1,25(OH)$_2$D$_3$ REGULATES RAT HSC ACTIVATION AND PROLIFERATION BY REGULATING IL-17-MEDIATED IMMUNE MICROENVIRONMENT

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ABSTRACT

Objective*: To analyze 1,25(OH)$_2$D$_3$ regulation of the activation and proliferation of rat HSCs by regulating the immune microenvironment mediated by IL-17.*

Methods: *Forty SD rats were randomly divided into normal (10 rats), model (10 rats), drug control (10 rats) and treatment (10 rats) groups. Rats in each group except the normal group were injected with 40% carbon tetrachloride (peanut oil solution was used as menstruum) subcutaneously to establish liver fibrosis models, and the rats in the treatment group were orally administered with 1,25(OH)$_2$D$_3$ solution (peanut oil solution was used as menstruum) from the day of modelling, and the drug control group was administered with an equal amount of peanut oil vehicle orally, 1 d/time. Four items of liver fibrosis, serum 25(OH)D$_3$ concentration and IL-17 level were detected in each group; HE staining was used to observe the pathological changes of liver tissue in each group of rats; A Western blot method and real-time quantitative PCR were used to detect the expression of IL-17, MIP3α, RORγt protein and mRNA in liver tissue of rats in the groups, respectively.*

Results: *The four fibrosis indicators of rats in both model and drug control groups were significantly higher than those in the normal group (P<0.05), and the indicators in the treatment group were significantly lower than those in the drug control group (P<0.05); The concentration of 25(OH)D$_3$ in both control and drug control groups was significantly lower than that in the normal group, and the level of IL-17 was significantly higher than that in the normal group (P<0.05). The concentration of 25(OH)D$_3$ in the treatment group was significantly higher than that in the drug control group. The level of IL-17 was significantly lower than that of the drug control group (P<0.05). The pathological changes of liver tissue in each group showed that the degree of inflammation and fibrosis of liver cells was significantly reduced in the treatment group compared with the drug control group. The expressions of IL-17, MIP3α, RORγt, mRNA and protein in liver tissue of rats in model group and drug control group were significantly higher than those in the normal group, and the difference was statistically significant (P<0.05). Those expressions in the treatment were significantly lower than those of the drug control group, and the differences were statistically significant (P<0.05).*

Conclusion: *1,25(OH)$_2$D$_3$ has a significant inhibitory effect on the formation of rat liver fibrosis and can significantly reduce the expression of IL-17 and its related factor proteins and genes in rat liver tissue, suggesting that 1,25(OH)$_2$D$_3$ may regulate the activation and proliferation of rat HSC and prevent the development of fibrosis by regulating the IL-17-mediated immune microenvironment.*

Keywords: *1,25(OH)$_2$D$_3$, IL-17, immune microenvironment, HSC, activation, proliferation.*

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Introduction

Hepatic fibrosis refers to the pathophysiological process of abnormal proliferation of connective tissue in the liver caused by various pathogenic factors that is a response of the body to chronic liver damage caused by various causes and is involved in any liver repair and healing process[(1)]. There are many causes of liver fibrosis. Viral hepatitis, fatty liver and autoimmune diseases are more common clinically. The lesions are essentially activated hepatic stellate cells (HSCs) that synthesize and secrete an extracellular matrix that can be deposited in cells, leading to the occurrence and development of liver fibrosis; hence, the activation of HSC is the central

link for liver fibrosis formation[2-3]. However, there is currently no clear and effective treatment method to reverse liver fibrosis in the clinic. In recent years, some scholars have discovered that fibroblasts can be activated by interaction with immune cells, and then participate in the occurrence and development of liver fibrosis[4]. Inflammation is an important part of the immune system. Relevant data[5] have demonstrated that the initiating factor of liver fibrosis is that the affected liver cells are attacked by lymphocytes, causing a large number of HSC to activate and proliferate; therefore, it was speculated that the immune cells may be potential therapeutic targets for liver fibrosis. IL-17 is an effector synthesized and secreted by helper T lymphocytes 17, and studies have confirmed that it positively correlated with liver fibrosis levels[6]. 1,25$(OH)_2D_3$ is the active form of vitamin D. Recent studies have found that cytokines such as IL-17 and IL-23 may be involved in the immune response of vitamin D in liver diseases[7]. This study aims to analyze how 1,25$(OH)_2D_3$ regulates the activation and proliferation of rat HSCs by regulating the immune microenvironment mediated by IL-17.

Materials and methods

Experimental animals

Forty SPF healthy male SD rats were selected, weighing 180-240 g, and all were purchased from Beijing Huafukang Biotechnology Co., Ltd. (animal license number: 170221). They were fed with standard pellets in the animal experiment centre of our hospital, with the room being well ventilated at 22~24 °C and 40%~70% RH. They were provided with free drinking and feeding, a 12-h light cycle and adaptive feeding for one week for this study.

Experimental reagents and instruments

1,25$(OH)_2D_3$ was purchased from the PeproTech company in the United States; carbon tetrachloride solution was obtained from the Shanghai Mecklin Biotechnology Co., Ltd.; chloral hydrate was purchased from the Shandong Baihong New Material Co., Ltd.; peanut oil was purchased from the Shandong Luhua peanut oil Co., Ltd .; ELISA kits were obtained from the Shenzhen NEOBIOSCIENCE Co., Ltd.; haematoxylin and eosin were bought from the Shanghai Gefan Biotechnology Co., Ltd.; Trizol reagents were purchased from the Invitrogen Company of the United States; IL-17, MIP3α, RORγt rabbit source polyclonal was bought from Abcam, USA. High-speed cryogenic centrifuges, pipettes and microplate readers were purchased from Eppendorf, Germany; -80 °C ultra-low temperature refrigerators and 4 °C refrigerators were obtained from the Qingdao Haier Co., Ltd.; an optical microscope was purchased from the Japanese Olympus Company; the electrophoresis, vertical electrophoresis tank and transfer tank were obtained via the Beijing Liuyi Instrument Factory; the PCR amplification instrument was purchased from the American ABI Company; the PVDF membrane was obtained from the American Millipore company; the spectrophotometer was purchased from the Thermo Fisher Scientific Co., Ltd.

Establishment and group intervention of experimental animal models

Forty rats were randomly divided into normal (10 rats), model (10 rats), drug control (10 rats) and treatment (10 rats) groups. All groups except the normal group were given a subcutaneous injection of 40% carbon tetrachloride (peanut oil solution as a vehicle) at 3 ml/kg to establish a liver fibrosis model, 2 times a week for a total of 8 weeks. From the day of modelling, rats in the treatment group were orally administered with 1,25$(OH)_2D_3$ solution (peanut oil solution as a solvent), once a day; the drug control group was administered with an equal amount of peanut oil solvent orally, once a day.

After 8 weeks of continuous intervention, all rats were fasted for 12 hours, and the liver was removed and stained routinely after rats had been anesthetized with chloral hydrate. Observation of obvious fibre strips suggested that the model had been successfully established. After the rats were sacrificed, blood was collected from the chest and was separated by centrifugation at 12,000 rpm for 5 minutes at 4 °C. The serum was stored at -80 °C for subsequent testing. The liver tissue in the middle of the right lobe of the liver was fixed with 4% paraformaldehyde, and the remaining liver tissue fluid was frozen using liquid nitrogen and stored in -80 °C for future use.

Observation indicators

• Four items of rat liver fibrosis: radioimmunoassay was used to detect the changes of serum hyaluronic acid enzyme (HA), laminin fibronectin (LN), type III procollagen (PCIII) and collagen type IV (CIV) levels.

• Serum 25$(OH)D_3$ concentration and IL-17 level: Tandem mass spectrometry was used to detect changes in serum 25$(OH)D_3$ concentration in each group of rats; ELISA was used to detect changes in IL-17 levels in each group of rats.

• The pathological changes of liver tissue in each group were detected by HE staining.

• A Western blot method was used to detect the expression of IL-17, macrophage inflammatory protein 3α (MIP3α) and retinoic acid-related orphan receptor (RORγt) protein in rat liver tissue.

• Real-time quantitative PCR was used to detect the expression of IL-17, MIP3α and RORγt and mRNA in liver tissue of each group of rats.

Statistical methods

All measurement data of this research data were expressed by ($\bar{x}\pm s$). An independent sample t test was used to compare the means between the two groups, and an analysis of variance was used to compare the means between groups. P<0.05 was considered to indicate statistically significant results. The research data were analyzed using the SPSS20.0 software package.

Results

Comparison of four indicators of liver fibrosis in each group

The four fibrosis indicators in both model and drug control groups were significantly higher than those in the normal group, and the differences were statistically significant (P<0.05).

The above indicators in the treatment group were significantly lower than the drug control group, with these differences also being statistically significant (P<0.05). See Table 1.

Groups	Cases	HA (ng/ml)	LN (ng/ml)	PCIII (ng/ml)	CIV (ng/ml)
Normal	10	136.03±7.74	87.53±5.44	115.14±10.83	88.12±6.21
Model	10	500.12±105.16*	280.89±12.13*	256.85±13.52*	180.12±8.55*
Drug control	10	524.59±50.05*	267.35±16.48*	254.59±9.51*	182.55±8.74*
Treatment	10	396.31±64.56#	239.37±7.68#	162.47±10.31#	161.02±5.21#

Table 1: Comparison of four indicators of liver fibrosis in each group ($\bar{x}\pm s$).
*Note: Compared with the normal group *P<0.05; compared with the drug control group #P<0.05.*

Comparison of serum 25(OH)D3 concentration and IL-17 level in each group

The 25(OH)D$_3$ concentration in both model and drug control groups was significantly lower than that in the normal group, and the level of IL-17 was significantly higher than that in the normal group. The differences were statistically significant (P<0.05). In the treatment group, 25(OH)D$_3$ concentration was significantly higher, and the level of IL-17 was significantly lower than that in the drug control group, with the differences being statistically significant (P<0.05). See Table 2.

Groups	Cases	25(OH)D$_3$ (ng/ml)	IL-17 (pg/ml)
Normal group	10	24.05±1.16	10.78±0.69
Model group	10	21.12±0.80*	94.30±3.41*
Drug control group	10	21.13±1.15*	93.16±2.92*
Treatment group	10	44.45±3.12#	71.79±4.53#

Table 2: Comparison of serum 25(OH)D3 concentration and IL-17 level in each group ($\bar{x}\pm s$).
*Note: Compared with the normal group *P<0.05; compared with the drug control group #P<0.05.*

HE staining to observe pathological changes of liver tissue in each group

The results of HE staining showed that hepatocytes in the normal group were arranged neatly around the central vein, and the surrounding blood vessels were infiltrated non-inflammatory cells. The hepatic lobular structure of both model and drug control groups disappeared; the hepatocytes were arranged disorderly, the pseudolobules were formed and many of the inflammatory cells infiltrated; compared with the drug control group, the degree of inflammation and fibrosis of liver cells was significantly reduced in the treatment group. See Figure 1.

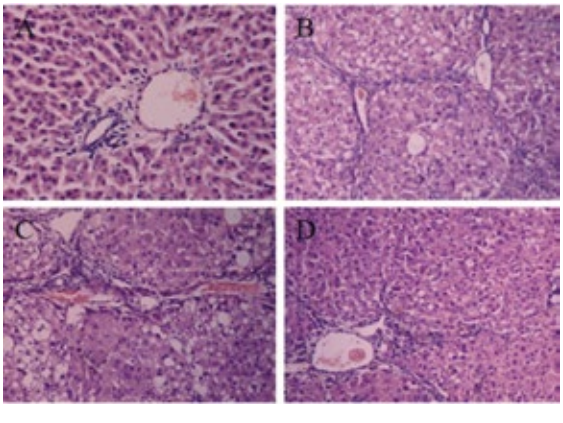

Figure 1: HE staining to observe pathological changes of liver tissue in each group.
A: Normal group; B: Model group; C: Drug control group; D: Treatment group.

Expression of IL-17, MIP3α and RORγt protein in liver tissue of each group tested by Western blot method

The expressions of IL-17, MIP3α and RORγt protein in the liver tissue of both model and drug control groups were significantly higher than those

in the normal group, and the differences were statistically significant (P<0.05). Those expressions in the treatment group were significantly lower than the drug control group, and the difference was statistically significant (P<0.05). See Table 3, Figure 2.

Groups	Cases	IL-17	MIP3α	RORγt
Normal group	10	0.35±0.05	0.41±0.02	0.52±0.04
Model group	10	0.54±0.06*	0.72±0.07*	0.82±0.03*
Drug control group	10	0.41±0.02*	0.75±0.10*	0.74±0.01*
Treatment group	10	0.35±0.01#	0.64±0.03#	0.65±0.02#

Table 3: Comparison of IL-17, MIP3α and RORγt proteins expression in liver tissue of each group ($\bar{x}\pm s$).
*Note: Compared with the normal group *P<0.05; compared with the drug control group #P<0.05.*

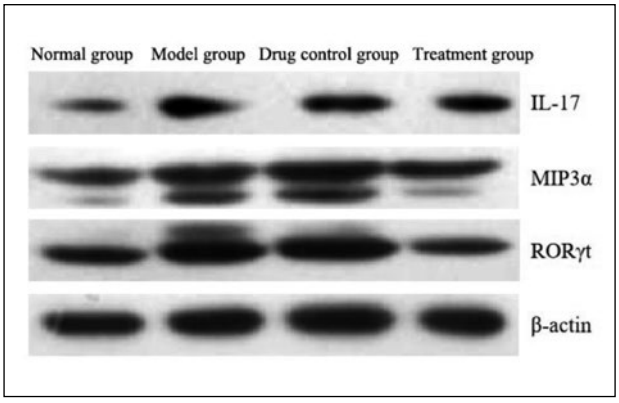

Figure 2: Expression of IL-17, MIP3α and RORγt proteins in liver tissue of each group.

Expression of IL-17, MIP3α, RORγt and mRNA in liver tissue of each group were detected by real-time quantitative PCR

The expressions of IL-17, MIP3α, RORγt and mRNA in the liver tissue of both model and drug control groups were significantly higher than those in the normal group, and the differences were statistically significant (P<0.05).

Those expressions in the treatment group were significantly lower than the drug control group, with this difference also being statistically significant (P<0.05). See Table 4.

Groups	Cases	IL-17	MIP3α	RORγt
Normal group	10	0.63±0.05	8.91±0.81	0.17±0.04
Model group	10	9.22±1.03*	91.30±3.52*	0.76±0.10*
Drug control group	10	9.22±0.84*	92.76±2.50*	0.77±0.13*
Treatment group	10	4.13±0.75#	58.63±1.06#	0.41±0.05#

Table 4: Expressions of IL-17, MIP3α, RORγt and mRNA in liver tissue of each group ($\bar{x}\pm s$).
*Note: Compared with the normal group *P<0.05; compared with the drug control group #P<0.05.*

Discussion

Liver fibrosis is a pathological process involving multiple cells and many factors and is a pre-stage lesion in which various chronic liver diseases develop into liver cirrhosis, which represents a liver injury repair response in excessive deposition of extracellular matrix in the liver. In recent years, liver fibrosis has maintained a high incidence and become one of the diseases that seriously threatens human health[8]. Hepatic fibrosis is often secondary to chronic inflammatory liver injury. During this process, a variety of immune cells can mediate immune inflammatory responses by synthesizing and secreting different cytokines. Damaged liver cells and activated HSCs, etc. can secrete a variety of chemokines and cytokines, which interact with and restrict immune cells to form a complex network system and participate in the body's inflammatory response in an active manner[9-10]. Therefore, the abnormal activation of the body's immune system is of high significance to the progress of liver fibrosis.

$1,25(OH)_2D_3$ is the final active form of vitamin D in the body and is often considered by clinicians to be an important hormone for maintaining bone health, mainly by activating biological receptors in the nucleus to exert biological effects, which play an important role in the proliferation and differentiation of many normal or tumours cells[11]. In recent years, studies have shown that $1,25(OH)_2D_3$ has various effects in a number of aspects of immune regulation, anti-inflammatory and anti-fibrosis[12]. Other scholars have found that $1,25(OH)_2D_3$ can inhibit the occurrence and development of liver fibrosis, but the further mechanism of action is not clear[13]. IL-17 is the main biological effector of Th17 cells mainly expressed by CD4+T cells and is an important inflammatory mediator that plays a role in inducing inflammatory cell infiltration and activation by inducing the expression of inflammatory cytokines, such as IL-6, chemokines and matrix metalloproteinases[14].

Recently, studies have suggested that IL-17 expression is significantly increased in liver tissues of various liver inflammatory diseases and is positively correlated with the degree of disease inflammation[15]. MIP3α is a small-molecular-weight chemokine produced by inflammatory cells and some tissue cells. The binding of its ligand and receptor can promote the accumulation of Th17 lymphocytes to the site of inflammation and aggravates the inflammatory response and promotes liver fibrogenesis. As a regulator, RORγt can differentiate Th17 lympho-

cytes and has a certain regulatory effect on the specific transcription of functions.

In this study, a hepatic fibrosis rat model was established, and 1,25(OH)$_2$D$_3$ was administered to intervene the rats. It was found that the four fibrosis indicators in both model and drug control groups were significantly higher than those in the normal group ($P<0.05$). Further, the treatment group had significantly lower values compared to the drug control group ($P<0.05$). The results of HE staining showed that, compared with the drug control group, the degree of inflammation and fibrosis of the hepatocytes was significantly reduced in the treatment group, which suggested that 1,25(OH)$_2$D$_3$ can reduce liver inflammation and fibrosis in rats. Further analysis of rat serum 25(OH)D$_3$ concentration and IL-17 levels revealed that the 25(OH)D$_3$ concentration in the treatment group was significantly higher than the drug control group, and the IL-17 level was significantly lower than the drug control group ($P<0.05$). There was a significant negative correlation of the two indicators, which suggested that IL-17 may participate in the occurrence and development of liver fibrosis, and 1,25(OH)$_2$D$_3$ has a significant inhibitory effect on the formation of liver fibrosis in rats.

At the same time, the results of Western blots and real-time quantitative PCR showed that the expressions of IL-17, MIP3α, RORγt and mRNA and protein in liver tissue of the treatment group were significantly lower than those in the model group, which suggested that the progress of 1,25(OH)$_2$D$_3$ inhibited liver fibrosis may be related to the regulation of Th17 cell function to improve the local microenvironment. In summary, 1,25(OH)$_2$D$_3$ has a significant inhibitory effect on the formation of rat liver fibrosis and can significantly reduce the expression of IL-17 and its related factor proteins and genes in rat liver tissues, suggesting that 1,25(OH)$_2$D$_3$ may regulate the activation and proliferation of rat HSCs and prevent the occurrence and development of fibrosis by regulating the IL-17-mediated immune microenvironment.

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