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The Protective Role of Hydrogen-Rich Saline in Experimental Liver Injury in Mice

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Abbreviations: Reactive oxygen species(ROS), hydroxyl radicals (·OH), peroxynitrite ($ONOO^-$), Hydrogen-rich saline(HS), Normal saline (NS), D-Galactosamine (GalN), Lipopolysaccharide (LPS), Carbon tetrachloride (CCl_4), Diethylnitrosamine (DEN), Acute hepatic failure (AHF), hydrogen (H_2), superoxide anion radical ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), Nitric oxide (NO·), immunohistochemical (IHC), dihydroethidine (DHE), hepatic stellate cells (HSC), regular chow (RC).

Abstract

***Background/Aims*:** Reactive oxygen species (ROS) are considered to play a prominent causative role in the development of various hepatic disorders. Antioxidants have been demonstrated effectively to protect against hepatic damage. Hydrogen (H_2), a new antioxidant, was reported to selectively reduce the strongest oxidants, such as hydroxyl radicals (·OH) and peroxynitrite ($ONOO^-$), and did not disturb metabolic oxidation-reduction reactions or disrupt ROS involved in cell signaling. In contrast to H_2 gas, hydrogen-rich saline (HS) may be more suitable for clinical application. We here aim to verify its protective effects in experimental models of liver injury.

Methods: H_2 concentration in vivo was detected by hydrogen microelectrode for the first time. Liver damage, ROS accumulation, cytokine levels and apoptotic protein expression were respectively evaluated after GalN/LPS, CCl_4 and DEN challenge. Simultaneously, CCl_4-induced hepatic cirrhosis and DEN-induced hepatocyte proliferation were measured.

Results: HS predominantly increased hydrogen concentration in liver and kidney tissues. Acute liver injury, hepatic cirrhosis and hepatocyte proliferation were reduced by quenching the detrimental ROS. Pro-apoptotic players such as JNK and caspase-3 activity were also inhibited.

Conclusions: HS could not only apparently protect against the liver injury but also inhibit the process of liver cirrhosis and hepatocyte compensatory proliferation.

Word account: 200

Keywords: hydrogen-rich saline, acute hepatic failure, hepatic cirrhosis, hepatocyte proliferation, reactive oxygen species, inflammation, apoptosis, JNK.

Introduction

Acute hepatic failure (AHF) is defined as the rapid onset of severe hepatocellular dysfunction with poor prognosis, and frequently resulted from hepatitis virus infection, the induction of drugs, toxins and hepatic ischemia-reperfusion injury. Oxidative stress has been regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis and hepatic cancer [1-3]. Moreover, it also represents an imbalance between the production of ROS and the activity of antioxidant defense systems [4]. Earlier reports have demonstrated that antioxidants were effective to protect against hepatic damage by inhibiting free radical generation or scavenging the free radicals generated by other biochemical reactions [5, 6].

Molecular hydrogen (H_2), the lightest and most abundant chemical element, has been defined as a novel anti-oxidant, which selectively quenches the detrimental ROS, such as ·OH and $ONOO^-$, while maintaining the metabolic oxidation–reduction reaction and other less potent ROS, such as superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and Nitric oxide (NO·) [7]. Hydrogen acts as a reductant for molecules that are strongly pro-oxidant [8, 9]. Unlike other most known antioxidants, which are unable to successfully target organelles, it has advantageous distribution characteristics for its capability to penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus [10]. It has been demonstrated that the inhalation of H_2 gas can

reduce brain, liver, heart ischemia-reperfusion injury and intestinal graft injury via its antioxidant effect [7, 11-13]. Moreover, inhalation of H_2 gas was more efficacious than a treatment currently approved for cerebral infarction [7]. These finding indicated that the beneficial effects of H_2 could be used in clinical practice. However, in clinical application, inhalation of H_2 gas is not convenient and is dangerous because of its flammable and explosive nature even at a concentration of 4.7% in air.

In contrast to H_2 gas, HS (H_2 saturated in saline) is easily administered and safe for clinical application. It has been reported that HS can not only prevent or reduce early pathological changes but also lead to long lasting functional improvement in neonatal hypoxia-ischemia rat model [14]. However, it remains unclear whether HS has similarly protective effects on acute hepatic injury, and prevents liver from ROS-induced cell death and inflammation. Here, we demonstrated that HS could alleviate liver injury in experimental Galn/LPS, CCl_4, or DEN-induced AHF models, and shed light on the clinical potential of HS for preventive and therapeutic anti-oxidative applications.

Materials and methods

Preparation of hydrogen-rich saline

The detailed information for the preparation of HS was described in our previous reports [15].

Animals

Male C57Bl/6 mice (20-25g) were obtained from the Model Animal Research Center of Nanjing University in Nanjing, China. They were maintained under controlled conditions (25°C, 55% humidity and 12h day/night rhythm) and fed a standard laboratory chow. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University (Shanghai, China).

Experimental model for hydrogen distribution studies in mice.

8ml/kg HS or NS was injected into mice via the peritoneal cavity. The mice were anesthetized with pentobarbital (0.7ug/g body weight, i.p.) and placed in a supine position. An incision was made on the midline of the abdomen under aseptic conditions. Heparin saline 0.5ml (50000U/L) was injected into the peritoneal cavity. Hydrogen microelectrode (dia. 50μm) was penetrated into the liver and kindey at the depth of 300μm.

Mice model of hepatic failure

GalN (Sigma, USA) was administered i.p. at 800mg/kg followed with lipopolysaccharide treatment (LPS,i.p., 20μg/kg; Sigma, USA). HS (8ml/kg) or NS of equivalent volume as control was given intraperitoneally every 1h after

the administration of GalN/ LPS. After stimulation of GalN/ LPS (800mg/kg.VS. 20μg/kg or 800mg/kg.VS. 5μg/kg body weight), survival rate of mice were measured (n=15 each group).

CCl_4 mixed with olive oil (1:19 v/v, 4ml/kg) was gavaged for acute hepatic injury and cirrhosis (3 times/week, 12 weeks) model [16, 17].

DEN (100mg/kg; Sigma, USA) was injected intraperitoneally for acute hepatic injury [18].

HS (8ml/kg) or NS of equivalent volume as control was given intraperitoneally every 3h after the administration of CCl_4 or DEN.

Histology of mice liver tissue

IHC analysis was performed with p-c-Jun antibody, F4/80 antibody, α-SMA antibody, using methods as described previously [19].

Measurement of transaminase activities

Activities of serum aminotransferases (ALT and AST) were determined by an automated procedure in Department of inspection, Eastem Hepatobiliary Surgery Hospital.

Cytokine measurement in murine serum

Levels of TNF-α and IL-6 of were measured with a commercial ELISA kit following the instructions of the manufacturer (Dakewe, Shenzhen, China) （Synergy 2 Multi-Mode Microplate Reader, BioTek, USA）.

Analysis of hepatocyte apoptosis

Apoptotic hepatocytes were detected by terminal deoxynucleotidyl

transferase dUTP nick end labeling (TUNEL) (Olympus BX51, Olympus, Japan) staining according to the manufacturer's recommendations of In Situ Cell Apoptosis Detection kit (Keygen, Nanjing, China) (Synergy 2 Multi-Mode Microplate Reader, BioTek, USA) .Caspase-3 activities were measured using fluorometric caspase activity detection kits (Keygen, Nanjing, China) (Synergy 2 Multi-Mode Microplate Reader, BioTek, USA) . The assays were performed as recommended by the manufacturer.

Measurement of ROS and GSH

Liver cryosections prepared 5h after GalN/LPS injection and 48h after CCl_4 lavage were incubated with 2mM dihydroethidine hydrochloride for 30 min at 37^{o}C. Cells staining positive for the oxidized dye were identified by fluorescence microscopy (Olympus IX70, Olympus, Japan). At the same time, liver homogenates prepared and analyzed for GSH content with a commercial ELISA kit (Jiancheng, Nanjing, China) according to the protocol provided by the manufacturer.

Analysis of liver fibrogenesis

mRNA was quantified by real-time PCR assay (7300 Real-Time PCR System, Applied Biosystems, USA) using double-stranded DNA-binding dye SYBR green-I(Trkara, Dalian, China), as described previously [20]. The expression of all the target genes was normalized to 18S. The liver sections were stained with picro-sirius red for collagen distribution [21] The content of hepatic hydroxyproline was determined by using the hydroxyproline kit

following the protocol provided by the manufacturer (Genmed, Shanghai, China).

Western Blot analysis

The anti-JNK, pJNK, PARP, α-SMA and GAPDH, monoclonal antibodies were purchased from Neomarker, Santacruz, Kangcheng, Sigma and Cell signaling, respectively. Protein concentration was determined by BCA method. Western blotting was performed as previously described [20].

Detection of hepatocytes proliferation

Hepatocyte proliferation was measured by Edu incorporation 72h after DEN challenge. The assays were performed as recommended by the manufacturer of Edu detection kits (Ribobio, Guangzhou, China) (Olympus IX70, Olympus, Japan).

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Differences between experimental and control groups were assessed by the analysis of variance (ANOVA) when applicable or nonparametric test using SPSS 16.0 (SPSS, Inc.). Recipient survival was plotted using the Kaplan-Meier method, and the differences between groups were analyzed using the log-rank test. A P-value of less than 0.05 was considered statistically significant.

Results

Intraperitoneal injection of HS predominantly increased H_2 concentration in liver and kidney tissues.

The H_2 levels in liver and kidney tissues were measured by H_2 microelectrode (Denmark–Unisense), a linear correlation was found between the current value of H_2 microelectrode and the hydrogen concentration (H_2 concentration:0-40μM, R^2=0.9977, fig 1A). As shown in fig 1B and 1C, concentrations of molecular H_2 peaked approximately 5min following HS injection in liver and kidney, and returned to normal levels 40min later. These results suggested that HS is an ideal tool for molecular H_2 induction, and intraperitoneal administration of HS could efficiently target hydrogen to liver and kidney. In addition, we respectively measured the pH value of HS and NS, which had no significant difference between the two solutions (NS, 7.35±0.02 vs. HS, 7.32±0.03).

GalN/LPS-induced liver injury was reduced by HS treatment

The effect of HS was tested in a widely accepted model of fulminant hepatitis i.e. in the model of GalN/LPS-induced liver injury. HS (8ml/kg) or NS of equivalent volume as control were injected every hour after GalN/LPS challenge. Liver injury was strongly reduced as determined by measurement of serum transaminase activities 5h after GalN/LPS administration (fig 2A). Histological examination of liver tissue by H&E staining revealed a prominent

preservation in liver structure of HS-treated animals (fig 2B). To characterize the inflammatory infiltration, sections of liver were subjected to immunohistochemical (IHC) staining to identify the presence and distribution of macrophages. As shown in fig 2C, GalN/LPS treatment resulted in the accumulation of macrophages in close vicinity to injured hepatocytes. However, the infiltration of macrophages was blunted in AHF mice followed with HS administration. In accordance with histological and biochemical findings, cytokine expression of injury markers was also blunted in HS-treated mice. As shown in fig 2D, GalN/LPS-induced increment of pro-inflammation cytokines TNF-α as well as IL-6 in serum were remarkably prevented by treatment with HS. Furthermore, the mortality in HS treated group with GalN/LPS-induced fulminant hepatic failure was decreased to 46.7% (73% in NS group) at 10h after GalN/LPS treatment (fig 2E, left panel). Similarly, HS also reduced the mortality of high dose GalN/LPS-treated mice (fig 2E, right panel).

These data demonstrated a notable improvement of GalN/LPS-induced acute hepatic failure followed with HS administration, as compared with that of control mice.

HS attenuated ROS-induced pro-apoptotic signaling and the activation of JNK in GalN/LPS- challenged mice

As GalN/LPS-induced liver injury is characterized by apoptosis of hepatocytes, the expression and activity of pro-apoptotic molecules were

examined 5h after GalN/LPS treatment to verify whether HS exerted its protective activity by preventing cell death. As shown in fig 3A and 3B, the administration of GalN/LPS resulted in a dramatic activation of caspase-3 and cleavage of PARP. However, these effects of caspase-3 activity and PARP cleavage in hepatocytes were markedly decreased in the presence of HS. Similar results were also observed in liver tissue samples by applying TUNEL-based IHC assay (fig 3C).

In the GalN/LPS model, TNF-α-induced ROS generation is the major mediator leading to apoptotic liver injury [22]. To verify whether the protective function of HS resulted from the reduction of ROS accumulation, we assessed the levels of hepatocyte superoxides by staining freshly frozen liver sections with dihydroethidine (DHE), whose oxidation gives rise to the fluorescent derivative ethidine [23] and detecting GSH levels of fresh liver tissue. As expected, the administration of HS remarkably decreased the amount of DHE-positive hepatocytes and increased the levels of GSH (fig 3D and 3E). Similarly, serum ALT level (fig 3F) was also reduced in GalN/LPS-sensitized mice fed with the antioxidant BHA-supplemented diet. Consistent with this notion, ROS-enhanced JNK activation which contributed to liver failure was prevented by HS administration (fig 3G) or BHA diet (data not shown). These data indicated that HS might exert its anti-apoptotic activity by preventing the effects of oxidative stress and JNK signaling.

HS reduced acute liver injury in the CCl_4 model of hepatitis

To examine whether HS also controlled ROS accumulation and in turn attenuated liver injury, we injected HS via the peritoneal into CCl_4-treated mice. CCl_4 challenge increased the serum levels of ALT and AST by approximately 24 and 6-fold, which were markedly lowered after administration of HS (fig 4A and 4B). Moreover, measurement of serum TNF-α and IL-6 also indicated the protective effects of HS on the release of injury-mediated cytokines (fig 4C and 4D). In addition, IHC and apoptosis analysis revealed the decrease of the amount of macrophages infiltration and TUNEL-positive hepatocytes in HS-treated group (fig 4E and 4F). Furthermore, we detected the reduced accumulation of superoxides by using the ROS indicator DHE, and the increase of GSH content in livers of CCl_4-treated mice followed with HS administration (fig 4G and 4H), Taken together, these data indicated that HS could abrogate CCl_4-sensitized acute liver injury as well.

Chronic CCl_4 treatment-induced hepatic cirrhosis was ameliorated in the presence of HS

To investigate whether HS has the same protective effects as in CCl_4-induced acute injury on CCl_4-induced chronic liver injury and cirrhosis, collagen deposition and hepatic stellate cells (HSC) activation were examined between groups treated with CCl_4 plus NS or CCl_4 plus HS injection. After CCl_4 administration, mice were injected intraperitoneally once every day with a single dose of HS. As shown in fig 5A and 5B, sirius red staining and

hydroxyproline content showed gradually increase in a time-dependent manner after chronic CCl_4 treatment, which were significantly attenuated in HS injection group. In addition, western blot and IHC analyses revealed similar reduction of α-SMA expression in liver sections (fig 5C and 5D). Furthermore, we also examined the mRNA expression of early markers of fibrogenesis including collagen-α1 (encoded by *Col1a1*) (fig 5E) and α-SMA (encoded by *Acta2*) (fig 5F) [24], and observed approximately 50% reduction upon HS injection in CCl_4 treatment model. These results suggested that HS has a protective capability on CCl_4-induced chronic liver injury and cirrhosis.

HS reduced liver injury and hepatocyte proliferation in DEN-challenged mice model.

DEN is the chemical procarcinogen, which is widely used to induce hepatocarcinogenesis in mouse and rat models. ROS accumulation has been suggested to be a major contributor to DEN-induced HCC by promoting inflammation and stimulating compensatory proliferation [18, 25]. As shown in fig 6A, the serum ALT and AST levels were increased upon DEN administration, whereas reduced after HS injection (HS vs. NS, ALT: 303.40/529.24 IU/L, AST: 237.17/371.64 IU/L). Moreover, the concentration of the tumor-promoting cytokine IL-6 was lower in HS group than in NS group (fig 6B). In addition, IHC analyses revealed that JNK activation was reduced in DEN plus HS model (fig 6C), which was detected by phosphorylation of c-Jun,

a specific JNK substrate, mostly occurred in hepatocytes that were involved in DEN metabolism and ROS production [3]. Interestingly, HS not only reduced acute liver injury, but also inhibited hepatocytes compensatory proliferation. As shown in fig 6D, the rate of Edu-positive hepatocytes was reduced in HS-treated mice 72h after DEN administration. Thus, HS showed the protective capability on DEN-induced acute liver injury and compensatory proliferation.

Discussion

ROS are important cytotoxic and signalling mediators in the pathophysiology of inflammatory liver diseases [26, 27], which include ·OH, $ONOO^-$, $O2^-$·, H_2O_2 and NO·. Among them, ·OH and $ONOO^-$ are much more reactive than others and have been regarded as major cytotoxic mediators of cellular oxidative damage [28-30]. Previous studies have reported that H_2 reacts only with the strongest oxidants (·OH and $ONOO^-$), which is advantageous for medical procedures, as it means that H_2 is mild enough not to disturb metabolic oxidation reduction reactions or to disrupt ROS involved in cell signaling—unlike some antioxidant supplements with strong reductive reactivity [7]. We now demonstrated that hydrogen-saturated saline also prevents ROS accumulation, cytokine production and cell death in various types of liver injury.

Gas chromatography-based technology has been applied to examine the concentration of H_2 in blood successfully, and it has been reported that H_2 dissolved in arterial and venous blood was increased by the inhalation of H_2 or the administration of H_2-water [31]. It was supposed that the elevated H_2 level in serum might lead to the incorporation of H_2 into organs, which plays the pivotal protective role in oxidative stress-induced tissues damage. The facts that H_2 protected mitochondria and nuclear DNA and the amount of H_2 dissolved in venous blood was less than that in artery blood provided indirect evidences that H_2 could penetrate most membranes and diffuse into

organelles. However, there is a lack of direct evidence in vivo that the concentration of H_2 was enhanced after H_2 inhalation or HS administration.

To verify whether the injection of HS could increase the organ levels of H_2, a real time dynamic method with glass-based H_2 microelectrode was developed to accurately and continuously monitor the concentration of H_2 in abdominal organs directly for the first time. After HS injection, the liver and kidney H_2 concentration reached a peak 5min later and gradually decreased to normal levels after 40min. In addition, we measured the arterial/venous blood pH after HS administration, and no significant difference was observed between HS and NS group (Supplementary fig 1), which suggested that the treatment of HS has no effect on the blood PH. Similarly, there was no difference between the pH of HS and NS solution. To our knowledge, it is the direct evidence of the diffusion of H_2 in organs for the first time. And these data also indicated that it is realizable to prevent the ROS accumulation by intraperitoneal administration of HS in organs, such as liver and kidney.

Oxidative stress activates various kinds of apoptotic signaling pathways, among which we focused particularly on JNK, as a number of recent reports have shown that JNK activation, following oxidative stress, induces apoptosis via activation of c-Jun, and through the caspase-dependent mitochondria pathway in liver. In a model of fulminant liver failure (GalN/LPS), a disease that is associated with many complications and high mortality in the clinic, administration of HS resulted in a marked reduction of liver injury. ROS down

regulation by HS or antioxidant BHA as shown here led to reduced apoptotic activity (PARP cleavage and caspase-3 activation) as well as decrease inflammatory cytokines release and tissue damage after GalN/LPS challenge. Importantly, phosphorylation and consecutively activation of the pro-apoptotic kinase JNK was blocked after HS administration (fig 3G) or BHA induction (data not shown), which indicated that HS might exert its protective role through preventing the activation of ROS-JNK-caspase-3 pathway. Moreover, the accumulation of kuffer cells in liver (fig 2C) was also attenuated after HS injection, which might lead to further decrease of inflammatory cytokines (such as TNF-α, IL-6) production and release.

CCl_4 and DEN-sensitized AHF are other two settings where ROS accumulation was thought to be responsible for liver damage [18, 25, 32, 33]. As shown in fig 4 and 6, HS resulted in a similar beneficial outcome as seen in the model of GalN/LPS-induced liver damage through scavenging ROS and inhibiting the activation of its downstream JNK pathway (some data no shown). The level of serum transaminases and the concentration of inflammatory cytokines in serum were lower in HS group than its counterpart group. Histopathological findings also demonstrated the HS protective effects to AHF.

Liver cirrhosis is a common scarring response to all forms of chronic liver injury and is always associated with inflammation that contributes to fibrogenesis. The usage of antioxidants, such as SAMe, vitamin E, has been reported successfully reducing the liver damage and delaying the progress of

hepatic cirrhosis [34]. In line with this notion, the effect of HS in the model of CCl_4-induced chronic liver damage was observed. Both the collagen deposition and nodule number were inhibited in the presence of HS. It is the first report that HS can play a protective effect to chronic liver injury, and suggested that HS could be used to prevent and retard fibrogenesis in medical application. Further studies with other models of cirrhosis should be warranted.

A causal link between ROS accumulation and cancer has been proposed. Previous results obtained in a mouse model in which HCC was induced by the chemical procarcinogen DEN suggest that DEN-induced oxidative stress leads to hepatocytes death, cytokine release, compensatory proliferation, and HCC development eventually [35]. We now showed that HS could reduce transaminase activities and inflammatory cytokines (IL-6) production upon DEN-induced liver injury. IL-6 is a multifunctional cytokine, which is largely responsible for compensatory hepatocyte proliferation that has a critical role in DEN-induced hepatocarcinogenesis [36]. Indeed, we also found the remarkable reduction in DEN-induced hepatocyte proliferation in HS group (fig 6B). Further investigation on the contribution of HS to the development of HCC should be performed.

In conclusion, we here presented a novel antioxidant-HS, which is easier and safer to apply than H2 gas, and could selectively remove ROS. We examined the impact of HS in the inflammatory models of GalN/LPS, CCl_4, DEN challenge, respectively. Not only apparent protection of the liver injury

was observed, but also the process of liver cirrhosis and hepatocytes compensatory proliferation was inhibited, which shed new light on the potential application of HS to target oxidative stress and alleviate liver injury clinically.

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Figure legends

Figure 1: Concentration of hydrogen in mice abdominal organs and blood samples.

(A) The standard curve represents the linear correlation of hydrogen concentration (μM) in saline and the current value (pA). (B) and (C) H_2 molecules concentration was changed after injected hydrogen-rich saline in mice abdominal organs (liver or kidney, n=8, *:p<0.05 vs. Time 0min).

Figure 2: HS leads to a significant decrease in acute liver injury 5h after GalN/LPS challenge.

(A) Transaminase levels (AST and ALT) of mice (n=8, mean±SD, **:p<0.01, *:p<0.05 vs. NS). (B) Hematoxylin-Eosin staining of mice liver sections. (C) IHC staining with F4/80 antibody of mice liver sections. (D) TNF-α and IL-6 serum levels of mice were determined by ELISA (n=8, mean±SD, **:p<0.01, *:p<0.05 vs. NS). (E) HS reduced the mortalities of low (left graph) and high dose (right graph) GalN/LPS-treated mice (CON group n=5, HS or NS groups n=15, Kaplan-Meyer, log-rank test, p<0.05 HS vs. NS).

Figure 3: HS attenuated ROS-induced pro-apoptotic signaling in GalN/LPS challenged mice.

(A) Caspase-3 like activity assay, controls were defined as 1.0 (n=8, mean±SD, **:p<0.01 vs. NS). (B) Hepatic expression of PARP, cleaved PARP, GAPDH. (C) Liver histology stained with TUNEL. (D) and (E) Accumulation of hepatocyte superoxides assessed by staining freshly frozen liver sections with

dihydroethidine (DHE) and measuring hepatic GSH levels(n=8, mean±SD, *:p<0.05 vs. NS). (F) Effects of BHA on serum transaminase activities in GalN/LPS-treated mice (n=8, mean±SD, *:p<0.05 vs. RC (regular chow)). (G) Hepatic expression of t-JNK, p-JNK and GAPDH.

Figure 4: Role of HS in acute liver injury 12h after CCl4 challenge.

(A) and (B) ALT and AST serum levels of mice (n=8, mean±SD, **:p<0.01, *:p<0.05, vs. NS). (C) and (D) TNF-α and IL-6 serum levels of mice determined by ELISA (n=8, mean±SD, **:p<0.01, *:p<0.05 vs. NS). (E) IHC staining with F4/80 antibody of mice liver sections. (F) Liver histology stained with TUNEL. (G) and (H) Accumulation hepatocyte superoxides assessed by staining freshly frozen liver sections with dihydroethidine (DHE) and measuring hepatic GSH levels (n=8, mean±SD, *:p<0.05 vs. NS).

Figure 5: HS reduced hepatic cirrhosis 12w after CCl4 treatment.

(A) and (B) Collagen deposition was evaluated by sirius red staining and hydroxyproline measurement 2W, 4W, 8W and 12W after CCl_4 challenge. (C) and (D) Expression of a-SMA was determined by western blot analysis and IHC. (E) and (F) Hepatic levels of Col1a1 (E), Acta2 (F) mRNA were measured by qPCR in HS group(n=8) and NS group (n=8) 72h after CCl_4 challenge and gene expression in control group was arbitrarily assigned the value of 1. (*:P<0.05 vs. NS)

Figure 6: Effects of HS treatment in acute liver injury after DEN challenge.

(A) ALT and AST levels in serum were determined 48h after DEN injection. (n=8, mean±SD, **:p<0.01 vs. NS). (B) Role of HS on serum IL-6 4h after DEN challenge. (n=8, mean±SD, **:p<0.01 vs. NS). (C) Expression of phospho-c-Jun in DEN-treated livers. (D) Hepatocyte proliferation (upper panel) was measured by Edu incorporation at 72 h and quantified by counting five randomly chosen high-power fields (*:P<0.05).

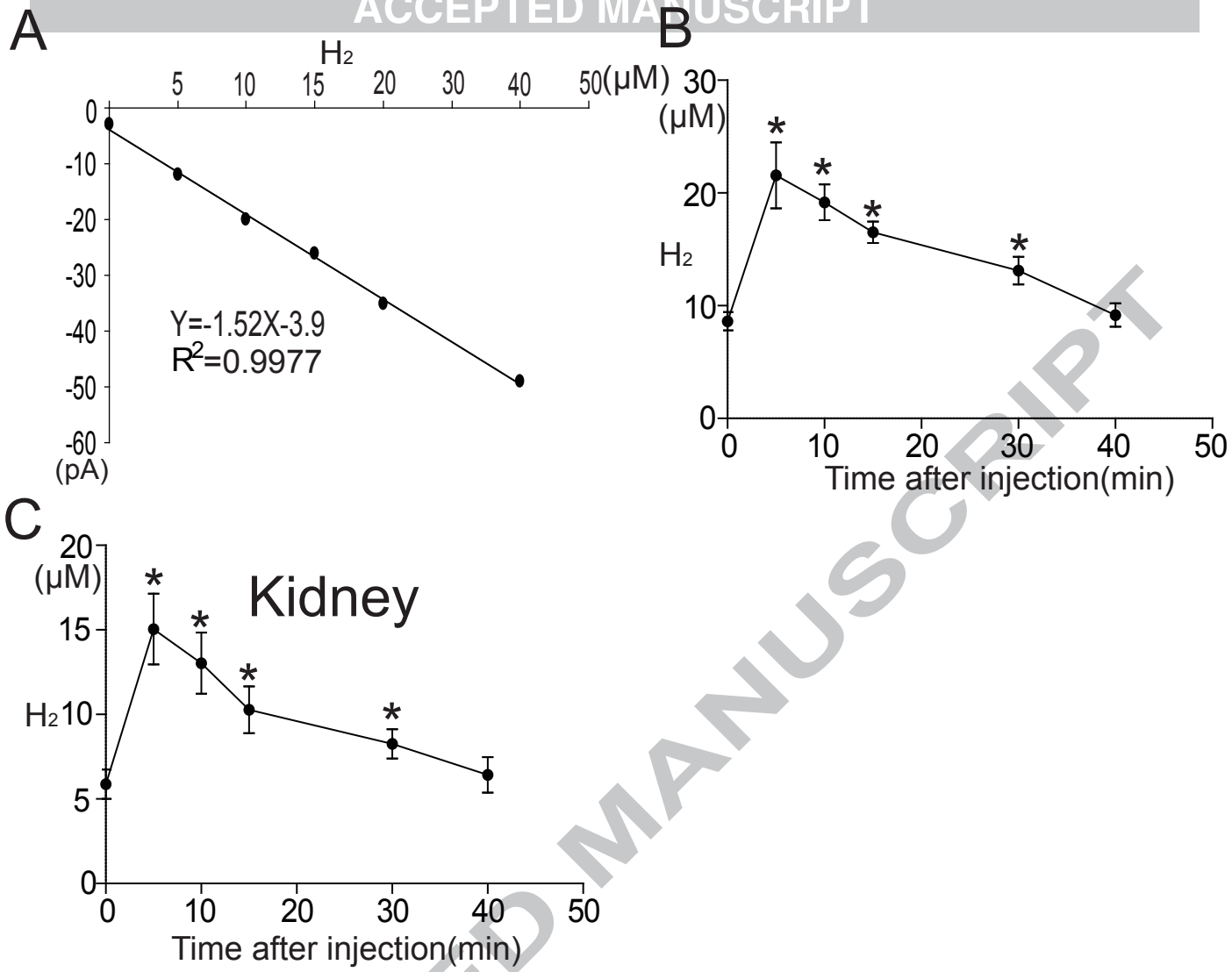

Figure 1

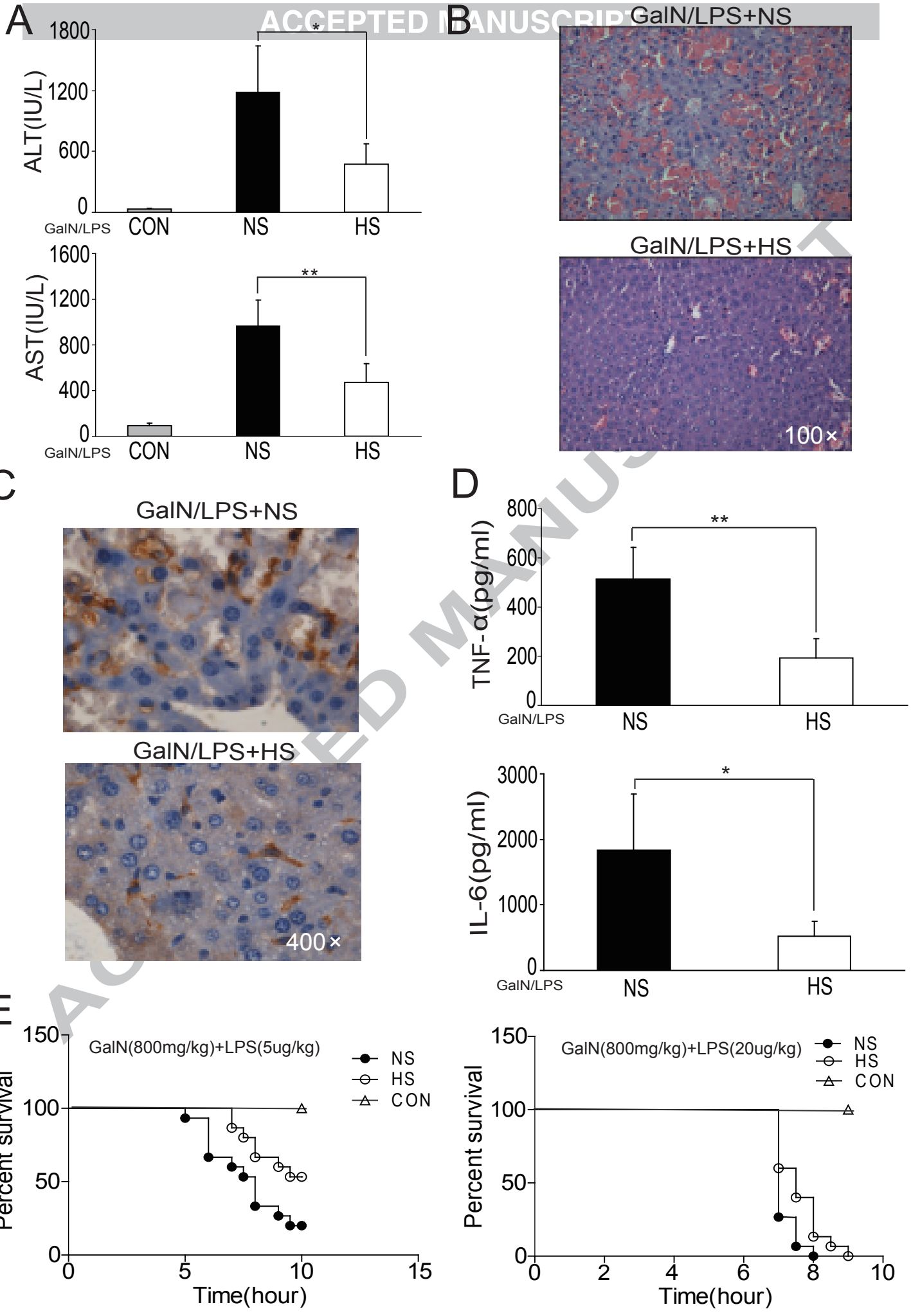

Figure 2

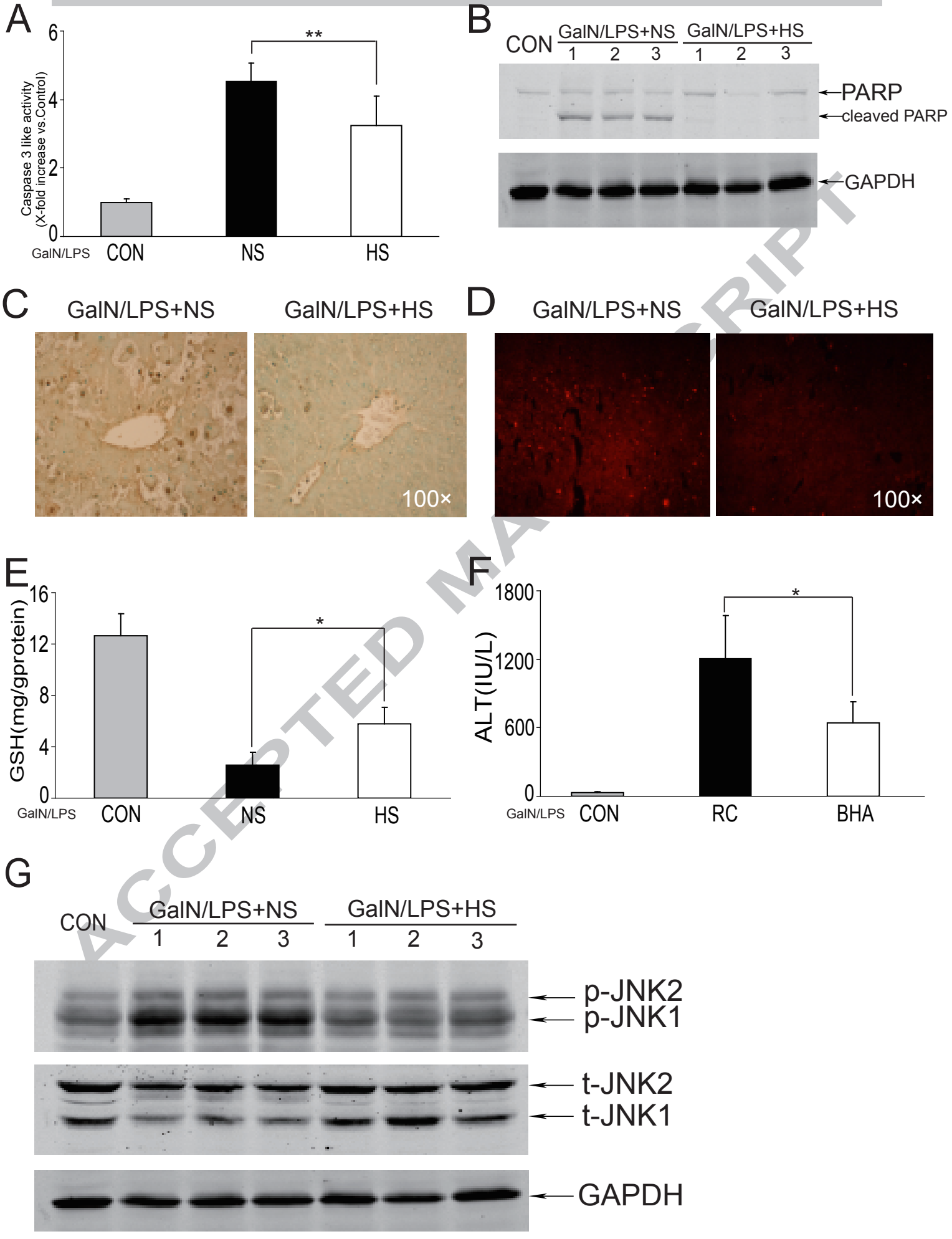

Figure 3

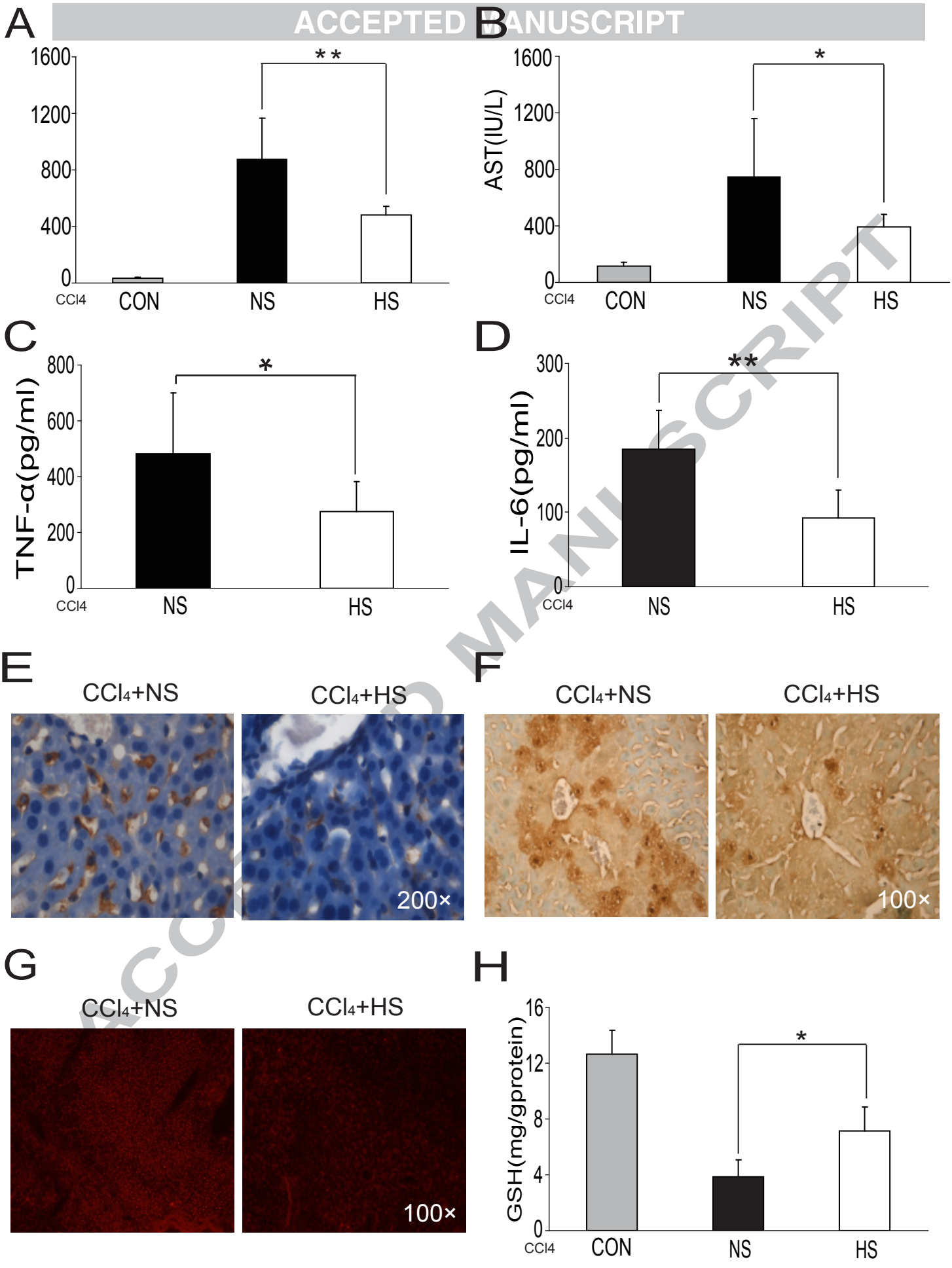

Figure 4

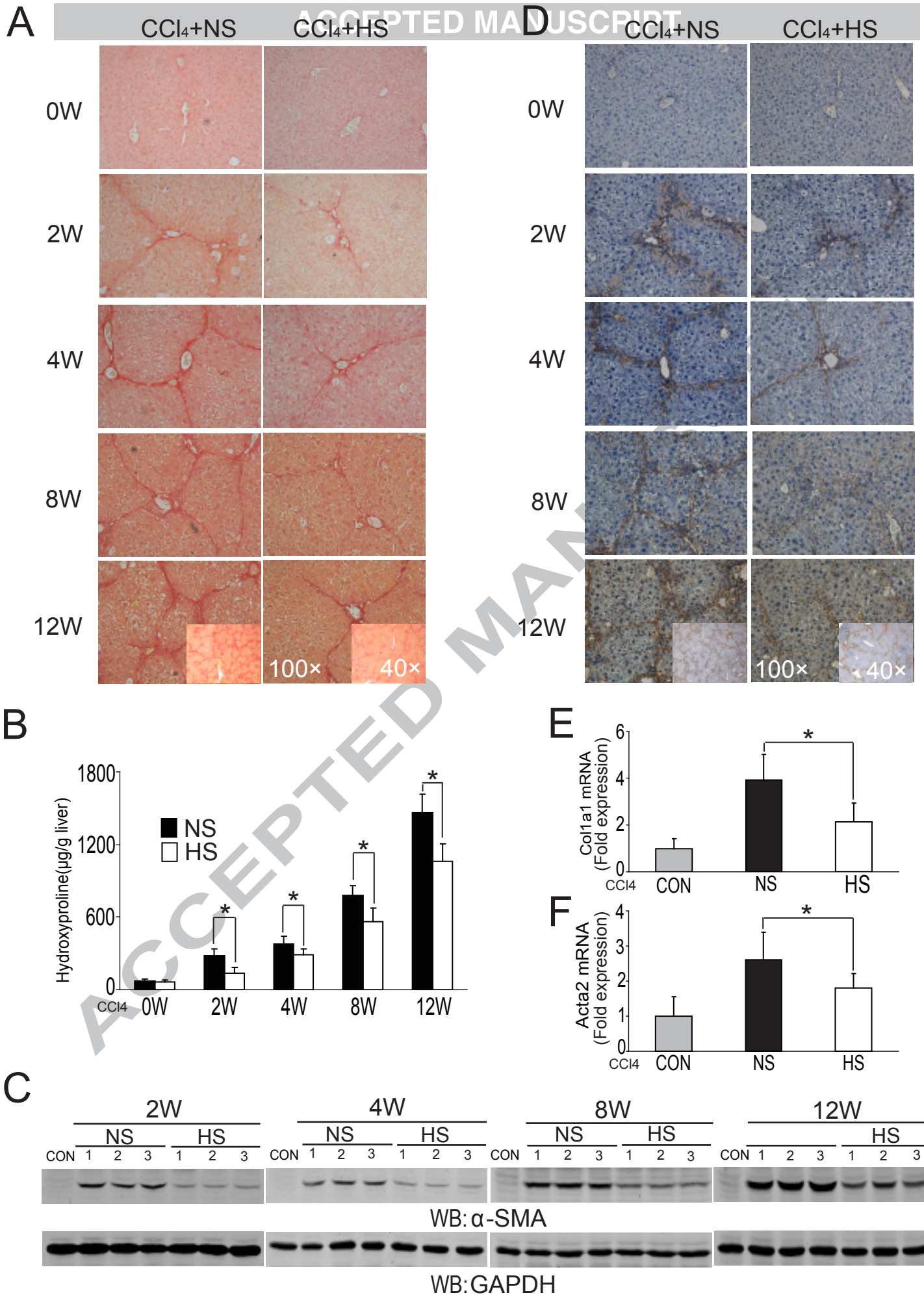

Figure 5

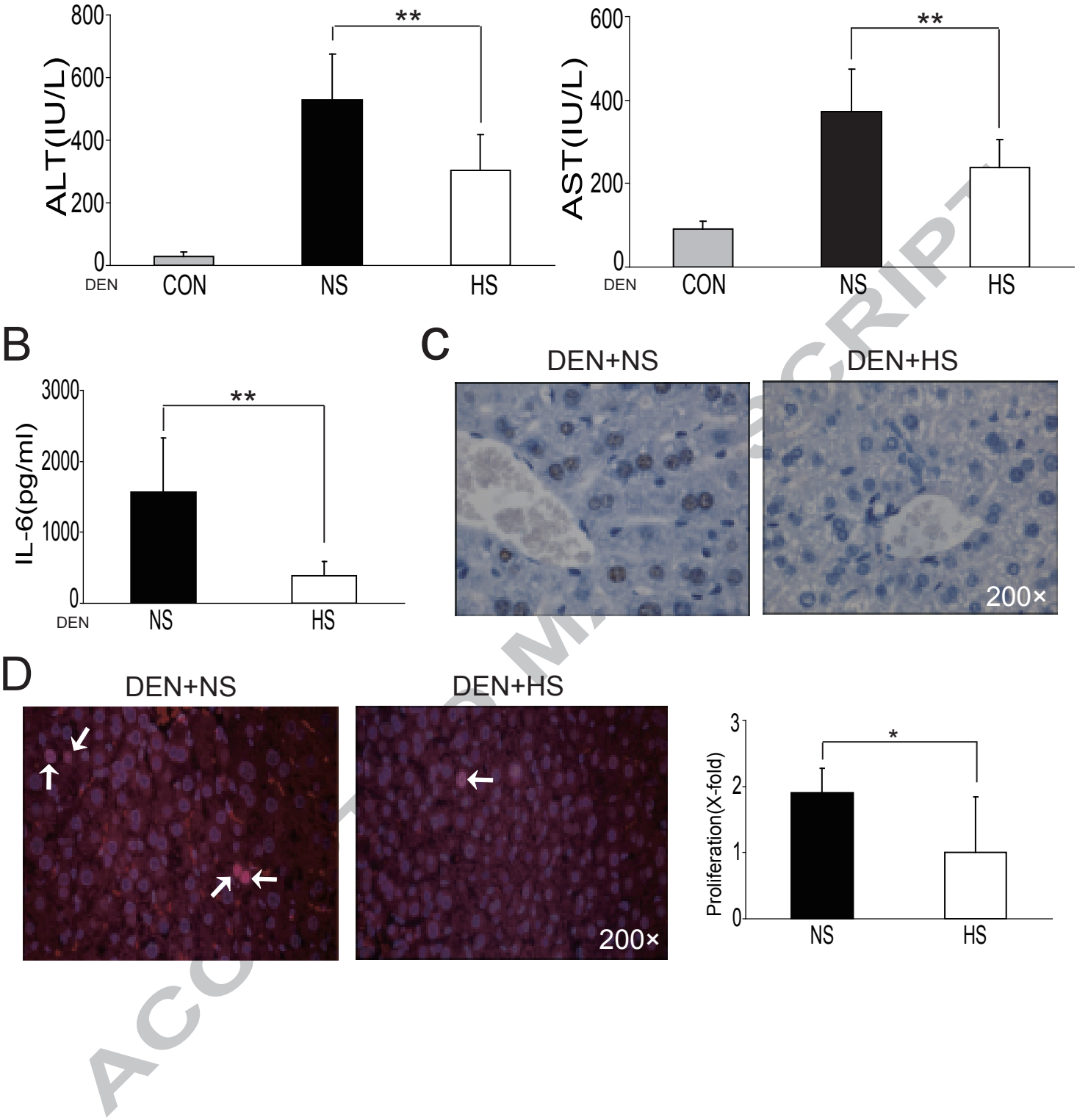

Figure 6