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ORIGINAL RESEARCH

Establishment of a standardized mouse model of hepatic fibrosis for biomedical research

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Abstract— Liver injury causes nodule and scar tissue formation and diffuse fibrosis, which are characteristic of liver cirrhosis. Since there are currently no efficacious therapies to prevent fibrosis, the development of animal models of liver fibrosis is necessary to facilitate further *in vivo* studies of this pathology. In this study, a mouse model of liver fibrosis was generated using Swiss mice and carbon tetrachloride (CCl_4) treatment. Induction of liver fibrosis was analyzed using 0.8, 1.0, or 1.2 mL/kg CCl_4 to determine the effective dose. In this study, we aimed to develop a standardized hepatic fibrosis mouse model by using CCl_4 induction to facilitate further studies in this field. In Swiss mice, we evaluated the dose of CCl_4 and the criteria of fibrosis, such as serum markers, fibrosis marker genes, and histopathology. Mice were administered CCl_4 three times per week for 8 consecutive weeks. Body weights, survival rates, levels of serum markers (aspartate aminotransferase/alanine aminotransferase [AST/ALT]) and fibrosis markers (fibronectin, procollagen, nt5e, transforming growth factor-beta [TGF-β], and integrin), and histopathology (using hematoxylin and eosin [H&E] staining) were analyzed to determine the optimal dose of CCl_4 for induction of liver fibrosis. Results showed that 1.0 mL/kg CCl_4 was the most efficient dose for the establishment of a liver fibrosis mouse model. In a standardized liver fibrosis model, mice were treated with 1.0 mL/kg CCl_4 three times per week for 11 consecutive weeks, and levels of serum markers (AST, ALT, bilirubin, and albumin), expression of fibrosis marker genes (using quantitative reverse transcription polymerase chain reaction [RT-PCR]), histopathology (using Hematoxylin and eosin staining), and connective tissue formation (using Massive trichrome staining) were analyzed. The outcomes showed that serum markers and the levels of fibrosis marker genes were significantly increased in the standardized liver fibrosis model. Additionally, we observed sharp increases in fibronectin and procollagen expression (1222.40 ± 4.20 and 241.35 ± 1.18, respectively), and the development of cirrhosis (fibrosis stage 3–5/6) in liver tissues of the standardized mouse model of hepatic fibrosis.

Keywords— Animal model of liver disease, Hepatic fibrosis, Liver cirrhosis, Liver fibrosis, Liver fibrosis mouse model

INTRODUCTION

Although there are many causes of chronic liver disease, such as liver cell injury (by alcohol, chemicals, viral hepatitis, etc.), bile duct injury, autoimmune disease/genetic disease, and metabolic dysfunction/disorder (SJ, 2009), the consequences are the same. Liver injury causes nodule and scar tissue formation and diffuse fibrosis, characteristics encompassing liver cirrhosis, which leads to reduced liver function and increased risk of cancer (SJ, 2009). End-stage liver disease (ESLD) is the final result of acute or chronic liver injury (Heidelbaugh JJ, 2006). Moreover, 400 million people are infected with the hepatitis B virus worldwide (Kapp, 2009). In Asian countries, the percentage of individuals infected with the hepatitis virus has been reported to be as high as 10% (Li et al., 2012). ESLD is classified as the tenth leading cause of death, and 500,000–1,200,000 deaths occur each year because of the progression of viral infection to ESLD (Kapp,

2009).

Liver fibrosis is characterized by necrosis and inflammation, which increase numbers of Kupffer cells and activation of hepatic stellate cells, thereby contributing to the degeneration of liver tissues (GI-PPEUM LEE, 2005). The liver normally exhibits limited cell turnover; however, as soon as cell loss or damage occurs, a regenerative process is rapidly induced, functioning to recover and maintain organ functions (MR Alison, 2009). This process then leads to enhancement of extracellular matrix (ECM) production and the proliferation of parenchymal and/or nonparenchymal cells, resulting in fibrogenesis and diffuse hepatic fibrosis. Because cirrhosis is the final stage of fibrosis, further studies are required to determine the molecular mechanisms of cirrhotic changes.

Regardless of the etiology of fibrosis, animal models of liver fibrosis are appropriate for studying hepatic fibrosis and cirrhosis. Although no model that specifically represent the etiologies of human liver cirrhosis have been developed, several animal models of human liver diseases are currently used, including models induced by hepatotoxins, carbon tetrachloride (CCl_4) (Domitrovic et al., 2009; Ming-Ling Chang, 2005), 3,5- diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Ming-Ling Chang, 2005), silica, allyl alcohol (AA), alpha-naphthyl-isothiocyanate (ANIT), and bile duct ligation. These models have provided support for our understanding of the mechanisms of hepatic fibrosis and have enabled us to evaluate the safety and effectiveness of novel potential therapies for liver fibrosis and cirrhosis (Ming-Ling Chang, 2005; Starkel and Leclercq, 2011; Yan Liu, 2013). Among hepatotoxins, CCl_4 is often used to induce hepatic fibrosis and cirrhosis in animals because the underlying biochemical mechanisms and histological characteristics are similar to those observed in human liver cirrhosis (Constandinou et al., 2005; Fujii et al., 2010; GI-PPEUM LEE, 2005; Li et al., 2012). CYP2E1, an enzyme expressed in perivenular hepatocytes, metabolizes CCl_4 into the CCl_3^+ radical, which causes centrilobular necrosis and alters the permeability of the plasma and mitochondrial membranes of hepatocytes (Fujii et al., 2010). This induces inflammation and fibrogenesis and increases the generation of ECM, thereby triggering wound healing. Chronic CCl_4 exposure results in formation of nodules and fibrosis, products of the wound healing process. Many studies have shown that long-term CCl_4 administration causes significant changes in histology (Ming-Ling Chang, 2005; Starkel and Leclercq, 2011). Specifically, CCl_4 treatment has been shown to cause fibrosis after 2–4 weeks, significant bridging fibrosis after 5–7 weeks, cirrhosis after 9–11 weeks, and micronodular cirrhosis after 10–20 weeks (Starkel and Leclercq, 2011). However, the dose and duration of CCl_4 treatment required to induce such lesions depend on the species and strain of the animal model used, as well as the method (i.e., intraperitoneal, oral, or inhalation) and frequency of administration (Ming-Ling Chang, 2005; Starkel and Leclercq, 2011). Therefore, we aimed to develop a mouse model of liver cirrhosis using induction by CCl_4. This study mainly concentrated on analyzing the results of oral administration of CCl_4 in Swiss mice, which are popular in Vietnamese laboratories.

MATERIALS – METHODS

CCl_4-induced liver fibrosis/cirrhosis in mice

This study was approved by our Institutional Ethical Committee (Laboratory of Stem Cell Research and Application). To determine the optimal dose of CCl_4, healthy male Swiss mice were randomly divided into four groups (10 mice/group). Mice in groups I, II, and III were given 0.8, 1.0, or 1.2 mL/kg CCl_4 (99.5% purity, UNI-CHEM Chemical Reagent, China), respectively, via oral administration three times per week for 8 consecutive weeks, while mice in the control group were treated with olive oil. At the end of the drug-treatment period, liver fibrosis was assessed based on the following criteria: body weight, survival rate (during the drug treatment period), liver function (serum aspartate aminotransferase [AST], alanine aminotransferase [ALT]), expression of fibrosis/cirrhosis-related genes, and histology (using hematoxylin and eosin [H&E] staining).

For the establishment of a standardized liver fibrosis model, 20 mice received CCl_4 at the optimal dose determined as described above. Mice were treated with CCl_4 for 11 consecutive weeks in order to induce hepatic cirrhosis (Starkel and Leclercq, 2011). To analyze changes in the expression of fibrosis-related genes, such as fibronectin, integrin, nt5e, transforming growth factor-beta (TGF-β), and procollagen, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was conducted using GAPDH as a housekeeping gene and internal control. Combined H&E and Massive trichrome staining were used to evaluate the formation of connective tissues in the liver.

Liver function analysis (serum markers: AST, ALT, total bilirubin, and albumin)

Venous blood was collected into 1.5-mL tubes and then centrifuged at 3000 rpm for 10 min. Plasma was obtained, and the activities of AST and ALT (Diagnosticum Zrt., Hungary), as well as the levels of total bilirubin (QuantiChrom Bilirubin Assay Kit, Bioassay Systems, CA, USA) and albumin (QuantiChrom BCG Albumin Assay Kit, Bioassay Systems), were evaluated according to the manufacturer's instructions.

Evaluation of the expression of fibrosis biomarkers

Mouse liver tissues were collected, and total RNA was extracted using Easy-BLUE Total RNA Extraction Kit (iNtON Biotechnology, Korea), according to the manufacturer's instructions. For investigation of the optimal dose of CCl_4, fibrotic gene expression was evaluated by RT-PCR. For the

establishment of a standardized liver cirrhosis model, fibrotic gene expression was assessed by quantitative RT-PCR (Brilliant II QRT-PCR Master Mix Kit, 1-Step, Agilent, CA, USA) using primers specific for fibronectin (forward: TGAAGAGGGGCACATGCTGA; reverse: GTGGGAGTTGGGCTGACTCG), procollagen (forward: CCTGGACGCCATCAAGGTCTAC; reverse: CCAAGTTCCGGTGTGACTCG), integrin (forward: GCCAGGGCTGGTTATACAGA; reverse: TCACAATGGCACACAGGTTT), nt5e (CD73) (forward: TTTGGAAGGTGGATTTCCTG; reverse: CCTCTCAAATCCAGGGACAA), TGF-β (forward: CTTCAGCTCCACAGAGAAGAACTGC; reverse: CACAATCATGTTGGACAACTGCTCC), and GAPDH (forward: AAGTTGTCATGGATGACC; reverse: ATCACCATCTTCCAGGAGC).

Histopathology

Liver tissues were collected and fixed in 4% paraformaldehyde (Merck Millipore, Germany). H&E and Massive trichrome staining were performed according to the procedures of the Department of Pathology and Anatomy (University of Medicine and Pharmacy, HCM). The interpretation of results was based on the Knodell-Ishak histological activity index (Ishak Modified HAI).

Statistical analysis

Data analysis was conducted using GraphPad Prism 6 software and Microsoft Excel 2011.

Results

CCl_4 (1.0 mL/kg) caused significant weight loss in mice after 8 weeks of treatment

At week 4, the body weights of mice in the CCl_4 treatment groups were reduced compared to that of the control group. Mice in group II continued to gradually lose weight from week 0 to week 8 (from 27.91 ± 2.2 to 23.90 ± 2.51 g), and similar weight loss was recorded in group III. Meanwhile, mice in group I and the control group gained weight after 8 weeks of drug administration (Fig. 1A–C).

All doses of CCl_4 were associated with low mortality rates

After 8 weeks, dead mice were noted in all CCl_4 treatment groups after 20 doses of CCl_4 (Fig. 1D). The highest survival rate was 85.71%, recorded in groups I and II. The death ratio increased significantly with the increase in CCl_4 dose.

Serum markers of liver damage increased significantly in mice treated with 1.0 or 1.2 mL/mg CCl_4

In the control group (treated with olive oil), serum AST and AST activities did not change after 8 weeks (p >0.05). In group I, serum AST levels did not change before, during, or after drug administration. However, serum AST and ALT levels were significantly increased after 8 weeks of CCl_4 oral gavage in groups II and III. Serum AST increased by nearly 2.5-fold, while serum ALT rose nearly 5.0-fold after drug treatment (**Fig. 2**).

Changes in liver morphology after CCl_4 treatment

After 8 weeks of CCl_4 treatment, in the control group, the liver surface was glossy and had a bright red color. In contrast, in all treatment groups, there were multiple liver nodules, and the liver was slightly swollen, with a darkish discoloration throughout. These results supported that 8 weeks of CCl_4 administration clearly altered the liver structure and health. The liver tissue surface was no longer smooth, but had multiple nodules and had a darkish color. This implied that CCl_4 affected liver cells, causing liver damage and changes to the external morphology of the mouse liver.

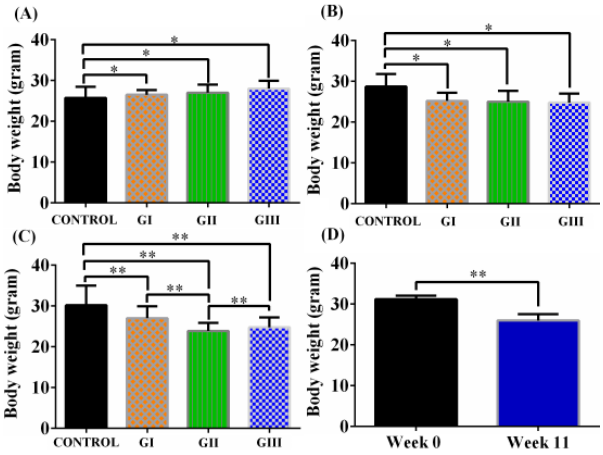

Figure 1. Body weight change in CCL4 treatment and control groups. A. Before CCL4 oral administration (Week 0); B. after 4 weeks of CCl4 treatment; C. after 8 weeks of CCL4 gavage administration. D. Body weight change before and after CCL4 (1.00 ml/kg/dose) treatment on standardized mouse model of hepatic fibrosis. Results are means and SD; Student's t-test, p<0.05: * non-significant difference; **: significant difference.

Expression of fibrogenesis- and ECM-related genes in the CCl_4-treated group

RT-PCR was performed to assess the expression of fibrogenesis- and ECM-related genes. After 8 weeks of drug treatment, fibronectin was expressed in the livers of mice in groups II and III, but not in those of the control group and group I. Procollagen α1 expression was higher in CCl_4-treated mice than in control mice (**Fig. 3**).

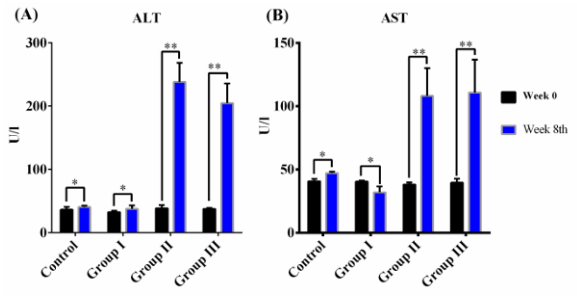

Figure 2. Level of serum AST and ALT in CCL4 treatment and control groups. Results are means and SD; Student's t-test, p<0.05: * non- significant difference; **: significant difference.

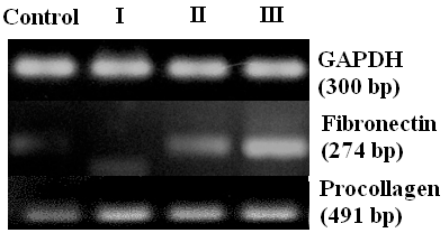

Figure 3. Reverse transcription –PCR analysis for GAPDH, fibronectin, and procollagen α1 was performed on mice.

Histopathology was altered in CCl_4-treated mice after 8 weeks of treatment

After 8 weeks, Hematoxylin and eosin staining results in all treatment groups showed different levels of liver fibrosis. Mice in the control group exhibited an inflammatory level of 1/18, without changes in the structures of blood vessels or bile ducts (**Fig. 4A**). Lymphocytes were present, but only mild inflammation and no signs of fibrogenesis were observed in the control group.

Table 1. Histological grading and staging of chronic hepatitis in experimental groups according to the Knodell-Ishak index (Ishak Modified HAI)

Group	Necroinflammatory Scores	Architectural changes, fibrosis and cirrhosis
Control	1/18	0/6
Group I	5/18	0/6, 1/6, 2/6
Group II	7/18	3–5/6
Group III	10/18	5/6

However, all mice in the CCl_4 treatment groups exhibited necrosis in some lobular areas and areas around the portal triad and central veins. Hematoxylin and eosin staining results showed fragmented nuclei, and lymphocytes and fibers were present (**Fig. 4B-D**). These data supported that liver tissues were replaced by connective tissues. All liver samples from mice treated with CCl_4 exhibited different levels of fibrosis, ranging from 3/6 to 5/6 according to the Knodell-Ishak index (Ishak Modified HAI) (**Table 1**).

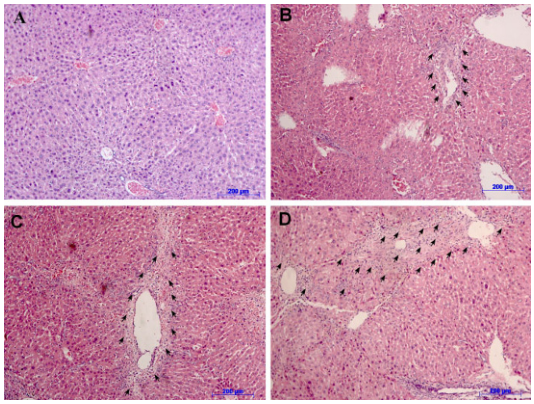

Figure 4. H&E staining in CCL4 treated – mouse livers and control liver after 8 weeks of CCL4 treatment. Black arrows indicate fiboris area in liver tissue.

From these results, we chose a CCl_4 dose of 1.0 mL/kg as the optimal dose for the standardized model since caused significant weight loss, low death rates, high levels of serum markers of liver damage, and marked changes in histopathology. Compared to other doses, 1.0 mL/kg CCl_4 was the best dose for further experiments. For establishment of a standardized liver fibrosis mouse model, we extended the CCl_4 treatment time to cause liver cirrhosis.

Table 2. Levels of serum markers in our mouse model of liver fibrosis after 11 weeks of treatment with 1.0 mL/kg CCl_4

	Control	Mouse model of liver fibrosis (CCL4 1.0ml/kg)
ALT (U/l)	40.639±2.018	412,181±90,640 *
AST (U/l)	47.153±1.116	242,584±126,325 *
Direct Bilirubin	0.1408±0.025	0.3283±0.053 *
Albumin	1.913±0.047	1.584±0.027 *

Results are means and SD; Student's t-test, p<0.05 (: There is significant difference with control group)*

Body weights were significantly reduced during 11 weeks of treatment with 1.0 mL/kg CCl_4 (Fig. 1D). Increase of ALT and AST activities demonstrated the effectiveness of CCl_4 for inducing liver toxicity. Additionally, levels of total bilirubin and albumin indicated the occurrence of liver dysfunction (**Table 2**).

Changes in the expression levels of fibrosis markers in fibrosis model mice

The results showed that the gene expression levels of fibronectin, TGF-β1, integrin, nt5e, and procollagen were altered compared to those of the control (Fig. 5). Integrin levels increased dramatically in our mouse model of liver fibrosis. Moreover, we observed sharp increases in fibronectin and procollagen expression (1222.40 ± 4.20 and 241.35 ± 1.18, respectively). The levels of TGF-β1 and nt5e expression were slightly elevated compared with those in the control group.

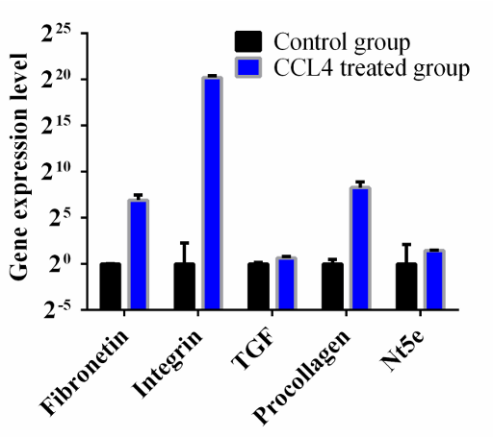

Figure 5. Gene expression analysis for fibronectin, integrin, TGF -β1, procollagen α1 and nt53 was performed on mouse model of liver fibrosis using quantitative RT-PCR. Analysis of relative gene expression data using Livak's method ($2^{-\Delta\Delta Ct}$ method). Gene expression level of control group was normalized.

Accumulation of fibers and connective tissue and changes in the histopathology of livers from our mouse model of hepatic fibrosis

The microstructure of livers from mice treated with CCl_4 differed significantly from that of normal livers from the control group (Fig. 6). Necrosis of hepatocytes was observed in the portal space and central lobular areas. Blood vessels and bile ducts exhibited accumulation of collagen fibers that gradually replaced healthy liver tissues. Collagen fibers occupied large areas of the liver, resulting in cytoplasmic shrinkage in hepatocytes and merging of cells, making it difficult to distinguish between cells. Fibrosis stages in these model mice were 3/6, 4/6, and 5/6. Massive trichrome staining also indicated that livers accumulated connective tissues, including collagen (Fig. 6). This illustrated that repetitive dosing of 1 mL/kg CCl_4 (three times a week for 11 weeks) caused hepatic cirrhosis in Swiss mice.

Discussion

Because CCl_4 is toxic and can cause mortality, establishment of a mouse model of liver fibrosis is challenging. According to our current study, gavage is suitable for CCl_4 administration as it resulted in low mortality compared to intraperitoneal administration of CCl_4 (Ming-Ling Chang, 2005). Moreover, estimating the degree of fibrosis in animal models is critical for determining whether therapeutic treatment could be effective. Traditionally, liver biopsy is considered the 'gold standard' for staging liver fibrosis. However, recent data have shown that there is a 30% failure/error rate in diagnoses by liver biopsy (Mahato, 2007). Therefore, we used other techniques to accurately determine the liver fibrosis stage (Mahato, 2007). To evaluate whether CCl_4 can cause acute liver injury in Swiss mice, we relied on indirect markers, such as serum AST, ALT, total bilirubin, and albumin. Additionally, to confirm liver fibrosis, we evaluated fibrosis biomarkers/direct markers by qRT-PCR and classified liver histology based on the Knodell-Ishak (Ishak Modified HAI) scoring system.

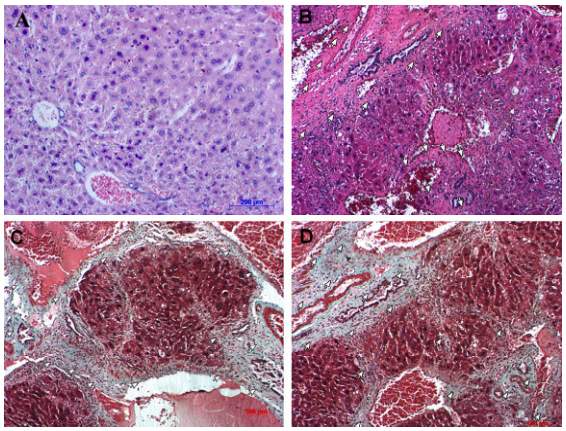

Figure 6. After administration of CCl4 (1ml/kg dose) for 11 weeks, liver morphology changed distinctly. The entire surface of liver became rough and shrink. A. H&E staining in control group: B. H&E staining in liver fibrosis mouse model; C&D. Massive Trichrome staining in liver fibrosis mouse model. White arrows show hepatic fibrosis area.

Increases in ALT and AST levels may indicate the occurrence of liver necrosis (Field et al., 2008). When liver cells are damaged, intracellular enzymes (including transaminase enzymes) leak into the blood and can be measured as indicators of cell necrosis. In our model, the level of serum ALT increased sharply in the context of hepatitis and acute liver injury. The results of AST and ALT levels in the control group were consistent with other reports (Domitrovic et al., 2009). In group I, we observed insignificant changes in serum ALT and AST before and after CCl_4 treatment, suggesting that injured liver cells were likely to be restored because of low CCl_4 concentrations (Mederacke, 2013). Moreover, increased AST and ALT levels in groups II and III were equivalent to those reported by Tsai et al (2009) (Tsai et al., 2009). These results demonstrated that acute liver injury and inflammation occurred in our mouse model. The increases in AST and ALT levels in liver fibrosis induced by 1.0 mL/kg CCl_4 supported that these mice exhibited substantial hepatic injury. A sharp increase in total bilirubin levels and a drop in albumin levels indicated loss of function in the mouse liver. These results were similar to those reported by Ohashi et al. (2012)(Ohashi et al., 2012). Furthermore, our data showed

that the ratio of AST/ALT in our mouse model was less than 1, supporting the occurrence of acute hepatitis (Thapa and Walia, 2007). In order to confirm the presence of hepatic fibrosis, fibrosis markers and histopathology should also be evaluated.

Fibronectin and other proteins of the ECM create a framework for fibroblast migration. Fibronectin also provides direction and chemical signals for fibroblast proliferation (Armbrust et al., 2004). Increased procollagen synthesis contributes to the increase in collagen levels observed in liver cirrhosis, and abnormal mRNA expression of procollagen α1 reflects the level of fiber in liver (Du WD, 1999). In this study, we observed increased procollagen expression in CCl_4-treated mice compared to that of normal mice. Because procollagen is a direct marker of fibrosis, abnormally high levels of procollagen indicate that liver tissues have accumulated scar nodules, accompanied by hepatitis and hepatic necrosis. In addition, high levels fibronectin (Attallah et al., 2013), TGF-β1 (Gressner et al., 2002), and integrin (Stickel, 2011) expression may activate cells, such as fibroblasts and hematopoietic stem cells (HSCs). Nt5e has been shown to contribute to increased adenosine levels, which have an important role in hepatic fibrosis (Peng et al., 2008). Indeed, synthetic ecto-5'-nucleotidase expression leads to increased levels of extracellular adenosine (Peng et al., 2008). The increase in adenosine in injured liver tissue activates the cells through the A2A receptor, enhancing collagen synthesis and expression (Che et al., 2007) and eventually leading to liver cirrhosis.

In this study, we applied the Ishak Modified HAI system to classify histological grading and staging of chronic hepatitis and fibrosis in mice. The results showed changes in hepatic histopathology, accumulation of ECM, and formation of nodules and fiber bridges. Our results of liver tissue histopathology were similar to the Massive trichrome staining results reported by Hao et al. (2012)(Hao et al., 2012).

Conclusion

Based on the results of biochemical tests, anatomical observations, histological staining, and gene expression analysis, we concluded that oral administration of 1.0 mL/kg CCl_4 for 11 weeks (three times per week, or once every two days) was sufficient for high-efficiency induction of liver fibrosis in Swiss mice. This mouse model of fibrosis had meaningfully changes in gene expression, as well as liver structure and function.

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Abbreviations

AST aminotransferase; ALT Alanine aminotransferase; CCl4: carbon tetrachloride; CYP2E1: Cytochrome P450 2E1; ESLD: End stage liver disease; ECM: extracellular matrix; H&E: Hematoxylin and Eosin; Nt5e: ecto-5'-nucleotidase; TGF-beta: transforming growth factor beta

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Truong Hai Nhung made substantial contributions to conception and design, acquisition of data and analysis and interpretation of data. Being corresponding author, Truong Hai Nhung gave final approval of the manuscript to be submitted and any revised version. Nguyen Hai Nam and Nguyen Thi Kim Nguyen who contributed to conduct some experiments, acquisition of data and participate in drafting the manuscript. Le Minh Huy and Tran Huong Giang made substantial contributions to analyze the histology change by using Knodell-Ishak (Ishak Modified HAI) scoring system. Huynh Nghia and Nguyen Van Thanh are supervisors of this study.

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