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ORIGINAL

"INTERLEUKIN-7 AS A POTENTIAL THERAPEUTIC TARGET FOR ALLEVIATING HEPATIC FIBROSIS IN A MICE MODEL INDUCED BY CARBON TETRACHLORIDE: IMPLICATIONS FOR FITNESS AND EXERCISE"

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Abstract

The effects of interleukin-7 (IL-7) on the carbon tetrachloride (CCL_4) induced hepatic fibrosis were investigated in this study. Thirty-six female BALB/C mice were randomized into group A (control group) injected with saline, group B (fibrotic model group) and group C (IL-7 intervention group). Histopathological changes were observed by HE, Masson as well as reticular fiber staining. The apoptosis cells and hepatic stellate cell (HSC) were detected from the tissues, and the expressions of *Bax* and *Bcl-2* gene were also detected. The results of histological HE, Masson and reticular fiber staining showed that compared with group B, the degree of inflammation and fibrosis of the tissue were statistically reduced in group C. Compared with sub-group B and C, the degree of reduce inflammation of the liver and inhibit hepatic fibrosis were more obviously with the extension of treatment time. The inflammatory activity and liver fibrosis score were statistical significant between groups ($P<0.05$), the highest score was group B, followed by group C. The apoptosis cells were similar between fibrotic model group and IL-7 intervention group, while the HSC count was obviously higher in group B compared to the other two groups. The *Bax* gene was up-regulated when intervened with IL-7 for hepatic fibrosis and *Bcl-2* showed to the contrary. IL-7 could inhibit hepatic fibrosis in mice induced by CCL_4 and **reduce** liver inflammation process. The anti-fibrosis mechanism

might be involved in inducing apoptosis through P53 pathway regulated *Bcl-2* and *Bax* genes.

KEYWORDS: carbon tetrachloride; hepatic fibrosis; Interleukins-7

1. INTRODUCTION

The pathological changes of fibrosis refer to the abnormal and overproduction of extracellular matrix (ECM) proteins, collagen (COL) and fibronectin in particular, secreted by fibroblasts (FBs) and FB-like cells. Unlike the wound healing process, the persistent presence of eosinophils, monocyte-macrophages, lymphocytes, and neutrophils results in elevated cytokine levels and suppressed enzymes that degrade ECM proteins (Shao, Suresh, Vakil, Gomer, & Pilling, 2008), all of which give rise to increased ECM protein deposition, a condition considered to be associated with repeated injury. Fibrosis leads to dysfunction of fibrotic lesions in tissues or organs, as well as changes in types and cells within tissues.

Fitness and exercise enthusiasts stand to benefit from advancements in hepatic fibrosis research, particularly regarding IL-7 as a potential therapeutic target. The liver plays a vital role in energy metabolism, including the storage and mobilization of glycogen, metabolism of fats, and detoxification processes. Any disruption in liver function, such as hepatic fibrosis, can impact an individual's ability to perform at their peak physical capacity. Therefore, identifying interventions that can alleviate hepatic fibrosis may contribute to the preservation of liver health and consequently enhance overall fitness and exercise performance.

Moreover, engaging in regular exercise has been shown to confer protective effects on liver health and reduce the risk of developing liver diseases. By exploring IL-7 as a therapeutic target for hepatic fibrosis, researchers may uncover insights into how exercise-induced mechanisms can potentially modulate IL-7 expression and activity. Such discoveries could pave the way for tailored exercise regimens that optimize liver health and mitigate the risk of hepatic fibrosis in fitness and exercise enthusiasts

(Wynn, 2004). These cytokines stimulate FBs-induced COL production and deposition, promoting FB activation and differentiation to myofibroblasts, the active cell type (Conlon et al., 1989). A number of fibrotic diseases, including systemic sclerosis, asthma, hepatic/cardiac fibrosis, rheumatoid arthritis, thermal injury, hypertrophic scars, and pulmonary-fibrosis pathologies, are shown to be related to Th-2 inflammatory disorders (Reiner & Locksley, 1995), and elevated levels of profibrotic cytokines like TGF-β, IL-4, IL-10, and IL-13 in these diseases seem to be correlated with disease progression.

Interleukin-7 (IL-7) is an important cytokine with extensive effects.

Studies have shown that IL-7 might inhibit fibrosis by interfering with the epithelial mesenchymal transition signal pathway (Wu et al., 2018). Previous studies indicated that IL-7 had anti-fibrotic action both in vivo and in vitro. IL-7 inhibited TGF-β pathway via inducing Smad7 (Powell et al., 1999; Seminario, 2019). In addition, TGF-β combined with active PKC-δ to stimulate several target cellular genes including pulmonary FBs (Dewald et al., 2004). At present, the effect and mechanism of IL-7 on liver fibrosis have not yet been reported. Therefore, in this study, we intended to build the mice model of hepatic fibrosis, intervened by using IL-7, and analyzed the effect of IL-7 on the pathological changes of liver tissue with hepatic fibrosis and its relevant factors.

2. Materials and methods

2.1 Animals used in this study

Thirty-six clean grade healthy female BALB/C mice, weighted 20±2g, were provided by the Department of animal science and technology of Nanchang University; IL-7 was provided by the Chimerigen company (United States).

2.2 Animal model construction

All the BALB/C mice were freely fed for one week prior to experiments. Thirty-six mice were randomized into 3 groups named group A, B and C. Furthermore, each group was stochastically separated into two sub-groups, named group A1, A2, B1, B2, C1 and C2. Group A: the normal control (n=12), all the mice were subcutaneous injected of saline 3 mL/kg, 2 times/week; group B: fibrosis model (n=12), the mice were subcutaneous injected of 20% CCL_4 (olive oil dilution) 3 mL/kg, 2 times/week; group C (n=12): intervention group. The animal model was constructed as mentioned above, then intramuscular injected of IL-7 10 μg, one time/day. Mice in subgroups A1, B1 and C1 were sacrificed after two weeks, while those in the other three subgroups were sacrificed after four weeks. All the animals were fasting prohibited before 12 h of the death. The mice were anesthetized by using ether, and the right lobe of the liver was fixed with 10% formalin, paraffin embedded and sliced for the test usage.

2.3 Histopathological examination

The liver tissue was performed HE staining to observe the pathological changes, the degree of type I COL hyperplasia was observed by Masson staining, and type III COL hyperplasia was shown by reticular fiber staining method. The histopathological criteria of inflammation activity and fibrosis degree were based on the revised standard for the prevention and treatment of viral hepatitis 2000-09. Ishak scoring system was used for analysis. Five visual fields of each slice were randomly selected and recorded the inflammation

grade, fibrosis stage and Ishak score (Friedman, 2000).

2.4 Histopathological detection for apoptotic cells and hepatic stellate cells

The mice were anesthetized mentioned above, and the livers were fixed with 10% formalin, paraffin embedded and sliced for the examinations. TUNEL method was used for labeling the apoptotic cells, and immune-histochemical method was used to detect hepatic stellate cell (HSC). All the experimental procedures were based on the manufacturers' instructions; the DeadEnd Colorimetric TUNEL system (Promega) and HSC specific antibody (Abcam production) were used respectively. Five visual fields counting positive cells were taken randomly under 10 times of microscope.

2.5 The expressions of Bax and Bcl-2 gene in liver tissues

Isolation of total RNA from cells was carried out following the manufacturer's recommendations of TRIzol (Invitrogen, Carlsbad, CA, USA). Then, with the use of SYBR Green PCR master mix and 7500 Fast Real-Time PCR System both supplied by Applied Biosystems, quantitative real-time PCR was performed with a final volume of 20 μl and under the amplification cycle of 95°C for 30 s, and 40 cycles of 95°C for 5 s, and 60°C for 30 s. A melting curve to confirm the amplification specificity was generated in dissociation step. Genes' relative expression relative to β-actin was shown as ΔCt =Ct gene-Ct reference, and the fold change was calculated using the formula 2-ΔΔCt. Each experiment was repeatedly determined three times.

See Table 1 for primer sequences.

gene	The primer sequences 5′→3′	product length（bp）
β-actin	Upstream:5'-GAGACCTTCAACACCCCAGC-3' Downstream:5'-CCACAGGATTCCATACCCAA-3'	446bp
Bax	Upstream:5'-CCAAGAAGCTGAGCGAGTGT-3' Downstream:5'-GTGTCCAGCCCATGATGGTT-3'	255bp
Bcl-2	Upstream:5'-GTCGCTACCGTCGTGACTTC -3' Downstream:5-CAGACATGCACCTACCCAGC-3'	284bp

Note: the whole sequence of the target gene was obtained by Pubmed Nucleotide in primer design, analyzed and designed by primer design software, and synthesized by Shanghai invitrogen Biological Engineering Co., Ltd.

2.6 Ethics statement

Animal experiments were performed in adherence to the principles proposed by the Second Affiliated Hospital of Nanchang University. The Institutional Review Board at the Second Affiliated Hospital of Nanchang University approved this study protocol without reserves.

2.7 Statistical analysis

The experimental data was analyzed by using SPSS17.0 software, the six groups of liver tissue inflammation and hepatic fibrosis staging were analyzed by using semi quantitative assessment system (Ishak) score, ANOVA analysis and multiple comparisons (least significant difference, LSD), with the significance level set as $P<0.05$.

3. RESULTS

3.1 Liver tissue macroscopic observations

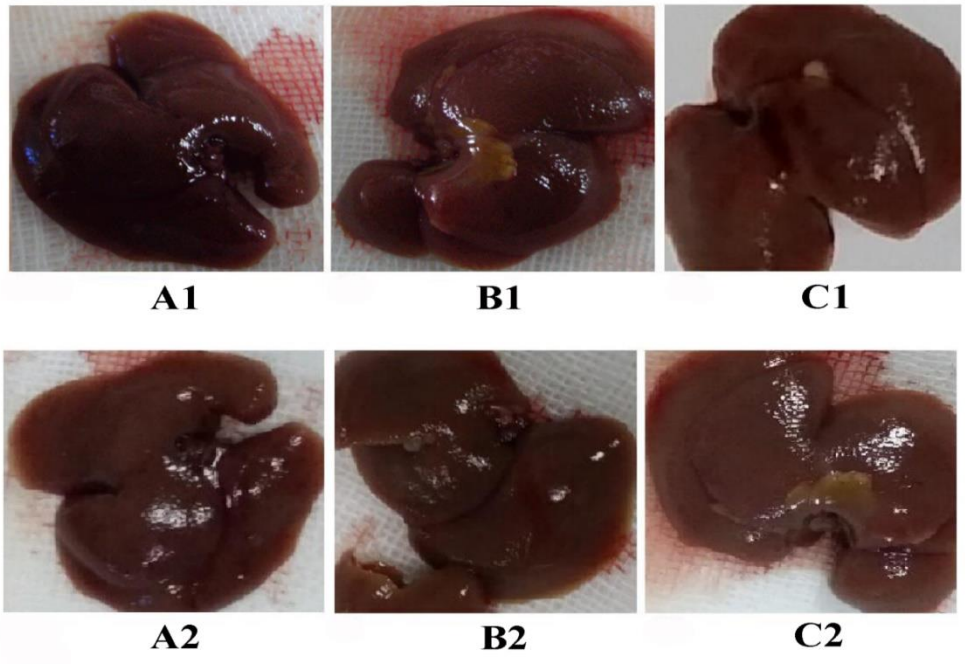


Figure 1. The observation of liver morphology for different experimental groups in this study.

The group A showed normal configuration of liver tissues, membrane finishing, ruddy color and soft texture; the liver was shown dark red, rough appearance, particles, and qualitative hard in model group (B1 and B2), B2 was more rough than B1; compared with group B, the liver appeared smooth, less particles, soft quality, and less bleeding in group C. The liver appearance was smooth, soft, small particles, less bleeding in group C2 compared with group C1 (Figure 1).

3.2 Light microscope examination

The results of light microscopy showed that there was no infiltration of inflammatory cells in group A for HE staining. The grade of inflammatory activity was in G_0 stage. No fibrosis was shown for Masson and reticular fiber staining, the fibrosis stage was in S_0 stage. In group B1, HE staining showed bile duct regeneration, portal area appeared severe hepatitis, and some lobular

confluent necrosis, necrosis lesions >10/10 times objective; the moderate periportal inflammation was also observed, and inflammation grade was in G_4 phase; Masson and reticular fiber staining showed the fibrous expansion near the portal area, there were manlike fibers and fiber spacing, and the degree of fibrosis staging in S_2 phase. The similar histopathological changes could be seen in group B2, further with obvious P-P and P-C bridging necrosis, and fibrosis staging was in S_3 phase. In group C1, HE staining showed small bile duct hyperplasia, visible hepatocyte ballooning degeneration, portal area had severe interface hepatitis, part of the region had three area of necrosis, spotty necrosis lesions >10/10 times objective, significant portal inflammation could be seen, and inflammation grade was in G_3 phase; Masson and reticular fibers staining showed fibrosis in portal area, and the degree of fibrosis staging was in S_1 stage. The similar histopathological changes could be found in group C2, while the inflammation grade was in G_2 phase, as shown in Figure 2.A-C.

A: HE staining; B: Masson staining; C: reticular fiber staining.

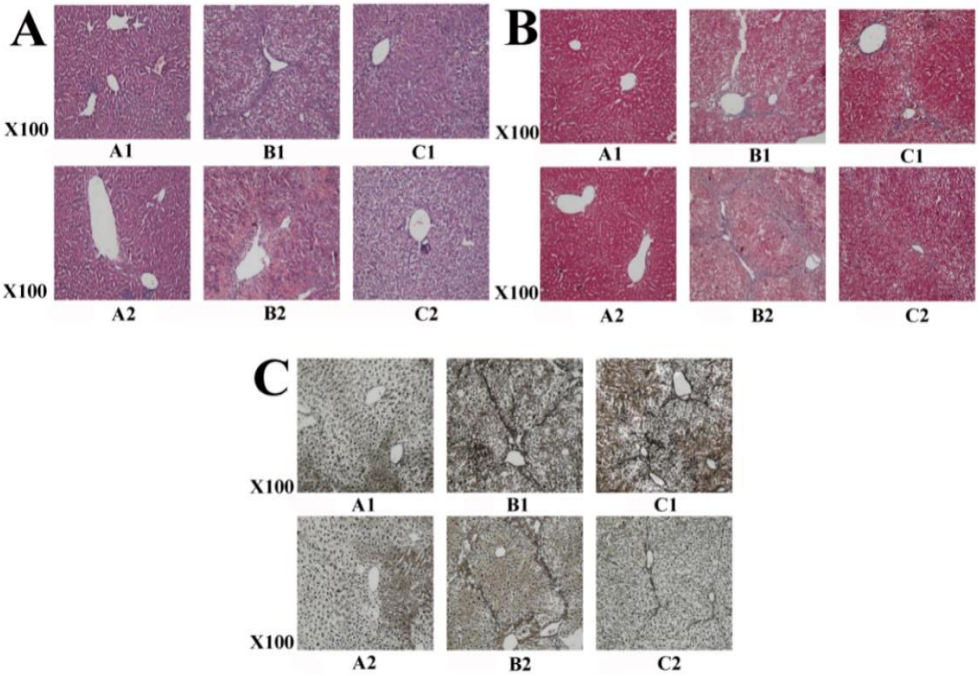


Figure 2. Histopathological examination for different experimental groups.

3.3 Ishak score

The results of inflammatory activity and liver fibrosis score were shown in Table 2 and Table 3. Statistical significance was present in inflammatory scores among groups A, B and C ($P<0.05$), with the highest score found in group B, followed by group C. For sub-group C, groups C1 and C2 showed significance difference ($P<0.05$), C2 group had lower inflammatory score than group C1. The liver fibrosis scores showed that group B2 and group C had statistical difference with group A ($P<0.05$), group B2 and group C showed difference with group B1 ($P<0.05$).

Table 2 The results of inflammatory activity score

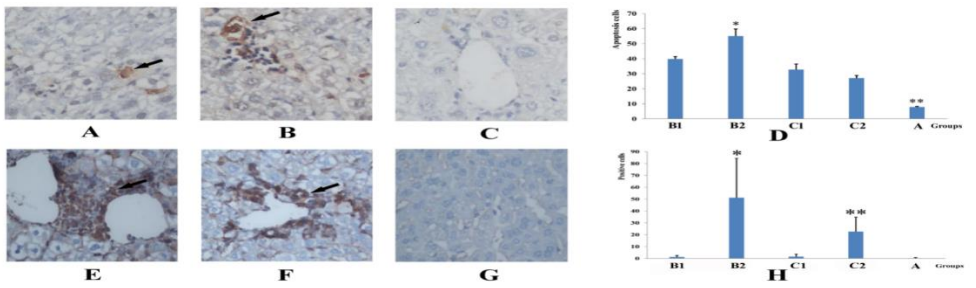
group		0-3	4-6	7-9	10-12	13-15	16-18
A	A1	5	1	0	0	0	9
	A2	4	2	0	0	0	0
B	B1	0	0	0	2	3	1
	B2	0	0	0	0	4	2
C	C1	0	0	2	2	2	0
	C2	0	1	2	2	1	0
Z		28.216					
P		0.001					

Table 3 The results of liver fibrosis score

group		0	1	2	3	4	5	6
A	A1	2	4	0	0	0	9	0
	A2	4	2	0	0	0	0	0
B	B1	0	0	0	0	3	1	2
	B2	0	0	0	0	1	2	3
C	C1	0	0	0	1	2	2	1
	C2	0	0	0	3	2	1	0
Z		26.312						
P		0.001						

3.4 Apoptotic cells and HSC detection

Under the microscope, there were yellow brown irregular cells in the hepatic and hepatic sinusoids as apoptotic cells, and the apoptosis index of each group was statistical different (Figure 3.A-D). Group B and C had statistical significance with group A, and group B2 showed the difference with group C1 and C2 ($P<0.05$). For HSC results, the positive expression of brown cells indicated the presence of activated hepatic stellate cells, as shown in Figure 3. E-H. Group B2 and C2 had statistical difference with other groups, and difference were still found between these two groups ($P<0.05$). The results indicated that apoptosis cells were similar between fibrotic model group and IL-7 intervention group, especially for group B2 with group C1 and C2; while for HSC, statistically higher HSC counts were determined in group B versus group C and control.



A:

Figure 3. Apoptotic cells and HSC detection results.

Apoptosis cells for fibrosis model group; B: Apoptosis cells for IL-7 intervention group; C: control; D: comparison the apoptosis cells for different groups; E: HSC for fibrosis model group; F: HSC for IL-7 intervention group; G: control; H: comparison the HSC for different groups.

* Indicated that difference with other groups in apoptosis cells and HSC ($P<0.05$).

3.5 The expression of Bax and Bcl-2 gene

The PCR results showed that proapoptosis gene *Bax* was down-regulated in group B compared with group C and control, which indicated that the *Bax* was up-regulated when intervened with IL-7 for hepatic fibrosis (Figure 4.A and B). However, the anti-apoptosis gene *Bcl-2* showed to the contrary. The *Bcl-2* gene was up-regulated in group B, and down-regulated in group C and control, as shown in Figure 4.A and C.

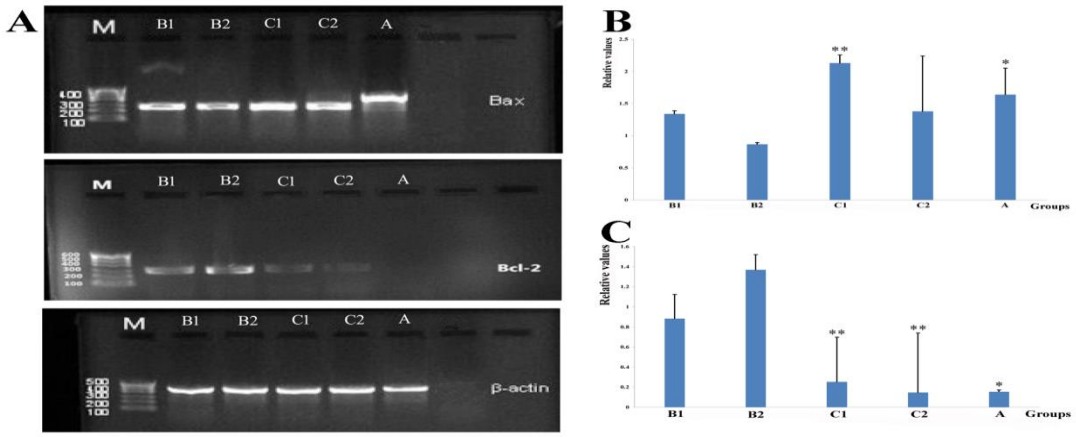


Figure 4. The expression of Bax and Bcl-2 gene in this study.

A: PCR electrophoresis results; B: The relative values for *Bax* gene expression; C: The relative values for *Bcl-2* gene expression.

* Indicated that statistical difference ($P<0.05$).

4. DISCUSSION

Fibrosis was the commonly chronic liver disease pathological basis, such as viral hepatitis, and alcoholic and autoimmune liver diseases. The intrahepatic fibrous connective tissue proliferated and synthesized of extracellular matrix (ECM) in the liver was greater than degradation, resulted in excessive ECM deposition. Local FBs migrated to the inflamed area and produced ECM proteins, inducing either fibrosis or wound healing (Bikker, Erik Hack, PJG Lafeber, & AG van Roon, 2012). There is a hypothesis indicating that bone marrow-derived precursors circulating in the blood were located at the site of injury, and they then differentiated into fibrocytes that mediated at least tissue repair and fibrosis (Hsieh et al., 2012).

Fibrocytes can express hematopoietic cells markers (CD45, CD34, MHC class II) as well as stromal cells makers (fibronectin, and COL types I and III) (Huang et al., 2002). Fibrocyte precursors can be differentiated from $CD14^+$ peripheral blood monocyte subsets. Fibrosis was a reversible pathological process, of which 25%-40% eventually developed into liver cirrhosis and liver cancer, seriously endangered people's physical and mental health. At present, studies on anti-fibrosis showed that several drugs or biological agents, such as interferon, lamivudine, colchicine, angiotensin II receptor blocker, hepatocyte growth factor could inhibit liver fibrosis to a certain extent (Ishak, 1995). However, the excessive toxicity or resistance ability of these drugs or agents limited the application in clinical usages. Therefore, it was urgently to find a safe and effective anti-fibrosis drug at the present stage.

IL-7, a bone marrow stroma cell-derived 25-kDa glycoprotein (Crouch, 1990), is defined as a pre-B lymphocyte growth factor, which has been subsequently revealed to enhance T lymphocyte growth (Bucala, Spiegel, Chesney, Hogan, & Cerami, 1994). Previous studies have indicated that IL-7 could stimulate T cell function and IFN-γ generation. In combination with IL-12, IL-7 induced the multiplication, cytotoxicity, as well as IFN-γ release of T cells (Abe, Donnelly, Peng, Bucala, & Metz, 2001). Consistent with our research results, IL-7 is reported to interfere with cell-mediated immunological responses of type I cytokines (Quan, Cowper, Wu, Bockenstedt, & Bucala, 2004). IL-7 has also shown to reduce TGF-β production in fibrosarcomas, macrophages, and melanomata (Bellini & Mattoli, 2007). And in murine macrophages, IL-7 down-regulated TGF-β gene transcription in an IFN-γ-independent manner (Kalluri & Weinberg, 2009). IL-7 was an important cytokine with broad effect, in the inflammatory process, macrophages, dendritic cells and FB cells could secrete IL-7, regulated the anti-tumor, anti-viral, immune response, and simultaneously had the function of anti-fibrosis. Recent studies also showed that IL-7 had the effect on anti-pulmonary fibrosis and renal fibrosis. At present, the related research achievements on IL-7 were mainly focused on the treatment of tumors, AIDS, autoimmune diseases, and the initial effect on inhibiting pulmonary fibrosis and renal fibrosis. However, the study on liver fibrosis has not been reported yet (McKleroy, Lee, & Atabai, 2013; Schuppan & Kim, 2013).

In this study, degrees of inflammation and fibrosis were significantly higher in group B than group C, and inflammatory activity score showed difference; the inflammation and fibrosis scores of group B2 were significantly higher than group C2, the differences were statistically significant. These results suggested that IL-7 could obviously reduced CCL_4 induced liver fibrosis, showing anti-fibrosis effect. Compared in model group, inflammation activity score of group B2 was slightly higher than group B1, while, the fibrosis score was significantly higher, which indicated that along with the time extending, liver fibrosis was more obviously by CCL_4 model construction. The inflammatory score of group C2 was significantly lower than group C1, which suggested that

the effect of IL-7 on liver inflammation and anti-fibrosis had positively correlated with the time of intervention. Apoptosis played an important role in tumorigenesis and immune response process. IL-7 signaling pathway was associated with survival and apoptosis in B and T cells. Several studies on IL-7 and apoptosis relations have focused on B and T cellular homeostasis, whereas few studies examined their role in hepatic fibrosis. In our study, the IL-7 intervened with hepatic fibrosis showed highly apoptosis ability and inhibited the HSC, and the anti-fibrosis mechanism might be involved in inducing apoptosis through P53 pathway regulated *Bcl-2* and *Bax* genes. The protective effects of IL-7 against liver fibrosis might be due to its ability to inhibit HSC activation, and influenced *Bax*, *Bcl-2* expressions. However, other mechanisms might be involved in the protective effects. IL-7 might be offer a novel therapeutic strategy for treating liver fibrosis, but there was still much work to do.

5. CONCLUSION

In conclusion, investigating the potential of IL-7 as a therapeutic target for alleviating hepatic fibrosis in a mice model induced by carbon tetrachloride not only holds promise for medical interventions but also carries implications for individuals involved in fitness and exercise activities. Understanding the interplay between IL-7, hepatic fibrosis, and exercise-related mechanisms may contribute to the development of targeted interventions that improve liver health, enhance fitness performance, and reduce the risk of liver-related complications in fitness and exercise enthusiasts.

Data Availability Statement

The labeled dataset used to support the findings of this study are available from the corresponding author upon request.

Conflict of interest

The author declares no competing interests.

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