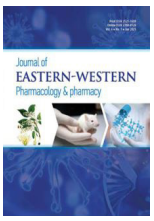


Original Article
https://doi.org/10.64269/jewpp.v6i2.4780

Journal of
EASTERN-WESTERN
Pharmacology & pharmacy

OPTIMIZATION OF CCL4 INDUCED LIVER FIBROSIS MODEL IN WISTAR RATS: DIFFERENT DOSES AND TIME PERIODS

Davaasambuu Tegshbayar[1*], Batchimeg Batbayar[1], Munkh-Erdene Ragchaasuren[1], Badamtsetseg Soyollkham[1], Lkhaasuren Ryenchindorj[1], Khurelbaatar Luvsan[1,2], Oyunchimeg Bayaraa[1]

[1] *Drug Research Institute, Ulaanbaatar, Mongolia*
[2] *Monos Group LLC, Ulaanbaatar, Mongolia*

KEYWORDS

Liver fibrosis; Carbon tetrachloride; Liver injury; Portal inflammation.

Received: 2025/05/19 Accepted: 2025/10/06
Revised: 2025/08/20 Published: 2025/11/20

ABSTRACT

The carbon tetrachloride (CCl_4)-induced liver fibrosis model is one of the most widely used experimental systems for investigating the mechanisms of hepatic fibrogenesis and evaluating antifibrotic therapies. However, the severity and reproducibility of fibrosis strongly depend on the applied dose and duration of exposure. In this study, Wistar rats were divided into three groups: healthy controls, a high-dose short-term group that received 2.0 ml/kg CCl_4 for 4 weeks, and a low-dose long-term group that received 1.0 ml/kg CCl_4 for 6 weeks. Biochemical markers, gross morphology, and histopathology were assessed to determine the optimal induction protocol. The 4-week group developed moderate fibrosis, characterized by hepatocellular vacuolation, periportal fibrous expansion, and a fibrosis score of 30% with a collagen volume fraction (CVF) of 21%, but biochemical changes remained mild and statistically nonsignificant. In contrast, the 6-week group exhibited advanced fibrosis, characterized by bridging septa, fatty infiltration, and extensive collagen deposition, as reflected by a fibrosis score of 42% and a CVF of 37%. A significant ($p<0.01$, $p<0.001$) elevation was observed in serum ALT, AST, GGT, and cholesterol levels, with marked increases in triglycerides. Gross pathology confirmed these findings, with pronounced nodularity and shrinkage of the liver surface in the 6-week group compared with the relatively preserved morphology of the 4-week group. Taken together, these results demonstrate that repeated administration of 1.0 ml/kg CCl_4 for 6 weeks provides an optimal balance between reproducibility, fibrosis severity, and survival, inducing advanced but non-cirrhotic fibrosis. This protocol, therefore, represents a reliable platform for mechanistic studies and the preclinical evaluation of antifibrotic interventions.

INTRODUCTION

Liver fibrosis is the pathologic result of ongoing chronic inflammatory liver diseases and is characterized by hepatic stellate cell (HSC) proliferation and differentiation to myofibroblast-like cells, which deposit extracellular matrix (ECM) and collagen[1-2].

**Drug Research Institute, Sonsgolon's road 32/b, Songinokhairkhan district, Ulaanbaatar, Mongolia, E-mail: davaasambuu@monos.mn*

Myofibroblasts, which are absent in a healthy liver[3-4], become activated in response to liver injury. These cells are the main source of ECM in a fibrotic liver[5-6], making them a key target for antifibrotic therapy. Myofibroblasts are characterized by a spindle or stellate shape and have high levels of specific intracellular proteins, including vimentin, α-smooth muscle actin (αSMA), and non-muscle myosin. The liver damage caused by carbon tetrachloride (CCl_4) in experimental fibrosis models closely resembles human liver fibrosis, especially regarding inflammation, regenerative response, and formation of fibrotic tissue. This model is frequently used to study acute liver injury, advanced fibrosis, and the reversal of fibrosis. Additionally, the CCl_4-induced liver fibrosis model is a highly reproducible, cost-effective, and physiologically relevant model for liver disease screening studies. But exceeding the dose and injection period of CCl_4 leads to irreversible pathological changes in hepatic tissue, such as liver cirrhosis, acute toxicity, and sudden death of animals. In this study, the objective was to determine the optimal dose and time period of CCl_4 injection based on histopathological evaluation and blood biochemical parameters to induce a liver fibrosis model in rats[6-8].

MATERIALS AND METHODS

Chemicals: ALT, AST, CHOL, TG, and GGT biochemical kits were purchased from Biobase, China. CCl_4 was purchased from Sigma Aldrich, China.

***Experimental animals*:** Twelve Male Wistar rats (8-week-old, 215-225 g) were randomly selected from the Vivarium of Drug Research Institute (Ulaanbaatar, Mongolia). Rats were housed in cages under a condition of a 12-h light/ dark cycle and maintained on standard rat food and water. The cage temperature and humidity were 23±2°C and 35-60% respectively.

***Experimental protocol*:** A total of three groups (n=10, each) of experiments were conducted. Wistar rats were i.p. injected with CCl_4 at doses of 1.0 ml/kg and 2.0 ml/kg, twice weekly, for time periods of 4 and 6 weeks, respectively. Thirty rats were randomly divided into 3 groups: Group 1 (Healthy), Group 2 (2 ml/kg, 4 weeks), and Group 3 (1 ml/kg, 6 weeks). To verify the liver fibrosis formation, blood samples were collected from the retro-orbital sinus vein, and serum was separated by centrifugation at 3000x g for 20 min. The serum level of ALT and the activity of the enzymes AST, CHOL, TG, and GGT were assayed according to the biochemical kit's manufacturer's methods. Liver histopathological changes were evaluated using Masson's trichrome staining to visualize collagen deposition and HE staining for enlargement of stellate cells in portal tracts with numerous septa formation[9-10].

***Liver tissue preparation for histological assessment*:** The liver of a sacrificed rat was fixed in 10% neutral buffered formalin at 4°C for 24 h and washed with tap water. Fixed samples were dehydrated through an ascending series of graded ethanol, cleared in xylene, and embedded in paraffin blocks. Subsequently, samples were cut either longitudinally or transversely into 5-µm-thick sections and mounted on silane-coated glass slides[11-12].

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA). GraphPad Prism 8.0 statistical program (USA) was used to conduct all of the analyses. The threshold for statistical significance was a probability value of less than 0.05.

RESULTS

In the group treated with 1 ml of CCl_4 for six weeks (Group 3), the serum concentrations of ALT, AST, cholesterol, triglycerides, and GGT were significantly elevated compared to healthy animals. However, in the other 2 groups, there were no significant differences for serum biochemical analysis.

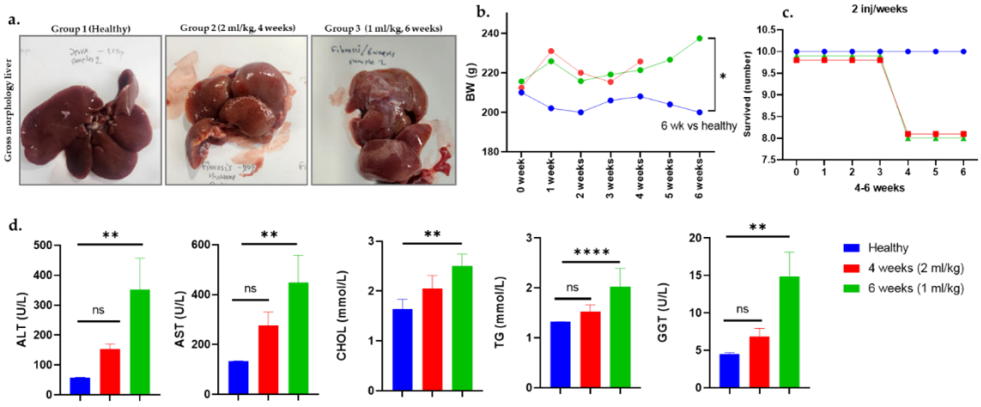
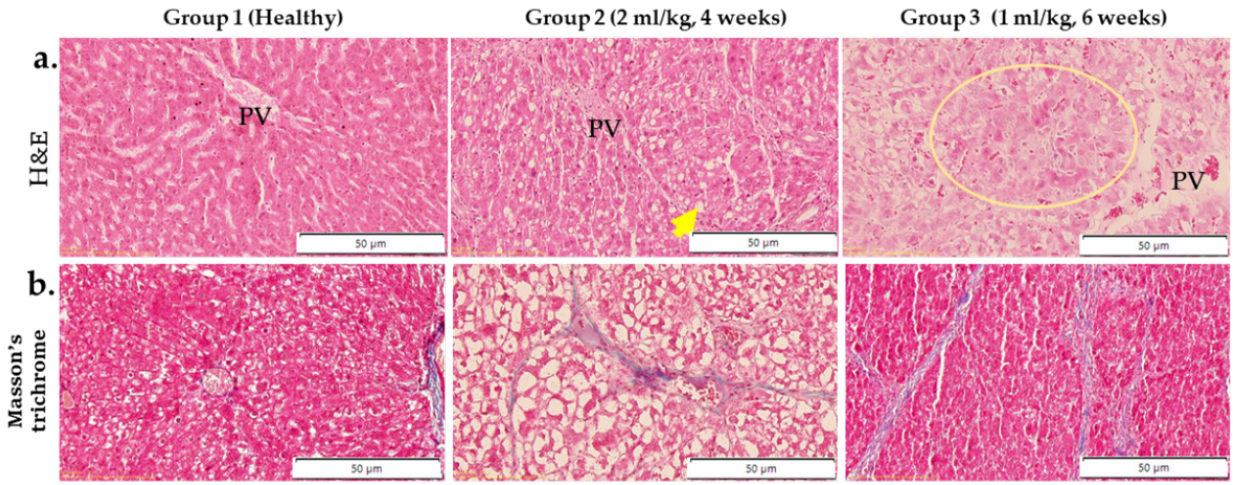


Figure 1. CCl_4-induced liver toxicity 4 or 6 weeks after administration was assessed by a) Liver gross morphology, b) Body weight for rats, c) Survival number, and d) Serum biochemical markers. Data are means±s.e.m. Values indicate significant differences when compared to the healthy group (*p < 0.05, **p < 0.01, ***p < 0.001).

Biochemical assays, along with gross and microscopic analyses, showed significant variations among groups based on both dose and duration. In the healthy control group, the liver appeared reddish-brown with a smooth surface and regular shape. In contrast, the 2 mL/kg for 4 weeks group displayed moderate shrinkage, surface irregularity, and mild discoloration. More pronounced changes were observed in the 1 mL/kg for 6 weeks group, where the livers showed severe shrinkage, an irregular nodular surface, and loss of smooth architecture, consistent with advanced fibrosis. While the normal livers exhibited an ordinary reddish-rufous color, a perfectly smooth surface, and a regular shape, the livers from the CCl_4-treated two groups showed varying degrees of shrinkage, abnormal coloration, irregular edges, and a strikingly uneven surface with protruding fibrotic nodules of mixed sizes (Fig. 1a). The initial body weight was not significantly different among the three groups. Although weight gain was observed in all groups, the animals in the 6-week group exhibited a significantly smaller increase compared to the control group ($p < 0.05$). Mortality was limited across groups, ranging from one to two animals per group, suggesting that both regimens were generally tolerated. Rats in the experimental groups treated with CCl_4 showed a non-significant increase in body weight. Rats treated with CCl_4 for six weeks showed significant differences in body weight and weight gain compared to the control group ($p < 0.05$), as illustrated in Fig. 1b. Rats mortality during the experimental period was very limited, ranging from 1 to 2 in all groups (Fig. 1c). After six weeks, Group 3 rats showed significantly ($p<0.01$, $p<0.001$) higher serum ALT, AST, CHOL, GGT, and TG levels compared with healthy controls (Fig. 1d). Serum biochemical assays revealed significant differences among groups, reflecting progressive hepatic injury. In the healthy control group of rats, the levels of ALT, AST, cholesterol (CHOL), triglycerides (TG), and GGT remained stable and within normal limits. In the group exposed to a dosage of 2 mL/kg for four weeks, serum ALT and AST levels showed a modest increase; however, these changes were not statistically significant when compared to the control group. Similarly, CHOL, TG, and GGT levels exhibited only modest increases, suggesting early biochemical disturbance without full establishment of functional impairment. In the group receiving 1 mL/kg for 6 weeks, significant biochemical changes were observed, indicating advanced liver cell damage and impaired metabolism. The activities of ALT and AST rose notably ($p < 0.01$ compared to controls), suggesting extensive leakage and necrosis of hepatocytes. CHOL and GGT levels were also significantly increased ($p < 0.01$), reflecting impaired bile flow and cholestatic injury. Most notably, TG levels were highly elevated ($p < 0.001$ vs. control), pointing to disrupted lipid metabolism and steatosis, which corresponded histologically with fatty degeneration in hepatocytes. Taken together, the biochemical results confirm that short-term high-dose treatment induces only modest and variable changes, whereas prolonged low-dose exposure produces consistent and statistically significant biochemical evidence of liver injury, correlating strongly with the observed histopathological findings. Sustained elevation of ALT, AST, GGT, cholesterol, and triglycerides was most pronounced in the 1.0 ml/kg for 6 weeks group, directly corresponding to histological evidence of hepatocyte necrosis, fatty degeneration, and bridging fibrosis, while the 2.0 ml/kg for 4 weeks group showed only modest biochemical disturbance with milder structural injury. These findings suggest that prolonged low-dose exposure maintains continuous hepatocellular stress and inflammatory signaling, thereby more effectively activating hepatic stellate cells and promoting extracellular matrix deposition compared with short-term high-dose treatment.



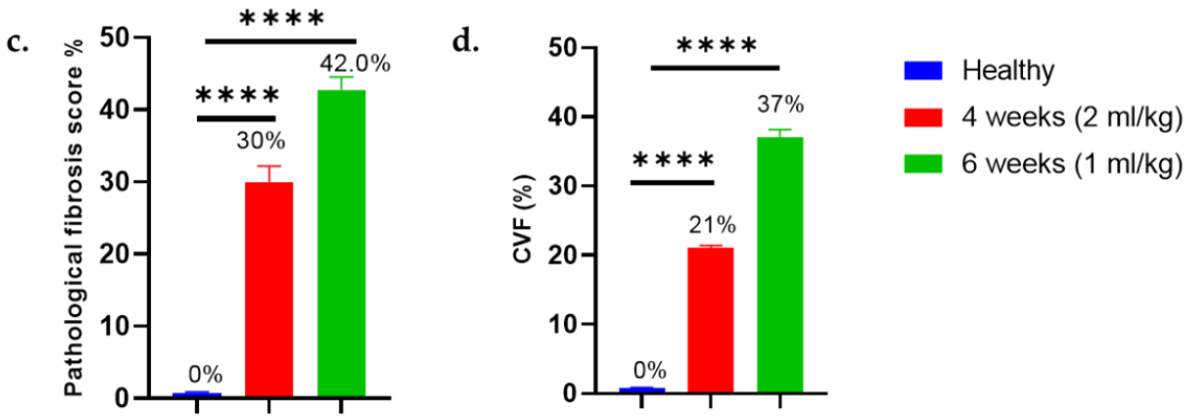


Figure 2. Histological assessments of liver tissue for experimental groups. a) Hematoxylin-eosin (H&E): *PV-portal vein, yellow arrow-liver vacuoles, white yellow circle- liver fibrosis area,* and b) Masson's Trichrome staining. c) Pathological fibrosis area d) CVF (%) area. The data were displayed as the mean ± SD. Note: ****p< 0.001 when compared to the control group; p < 0.001 when compared to the model groups.

Histopathological evaluation revealed that the liver tissues from the control group exhibited normal histological features. In contrast, H&E-stained sections from rats treated with 2 mL/kg of CCl_4 for four weeks showed significant stromal and parenchymal damage. Key findings included thickening of the liver capsule and connective tissue septa, the presence of numerous vacuoles of varying sizes and shapes within these areas, and distortion of the hepatic parenchyma resulting in the loss of normal architecture. In contrast, hepatofibrotic changes such as an increase in collagen deposition around the central vein and portal areas, endothelial lining of the central vein, cellular and fatty infiltration in-between hepatocytes, and congested blood sinusoids were notably observed in the CCl_4-treated groups, especially 1ml/kg for 6 weeks (Fig. 2a). Fibrosis was evaluated using the METAVIR scoring system based on image analysis: Healthy – F0, 4 weeks (2 ml/kg) – F2, and 6 weeks (1 ml/kg) – F3 (Fig 2c). A quantitative assessment of fibrosis severity revealed a clear dose- and time-dependent progression in the CCl_4-treated groups compared to healthy controls. The pathological fibrosis score was 0% in the control group, but increased markedly to 30% following 4 weeks of high-dose CCl_4 administration (2 ml/kg). Prolonged exposure at a lower dose (1 ml/kg for 6 weeks) resulted in a further rise to 42%, representing a 12% absolute increase relative to the 4-week regimen. Masson's trichrome staining revealed a significant accumulation of collagen fibers in the stroma, especially around the central veins and in the portal areas (Fig. 2b). Morphometric analysis of the collagen volume fraction confirmed a notable increase in collagen deposition in the CCl_4-treated groups when compared to the healthy controls (Fig. 2d). Similarly, morphometric evaluation of collagen deposition, expressed as collagen volume fraction (CVF), confirmed progressive fibrosis. The CVF remained 0% in healthy controls, while the 4-week high-dose group exhibited 21% collagen deposition. In contrast, the 6-week low-dose group demonstrated a significantly higher CVF of 37%, corresponding to a 16% absolute increase compared with the 4-week group. These findings were consistent with the qualitative histopathological observations. Group 2 displayed hepatocyte vacuolization, capsule thickening, and septa formation (METAVIR F2), whereas Group 3 exhibited bridging fibrosis, sinusoidal congestion, and marked collagen accumulation (METAVIR F3). Collectively, these results indicate that extended lower-dose exposure is more effective in generating reproducible advanced fibrosis than short-term high-dose treatment[13].

DISCUSSION

The liver fibrosis model induced by CCl_4 in rats is a well-established and reproducible preclinical system that closely mimics the pathological features of human hepatic fibrosis[3]. CCl_4 is metabolized by cytochrome P450 enzymes, particularly CYP2E1, into highly reactive trichloromethyl radicals, which initiate lipid peroxidation, leading to hepatocyte degeneration and necrosis[14,16]. This hepatocellular damage was clearly confirmed in our study through histological examination with hematoxylin–eosin staining, which revealed hepatocyte ballooning, fatty infiltration, and lobular disorganization in the treatment groups, particularly in the low-dose 6-week regimen. The liver's response to ongoing hepatocellular injury involves inflammatory reactions, activation of hepatic stellate cells (HSCs), and the accumulation of extracellular matrix (ECM), particularly fibrillar collagen[2,5]. In our study, quantitative analysis supported this mechanism: the fibrosis score increased from 30% at 4 weeks (2.0 ml/

kg) to 42% at 6 weeks (1.0 ml/kg), while collagen volume fraction (CVF) increased from 21% to 37% over the same regimens. These findings demonstrate that fibrosis severity is more dependent on the duration of exposure than on the intensity of dose, suggesting that chronic low-level oxidative stress more effectively maintains HSC activation and collagen synthesis compared with short-term high-dose injury[7]. Our biochemical results further corroborate this mechanism. In the 6-week group, serum ALT and AST were significantly elevated, reflecting hepatocellular leakage and necrosis, while GGT and cholesterol increases indicated cholestatic dysfunction. The most striking change was observed in triglyceride levels ($p < 0.001$), consistent with lipid metabolic disruption and histological evidence of steatosis. These alterations were far less pronounced in the 4-week high-dose group, again emphasizing the importance of chronicity in fibrogenesis[17]. Together, these results confirm that prolonged low-dose administration of CCl_4 more reliably models the progressive biochemical and histological hallmarks of liver fibrosis than short-term high-dose exposure.

Our findings also align with previous studies demonstrating that evidence of liver fibrosis becomes detectable after four weeks of CCl_4 exposure, with cirrhosis developing after approximately eight weeks[15,19]. By selecting 4 and 6 weeks as experimental endpoints, we were able to capture both the moderate (F2) and advanced (F3) stages of fibrosis without progressing to cirrhosis, which is often associated with high mortality and irreversibility[1]. This is an advantage of our study design, as it provides an optimized timeframe for preclinical evaluation of antifibrotic therapies. Furthermore, our methodology is consistent with established research protocols, which commonly employ intraperitoneal injections of CCl_4 two to three times weekly for 4–6 weeks[16,19].

In summary, the present study demonstrates that 1.0 ml/kg of CCl_4 administered twice weekly for 6 weeks produces advanced, yet non-cirrhotic fibrosis with strong biochemical and histological correlation. This protocol strikes a balance between reproducibility, disease severity, and animal survival, making it highly suitable for mechanistic studies and preclinical testing of antifibrotic interventions.

CONCLUSIONS

Our results showed that a dose of 1 ml/kg injection for 6 weeks effectively leads to liver fibrosis. Fibrosis severity is increasing depending on the dosage and duration of exposure. High dosage and injection time periods lead to severe liver cirrhosis, which is untreatable. These findings may serve as a valuable reference for studies on hepatofibrosis.

References

1. Mohammed, O.S., Attia, H.G., Mohamed, B., et al (2023). Current investigations for liver fibrosis treatment: Between repurposing the FDA-approved drugs and other emerging approaches. J. Pharm. Pharm. Sci (26). 11808.
2. Horn P., Tacke F. (2024). Metabolic reprogramming in liver fibrosis. Cell Metab (36). 2024.p.1439–1455.
3. Bataller R., Brenner D. A. (2005). Liver fibrosis. J. Clin. Invest. 115,.209–218.
4. Kisseleva T., Brenner D. A. (2008). Mechanisms of fibrogenesis. Exp. Biol. Med. 233.p.109–122.
5. Schmitt-Graff A., Kruger S., et al. (1991). Modulation of alpha smooth muscle actin and desmin expression in perisinusoidal cells of normal and diseased human livers. Am. J. Pathol. 138, 1233–1242.
6. Leeming, D. J., Byrjalsen, I., Jiménez, W., Christiansen, C., and Karsdal, M. A. (2013). Protein fingerprinting of the extracellular matrix remodelling in a rat model of liver fibrosis–a serological evaluation. Liver Int. 33, 439–447. doi: 10.1111/liv.12044
7. Marfà, S., Morales-Ruiz, M., Oró, D., Ribera, J., Fernández-Varo, G. y Jiménez, W. (2016). Sipa1l1 is an early biomarker of liver fibrosis in CCl4-treated rats. Biol. Open 5, 858–865. doi: 10.1242/bio.018887
8. Yu-long Bao, Li Wang, Hai-ting Pan, Tai-ran Zhang et al. (2021). Animal and Organoid Models of Liver Fibrosis. Animal and Organoid Models of Liver Fibrosis. Front. Physiol. 12:666138. doi: 10.3389/fphys.. 2021.666138.
9. Mandal, S., Maity, T., Das, J., Pal, M., & Saha, B. (1999). Hepatoprotective activity of Ficus racemosa leaf extract on liver damage caused by carbon tetrachloride in rats. Phytotherapy Research, 13, 430– 432.
10. Sana Janakata, Hela Al-Merieb. (2002). Optimization of the dose and route of injection, and characterisation of the time course of carbon tetrachloride-induced hepatotoxicity in the rat. Journal of Pharmacological and Toxicological Methods 48. 41– 44.
11. Keywan Mortezaee, Fatemeh Sabbaghziarani, Ameneh Omidi et al. (2016). Therapeutic value of melatonin post-treatment on CCl_4-induced fibrotic rat liver. An. J. Physiol. Pharmacol. 94: 1–12 dx.doi.org/10.1139/cjpp-2015-0266

12. Ding J, Yu J, Wang C, Hu W et al. (2005). Ginkgo biloba extract alleviates liver fibrosis induced by CCl_4 in rats. Liver International: 25: 1224–1232.
13. Haytham EL., Sayed Ali EL Sayed., Lotfy EL Sayed Morsy. (2019). Effect of Carbon Tetrachloride (CCL_4) on Liver in Adult Albino Rats: Histological study. The Egyptian Journal of Hospital Medicine Vol. 76 (6), Page 4254-4261.
14. Kisseleva T, Brenner DA. (2008). Mechanisms of fibrogenesis. Exp Biol Med;233:109–122.
15. Leeming DJ, Byrjalsen I, Jiménez W, et al. (2013). Protein fingerprinting of extracellular matrix remodeling in a rat model of liver fibrosis. Liver Int;33:439–447.
16. Sana J, Al-Merieb H. (2002). Optimization of dose and route of injection, and characterisation of the time course of CCl_4-induced hepatotoxicity in rats. J Pharmacol Toxicol Methods;48:41–48.
17. Dong S, Chen QL, Song YN, et al. (2016). Mechanisms of CCl_4-induced liver fibrosis with combined transcriptomic and proteomic analysis. J Toxicol Sci;41:561–572.
18. Bubnov RV, Drahulian MV, Buchek PV, et al. (2018). High regenerative capacity of the liver and irreversible injury of the male reproductive system in CCl_4-induced fibrosis. EPMA J;9:59–75.
19. Ding J, Yu J, Wang C, et al. (2005). Ginkgo biloba extract alleviates liver fibrosis induced by CCl_4 in rats. Liver Int;25:1224–1232.