

# Chronic restraint stress reduces carbon tetrachloride-induced liver fibrosis

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**Abstract.** Stress as a cofactor has been reported to affect the progression and severity of liver diseases. The present study investigated the effect of chronic restraint stress on carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis. A total of 30 male BALB/c mice were randomly divided into three groups: Oil-treated control group; CCl<sub>4</sub>-treated group; and CCl<sub>4</sub> + restraint-treated group. CCl<sub>4</sub> was administered via intraperitoneal injection once every 3 days over a period of 42 days. In the CCl<sub>4</sub> + restraint-treated group, mice were immobilized using 50 ml centrifuge tubes for 0.5 h to inflict chronic restraint stress immediately after the injection of CCl<sub>4</sub>. On day 42, blood and liver tissue samples were collected for analysis. The effect of restraint on CCl<sub>4</sub>-induced liver fibrosis in mice was evaluated by analyzing the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Histopathological examination of liver samples was performed using hematoxylin and eosin (HE), Masson's trichrome, 5-hydroxytryptamine 2B (5-HT<sub>2B</sub>) receptor and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) immunohistochemical staining. ALT, AST, 5-HT<sub>2B</sub> receptor and  $\alpha$ -SMA expression levels were significantly increased in mice exposed to CCl<sub>4</sub> in comparison with those in the oil-treated control mice (P<0.01). However, these increases were significantly reduced by exposure to restraint (P<0.05). HE and Masson's trichrome staining revealed that restraint can alleviate CCl<sub>4</sub>-induced liver fibrosis. These results suggest that chronic restraint stress reduces the development of liver fibrosis by inhibiting the activation of hepatic stellate cells via 5-HT<sub>2B</sub> receptor. Therefore, restraint

may be a useful therapeutic approach in the management of liver fibrosis.

## Introduction

Stress is a common factor in everyday life and is known to induce circulatory diseases and ulceration of the digestive tract (1). A number of early clinical reports have suggested that stress serves a major role in the initiation, course and outcome of liver diseases. Hirose *et al* (2) demonstrated that emotional stress significantly decreases hepatic blood flow. In addition, Tissari *et al* (3) reported that electric foot-shock stress exacerbates liver injury in mice treated with carbon tetrachloride (CCl<sub>4</sub>). Furthermore, it has been observed that stress can aggravate  $\alpha$ -galactosylceramide-induced hepatitis (4). Therefore, growing evidence continues to demonstrate that stress can influence the progression of liver disease.

The term 'restraint stress' refers to a specific type of stress resulting from the restriction of movement. Two primary experimental restraint stress animal models have been established: The first model involves confinement when the animal's movement is limited within a restricted space (5). The second model utilizes immobilization of the limbs and body of the animal using tape or plaster (6). Panuganti *et al* (7) demonstrated that restraint for periods of 0.5 or 1.5 h did not significantly enhance liver injury in healthy animals, whereas restraint for 2.5 h caused a significant increase in liver injury. However, whether restraint for 0.5 or 1.5 h has an effect on CCl<sub>4</sub>-induced liver injury in animals remains unknown.

In the present study, mice were restrained in 50 ml centrifuge tubes in order to investigate the effects of chronic restraint stress on CCl<sub>4</sub>-induced liver fibrosis. A previous study reported that stimulation of 5-hydroxytryptamine 2B (5-HT<sub>2B</sub>) receptor on hepatic stellate cells (HSCs) promotes HSC activation, and that antagonism of 5-HT<sub>2B</sub> receptor attenuates fibrogenesis (8). Therefore, in the present study, the effects of chronic restraint stress on 5-HT<sub>2B</sub> expression and HSC activation were investigated.

## Materials and methods

**Chemicals.** CCl<sub>4</sub>, hematoxylin and eosin (HE) solution and a Masson's trichrome staining kit were obtained from

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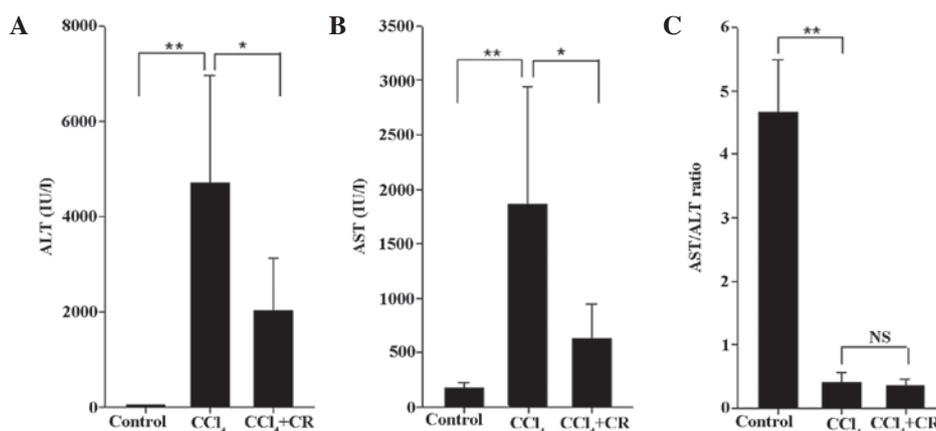


Figure 1. Serum levels of biochemical parameters. (A) ALT, (B) AST, and (C) AST/ALT ratio of the control, CCl<sub>4</sub>, and CCl<sub>4</sub> + CR groups. n=10; error bars represent standard deviation; \*P<0.05, \*\*P<0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl<sub>4</sub>, carbon tetrachloride; CR, chronic restraint; NS, no significant difference.

Sigma-Aldrich (Shanghai, China). Peanut oil was obtained from Shandong Luhua Group Co., Ltd. (Beijing, China).

**Animals and chronic restraint stress model.** A total of 30 male BALB/c mice (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China), weighing 18-20 g and aged 6-8 weeks, were acclimatized for 7-10 days in the Capital Medical University (Beijing, China) animal care facility. Throughout the experiments, animals were housed under a 12 h light/dark cycle with controlled temperature (24°C) and humidity (50%). Animals were allowed access to food and water *ad libitum*, except when restrained. All procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University of China. On day 1, 30 male BALB/c mice were randomly divided into three groups (n=10 each): Oil-treated control group; CCl<sub>4</sub>-treated group; and CCl<sub>4</sub> + restraint-treated group, which mice were treated with CCl<sub>4</sub> and exposed to chronic restraint stress. Mice in the oil- and CCl<sub>4</sub>-treated groups were injected intraperitoneally with 10 μl/g peanut oil or 0.5% CCl<sub>4</sub> solution once every 3 days over a 42-day experimental period. Following injection of 0.5% CCl<sub>4</sub> solution, animals in the restraint group were placed in 50 ml centrifuge tubes (Axygen; Corning Incorporated, Corning, NY, USA) for 0.5 h every 3 days over the 42-day experimental period. The restraint cage was well ventilated and prevented animals from turning or ambulating, but did not squeeze them. Following exposure to restraint, the mice were transferred to their home cages.

Immediately after restraint on day 42 of the experimental period, all mice were sacrificed via CO<sub>2</sub> narcosis. Blood samples were collected from the vena cava. The livers were removed and fixed in 2.5% glutaraldehyde-polyoxymethylene solution (Leagene Biotech Co., Ltd., Beijing, China) for 72 h for analysis. Liver tissues were then processed into paraffin and 5-μm sections were prepared for HE, Masson's trichrome, 5-HT<sub>2B</sub> (Abgent Biotech Co., Ltd., Suzhou, China) and α-smooth muscle actin (α-SMA; Abcam, Shanghai, China) immunohistochemical staining.

**Liver injury assessment.** A total of 0.1 ml serum was isolated from blood samples by centrifugation at 2,500 × g for

15 min. The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) expression levels in serum samples were measured using a commercially available colorimetric assay kits (Kinghawk Pharmaceutical Co., Ltd., Beijing, China). A highly colored end product from colorimetric assay was detected at 490-520 nm by a spectrophotometer (736-10; Hitachi, Ltd., Beijing, China), as the absorbance of each end product is proportional to the activity of the enzyme.

**HE staining.** Liver sections were stained with HE, and the degree of fibrosis in each section was classified according to grades 0-4, as previously described (9), where grade 0, 0% fibrosis; grade 1, <10% fibrosis; grade 2, <30% fibrosis; grade 3, <50% fibrosis; and grade 4, ≥50% fibrosis. Each tissue section was examined using an Olympus BH-2 microscope (Olympus Optical Co. Ltd., Beijing, China). In addition, histopathological changes were investigated using a Motic Images 2000 microscope (Motic China Group Co. Ltd, Guangzhou, China).

**Masson's trichrome staining for collagen level detection.** A Masson's trichrome staining kit (Sigma-Aldrich) was used to detect the collagen levels in the liver tissue. The blue-stained areas in the tissue sections were assessed using an Image-Pro Plus image analyzer (Media Cybernetics, Inc., Rockville, MD, USA) for semi-quantitative analysis. Results were expressed as the area density, which was defined the area of the positive cells / area of the entire field.

**Measurement of 5-HT<sub>2B</sub> receptor and α-SMA expression levels.** Immunohistochemical staining for 5-HT<sub>2B</sub> receptor and α-SMA was performed using Streptavidin Biotin Complex immunohistochemistry kits (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The yellow-stained areas in the tissue sections were assessed and area density was recorded using a similar method as for collagen levels.

**Statistical analysis.** Experimental data were analyzed by one-way analysis of variance using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The results are expressed as the

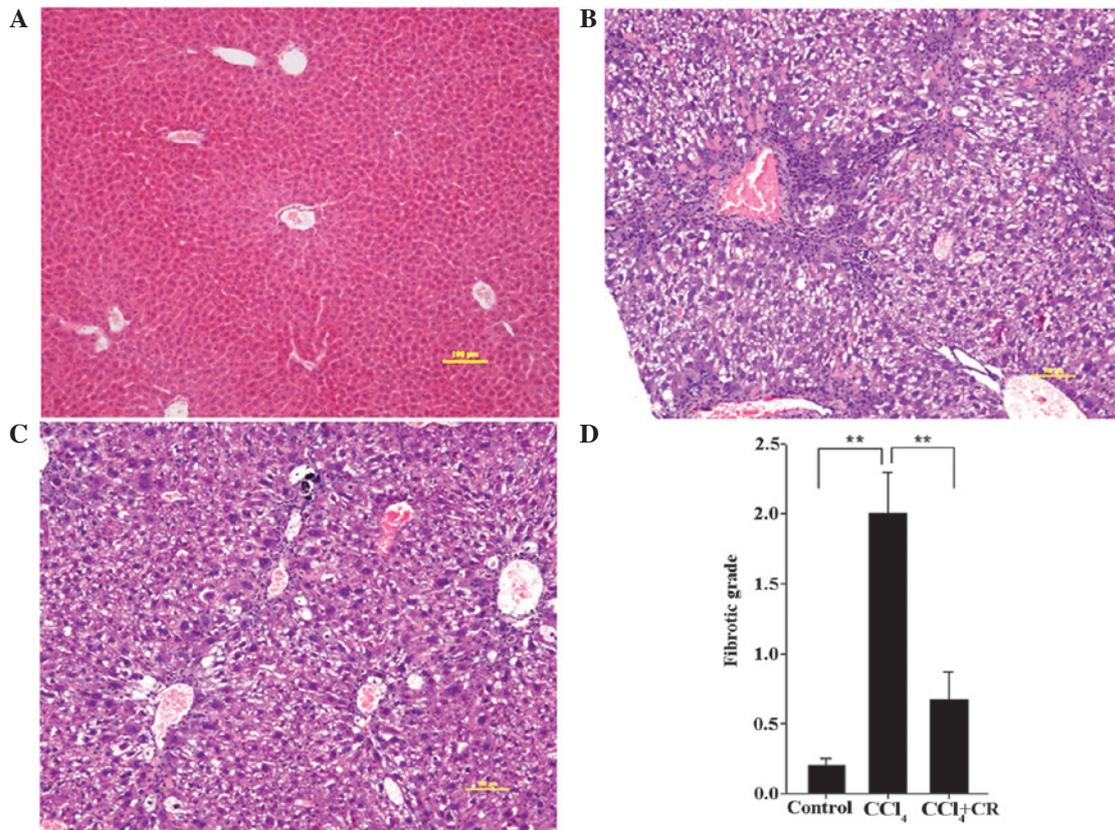


Figure 2. Gross changes in liver sections. Representative images of hematoxylin and eosin staining from liver sections of the (A) control, (B) CCl<sub>4</sub> and (C) CCl<sub>4</sub> + CR groups (bar=100 μm). Magnification, x100. (D) Fibrosis degree score of the control, CCl<sub>4</sub> and CCl<sub>4</sub> + CR groups (n=10 each; error bars represent standard deviation). \*\*P<0.01. CCl<sub>4</sub>, carbon tetrachloride; CR, chronic restraint.

mean ± standard deviation. Differences were considered as statistically significant at P<0.05.

## Results

**Serum biochemistry.** On day 42, no mortalities were reported in any of the experimental groups. However, all CCl<sub>4</sub>-treated mice displayed progressive jaundice, ascites and hepatomegaly, whereas these conditions were observed to a lesser degree in the oil-treated control and CCl<sub>4</sub> + restraint-treated mice. Liver injury was assessed by determining the serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the AST/ALT ratio. As presented in Fig. 1, the serum levels of AST and ALT, and the AST/ALT ratio were significantly higher in CCl<sub>4</sub>-treated mice in comparison with those in the oil-treated control mice (P<0.01). Treatment with restraint significantly reduced the expression levels of AST and ALT compared with the CCl<sub>4</sub>-treated mice (P<0.05; Fig. 1), but did not reduce the AST/ALT ratio.

**HE staining.** As presented in Fig. 2, the fibrotic grade of the livers from CCl<sub>4</sub>-treated mice was significantly higher on day 42 in comparison with the grade in the control mice (P<0.01), while restraint significantly reduced this fibrotic grade (P<0.01). HE staining detected centrilobular necrosis and macrovesicular lipid droplets in the CCl<sub>4</sub>-treated group (Fig. 2B), in which collagen had accumulated around the blood vessels and pseudolobuli had formed by thin fibrous septa. In

the CCl<sub>4</sub> + restraint group, the area of centrilobular necrosis and the degree of macrovesicular lipid droplets were decreased (Fig. 2B). In addition, collagen accumulation surrounding the blood vessels was reduced in the CCl<sub>4</sub> + restraint group compared with that in the CCl<sub>4</sub>-treated group, and no evident pseudolobuli had formed (Fig. 2C).

**Masson's trichrome staining.** As presented in Fig. 3, the area density of collagen in liver sections from CCl<sub>4</sub>-treated mice was significantly higher on day 42 compared with that in sections from the oil-treated control mice (P<0.01). In addition, the density of collagen in CCl<sub>4</sub> + restraint-treated mice was significantly lower compared with that in CCl<sub>4</sub>-treated mice (P<0.01), indicating that restraint significantly reduced the CCl<sub>4</sub>-induced collagen production.

In CCl<sub>4</sub>-treated mice, collagen fibers were more abundant in the centrilobular area and neighboring central veins were bridged by fibrous septa (Fig. 3B). In addition, pseudolobuli actively formed, macrovesicular lipid droplets were detected and the collagenous septa were much thicker compared with the oil-treated control group (Fig. 3A and B). Following restraint stress treatment, the prevalence of collagen fibers and macrovesicular lipid droplets was reduced and no pseudolobuli were identified (Fig. 3C).

**Expression levels of 5-HT<sub>2B</sub> receptor and α-SMA.** As presented in Figs. 4 and 5, the area densities of 5-HT<sub>2B</sub> receptor and α-SMA in liver sections from CCl<sub>4</sub>-treated

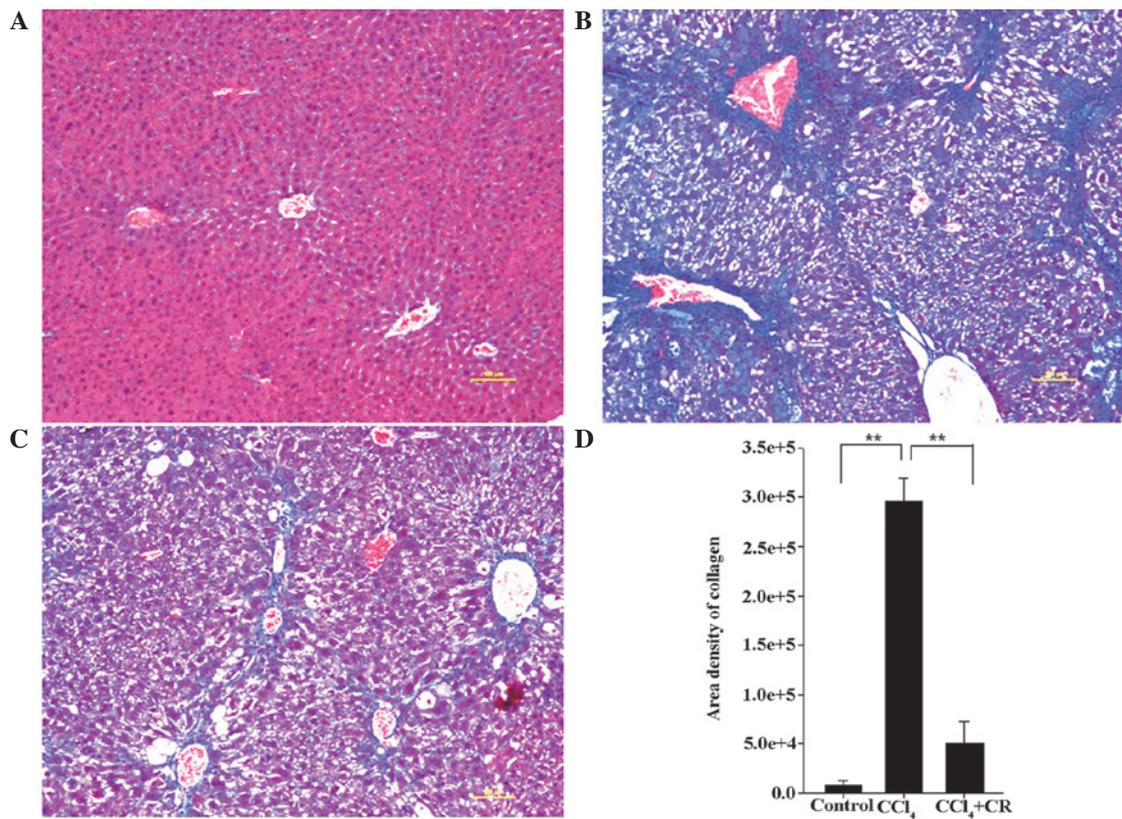


Figure 3. Masson's trichrome staining of collagen in liver sections. Representative images of hematoxylin and eosin staining of liver sections from the (A) control, (B) CCl<sub>4</sub> and (C) CCl<sub>4</sub>+CR groups (bar=100  $\mu$ m). Magnification,  $\times 100$ . (D) Area density of collagen in representative images of each group (n=10; error bars represent standard deviation). \*\*P<0.01. CCl<sub>4</sub>, carbon tetrachloride; CR, chronic restraint.

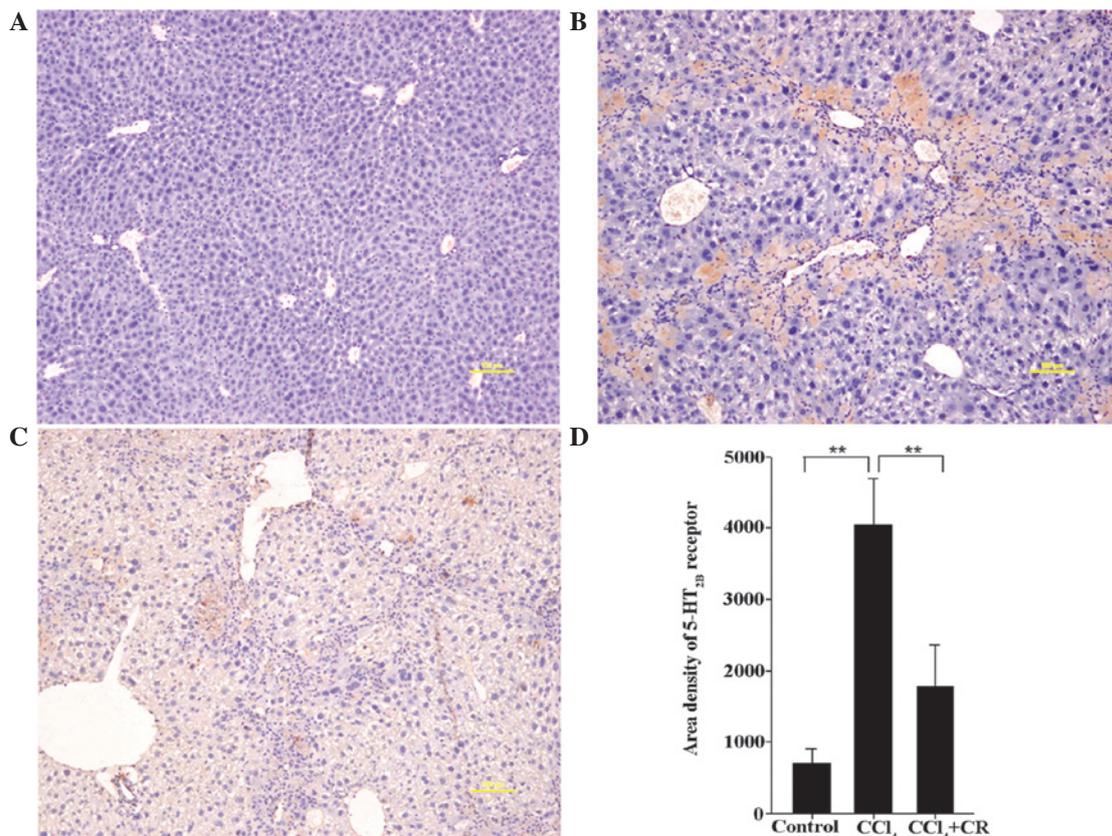


Figure 4. Immunochemical staining of 5-HT<sub>2B</sub> receptor in liver sections. Representative images of 5-HT<sub>2B</sub> receptor immunochemical staining in liver sections from the (A) control, (B) CCl<sub>4</sub>, and (C) CCl<sub>4</sub>+CR groups (bar=100  $\mu$ m). Magnification,  $\times 100$ . (D) Area density of 5-HT<sub>2B</sub> receptor in representative images of each group (n=10; error bars represent standard deviation). \*\*P<0.01. CCl<sub>4</sub>, carbon tetrachloride; CR, chronic restraint.

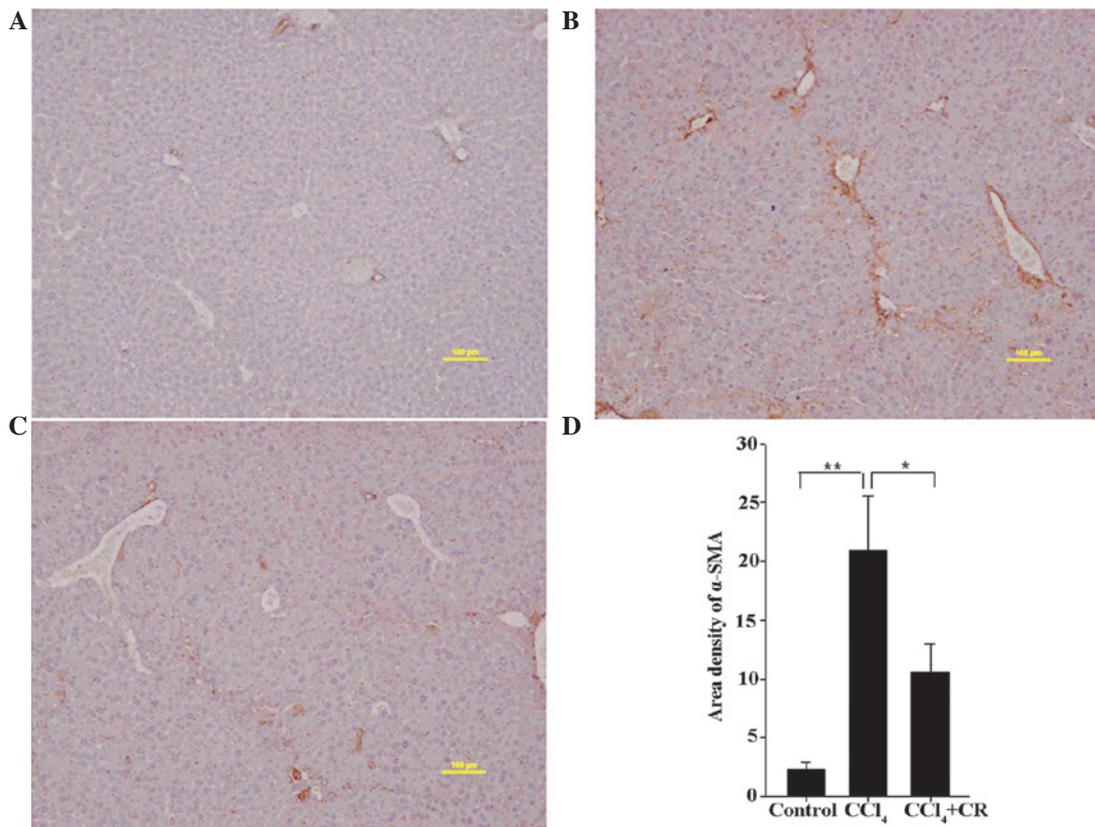


Figure 5. Immunochemical staining of  $\alpha$ -SMA in liver sections. Representative images of  $\alpha$ -SMA immunochemical staining of liver sections from the (A) control, (B)  $\text{CCl}_4$ , and (C)  $\text{CCl}_4$  + CR groups (bar=100  $\mu\text{m}$ ). Magnification, x100. (D) Area density of  $\alpha$ -SMA in representative images of each group (n=10; error bars represent standard deviation). \*\* $P<0.01$ , \* $P<0.05$ .  $\alpha$ -SMA;  $\alpha$ -smooth muscle actin;  $\text{CCl}_4$ , carbon tetrachloride; CR, chronic restraint.

mice were significantly higher on day 42 in comparison with oil-treated control mice ( $P<0.01$ ). In addition, the densities in  $\text{CCl}_4$ -treated mice were significantly higher compared with  $\text{CCl}_4$  + restraint-treated mice ( $P<0.05$ ), indicating that restraint can significantly reduce the expression levels of 5-HT2B receptor and  $\alpha$ -SMA that were induced by  $\text{CCl}_4$  treatment. The expression of 5-HT2B receptor and  $\alpha$ -SMA in the  $\text{CCl}_4$ -treated group was highly localized within the portal area, confirming that the increased expression levels were induced by  $\text{CCl}_4$  (Figs. 4B and 5B). In comparison with the  $\text{CCl}_4$ -treated group, 5-HT2B receptor and  $\alpha$ -SMA staining in the  $\text{CCl}_4$  + restraint group was significantly reduced and covered a smaller area (Figs. 4C and 5C), indicating that the expression levels of 5-HT2B receptor and  $\alpha$ -SMA are associated with the effects of restraint.

## Discussion

In the present study, animals were restrained in 50 ml centrifuge tubes for 0.5 h following the injection of  $\text{CCl}_4$  once every 3 days for 42 consecutive days, in order to evaluate the effect of chronic restraint stress on  $\text{CCl}_4$ -induced liver injury. Notably, chronic restraint stress was found to reduce  $\text{CCl}_4$ -induced increases in the ALT and AST expression levels. Furthermore, liver fibrosis was alleviated by chronic restraint stress treatment, which may prove to be a novel therapy for chronic liver disease.

Stress has been associated with a number of definitions in scientific literature. Certain life-changing or threatening

events are considered to be 'stressors' that can be acute or chronic factors, depending on their duration (10). In addition, stressors have been associated with a number of immune system dysfunctions, independent of whether an individual is affected by a chronic or acute disease (11).

Stress has been suggested to result in liver damage, since it affects hepatic blood flow by inducing vasospasm and centrilobular hypoxia (12). As the understanding of stress mediators increases, research focuses on the effect of stress on the onset and development of liver damage in acute and chronic liver diseases (13,14). Several animal models have demonstrated that there is a close association between stress and liver disease. For instance, electric foot-shock stress was found to exacerbate liver injury in mice treated with  $\text{CCl}_4$ , and to aggravate  $\alpha$ -galactosylceramide-induced hepatitis (4) that is associated with malaria and Salmonella infection-induced liver injury, and viral hepatitis B and C (15-18). In addition, restraint and electric foot-shock stress were demonstrated to induce mild liver injury in healthy rodents, which was confirmed by slightly elevated ALT expression levels (19,20). Furthermore, social isolation stress has been demonstrated to increase the spontaneous hepatocellular carcinoma incidence in transforming growth factor- $\alpha$  transgenic mice (21) and to accelerate the development of liver metastasis in the colon of mice injected with carcinoma cells (22-24).

In the present study, it was observed that 0.5 h of restraint was able to reduce  $\text{CCl}_4$ -induced liver fibrosis. This may be associated with the fact that the restraint model in the present study limited the animals' movement by restricting the amount

of available space, however the animals' limbs and body were not completely immobilized. Furthermore, the time and frequency of restraint were lower than those applied in a previous study (6).

The activation of the hypothalamic-pituitary-adrenal (HPA) axis that is induced by stress results in an inhibitory effect on the immune and inflammatory responses, as all the immune response components can be inhibited by glucocorticoids (25). Glucocorticoids are the final HPA axis effector molecules released from the adrenal cortex that participate in the regulation of homeostasis in each organ (26). Thus, the ability of chronic restraint stress to reduce liver fibrosis may result from changes in glucocorticoid levels. Further research is, therefore, required to evaluate the role of glucocorticoids in the reduction of CCl<sub>4</sub>-induced liver fibrosis following the application of restraint.

A previous study reported that stimulation of 5-HT<sub>2B</sub> receptor on HSCs promotes HSC activation. The present study demonstrated that 5-HT<sub>2B</sub> receptor and  $\alpha$ -SMA expression levels in the CCl<sub>4</sub> + restraint-treated mice were significantly reduced compared with those in CCl<sub>4</sub>-treated mice. These results indicate that chronic restraint stress may inhibit HSC activation via the 5-HT<sub>2B</sub> receptor.

In conclusion, restraint has been identified as an important factor in the progression and outcome of liver pathologies. The results of the present study demonstrated that proper restraint stress may be a potential therapeutic strategy for the treatment of chronic liver disease. However, the effects of chronic strain stress may vary depending on the time, type, or equipment of restraint. An increased understanding of how restraint alters hepatic inflammation will provide important information for the development of novel therapies for managing liver diseases.

### Acknowledgements

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