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# Glycyrrhizin prevents liver injury by inhibition of HMGB1 production by kupffer cells after ischemia-reperfusion in rats

by

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Running title: **Glycyrrhizin mediates I/R-induced liver injury**

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**Abbreviations:** ALT: alanine aminotransferase; I/R: ischemia-reperfusion; ELISA: enzyme linked immunosorbent assay; HMGB1: High mobility group box 1; IL: interleukin; lipo-MDP: liposome-entrapped dichloromethylene diphosphonate

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## Abstract

High mobility group box 1 (HMGB1) acts as an early mediator of inflammation and organ damage in hepatic ischemia-reperfusion (I/R) injury. Glycyrrhizin is a natural anti-inflammatory and antiviral triterpene in clinical use. The purpose of this study was to investigate the effect of glycyrrhizin on liver injury caused by I/R and production of HMGB1 by Kupffer cells in rats. In the first test period, rats were given saline or glycyrrhizin 20 minutes before segmental hepatic warm I/R. Serum ALT and HMGB1 levels and hepatic histopathological findings were evaluated after I/R. Furthermore, expression of HMGB1 in the liver was assessed by immunohistochemical staining after I/R. Kupffer cells (KC) were isolated by collagenase digestion and differential centrifugation, and production of HMGB1 was assessed. In another set of experiments, the effect of inhibition of KCs by injection of liposome-entrapped dichloromethylene diphosphonate (lipo-MDP) on liver injury and expression of HMGB1 were investigated after I/R. Liver injury was prevented in the glycyrrhizin group compared with the control group. Furthermore, serum HMGB1 levels were also significantly blunted in the glycyrrhizin group compared with the control group. Cells expressing HMGB1 were detected in the hepatic sinusoid by immunohistochemistry

and were recognized morphologically as KCs. Furthermore, the expression of HMGB1 was reduced in the glycyrrhizin group compared with the control group. Production of HMGB1 was reduced in KCs isolated from the glycyrrhizin group compared with the control group. Importantly, treatment with lipo-MDP significantly blunted serum HMGB1 levels and prevented liver injury after I/R. These results suggest that glycyrrhizin has the therapeutic potential to prevent warm I/R-induced injury during hepato-biliary surgery.

## Introduction

Hepatic ischemia-reperfusion injury is encountered during liver surgery, trauma, and hypovolemic shock. Liver injury induced by ischemia-reperfusion (I/R) has two distinct phases (Jaeschke. 1999; Lentsch et al., 2000). The initial phase is characterized by activation of Kupffer cells, their production of reactive oxygen species (ROS) (Jaeschke. 1999), and the release of proinflammatory chemokines and cytokines, including interleukin-12 and tumor necrosis factor alpha (Colletti et al.,1990; Lentsch et al., 1999; Rudiger and Clavien., 2002) . The expression of these mediators leads to the second phase characterized by the recruitment and extravasation of neutrophils into the parenchyma, significant ROS production, and release of proteases, leading to more severe hepatic injury (Gujral et al., 2003; Jaeschke and Hasegawa., 2006).

High mobility group box 1 (HMGB1) was originally discovered as a nuclear transcription factor (Goodwin and Sanders., 1973). HMGB1 is actively released into the serum from monocyte/macrophages and passively diffuses from necrotic cells (Klune et al., 2008). Recently, HMGB1 was identified as an inflammatory cytokine that is a late mediator of lethality in sepsis and is a promising therapeutic target (Wang ., 2004; Yang., 2004). In a previous study, HMGB1 acted an early mediator of

inflammation and organ damage in hepatic I/R injury (Tsung et al., 2005). Furthermore, anti-HMGB1 antibody treatment reduced liver damage in hepatic I/R. However, the source of HMGB1 in I/R-induced liver injury remains unclear.

Glycyrrhizin, a glycoconjugated triterpene produced by the licorice plant, Glycyrrhiza inflata, is an anti-inflammatory that has been used in the treatment of patients with chronic hepatitis B and C (Iino et al., 2001; Miyake et al., 2002; Matsui et al., 2006; Yoshida et al., 2007) . Furthermore, glycyrrhizin reduces I/R-induced liver injury (Nagai et al., 1992) and inhibits elevated circulating levels of HMGB1 in the acute phase (Mabuchi et al., 2009).

Accordingly, the purpose of this study was to investigate the effect of glycyrrhizin on liver injury caused by I/R and production of HMGB1 by Kupffer cells in rats.

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## Materials and methods

### Animals.

Male Sprague-Dawley rats (200g body weight, Japan SLC Inc., Shizuoka, Japan) were used in these experiments. The experimental protocol followed the Institutional and the National Research Council's criteria for the care and use of laboratory animals in research. All animals used for this study were housed in sterilized cages in a facility with a 12-hour night/day cycle. Temperature and relative humidity were held at 23 ± 2°C and 50 ± 10%, respectively. The University of Yamanashi staff maintains these animal facilities, and veterinarians are always available to ensure animal health. All animals were given humane care in compliance with governmental regulations and institutional guidelines, and studies were performed according to protocols approved by the appropriate institutional review board.

### Effect of glycyrrhizin on I/R-induced liver injury (Experimental series 1).

A nonlethal model of segmental (70%) hepatic warm ischemia was used. Rats were anesthetized using sodium pentobarbital (50 mg/kg, i.p.). The liver hilum was dissected free of surrounding tissue. All structures in the portal triad (hepatic artery,

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portal vein, bile duct) to the left and median liver lobes were occluded with a microvascular clamp for 60 min; reperfusion was initiated by removal of the clamp (Tsung et al., 2005). This method of segmental hepatic ischemia prevents mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. The clamp was removed, and gross evidence of reperfusion based on immediate color change was assured before closing the abdomen. After surgery, rats were placed on a heating pad until they recovered from the anesthesia. At 120 minutes after reperfusion, tissue and blood samples were collected. Prior to ischemia or sham operation, glycyrrhizin (60 mg/kg, i.v.) or an equivalent volume of saline was administered over 20 minutes using an infusion pump[18] (n = 6 in each group). Glycyrrhizin was purchased from Minophagen Pharmaceutical Co. (Tokyo, Japan)

**Effect of glycyrrhizin and inhibition of Kupffer cell by lipo-MDP on I/R-induced liver injury (Experimental series 2).**

Animals received intravenous administration of 1 ml of lipo-MDP to eliminate the Kupffer cell 72 hours before warm ischemia since intravenous injection of 1ml of lipo-MDP eliminated the Kupffer cell but not the splenic macrophage (Boulton et al., 1998; Kono et al., 2011). Non-entrapped liposome (lipo; 1 ml/animal) was

administered as a control. Animals were randomly divided into 4 groups (n = 6 in each group): Group I, lipo + saline + I/R; Group II, lipo + glycyrrhizin + I/R; Group III, lipo-MDP + saline + I/R; and Group VI, lipo-MDP + glycyrrhizin + I/R. Serum and tissue samples were collected 2 hours after reperfusion.

**Liver sample collection.**

Liver samples were collected 2 hours after reperfusion or a sham operation. The samples were fixed in formalin, embedded in paraffin and sectioned (5μm thick). Some sections were stained with hematoxylin-eosin to assess sinusoidal congestion and necrosis. Values were expressed as the number of hemolysis in the hepatic sinusoid per 400 hepatocytes (n = 6 in each group). The sections were evaluated in a blind manner under light microscopy by 2 investigators.

**Measurement of serum HMGB1.**

Blood samples were collected via the aorta at 2 hours after reperfusion or the sham operation (n = 6). The samples were centrifuged at 1200×g for 10 min at 4°C, and serum was stored at -80°C until the assays. Determination of serum HMGB1 levels was performed using enzyme-linked immunosorbent assay kits (ELISA; Central

Institute, Shino-Test, Kanagawa, Japan).

**Measurement of plasma alanine aminotransferase.**

Plasma alanine aminotransferase (ALT) levels were measured to assess hepatic parenchymal damage using FUJI DRI-CHEM analyzers (Fujifilm Co., Tokyo, Japan).

**Immunohistochemistry for HMGB1.**

Formalin-fixed, paraffin-embedded tissue specimens were cut into 4-μm sections. Each section was mounted on a silane-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 0.3% $H_2O_2$/methanol to block endogenous peroxidase. Antigen retrieval was performed with 10 mM citrate buffer at pH 6.0 (Dako, Carpinteria, CA) for 40 min at 94 °C in water bath. Endogenous avidin and biotin blocking were carried out with a biotin blocking system (Dako). A rabbit anti-HMGB1 monoclonal antibody (dilution 1:250, Abcam, Cambridge, UK) was applied for 12 hours at 4°C. Peroxidase-linked secondary antibody and diaminobenzidine (Vectastain ABC elite kit, Vector Laboratories, Burlingame, CA) were used to detect specific binding. The slides were rinsed twice with PBS containing Tween 20 between each incubation, and sections were counterstained with hematoxylin as described elsewhere. Sections from the same

rats were processed without the primary antibody and then examined using the procedure detailed above as a control for nonspecific binding of the secondary antibody. Values were expressed as the number of positive cells per 400 hepatocytes (n = 6 in each group).

**Isolation of Kupffer cells.**

Kupffer cells were isolated by collagenase digestion and differential centrifugation using Nycodenz (Nycomed Pharma AS, Oslo, Norway) as described in our previous work with some modifications (Kono et al., 2000; Tsuchiya et al., 2008). Briefly, saline-treated or glycyrrhizin-treated rats underwent I/R before isolation of Kupffer cells. Rats were anesthetized using sodium pentobarbital (50 mg/kg, i.p.), the abdomen was opened, and the portal vein was cannulated. The liver was perfused *in situ* for 5 min with $Ca^{2+}$/$Mg^{2+}$-free liver perfusion medium (LPM-1: 8,000 mg/L NaCl, 400 mg/L KCl, 88.17 mg/L $NaH_2PO_4$-$2H_2O$, 120.45 mg/L $Na_2HPO_4$, 2380 mg/L HEPES, 350 mg/L $NaHCO_3$, 190 mg/L EDTA, 900 mg/L glucose, 6 mg/L Phenol red; pH 7.4, 37°C) and then was perfused with complete liver perfusion medium (LPM-2: same as LPM-1 except without EDTA and glucose, but with 560 mg/L $CaCl_2$-$2H_2O$; pH 7.4, 37°C) containing 0.06% collagenase type IV (Sigma, St. Louis, MO) for an

additional 15 min. After perfusion, the liver subjected to I/R was removed, cut into small pieces, and homogenized. After passing through a gauze filter (mesh size -60 μm), cells were washed twice with warm Gey's balanced salt solution (GBSS-B: 370 mg/L KCl, 210 mg/L $MgCl_2$-$6H_2O$, 70 mg/L $MgSO_4$-7 $H_2O$, 150 mg/L $NaH_2PO_4$-$2H_2O$, 30 mg/L $KH_2PO_4$, 1090 mg/L glucose, 227 mg/L $NaHCO_3$, 225 mg/L $CaCl_2$-$2H_2O$, 6 mg/L Phenol red, 8,000 mg/L NaCl, 100 U/l streptomycin, 105 U/l penicillin G; pH 7.4) and centrifuged over a 16% (wt/vol) Nycodenz (Axis-Shields, Oslo, Norway) gradient for 20 min at 1900 × g at 4°C (Kawada et al., 1998). Kupffer cells were collected from under the interface, washed with GBSS-B, and resuspended at $1 \times 10^6$ cells/mL in D-MEM media (Invitrogen, Carlsbad, CA).

**Production of HMGB1 by isolated Kupffer cells.**

Isolated Kupffer cells were seeded onto 24-well plates ($1 \times 10^6$/well) and cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in the presence of 5% $CO_2$. Cells were incubated for 1, 3, and 6 hours. Medium was collected and kept at -80°C until assay. HMGB1 levels were measured using ELISA (Shino-Test) according to the manufacturer's instructions (n = 4 in each group).

**Preparation of liposome-entrapped dichloromethylene diphosphonate (lipo-MDP).**

Multilamellar liposomes were prepared as described previously (Kiwada et al., 1985). In brief, 58.72 mg phosphatidylcholine (PC; Nihon Seika, Hyogo, Japan), 8.75 mg dehexadecyl phosphate (DCP; Nacalai Tesque, Kyouto, Japan), and 24.75 mg cholesterol (CH; Wako, Tokyo, Japan) were dissolved in chloroform in a round-bottom flask and dried under reduced pressure. The lipids were then hydrated in 5 ml PBS with or without 650 mM dichloromethylene diphosphonate (MDP, Sigma, St. Louis, MO) and vortexed at room temperature. The liposomes were extruded through polycarbonate membranes (Costar, Pleasanton, CA) with a pore size of 0.8 μm. Non-encapsulated MDP was removed by ultracentrifugation. The liposomes were comprised of PC/DCP/CH at a molar ratio of 5:1:4.

**Statistical analysis.**

Data are expressed as mean ± SEM. Survival was compared by Kaplan-Meier analysis and log rank statistics, and the comparisons between other data were made with ANOVA with Bonferroni's post-hoc test, with a P value of less than 0.05 considered to indicate a statistically significant difference.

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## Results

### Experimental series 1

#### Serum ALT levels.

Serum ALT levels were minimal in the sham operation groups but increased in the saline-treated group after I/R (Fig. 1). On the other hand, the values were significantly blunted by about 70% in the glycyrrhizin-treated group compared with the saline-treated group.

#### Pathological findings in the liver.

In the sham operation groups, no pathological changes were observed in the liver (data not shown). In the saline-treated rats, severe sinusoidal congestion and hemolysis was observed 2 hours after I/R (Fig. 2A). In contrast, these pathological changes were prevented in the glycyrrhizin-treated rats (Fig. 2B). The number of hemolysis in the hepatic sinusoid was reduced in livers from glycyrrhizin-treated rats compared to saline-treated rats (Fig. 3)

**Serum HMGB1 levels after I/R.**

The serum HMGB1 levels were minimal in the sham operation groups (Fig. 4). In contrast, the values increased in the saline-treated rats after I/R. However, glycyrrhizin treatment significantly blunted the values by about 80% after I/R.

**Immunohistochemical staining for HMGB1 in the liver.**

To evaluate the expression of HMGB1 in the liver, immunohistochemistry was performed (Fig. 5). The expression of HMGB1 was not detected in the sham operation groups (Figs. 5A and 5B). In contrast, the expression was observed in the hepatic sinusoid in the saline-treated rats subjected to I/R (Fig. 5C). They were recognized morphologically as Kupffer cells. This expression was reduced in livers from glycyrrhizin-treated rats compared to saline-treated rats (Fig. 5D). The number of HMGB1-positive cells in the hepatic sinusoid was reduced in livers from glycyrrhizin-treated rats compared to saline-treated rats (Fig. 6)

**Production of HMGB1 by isolated Kupffer cells after I/R.**

The production of HMGB1 by isolated Kupffer cells from animals without I/R

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was minimal at all points in time (data not shown). In contrast, the production increased dramatically in cells isolated from I/R groups after 1 hour of incubation; however, there were no significant differences between the two groups (Fig. 7). On the other hand, the production was significantly greater in cells isolated from the saline-treated group than cells isolated from the glycyrrhizin-treated group after incubation for 3 (24.6 ± 0.9 vs. 20.7 ± 0.8 ng/ml) or 6 hours (42.5 ± 2.1 vs. 24.9 ± 1.4 ng/ml).

**Experimental series 2**

**Effects of MDP on serum ALT levels after I/R.**

Serum ALT levels were 5200 U/L in the saline-treated rats (VEH) administered lipo after I/R (Fig. 8). In contrast, in the saline-treated rats administered MDP, the values were significantly reduced by about 70%. Furthermore, in MDP-treated groups, there were no significant differences in serum ALT levels between the saline-treated rats and the glycyrrhizin-treated rats.

**Effects of MDP on serum HMGB1 levels after I/R.**

Serum HMGB1 levels were 28 ng/ml in the saline-treated rats administered lipo

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(Fig. 9). In contrast, in the saline-treated rats administered MDP, the values were significantly reduced by about 60%. There were no significant differences in serum HMGB1 levels between the glycyrrhizin-treated rats administered MDP and the saline-treated rats administered MDP.

**Effect of glycyrrhizin on production of HMGB1 by isolated Kupffer cells.**

The effect of glycyrrhizin on production of HMGB1 by isolated Kupffer cells was investigated *in vitro*. Production of HMGB1 did not change when concentrations of glycyrrhizin ranging from 0 to 100 μg/ml were administered (Fig. 10), suggesting that glycyrrhizin has no effects on production of HMGB1 by Kupffer cells.

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## Discussion

### Effects of glycyrrhizin on ischemia-reperfusion injury in the liver.

In the present study, glycyrrhizin significantly prevented increased serum ALT levels and I/R-induced liver injury, consistent with previous reports (Nagai et al., 1992; Mabuchi et al., 2009). It was reported that expression of HMGB1 induces microvascular thrombosis in a model of thrombin-induced disseminated intravascular coagulation (DIC) in rats (Hatada et al., 2005; Ito et al., 2007). Furthermore, it was reported that glycyrrhizin inhibits blebosome formation of the hepatocyte and prevents microcirculatory disturbances in the sinusoidal blood flow (Miyoshi et al., 1996; Jaeschke and Lemasters., 2003; Mabuchi et al., 2009). In the present study, sinusoidal congestion and serum HMGB1 levels were significantly reduced in the glycyrrhizin-treated rats (Fig. 2, 3 and 4). These results support the hypothesis that glycyrrhizin most likely prevents hepatic microcirculatory disturbances by inhibition of formation of blebosomes after I/R.

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**Possible source of HMGB1 after I/R in the liver.**

Predominant sources of HMGB1 after reperfusion are still unclear. Activated macrophages are a possible source of HMGB1. Indeed, isolated Kupffer cells activated by lipopolysaccharide produced HMGB1 *in vitro* (unpublished data). In the present study, production of HMGB1 increased significantly in the Kupffer cells isolated from animals that had undergone I/R compared with cells isolated from sham-operated animals (values of production of HMGB1 by Kupffer cells isolated from animals without I/R;1h, 3.7 ± 0.6; 3h, 5.8 ± 1.1; and 6h, 4.6 ± 0.8 ng/ml) (Fig. 7). Furthermore, liver injury and serum HMGB1 levels after I/R decreased in animals whose Kupffer cells were deleted by administration of MDP (Fig. 8 and 9). These results support the hypothesis that activated Kupffer cells are a major source of HMGB1 after reperfusion.

Alternatively, it was reported that HMGB1 is released from necrotic tissue and the hepatocytes in response to hypoxia after I/R (Scaffidi et al., 2002; Tsung et al.,

2007). Indeed, hypoxia induces acetylation of intranuclear HMGB1 during ischemia. Acetylated HMGB1 is translocated to the cytoplasm and released into serum after reperfusion (Evankovich et al., 2010). It was reported that glycyrrhizin protects the liver from I/R-induced injury by stabilization of the cellular membrane structure of the hepatocytes (Shiki et al., 1992). Thus, this stabilization of the cellular membrane may inhibit extracellular release of HMGB1 from hepatocytes with oxidative stress. To evaluate the effect of glycyrrhizin on oxidative stress in the liver, the expression of nitrotyrosine was investigated by immunohistochemistory. As a result, the expression of nitrotyrosine was not detected in both groups (data not shown). Since glycyrrhizin had no direct effects on production of HMGB1 by isolated Kupffer cells *in vitro* (Fig. 10), a predominant effect of glycyrrhizin on serum levels of HMGB1 may be due to protection of the hepatocyte from reperfusion injury.

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**Conclusion**

In summary, the present study demonstrated that activated Kupffer cells are involved in I/R-induced liver injury and HMGB1 production. In addition, glycyrrhizin inhibited HMGB1 production by Kupffer cells and prevented I/R-induced liver injury. Thus, glycyrrhizin has the therapeutic potential to reduce warm I/R-induced injury during hepato-biliary surgery.

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**Authorship Contributions.**

Participated in research design: Kono, Fujii, Ogiku, Tsuchiya and Hara

Conducted experiments: Ogiku

Contributed new reagents or analytic tools: Ogiku

Performed data analysis: Ogiku

Wrote or contributed to the writing of the manuscript: Ogiku

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## Figure legends

**Figure 1. Serum ALT levels after I/R.**

Blood was collected via the aorta 2 hours after I/R, and serum ALT levels were determined as described in Materials and Methods. Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion: I/R; Sham operation: Sham; saline vehicle: VEH; and glycyrrhizin: GL. $^{*}$, $P < 0.01$ compared with saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

**Figure 2. Pathological findings of the liver after I/R.**

Liver tissues were harvested 2 hours after I/R. A, the liver in saline-treated rats subjected to I/R; and B, the liver in glycyrrhizin-treated rats subjected to I/R. Ischemia-reperfusion, I/R; saline vehicle, VEH; and glycyrrhizin, GL. Original magnification, 400×.

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**Figure 3. The number of hemolysis in the hepatic sinusoid after I/R.**

The number of hemolysis in the hepatic sinusoid were evaluated as described in the Materials and Methods (400×, per 400 hepatocytes). Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion, I/R; saline vehicle, VEH; and glycyrrhizin, GL. *, $P < 0.05$ compared with saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

**Figure 4. Serum HMGB1 levels after I/R.**

Blood was collected via the aorta 2 hours after I/R, and serum HMGB1 levels were determined as described in Materials and Methods. Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion, I/R; sham operation, Sham; saline vehicle, VEH; and glycyrrhizin, GL. *, $P < 0.01$ compared with saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

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**Figure 5. Immunohistochemical staining using anti-HMGB1 antibody in the liver after I/R.**

Immunohistochemical staining for HMGB1 was performed as described in Materials and Methods. A, liver in saline-treated rats subjected to sham operation; B, liver in glycyrrhizin-treated rats subjected to sham operation; C, liver in saline-treated rats subjected to I/R; and D, liver in glycyrrhizin-treated rats subjected to I/R. Ischemia-reperfusion, I/R; sham operation, Sham; saline vehicle, VEH; and glycyrrhizin, GL. Representative photomicrographs. Original magnification, ×200.

**Figure 6. The number of HMGB1-positive cells in the hepatic sinusoid after I/R.**

The number of HMGB1-positive cells in the hepatic sinusoid was evaluated as described in the Materials and Methods (400×, per 400 hepatocytes). Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion, I/R; sham operation, Sham; saline vehicle, VEH; and glycyrrhizin, GL. $^{*}$, $P < 0.01$ compared with saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

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**Figure 7. Production of HMGB1 by isolated Kupffer cells after I/R.**

Cells were isolated as described in Materials and Methods and then incubated with fresh media for 1, 3, or 6 hours. HMGB1 production was determined as described in Materials and Methods. Data represent mean ± SEM (n = 4 in each group). Ischemia-reperfusion, I/R; saline vehicle, VEH; and glycyrrhizin, GL. $^{*}$, $P < 0.05$ compared with HMGB1 production by cells isolated from saline-treated rats subjected to I/R for 3 hours of incubation; and $^{**}$, $P < 0.01$ compared with HMGB1 production by cells isolated from saline-treated rats subjected to I/R for 6 hours of incubation by two way ANOVA with Bonferroni's post-hoc test.

**Figure 8. Effects of MDP on serum ALT levels after I/R.**

Blood was collected via the aorta at 2 hours after I/R following treatment with MDP, and serum ALT levels were determined as described in Materials and Methods. Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion, I/R; saline vehicle,

VEH; glycyrrhizin, GL; MDP-liposome, lipo-MDP; and non-entrapped-liposome, lipo. $^{*}$, $P < 0.01$ compared with lipo and saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

**Figure 9. Effects of MDP on serum HMGB1 levels after I/R.**

Blood was collected via the aorta at 2 hours after I/R, and serum HMGB1 levels were determined as described in Materials and Methods. Data represent mean ± SEM (n = 6 in each group). Ischemia-reperfusion, I/R; saline vehicle, VEH; glycyrrhizin, GL; and MDP-liposome, lipo-MDP; and non-entrapped-liposome, lipo. $^{*}$, $P < 0.01$ compared with lipo and saline-treated rats subjected to I/R, and $^{**}$, $P < 0.05$ compared with lipo and saline-treated rats subjected to I/R by two way ANOVA with Bonferroni's post-hoc test.

**Figure 10. Effects of glycyrrhizin on production of HMGB1 by isolated Kupffer cells from rats subjected to I/R.**

Cells were isolated as described in Materials and Methods. They were then incubated

with fresh media containing glycyrrhizin for 6 hours. HMGB1 production was determined as described in Materials and Methods. Data represent mean ± SEM (n = 4 in each group).

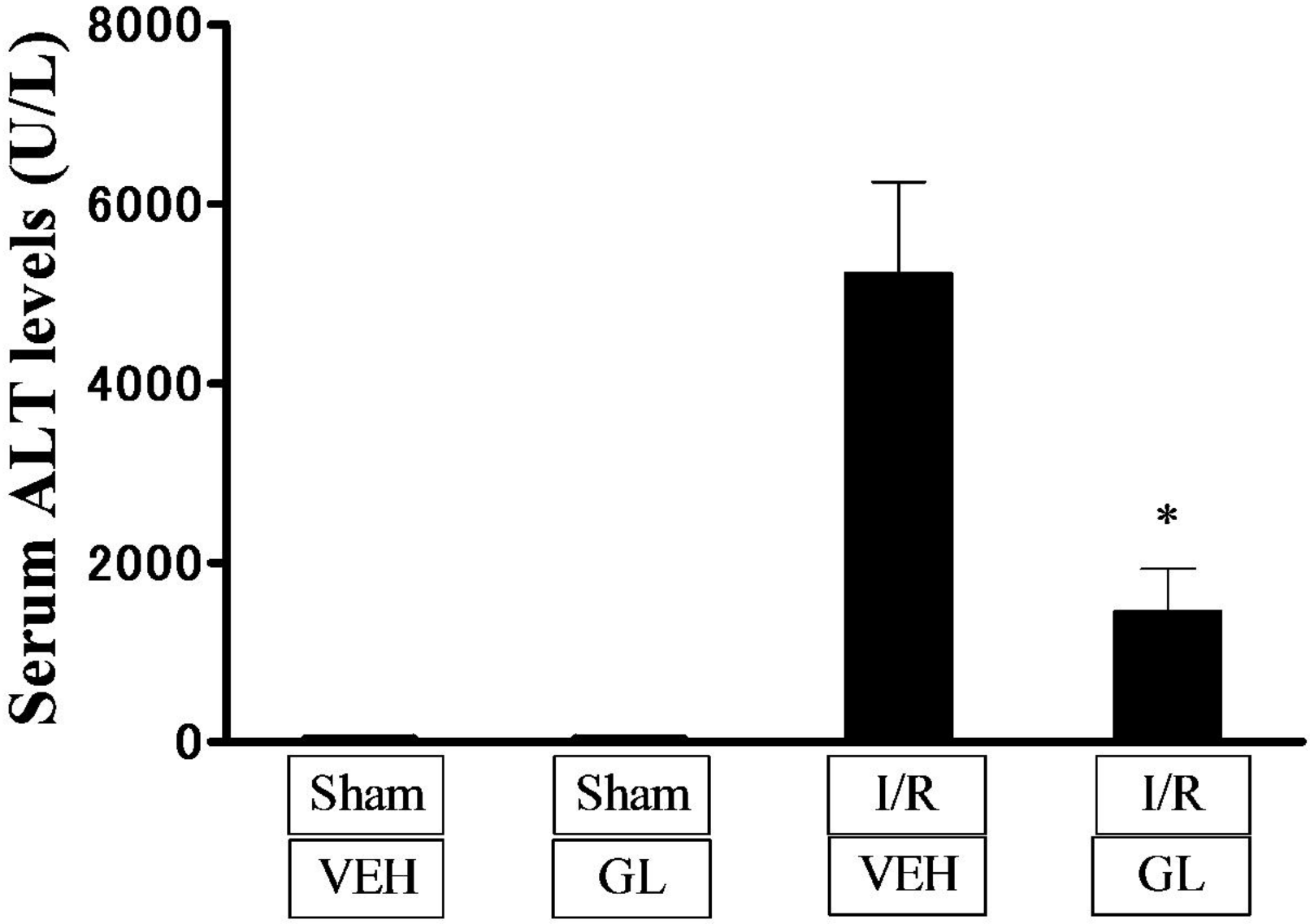
Serum ALT levels (U/L)
8000
6000
4000
2000
0
*
Sham
VEH
Sham
GL
I/R
VEH
I/R
GL

**Figure 1.**

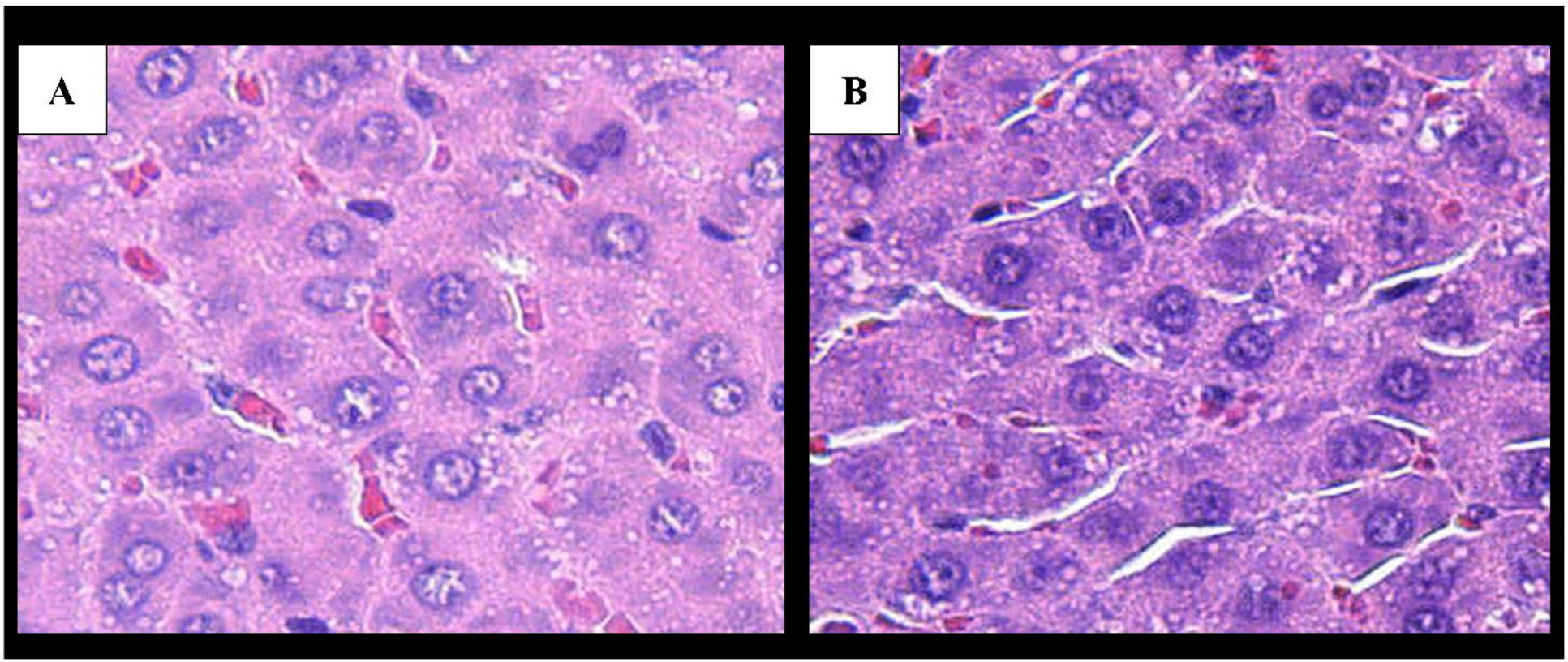


**Figure 2.**

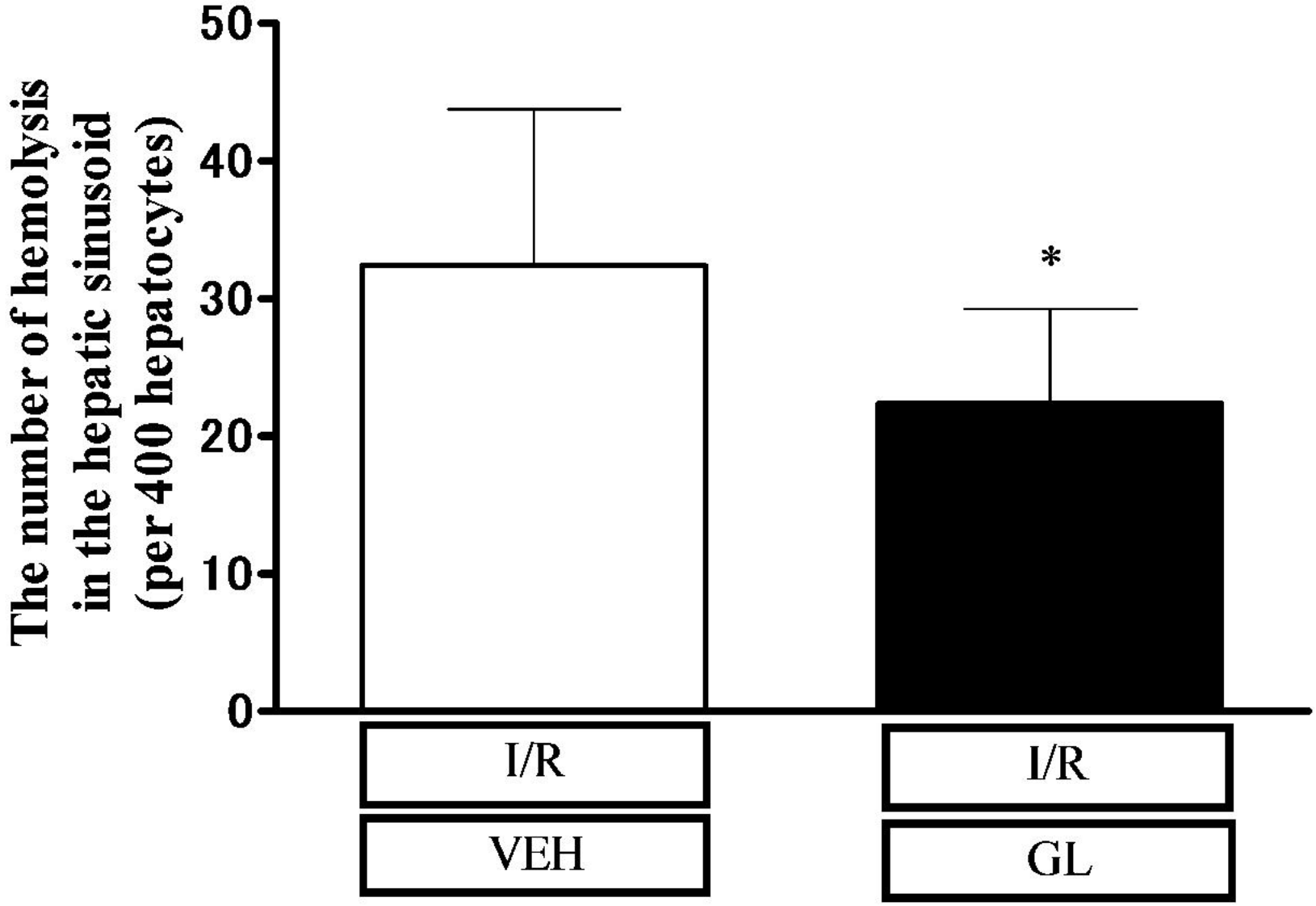
The number of hemolysis in the hepatic sinusoid (per 400 hepatocytes)
50
40
30
20
10
0
*
I/R
VEH
I/R
GL

**Figure 3.**

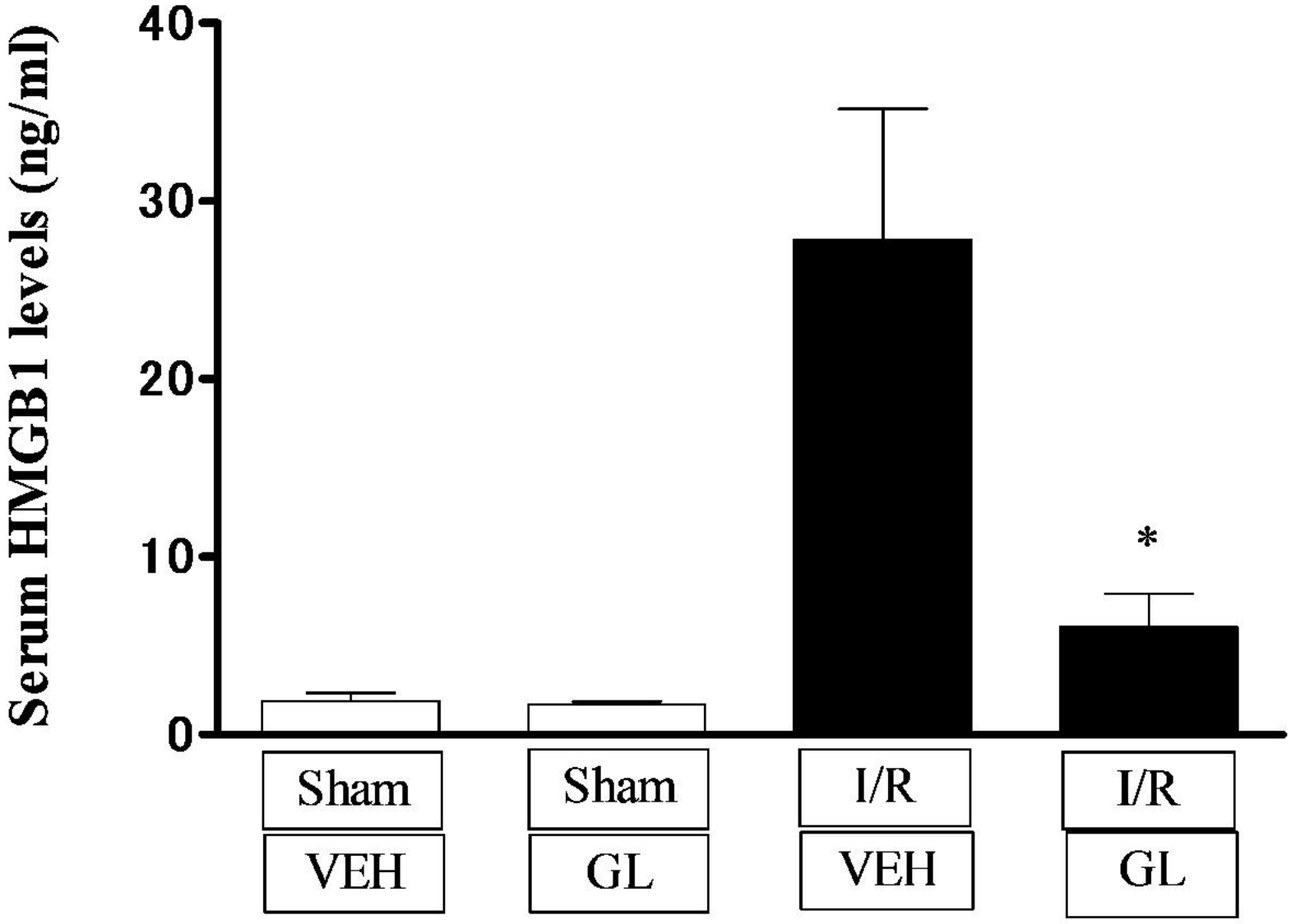
Serum HMGB1 levels (ng/ml)
40
30
20
10
0
*
Sham
VEH
Sham
GL
I/R
VEH
I/R
GL

**Figure 4.**

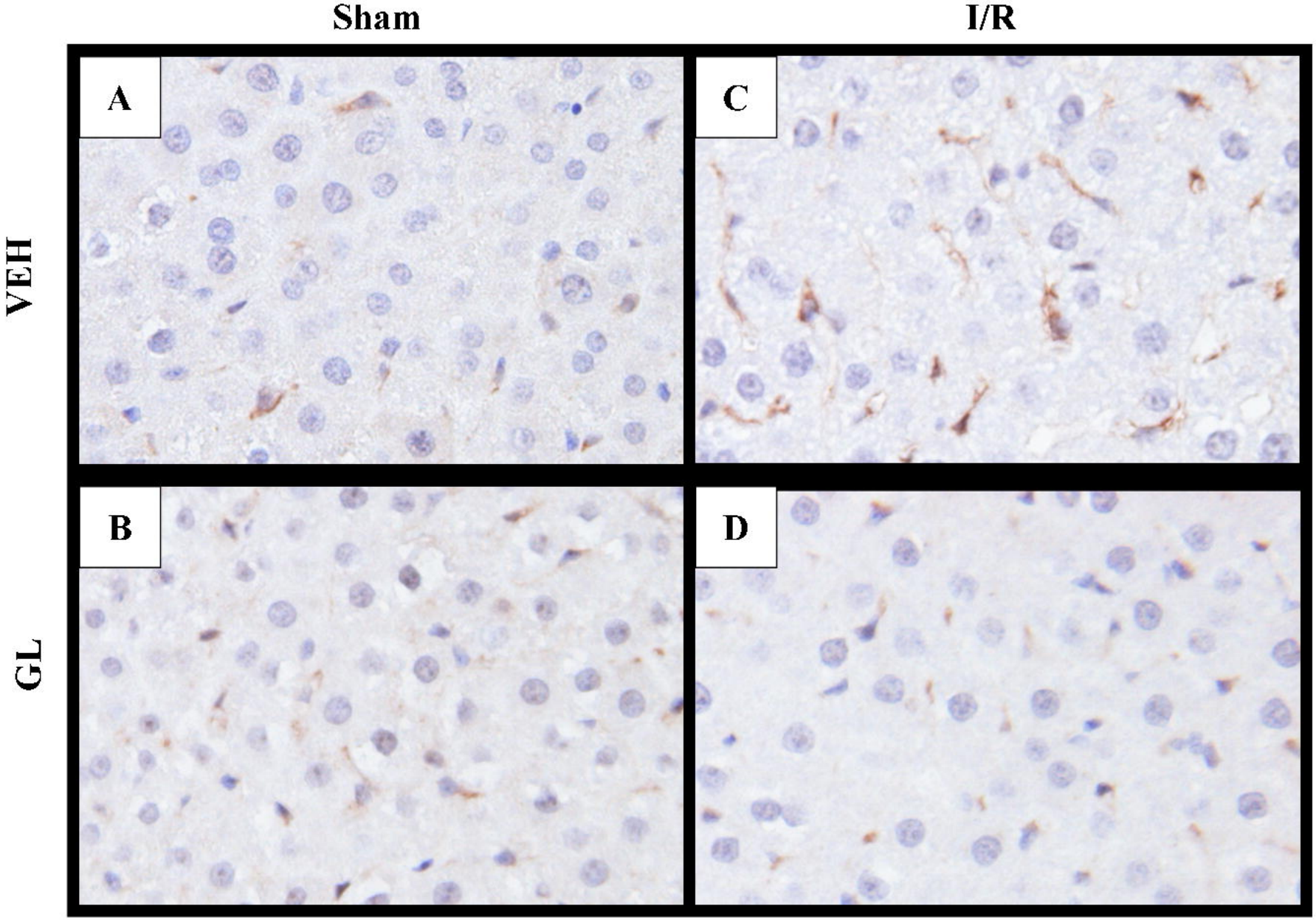


Figure 5.

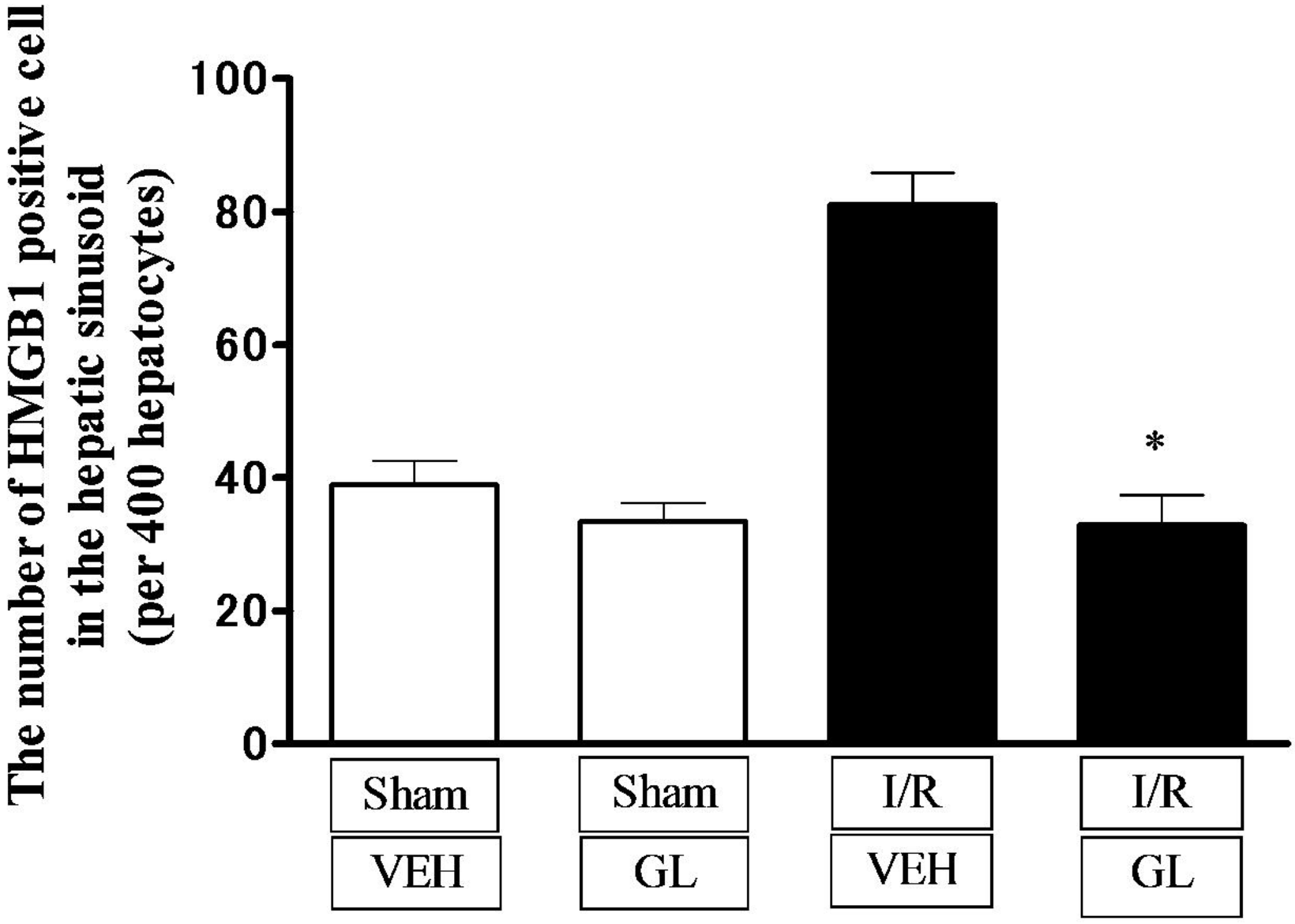
The number of HMGB1 positive cell in the hepatic sinusoid (per 400 hepatocytes)
100
80
60
40
20
0
*
Sham
VEH
Sham
GL
I/R
VEH
I/R
GL

**Figure 6.**

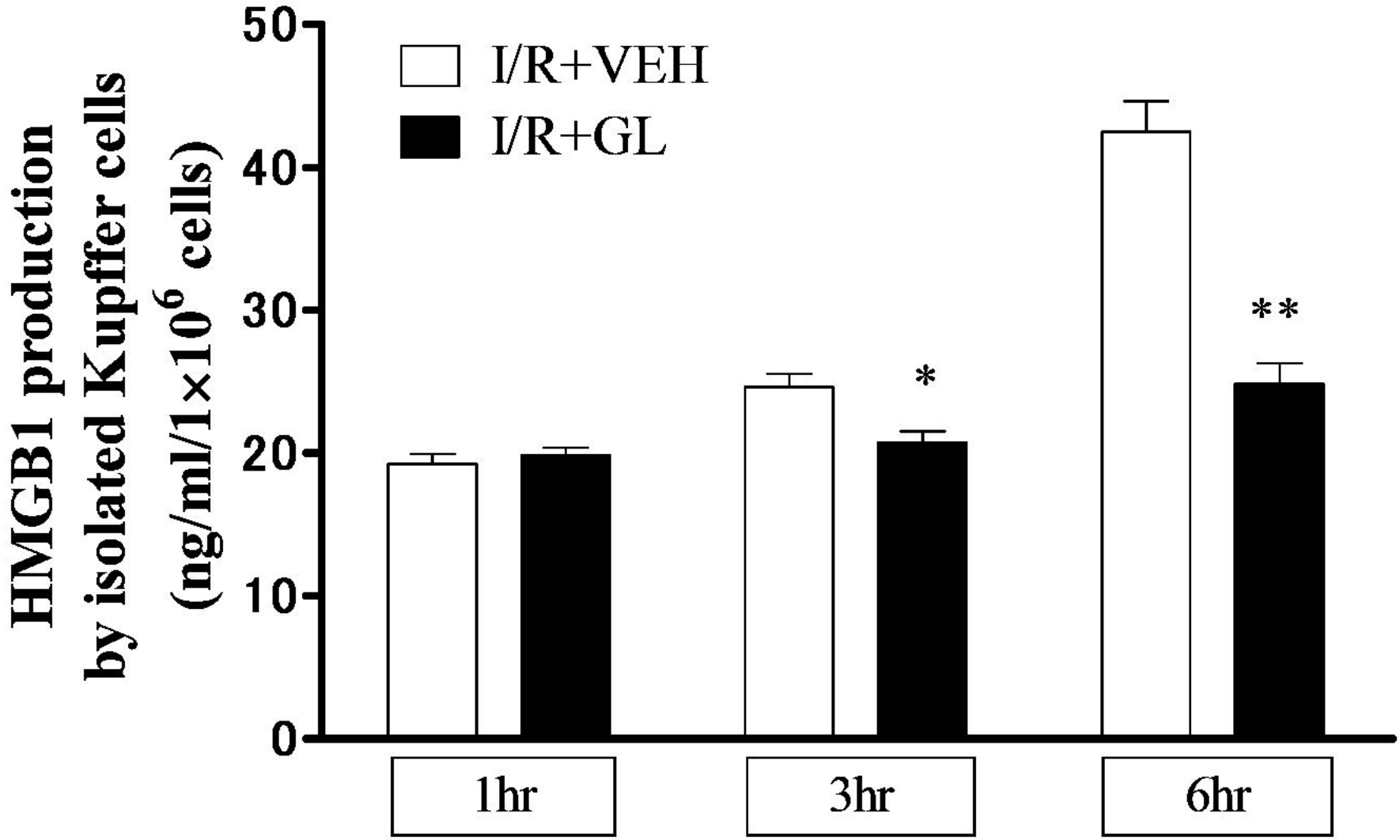
HMGB1 production
by isolated Kupffer cells
(ng/ml/1×10^6 cells)
50
40
30
20
10
0
I/R+VEH
I/R+GL
*
**
1hr
3hr
6hr

**Figure 7.**

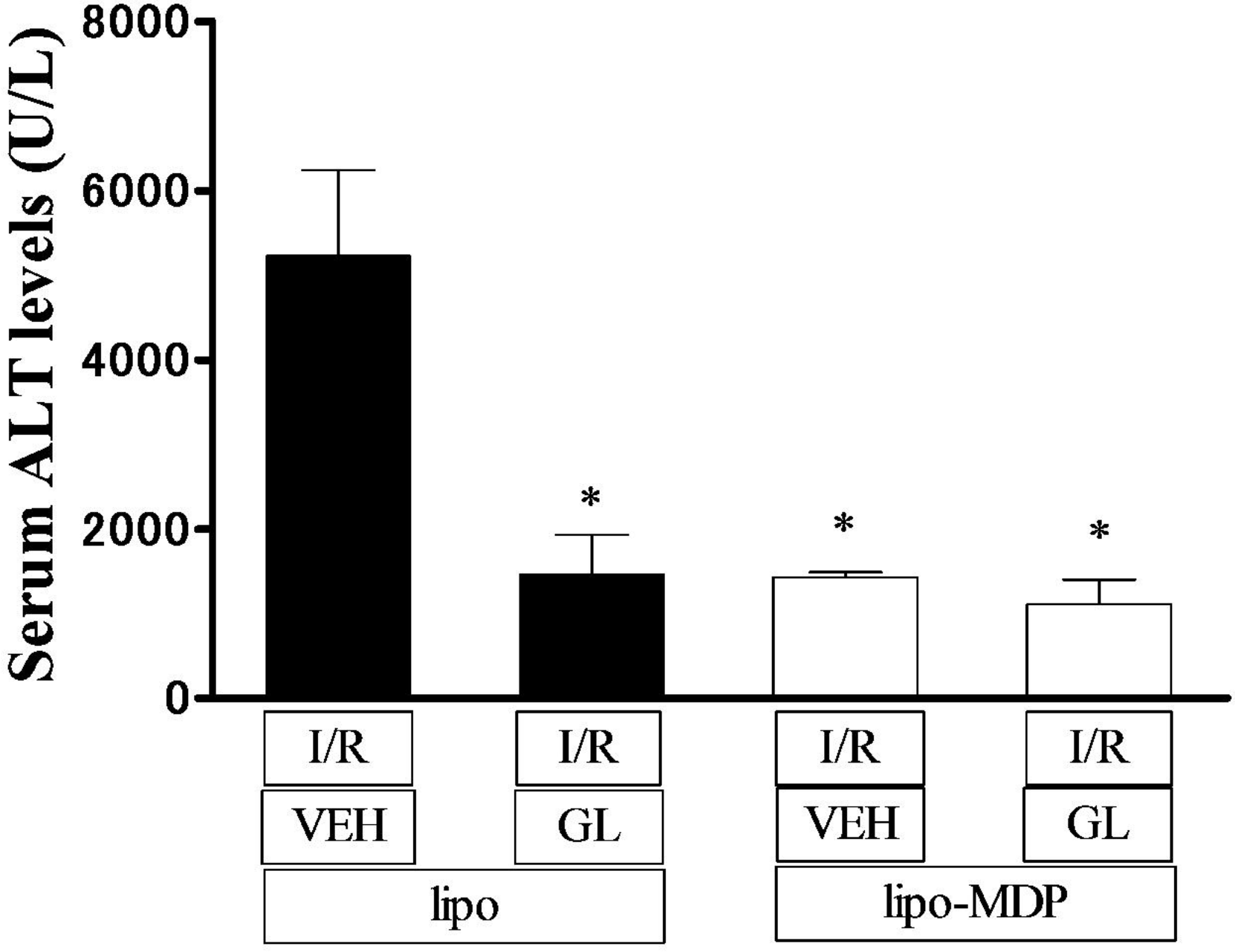
Serum ALT levels (U/L)
8000
6000
4000
2000
0
*
*
*
I/R
I/R
I/R
I/R
VEH
GL
VEH
GL
lipo
lipo-MDP

**Figure 8.**

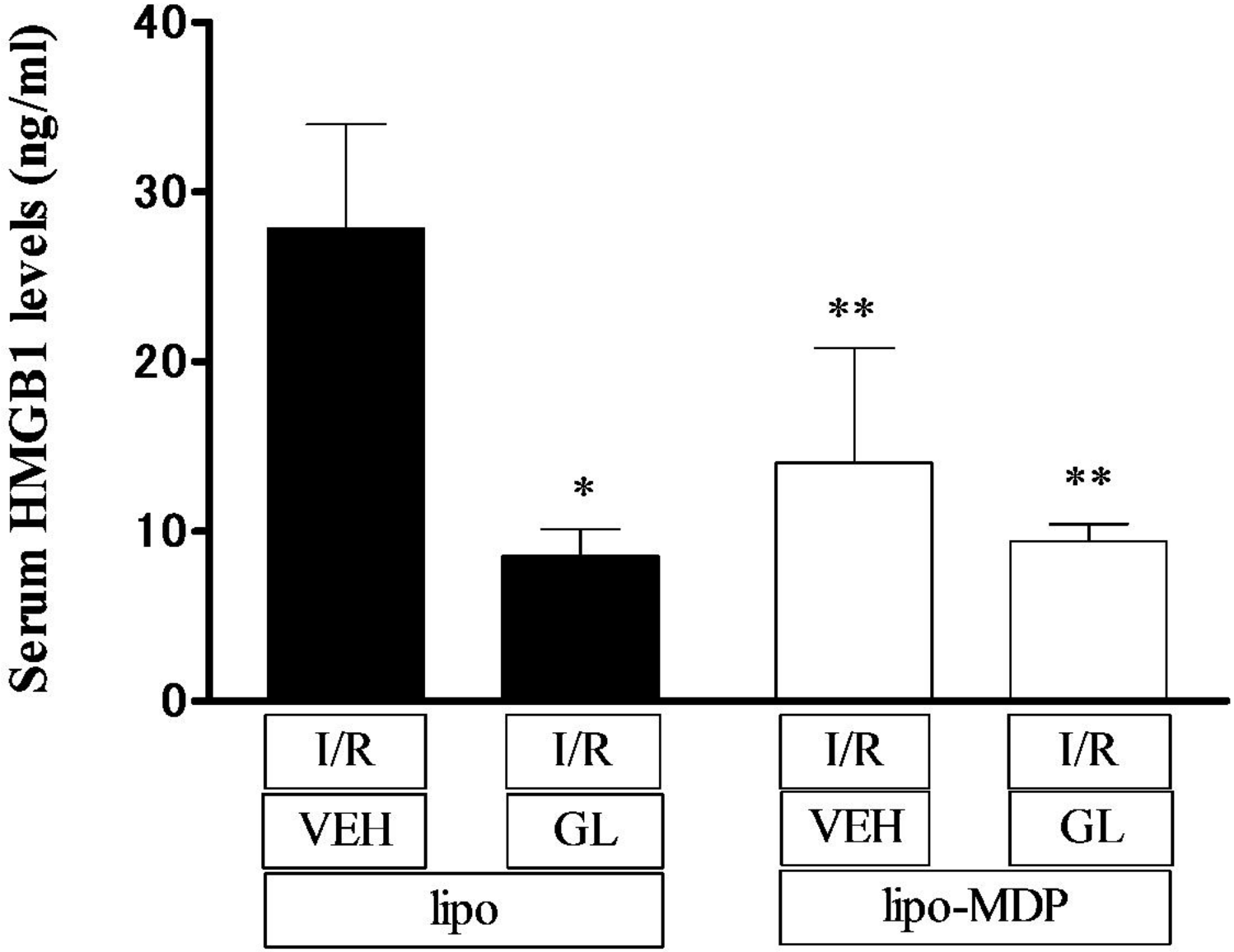
Serum HMGB1 levels (ng/ml)
40
30
20
10
0
*
**
**
I/R
I/R
I/R
I/R
VEH
GL
VEH
GL
lipo
lipo-MDP

**Figure 9.**

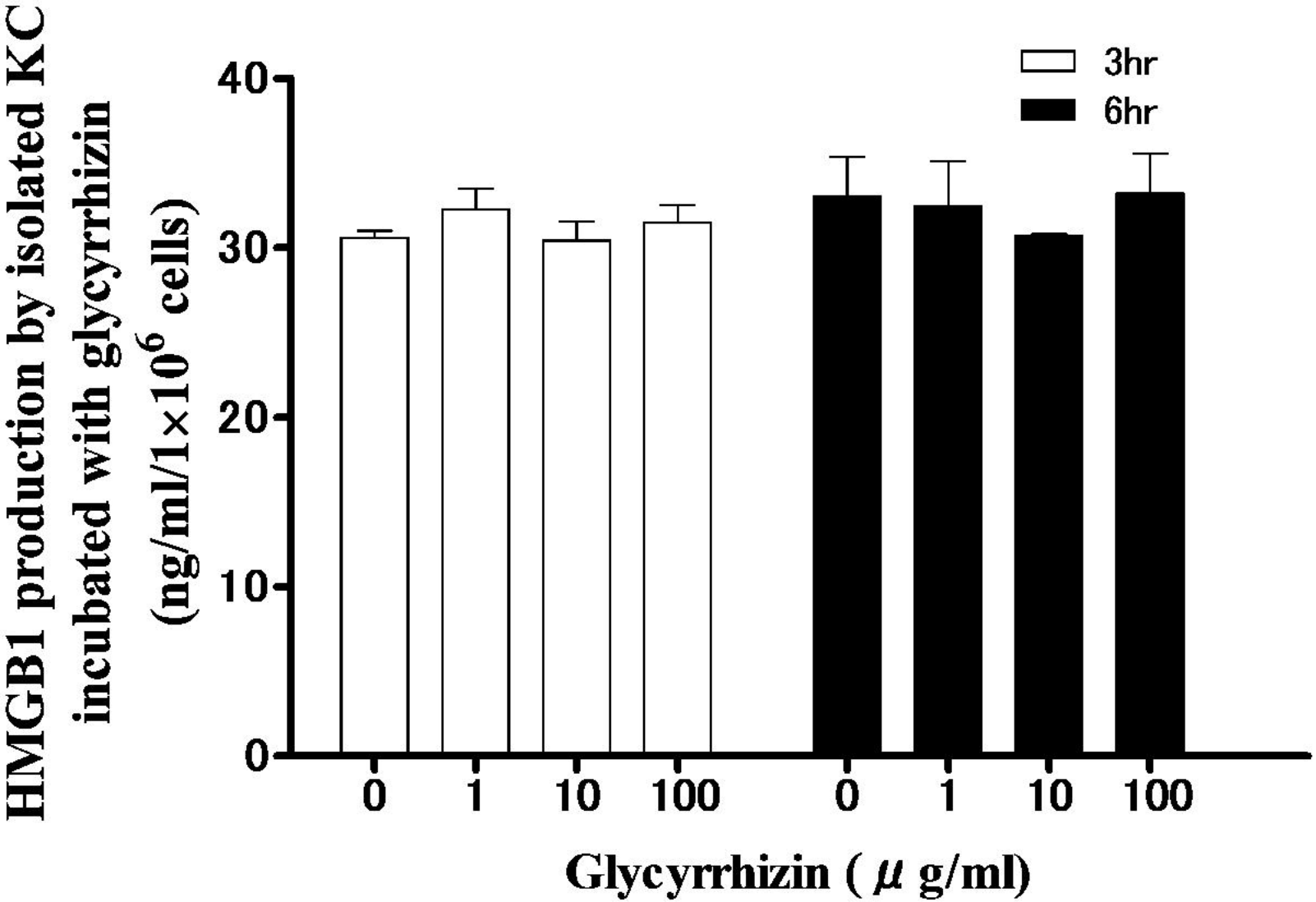
HMGB1 production by isolated KC
incubated with glycyrrhizin
(ng/ml/1×10^6 cells)
40
30
20
10
0
3hr
6hr
0
1
10
100
0
1
10
100
Glycyrrhizin ( μ g/ml)

Figure 10.