 6-h hypoxia and 4-h reoxygenation. After 24-h pretreatment, liver cells were randomly divided into the hypoxia-reoxygenation injury model group and low-, moderate-, and high-MgIG-concentration groups (0.1, 1, 10 mg/mL, respectively), and hypoxia and reoxygenation were simulated for 6 and 4 h, respectively. Cell morphology was observed by light microscopy. Nuclear factor- κ B gene expression was analyzed by quantitative reverse transcription-polymerase chain reaction. MTT results showed that MgIG (0.1, 1, 10 mg/mL) improved the A-value of anoxia-reoxygenation injury in liver cells ($P < 0.01$) compared with that of the model group. Cells did not survive when the MgIG concentration was 100 mg/mL. At an MgIG concentration

lower than 0.01 mg/mL, the A-value of the MTT group was higher than that of the model group ($P > 0.05$). Nuclear factor-kB mRNA expression (0.597 ± 0.062 , 0.248 ± 0.067 , 0.141 ± 0.029) in the low-, moderate-, and high-concentration groups was lower than that in the model group ($P < 0.01$). MgIG reduced hypoxia-reoxygenation injury of liver cells, indicating that it improved hepatic cell activity, inhibited lipid peroxidation and inflammatory reactions, and decreased nuclear factor-kB mRNA expression.

Key words: Hypoxia; Liver cell; Magnesium isoglycyrrhizinate; Reoxygenation

INTRODUCTION

Magnesium isoglycyrrhizinate (MgIG) is a type of glycyrrhizin and liver cell protective agent, which has a variety of effects, including anti-inflammatory, detoxifying, antioxidative, and lipid peroxidation effects. In addition, MgIG can protect the membrane of liver cells and improve the function of the liver (Yan et al., 2014). Previous studies have shown that MgIG has a clear protective effect against liver injury and is induced by a variety of chemicals, such as carbon tetrachloride, lipopolysaccharide, and concanavalin A (Wang and Liu, 2008; Jin et al., 2009), but the protective effects of hypoxic-oxygen injury have not been reported. In this study, we developed a liver-cell hypoxia-reoxygenation injury model, which simulated the ischemia-reperfusion injury model of the liver *in vivo*, and investigated the protective effect of and mechanism underlying the effect of MgIG on hypoxia-reoxygenation injury in liver cells. Our results provide a foundation and theoretical basis for the clinical application of hepatic ischemia reperfusion (HIRI).

MATERIAL AND METHODS

Materials

We used adult male Sprague-Dawley rats of clean grade with body masses of 200-220 g [animal license number: SCXK (Jiangxi) 2005-0001]. The following were used in this study: magnesium isoglycyrrhizinate powder (Nanjing Zhengda Tianqing Pharmaceutical Co. Ltd., Nanjing, China), 95% N₂ + 5% CO₂ gas (Shenzhen Gaofa Special Type Industrial Gas Co. Ltd., Guangdong, China), RNASE-free water (Takara, Shiga, Japan), TRIzol (Invitrogen, Carlsbad, CA, USA), reverse transcriptase (RT) reaction kit (Takara), fluorescence quantitative polymerase chain reaction (qPCR) kit (Takara), primer synthesis kit (Beijing Genomics Institute, Beijing, China), malondialdehyde (MDA) test kit, superoxide dismutase (SOD) test kit, alanine aminotransferase (ALT/GPT) test kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China), enzyme-linked immunosorbent assay (ELISA) kits with precoated tumor necrosis factor (TNF)- α (Shenzhen Dakewe Biological Engineering Co., Ltd., Guangdong, China) or interleukin (IL)-1 β .

Methods

Isolation, culture, and identification of liver cells

We used an improved 2-step method for *in situ* perfusion, and collagenase was primed

in situ until the liver became soft. The liver cells were separated and collected using DMEM (4°C), followed by filtration through 100- and 200-mesh stainless steel screen filters and low-speed centrifugation at 50 *g* for 3 min; this process was repeated 3 times. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum, with 1×10^3 /mL liver cells in the suspension. Dynamic changes in cell morphology using periodic acid-Schiff glycogen staining were observed under a light microscope and by hematoxylin and eosin staining.

Effects of MgIG on the vitality of hypoxia-reoxygenation-injured liver cells with methyl thiazolyl tetrazolium (MTT) detection

Liver cells in the logarithmic growth phase were adjusted to a cell density of 4×10^4 /mL and 200 μ L of each was inoculated into the wells of 96-well plates. The liver cells were randomly divided into the hypoxia-reoxygenation injury model group and the MgIG pretreatment group (0, 0.01, 0.1, 1, 10, 100 mg/mL) and pretreated for 24 h; 6 wells were used for each group and the hypoxia-reoxygenation injury model was prepared as described previously (Gao et al., 2004; Wang et al., 2009). Liver cells were cultured under hypoxic conditions in a closed container filled with 95% N₂ and 5% CO₂; the flow rate was 5 L/min. After inflation for 5 min, the measured O₂ concentration in the container was less than 1%. Krebs-Henseleit-Hepes buffer culture solution was also used for culture. Next, we arranged the sealed container and cultured the cells under hypoxic conditions at 37°C for 6 h to stimulate the hepatic ischemia state *in vivo*. For reoxygenation of the culture, each group of cells in the culture bottle was removed from the closed container, added to RPMI 1640 containing fetal bovine serum, and cultured under aerobic conditions with 5% CO₂ at 37°C for 4 h to stimulate the hepatic reperfusion state *in vivo*. After 48-h culture, we added 20 μ L MTT solution to each well of the plate and then incubated the plate at 5% CO₂, saturated humidity, and 37°C for 4 h. The supernatant was removed and 150 μ L dimethyl sulfoxide was added to each well. The plate was oscillated for 10 min to dissolve the purple-blue crystals and absorbance was measured at a wavelength of 492 nm. The absorption value (A value) for each well was compared to a standard value to determine the reaction activity of the cells.

Effects of MgIG on cell morphology and ALT, SOD, MDA, IL-1 β , TNF- α levels in each group

Liver cells in the logarithmic growth phase were randomly divided into a hypoxia-reoxygenation injury model group and low, moderate, and high concentration MgIG groups (0.1, 1, 10 mg/mL, respectively) after 24-h pretreatment. Hypoxia and reoxygenation conditions were simulated for 6 and 4 h, respectively, as described above. Morphological changes were observed using an inverted microscope and the supernatant was collected. The ALT, SOD, MDA, IL-1 β , and TNF- α levels were detected with the corresponding kit.

Effects of MgIG on nuclear factor (NF)- κ B expression in each group, detected by RT-qPCR

Cell grouping and treatment were as described above. Total RNA was extracted from liver cells using the TRIzol kit and the concentration and purity of RNA were determined. RT and PCR were conducted as described by the manufacturer. Primer sequences were as follows: NF- κ B upstream

5'-TCCGTTATGTATGTGAAGGC-3' and downstream 5'-TTTGCTGGTCCCACATAGTTGC-3' (amplified product, 112 bp); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) upstream 5'-CAGGGCTGCTTTAACTCTGG-3' and downstream 5'-TGGGTGGAATCATATTGGAACA-3' (amplified product, 102 bp). The experiment was repeated 3 times. An amplification curve and a melting curve were prepared to determine the mRNA expression and Ct values of GAPDH in the sample. The $2^{-\Delta\Delta Ct}$ relative quantitation method was used to determine sample concentrations. $\Delta\Delta Ct = (\text{average Ct value of experimental group} - \text{average Ct value of GAPDH gene}) - (\text{average Ct value of control group} - \text{average Ct value of GAPDH gene})$. We determined the difference ratio of gene expression in each experimental and control group.

Statistical analysis

All data were analyzed using the SPSS software 17.0 (SPSS, Inc., Chicago, IL, USA), and measurement data are reported as means \pm standard deviation. Two samples were compared using the *t*-test, while multiple samples were compared using single-factor analysis of variance. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Morphologic observation of hepatic cells after isolation, culture, and PAS glycogen staining

Freshly isolated liver cells were in a decentralized state and had a spherical or ovoid three-dimensional shape. Liver cells were affixed to the wall after 3-4 h; they were adherent after 6 h, and the cells were oval and some were larger and flat. Scattered mononuclear and binuclear liver cells were seen. After 24 h, the liver cells were flat or had a polygonal shape, their volume increased significantly, and the adjacent cells started adhering to each other. After liver cells were cultured for 48 h and subjected to PAS glycogen staining, the glycogen in intracytoplasmic liver cells stained purple and red and showed granular distribution (Figure 1A-D).

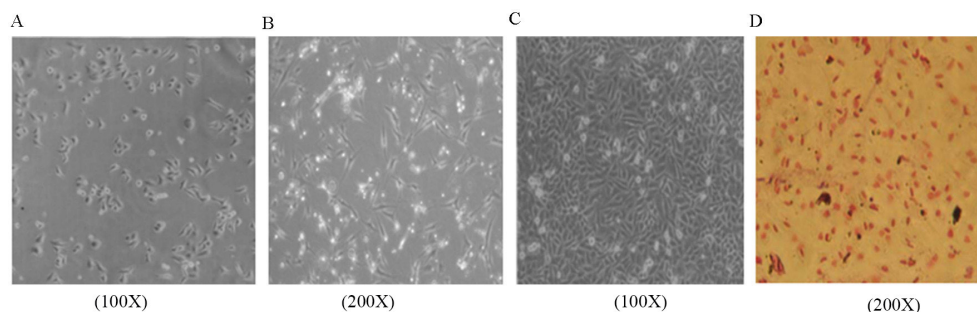


Figure 1. Isolation, culture, and identification of liver cells showing PAS glycogen staining. **A.** Liver cells isolated and cultured for 12 h. **B.** Liver cells cultured for 48 h. **C.** Liver cells cultured for 48 h. **D.** PAS glycogen staining of liver cells.

Morphological observation of the protective effects of MglG against hypoxia-reoxygenation damage to liver cells

The morphology and outline of liver cells in two groups were regular and clear; most cells

adhered to the dish wall and a few floating cells were noted. The cell morphology was generally regular in the model group after 6-h hypoxia, and floating cells slightly increased before hypoxia. The cells had shrunk after reoxygenation for 4 h, and the morphology of most cells was round; most cells were floating and few cells were adherent. After 1.0 mg/mL MgIG pretreatment for 24 h, minimal changes in morphological characteristics were observed, and after hypoxia for 6 h and reoxygenation for 4 h, only a few floating cells were noted (Figure 2A-F).

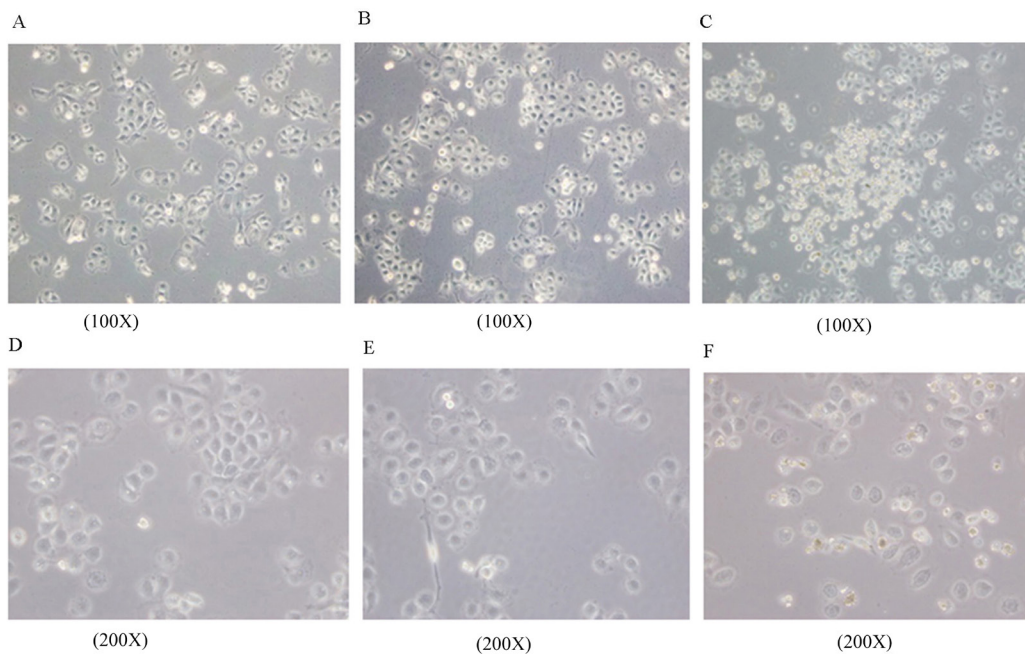


Figure 2. Morphological observation of the protective effects of MgIG on liver cells under hypoxia. **A. B. C.** Hypoxia reoxygenation injury model group. **D. E. F.** MgIG (1.0 mg/mL) pretreatment group. **A. D.** Pre-hypoxia and reoxygenation hepatocytes, respectively; **B. E.** Liver cells cultured under hypoxia conditions for 6 h; **C. F.** Liver cells cultured under reoxygenation conditions for 4 h.

Effects of MgIG on hypoxia-reoxygenation activity in liver cells

MgIG increased the viability of liver cells subjected to hypoxia-reoxygenation when used at concentrations of 0.1, 1, 10 mg/L (A values: 0.285 ± 0.017 , 0.320 ± 0.008 , 0.341 ± 0.019 , respectively); in the model group (0.227 ± 0.013), the difference was statistically significant ($P_{0.1} = 0$, $P_1 = 0.000$, $P_{10} = 0.000$, respectively). When the concentration was lower than or equal to 0.01 mg/mL, the A-value of the MTT group was higher than that of the model group ($P > 0.05$). Although liver cell viability after ischemia-reperfusion injury improved in 0.01 mg/mL MgIG concentration group (A value, 0.235 ± 0.014), the difference was not statistically significant ($P = 0.330$); when the MgIG concentration was high (100 mg/mL), the cells did not survive (Table 1).

Effects of MglG on ALT, IL-1 β , TNF- α , SOD, and MDA levels of hypoxia-reoxygenation-injured liver cells

The ALT level of the low-, moderate-, and high-concentration MglG pretreatment groups were 21.27 ± 1.58 , 17.03 ± 0.87 , and 14.79 ± 1.54 U/L, respectively, in the supernatant culture. The values were all lower than those in the model group (24.65 ± 1.22 U/L), and the difference was statistically significant ($F = 64.98$, $P < 0.01$). The SOD level in the cells (16.99 ± 1.25 , 20.22 ± 1.35 , 23.76 ± 0.99 U/mL, respectively) was higher than in the model group (12.52 ± 1.80 U/mL), and the difference was statistically significant ($F = 72.35$, $P < 0.01$). The MDA level (9.84 ± 1.14 , 5.64 ± 0.87 , 3.93 ± 0.59 μ M, respectively) was lower in the model group (11.89 ± 0.82 μ M), and the difference was statistically significant ($F = 105.19$, $P < 0.01$). The IL-1 β (33.34 ± 1.30 , 30.51 ± 0.76 , 28.35 ± 1.24 pg/mL) and TNF- α (58.75 ± 4.05 , 40.04 ± 4.74 , 29.22 ± 2.31 pg/mL) levels were lower than those in the model group (37.07 ± 1.18 and 66.43 ± 3.28 pg/mL, respectively). The difference was statistically significant ($F = 65.34$, 126.44 , $P < 0.01$) (Table 2).

Table 1. Effect of different concentrations of MglG on the activity of HL-7702 liver cells (means \pm SD) subjected to ischemia reperfusion injury.

Groups	N	A492 value
Model (0 mg/mL MglG)	6	0.227 ± 0.013
0.01 mg/mL MglG	6	0.235 ± 0.014^M
0.1 mg/mL MglG	6	0.285 ± 0.017^N
1.0 mg/mL MglG	6	0.320 ± 0.008^N
10 mg/mL MglG	6	0.341 ± 0.019^N
100 mg/mL MglG	6	After reperfusion, no living cells were seen under a microscope
F value		68.06
P		<0.01

Compared with the model group, ^MP > 0.05, ^NP < 0.01.

Table 2. ALT, IL-1 β , TNF- α , intracellular SOD, and MDA levels in the culture supernatant of liver cells (N = 6).

Group	ALT (U/L)	SOD (U/mL)	MDA (μ M)	IL- β (pg/mL)	TNF- α (pg/mL)
Model	24.65 ± 1.22	12.52 ± 1.80	11.89 ± 0.82	37.07 ± 1.18	66.43 ± 3.28
Lower	21.27 ± 1.58^a	16.99 ± 1.25^a	9.84 ± 1.14^a	33.34 ± 1.30^a	58.75 ± 4.05^a
Moderate	17.03 ± 0.87^a	20.22 ± 1.35^a	5.64 ± 0.87^a	30.51 ± 0.76^a	40.04 ± 4.74^a
Higher	14.79 ± 1.54^a	23.76 ± 0.99^a	3.93 ± 0.59^a	28.35 ± 1.24^a	29.22 ± 2.31^a
F value	64.98	72.35	105.19	65.34	126.44
P	<0.01	<0.01	<0.01	<0.01	<0.01

^aCompared to the model group, $P < 0.01$.

Effects of MglG on NF- κ B mRNA expression in hypoxia-reoxygenation-injured liver cells

RT-qPCR was used to construct amplification curves for NF- κ B and GAPDH mRNA in all samples. Dissolution curves were unimodal, while the amplification curves were S-shaped, indicating that the primers were specific and that amplification was quite efficient. NF- κ B mRNA expression in the low-, moderate-, and high-concentration MglG pretreatment groups was 0.597 ± 0.062 , 0.248 ± 0.067 , and 0.141 ± 0.029 , respectively. The values were lower than that in the hypoxia-reoxygenation injury model group (0.962 ± 0.034), and the difference was statistically significant ($F = 160.327$, $P < 0.01$) (Figure 3A-D).

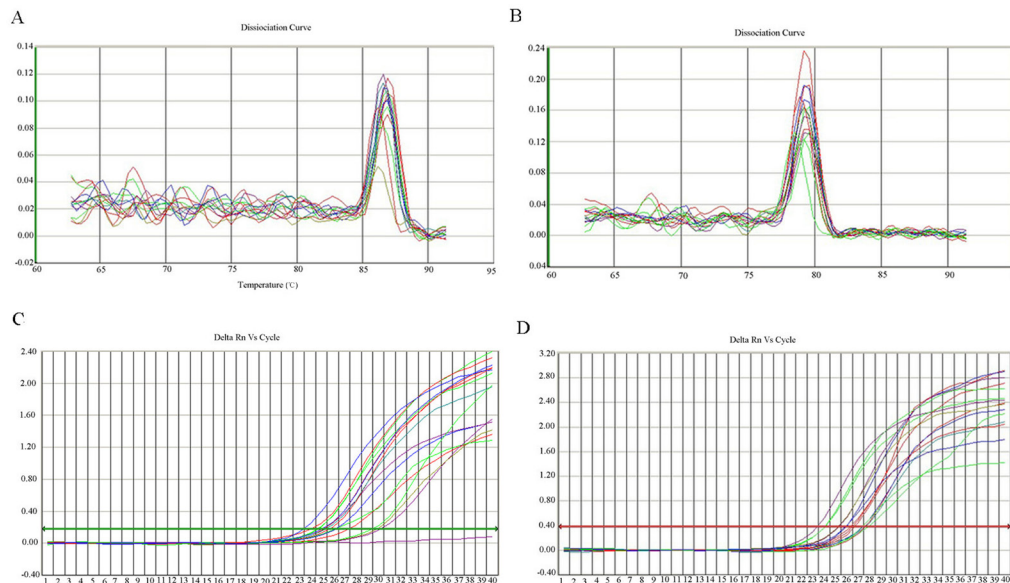


Figure 3. Effects of MgIG on NF- κ B mRNA expression in hypoxia reoxygenation injury liver cells. **A. B.** Dissolution curve and amplification curve of NF- κ B, respectively. **C. D.** Dissolution curve and amplification curve of GAPDH, respectively.

DISCUSSION

MgIG is a 4th-generation glycyrrhizic acid agent extracted from glycyrrhiza plants by alkali isomerization catalysis and using refined salt. Its effective component is a single 18 α isomer of glycyrrhizic acid according to Gao et al. (2004). MgIG strongly targets the liver and shows good lipophilicity. It combines easily with receptor proteins and the target cell receptor of steroid hormones *in vivo*, and thus acts as a hormone. MgIG also has anti-inflammatory, antioxidant, and lipid peroxidation effects and protects the membrane of liver cells to improve liver function. Previous studies showed that MgIG protects liver cells *in vitro* (Wang and Liu, 2008; Jin et al., 2009; Yan et al., 2014). Recent studies found that MgIG can significantly reduce inflammation and liver cell apoptosis induced in HIRI rats (Wang and Liu, 2008; Jin et al., 2009; Hua et al., 2010; Yan et al., 2014). Therefore, MgIG can improve liver function. However, the protective effect of MgIG on liver cells in an *in vitro* simulated ischemia reperfusion injury (anoxia/reoxygenation injuries) model has not been reported.

In this study, we established a model of liver hypoxia-reoxygenation injury after separation and culture of rat liver cells. The results showed that pretreatment with 0.1-10 mg/mL MgIG improved the vigor of liver cells subjected to hypoxia-reoxygenation and protected against hypoxia-reoxygenation injury. Morphological observation also showed that MgIG maintained the normal liver cell morphology and reduced the death of liver cells.

Under hypoxia-reoxygenation injury conditions, liver cells can release a large number of oxygen free radicals and induce lipid peroxidation, resulting in cell membrane damage and exacerbating cell injury. MDA is the end-product of lipid peroxidation and can indirectly reflect the extent of cell injury. While SOD is a superoxide anion free radical scavenger, which inhibits lipid

peroxidation in the body, its levels indirectly reflect the ability of the organism to scavenge oxygen free radicals. Licorice compounds are potent antioxidants (Cheng et al., 2009), and studies have showed that MglG can significantly improve rat liver SOD levels and decrease MDA levels in the case of ischemia-reperfusion, thereby reducing the degree of damage to liver cells and improving liver function (He et al., 2010). We showed that MglG increased the SOD level in liver cells subjected to hypoxia-reoxygenation, thus reducing the damage caused by oxygen free radicals to liver cells and reducing MDA levels, thereby effectively protecting the liver cells from hypoxia-reoxygenation injury.

HIRI is an aseptic inflammatory reaction process involving many inflammatory cells and inflammatory factors (Inoue et al., 2013). NF- κ B is one of the most important transcription factors in the cell, and can regulate the transcription of a variety of inflammatory factors and adhesion molecules that participate in HIRI; for example, it regulates the transcription and expression of the TNF- α and IL-1 β genes. These inflammatory factors initiate the inflammation process, causing liver cell injury both individually and synergistically. Wang et al. (2011) found that MglG can block the generation of inflammatory pathways downstream of relevant inflammatory mediators by decreasing the starting level of the inflammatory response signal. MglG can inhibit the inflammatory response reaction caused by various factors. In this study, MglG downregulated NF- κ B mRNA expression in cells subjected to hypoxia-reoxygenation and also reduced TNF- α and IL-1 β levels in hypoxia-reoxygenation injury liver cells.

We simulated HIRI in cells and confirmed that a certain concentration of MglG can reduce HIRI in several ways. MglG may increase the proliferation activity of liver cells and confer anti-inflammatory, antioxidant, lipid peroxidation, and antiapoptotic effects. However, additional studies should be conducted to investigate the effects of MglG on liver cell apoptosis.

Conflicts of interest

The authors declare no conflict of interest.

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