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Comparison of the Effects of Intraperitoneal Injection with Carbon Tetrachloride on Acute Liver Toxicity in Male and Female Kunming Mice

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Statistical Analysis C
Data Interpretation D
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Background: Acute chemical liver injury needs to be further explored. The present study aimed to compare the effects of intraperitoneal injection with carbon tetrachloride on acute liver toxicity after 24 h in male and female Kunming mice.

Material/Methods: In this study, female and male mice were simultaneously divided into 3 different groups. Each group was treated differently, and after 24 h, blood samples were collected to check for changes in the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were used to assess liver toxicity. Liver samples were used for hematoxylin-eosin staining, and periodic acid Schiff reagent staining was performed to detect the pathological changes of each group. The expression level of biomarker molecules in liver cells was also systematically analyzed.

Results: Our results showed that, compared with male mice, female mice showed more serious damage: reduced glycogen and higher degree of necrosis, and the levels of heatshock protein 27 (HSP27), heat-shock protein 70 (HSP70), proliferating cell nuclear antigen (PCNA) and B cell lymphoma/lewkmia-2 (Bcl-2) were significantly lower than in the male group ($P<0.05$ or $P<0.01$), while the results of Bcl-2-associated X protein (Bax), cysteinyl aspartate specific proteinase 3 (Caspase3), and cytochrome P450 2E1 (CYP2E1) were the opposite ($P<0.05$ or $P<0.01$).

Conclusions: The findings from this study showed that, compared with male mice, at 24 h after CCl_4 toxicity, female mice showed more severe changes of hepatocyte necrosis and PAS-positivity, with significantly reduced expression of HSP27, HSP70, PCNA, and Bcl-2, and significantly increased expression of Bax, caspase-3, and CYP2E1.

Keywords: Carbon Tetrabromide • Chemical and Drug Induced Liver Injury • Interpersonal Relations • MICE Regimen

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Background

Acute chemical liver injury is caused by a range of drugs, alcohol, poisons, and other chemicals or their metabolites, resulting in direct or indirect liver injury [1]. In the present study, carbon tetrachloride (CCl_4) was used as the agent to induce acute chemical liver injury. CCl_4 is a common hepatotoxic substance that is widely used to induce toxic hepatic injuries [2]. After entering the body through the respiratory and digestive tracts, it causes liver cell injury and liver inflammation [3]. Liver inflammation is not a simple process [4], and regulating the processes of acute liver injury may exert a protective or harmful role in the liver. Mild inflammatory response assists in tissue repair, helping to restore the balance in vivo, whereas excessive and permanent inflammation leads to a massive loss of hepatocytes, heightening the severity of liver parenchyma injury [5] and causing an irreversible decrease in liver function [6].

It is often desirable to evaluate sex differences (ie, male vs female) in CCl_4-induced liver injury, although any potential differences in the molecular mechanism of CCl_4-induced liver injury by comparing men vs women have rarely been studied. This study aimed to compensate for this deficiency. In the present study, acute chemical liver injury was induced through intraperitoneal injection of CCl_4 into mice, aiming to simulate the situation of acute chemical liver injury in humans. Differences in oxidative stress, proliferation, apoptosis, and metabolism were evaluated in male vs female mice to elucidate the molecular mechanism underpinning the important effect of sex differences on acute chemical liver injury. It has been observed from prospective clinical trials that women usually have a higher risk of adverse drug responses compared with men, and there is also evidence suggesting that women may be more likely to develop drugrelated acute liver failure and autoimmune hepatitis [7]. In addition, a previous study has shown that the effects of hepatotoxicity induced by inorganic mercury are influenced by sex [8]. Mercury exposure elicits changes in liver function biomarker molecules, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase activity, leading to an increase in pathological liver damage; women have been shown to be more adversely affected than men [8], suggesting that exposure to the same levels of drugs or toxins may lead to higher levels of damage in women. Therefore, this study aimed to compare the effects of intraperitoneal injection with carbon tetrachloride on acute liver toxicity after 24 h in male and female Kunming mice.

Material and Methods

Experimental Animals

We obtained 120 healthy adult Kunming mice (60 males and 60 females) weighing 22±2 g and aged 6 weeks (pure background) from the Henan Experimental Animal Research Center. The mice were raised in the Experimental Animal Center of the Medical College of Henan University of Science and Technology. The license number for the grant obtained for these experimental studies was SCXK (Yu) 2016-0004. Mice were kept in an environment with temperature (22±2°C) and humidity (54±10%) maintained at a constant level, and the mice were housed in clean and hygienic conditions (with normal ventilation, alternation of day and night, and free access to drinking water). All procedures involving animals care and study were approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology (approval number HNKJDXDYFSYY20150009).

Experimental Reagents and Methods

The mice (60 females and 60 males) were housed in the animal room of the Experimental Center for a period of approximately 1 week. The female mice were subsequently divided equally into 3 groups (n=20 mice per group) as follows: i) the normal group, without any treatment; ii) the normal control group, wherein the mice received an intraperitoneal injection of olive oil, the quantity of which was calculated according to the formula of 0.1 ml olive oil per 10 g body weight; and iii) the CCl_4 group, which received an intraperitoneal injection of a 0.1% CCl_4-containing olive oil solution (0.1 ml olive oil per 10 g body weight). The CCl_4 was purchased from Tianjin Fengchuan Chemical Reagent Technology Co., Ltd. (cat. no. 56-23-5). The male mice were divided into 3 identical groups, exactly the same as the female mice. At 24 h after the intraperitoneal injections, the mice were anesthetized with cotton dipped in ether in a relatively closed space. After the mice fell asleep, extraction of the eyeballs was performed and 0.9 ml of blood was collected from each mouse. The serum was isolated prior to detection of the levels of the enzymes ALT and AST (see below). The mice were sacrificed by CO_2 asphyxiation using a fixed flow rate of 20% chamber volume/min, and their livers were extracted. The livers were divided into 2 parts: one for subsequent histological analysis, and the other for western blot analysis. This study was conducted in accordance with the experimental Animal Care and Use guidelines (version 8) [9] approved by the Animal Experiment Committee of Henan University of Science and Technology.

Determination of the levels of ALT and AST activity in the sera of mice

Blood was collected from the mice at 24 h after the CCl_4 injection, and the blood was subsequently allowed to clot at room temperature for 15-30 min. After it had completely clotted, the blood was rimmed using an applicator stick and then centrifuged for approximately 5-10 min at 590×*g* and 4°C. The serum was then separated from the pellet, and serum

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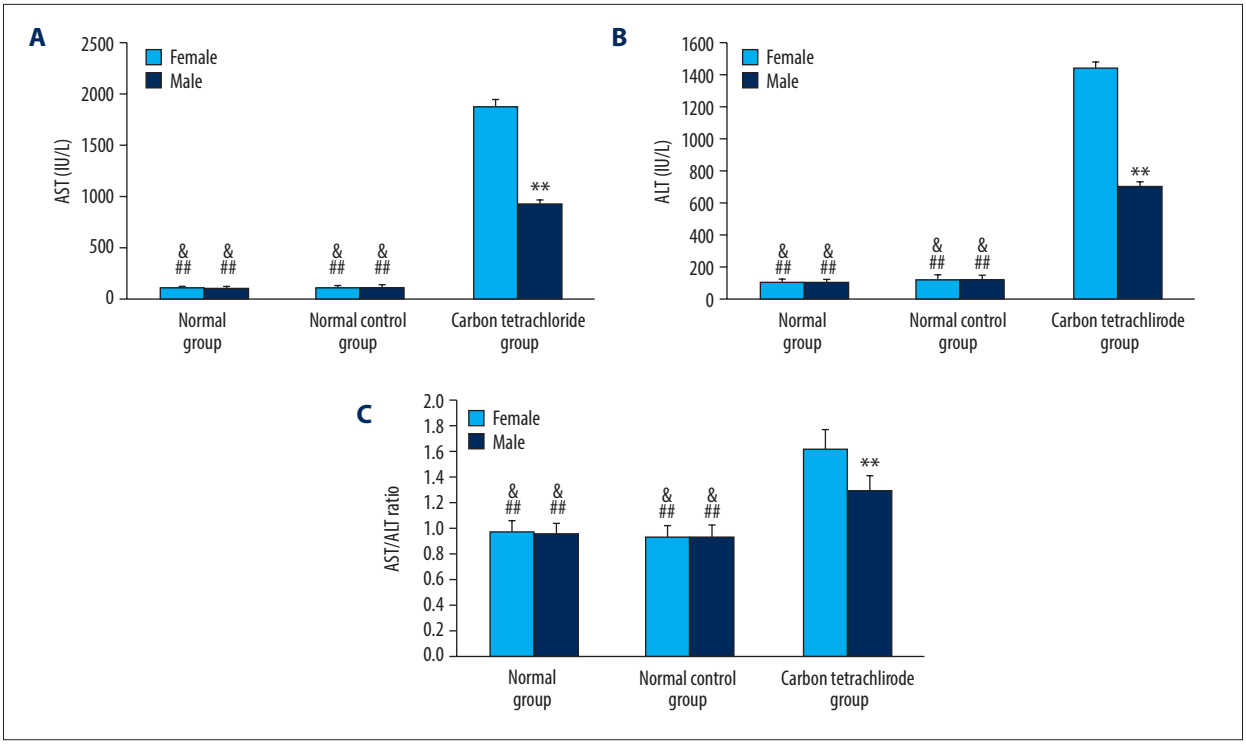

Figure 1. (**A**) Serum aspartate aminotransferase (AST), (**B**) alanine aminotransferase (ALT) levels, and (**C**) AST/ALT ratio in mice at 24 h after intraperitoneal injection of carbon tetrachloride (CCl_4). Male or female mice that were fed normally formed the normal group, mice that were treated with normal feeding plus olive oil intake by intraperitoneal injection formed the normal control group, and the CCl_4 group comprised normal feeding plus 0.1% CCl_4, which was diluted with olive oil intake (0.1 ml per 10 g body weight) by intraperitoneal injection. All data are presented as the mean±standard deviation (SD). ** $P<0.01$: there was a significant difference between the female group and the male group. ## $P<0.01$: there was a significant difference between the female CCl_4 group and the normal group or the normal control group. & $P<0.05$: there was a significant difference between the male CCl_4 group and the normal group or the normal control group. Experiments were repeated in triplicate.

AST and ALT activity levels were determined using an AST assay kit (Nanjing Jiancheng Biological Technology, Inc.; cat. no. C010-3-1) and an ALT assay kit (Nanjing Jiancheng Biological Technology, Inc.; cat. no. C009-3-1), respectively. Enzyme activities are expressed in International Units (IU)/l. The serum AST/ALT ratios were also calculated in each group.

Histological Examination

As mentioned above, liver specimens were obtained from the mice at 24 h after the intraperitoneal injections. Samples of liver were fixed in 10% formaldehyde (Tianjin Fengchuan Chemical Reagent Technology Co., Ltd.; cat. no. 50-00-0) for 24 h, and then dehydrated and embedded in paraffin (Shanghai Specimen and Model Factory; cat. no. Q/SOCC 07). Sections (6-μm thickness) were cut from each paraffinembedded tissue and stained with hematoxylin and eosin (H&E) (Shanghai Lanji Technology Development Co., Ltd; cat. no. 517-28-2) [10,11]. Then, the sections were viewed at 200× under a light microscope (Olympus, Tokyo, Japan). The hepatocyte necrosis rate score was then assigned as follows: 0 points, no lesions identified; 1 point, <2 lesions/visual field; 2 points, 2-4 lesions/visual field; and 3 points, >4 lesions/visual field.

Periodic Acid-Schiff (PAS) Staining

Glycogen staining of the liver was performed using a PAS staining kit (Cat. no. 1016460001) (Merck KGaA, Darmstadt, Germany), as described by Li et al [12]. Shortly afterwards, tissue sections were incubated in 0.8% periodic acid, followed by incubation in Schiff reagent at 25°C. Sections were counterstained with hematoxylin.

Western Blot Analysis

Protein samples (70 μg) from mice in the different groups were added to the electrophoresis sample buffer [50 mM Tris/HCl (Guangzhou Saiguo Biotech Co., Ltd.; cat. no. 1115GR500), pH 6.8, 10% glycerol (Sigma-Aldrich; Merck KGaA; cat. no. G7757), 5% β-mercaptoethanol (Ameresco, Inc.; cat. no. M8210), 2% sodium dodecyl sulfate (SDS) (Guangzhou Saiguo Biotech Co., Ltd.; cat. no. 3250GR500), and 0.1% bromophenol blue

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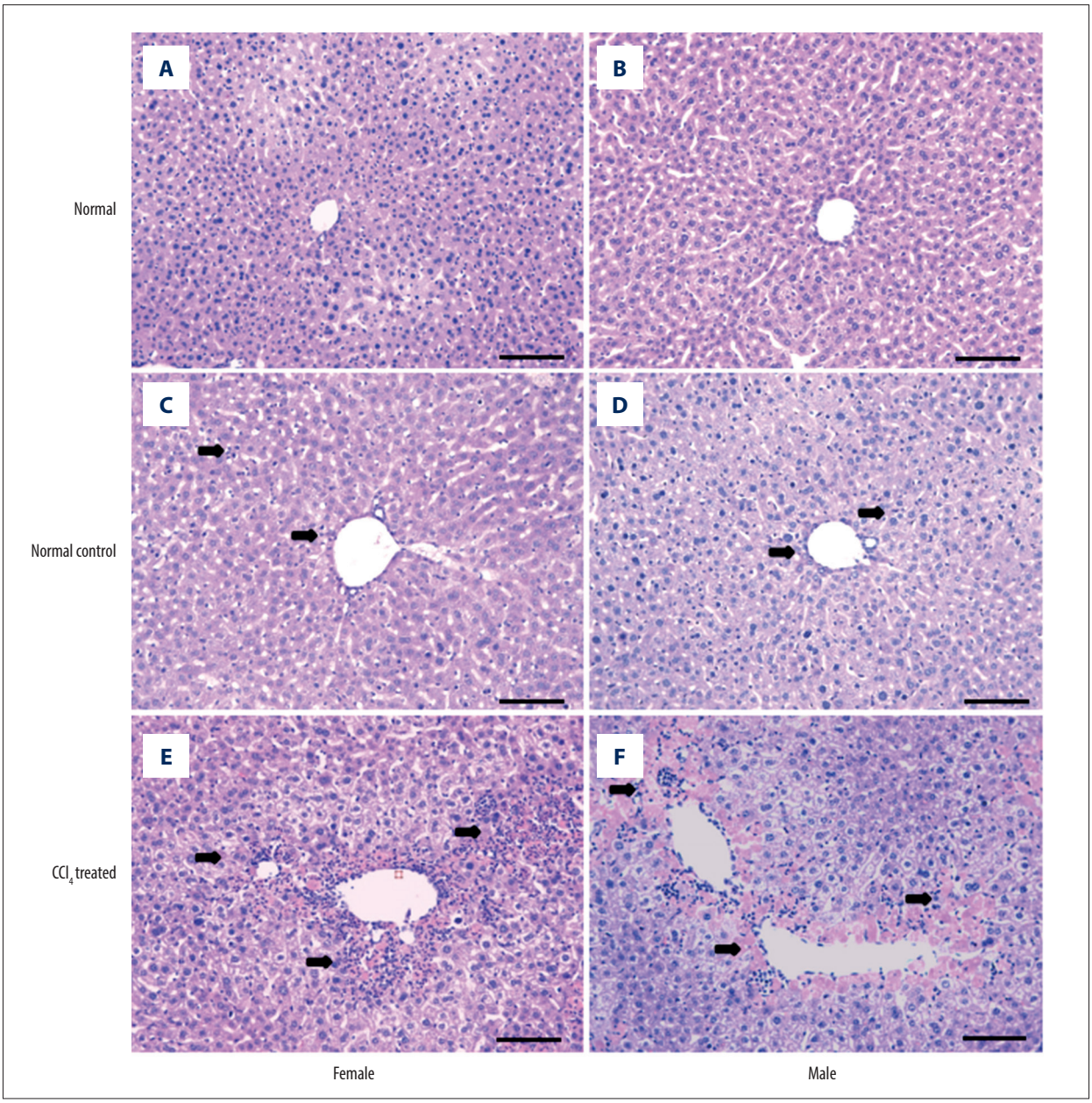
(Ameresco, Inc.; cat. no. 115-39-9)] and boiled for 5 min prior to analysis. Dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDSPAGE) (10% polyacrylamide gels) in 1-mm slab gels was performed as described by Li et al [13]. The proteins were transferred from the gel to a nitrocellulose membrane (Pall Life Sciences; cat. no. 35258530), and membranes were subsequently probed with monoclonal antibodies against mouse PCNA (Santa Cruz Biotechnology, Inc.; 1: 500 dilution, cat. no. sc-56), Bcl-2 (Santa Cruz Biotechnology, Inc.; 1: 500, cat. no. sc-7382), Bax (Santa Cruz Biotechnology, Inc.; 1: 500, cat. no. sc-7480), Caspase3 (Abcam; 1: 500, cat. no. EPR21032), HSP70 (Santa Cruz Biotechnology, Inc.; 1: 500, cat. no. sc-24), HSP27 (Santa Cruz Biotechnology, Inc.; 1: 500, cat. no.sc-59562), and CYP2E1 (Wuhan Sanying Biotechnology Co., Ltd.; 1: 500, cat. no. 67263-1-Ig), respectively. The signal was detected with a horseradish peroxidase detection system using DAB (SigmaAldrich, Merck KGaA; cat. no. D12384), protein bands were quantified using Gel Pro Analyzer software 4.0 (Media Cybernetics, Inc.) [14-17], and the intensities of the bands were normalized against β-actin. Each experiment was repeated 3 times.

Statistical Analysis

All data are presented as the mean±standard deviation. Statistical comparisons were made using oneway ANOVA with



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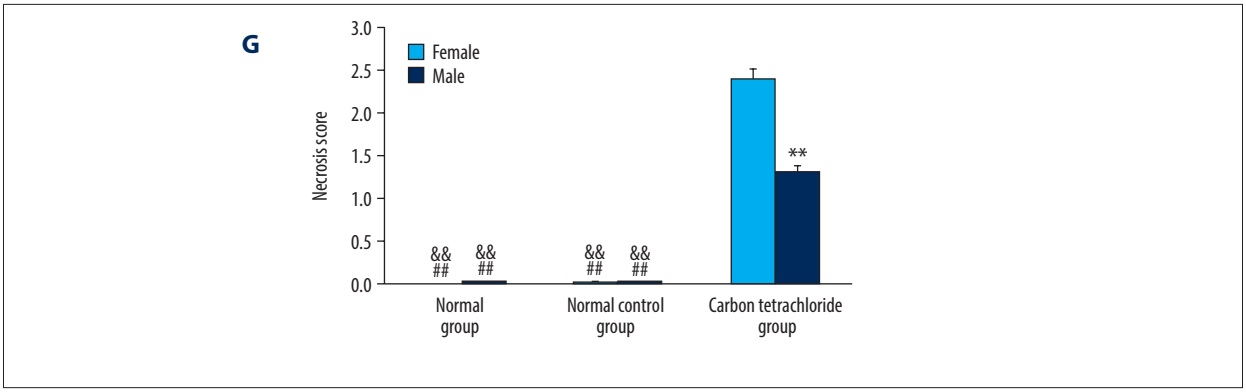

Figure 2. Detection of liver damage in mice by hematoxylin-eosin staining (H&E staining) at 24 h after intraperitoneal injection of CCl_4. (**A-F**) Liver damage of the female normal group, the male normal group, the female normal control group, the male normal control group, the female CCl_4 group, and the male CCl_4 group, respectively. (**G**) Area of necrosis. Measurements of liver sections of at least 10 mm^2 per mouse were performed using ImagePro Plus 6.0 software. ** $P<0.01$: there was a significant difference between the female group and the male group. ## $P<0.01$: there was a significant difference between the female CCl_4 group and the normal group or the normal control group. && $P<0.01$: there was a significant difference between the male CCl_4 group and the normal group or the normal control group. Experiments were repeated in triplicate (scale bar, 50 μm). H&E – hematoxylin and eosin.

the Tukey post hoc test for multiple comparisons. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc.).

Results

Differences in Serum AST and ALT Levels Comparing Between Female and Male Mice

Compared with the normal and the normal control groups, the levels of AST and ALT, and the AST/ALT ratio in the CCl_4 group were significantly higher in female and male mice ($P<0.01$ or $P<0.05$). In addition, the levels of AST and ALT, and the AST/ALT ratio in the female group were significantly higher compared with those in the male group following CCl_4 injection ($P<0.01$) (**Figure 1**).

Results of H&E Staining in Liver Tissue of The Mice in Each Group

Concerning the liver tissue of the mice in the normal and the normal control groups, the liver structure was observed to be complete, with the hepatic cord of hepatocytes around the central vein arranged radially. By contrast, in the CCl_4 group, the arrangement of hepatocytes was disordered, the hepatocytes appeared with a patchy necrosis area, and the hepatocytes around the central vein were more concentrated, accompanied by the infiltration of a large number of inflammatory cells. Compared with the normal and the normal control groups, liver injury had occurred in the CCl_4 group ($P<0.01$), and the degree of liver injury with the hepatocytes in the female group was more extensive compared with that in the male group ($P<0.01$). The hepatocyte necrosis rate scores of the female mice were also significantly higher compared with those in the male mice ($P<0.01$) (**Figure 2**).

Results of PAS Staining in the Liver Tissue of Mice in Each Group

At 24 h after intraperitoneal injection of CCl_4, the livers of both the female and male mice in the CCl_4 group exhibited a decrease in the level of glycogen compared with the normal and the normal control groups ($P<0.01$), and the content of glycogen in the livers of the male group was higher than in the female group ($P<0.05$) (**Figure 3**).

L2 Protein Expression Levels of HSP27, HSP70, Caspase3, PCNA, Bax, Bcl-2, and CYP2E1 in the Liver Tissues of Mice in Each Group, as Determined by Western Blot Analysis

At 24 h after intraperitoneal injection of CCl_4, the expression levels of HSP27, HSP70, Bax, Caspase3, and CYP2E1 in the livers of the CCl_4 group were found to be significantly upregulated compared with the normal and the normal control groups ($P<0.05$ or $P<0.01$), whereas the protein expression levels of Bcl-2 and PCNA were significantly downregulated. In addition, the levels of HSP27, HSP70, Bcl-2, and PCNA were significantly lower in the female group compared with the male group, whereas the opposite results were identified with Bax, Caspase3, and CYP2E1 (**Figure 4**).

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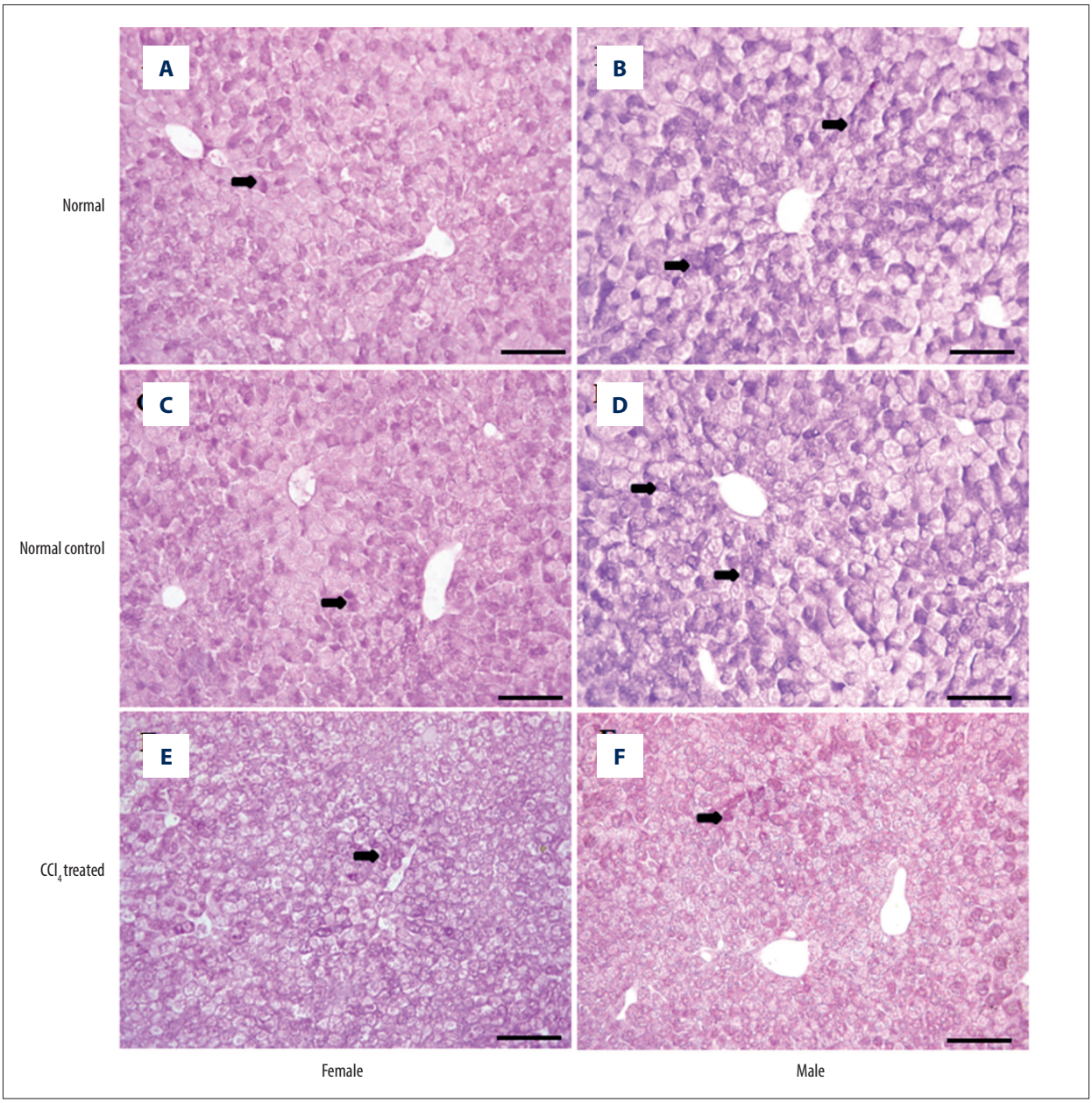
Discussion

Sex-associated differences in druginduced liver injury have been well documented, as determined from clinical and epidemiological data, and, in general, women are more susceptible to drug-induced liver injury [18-27]. However, the specific underlying mechanism requires further exploration. The present study systematically analyzed the molecular mechanism of the effects of sex differences on acute chemical liver injury in mice.

A previous study demonstrated that female Sprague-Dawley (SD) rats responded more severely to acute CCl_4 hepatotoxicity compared with male SD rats [28], a finding that was consistent with the results of the present study. The results of the serum AST and ALT enzyme assays revealed that liver damage was more extensive in female mice than in male mice following CCl_4 induction (**Figure 1**). A previous study [28] highlighted that the extent of tissue repair and cellular regeneration was greater in female rats. However, our study revealed that the expression level of PCNA in the female group was significantly lower compared with that in the male group (**Figure 4C**). PCNA is the core component of the eukaryotic replication complex, which has a cyclic tertiary structure, enabling it to participate in eukaryotic cell polymerization. PCNA binds to different replication-associated proteins, coordinates the replication process, and



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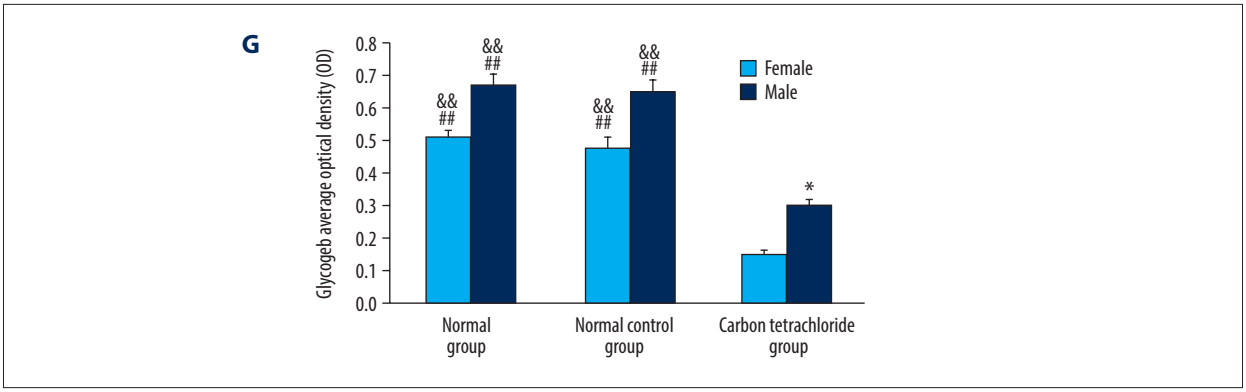

Figure 3. Detection of glycogen expression by periodic acid Schiff reagent staining (PAS staining) assay of staining at 24 h after intraperitoneal injection of CCl_4. (**A-F**) Liver PAS staining of the female normal group, the male normal group, the female normal control group, the male normal control group, the female CCl_4 group, and the male CCl_4 group, respectively. (**G**) Uniform white balance processing, two-color segmentation, and optical density analysis using Motic Images Advanced 3.2 software. At least 12 mm^2 of liver tissue sections were tested for each mouse. ** $P<0.05$: there was a significant difference between the female group and the male group. ## $P<0.01$: there was a significant difference between the female CCl_4 group and the normal group or the normal control group. && $P<0.01$: there was a significant difference between the male CCl_4 group and the normal group or the normal control group. Experiments were repeated in triplicate (scale bar, 50 μm). PAS – periodic acid-Schiff.

acts as a functional conversion factor [29]. It also participates in a large number of other important cellular events, including injury repair, cell cycle regulation, and apoptosis, through various regulatory methods and various cytokines [29]. In addition, PCNA has been shown to be a good marker for distinguishing proliferating cells [30-32]. The results of the present study suggest that the proliferative ability of hepatocytes of female mice was poorer compared with that of the male mice; indicating that the ability to repair liver damage in female mice was lower than that of the male mice, which may be one of the reasons why chemical liver damage in female mice was more severe compared with that of the male mice.

The present study also showed that in mice with CCl_4-induced liver injury the expression levels of HSP27 and HSP70 in male mice were significantly higher compared with that of female mice (**Figure 4A, 4B**). The main role of HSPs is to protect cells, and they fulfill important roles in protein folding, apoptosis, and signal transduction [33]. HSP27 and HSP70 act as important molecular chaperones in the body, protecting cells under stress from damage by preventing both protein denaturation and the renaturation of denatured proteins [34,35]. This may help us to understand why the degree of hepatocyte damage in male mice was less severe than that in female mice.

Previous studies have shown that CCl_4 induces acute liver injury in mice via induction of hepatocellular apoptosis [36] and necrosis [37]. Apoptosis is an active and programmatic process in which cells die according to a mechanism that is precisely regulated by various genes under certain physiological or pathological conditions, providing an important mechanism of homeostasis regulation [38]. The occurrence and development of apoptosis may be broken down into 3 stages: apoptosis induction, regulation, and execution. Of the Bcl-2 family members, the Bcl-2 and Bax genes are the most important regulatory genes in apoptosis, and they are able to mediate the release of cytochrome *c* (Cyt *c*) and other substances through the mitochondrial pathway (with Cyt *c* being a component of the mitochondrial electron transport chain). The release of Cyt *c* from mitochondria is a critical step in the initiation of apoptosis [39]. Caspase-3 is the most critical apoptotic executor downstream of the caspase cascade. Compared with the normal and the normal control groups, the expression of Bax and caspase-3 in the liver of the CCl_4 group was significantly upregulated (**Figure 4D, 4F**). Bax has a role mainly in promoting apoptosis; however, Bcl2 has an antiapoptotic role. The expression levels of Bax and caspase-3 in the female group were higher compared with those in the male group, whereas that of Bcl-2 occurred the other way around (**Figure 4D-4F**). The higher expression levels of Bax and cleaved caspase3, and the lower expression level of Bcl-2, in female mice compared with male mice were important factors contributing to the sex differences of hepatocyte apoptosis in CCl_4injured mice. Taken together, our findings show that CCl_4 treatment influenced the apoptosis of hepatocytes via regulating the expression of caspase-3, Bax, and Bcl-2 in female and male mice, which further influenced the differences in liver injury during CCl_4 intake observed in female and male mice.

The CYP2E1 enzyme is a member of the cytochrome P450 enzyme system, and is part of the core system of drug metabolism. The CYP2E1 enzyme is mainly distributed in the liver [40-42]. It has been found that the induction/overexpression of CYP2E1 not only promotes the activation of cancer cell oncoproteins,

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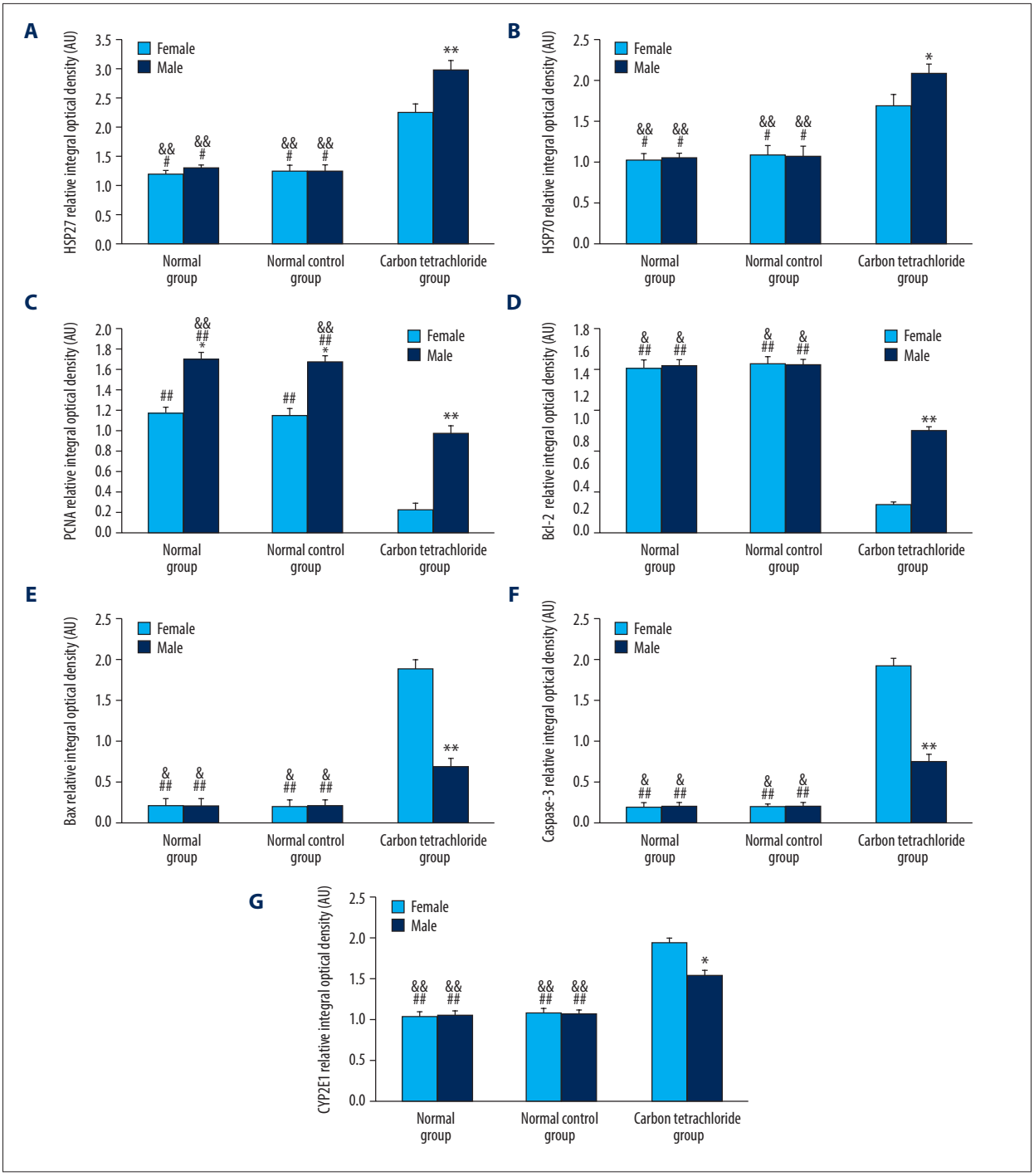

Figure 4. Expression of HSP27, HSP70, PCNA, Bcl-2, Bax, caspase-3, and CYP2E1 in the liver of mice at 24 h after intraperitoneal injection of CCl_4. Expression of HSP27, HSP70, PCNA, Bcl-2, Bax, pro-caspase-3, cleaved caspase-3 and CYP2E1 protein was detected by western blot analysis. The protein bands were quantified for (**A**) HSP27, (**B**) HSP70, (**C**) PCNA, (**D**) Bcl-2, (**E**) Bax, (**F**) caspase-3, and (**G**) CYP2E1 with Gel-Pro Analyzer 4.0 software (Media Cybernetics Inc.), and the intensities of the bands were normalized against beta-actin. Experiments were performed in triplicate, and all experimental data are expressed as the mean±standard deviation (SD). ** $P<0.01$ or * $P<0.05$: there was a significant difference between the female group and the male group. ## $P<0.01$ or # $P<0.05$: the female carbon tetrachloride group was significantly different from the normal group or the normal control group. && $P<0.01$ or & $P<0.05$: there was a significant difference between the male carbon tetrachloride group and the normal group or the normal control group. AU – arbitrary unit; NG – normal group; NCG – normal control group; CTG – intraperitoneal injection of CCl_4 group.

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but it also leads to the formation of oxidative stress and the release of inflammatory cytokines [including tumor necrosis factor-α, interleukin-6 (IL-6) and IL-1], which may stimulate cancer, leading to more severe damage [43-45]. Wong et al [46] demonstrated that CYP2E1 has a major role in CCl_4 toxicity, based on previous studies of Cyp2e1 null mice. The results of the present study indicate that CYP2E1 was significantly upregulated in the CCl_4 group compared with the normal and the normal control groups, and that the level of CYP2E1 was also significantly higher in the female CCl_4 group compared with the male CCl_4 group (**Figure 4G**), indicating that the oxidative stress caused by liver injury in the female CCl_4 group was more severe and that it also produced a higher level of inflammatory cytokines, factors that may help us to understand the more severe liver damage identified in the female mice.

There was also a significant difference in glycogen content identified in CCl_4-induced liver injury by comparing the sexes: Although the glycogen level was decreased in the livers of both female and male mice in the CCl_4 group, the content of glycogen in the male group was higher compared to that in the female group (**Figure 3**). This indicates that the male group had an improved ability to repair liver damage compared with the female group, since liver glycogen provides an important energy store in the liver. In liver injury, changes in the hepatic glycogen content can affect liver cell regeneration and repair.

Although we have found that some molecules play a key role in the influence of sex differences on acute chemical liver injury, we have not studied the signal pathway that plays key roles in the influence of sex differences on acute chemical liver injury. We will further study which signal pathway plays key roles in the influence of sex differences on acute chemical liver injury by use of gene chip technologies.

Conclusions

In summary, the findings from this study showed that, compared with male mice, at 24 h after CCl_4 toxicity, female mice showed more severe changes of hepatocyte necrosis and PAS-positivity, with significantly reduced expression of HSP27, HSP70, PCNA, and Bcl-2 and significantly increased expression of Bax, caspase-3, and CYP2E1. Although the important role of sex differences in acute chemical liver injury in mice has been well explained, whether it is applicable to human clinical practice still needs more in-depth research.

Acknowledgments

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

None.

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