Chronic Diseases Prevention Review 7 (2018) 11-16

Available at http:// www.cancercellresearch.orgISSN 2158-0820

Protective effects of Vitamin D on isoniazid and rifampicin induced liver injury in rats

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Abstract: **This study was designed to investigate the effects of vitamin D (VD) on liver injury induced by isoniazid (INH) and rifampicin (RFP) in rats and whether VD could be used as a nutritional supplement to assist the treatment of ATLI. 50 male Sprague-Dawley (SD) rats were randomly divided into 5 groups according to their body weight: normal control (control) group, model group, VD low dose (VD-L) group, VD medium dose (VD-M) group, VD high dose (VD-H) group. All rats were given intragastric administration twice a day: firstly, control group was given 0.5% sodium carboxymethyl cellulose (CMC-Na); model, VD-L, VD-M and VD-H groups were given isoniazid (100mg/kg/d) + rifampicin (150mg/kg/d). Secondly, control and model groups were fed with soybean oil, VD-L, VD-M and VD-H groups were fed with 1ug/kg/d, 5ug/kg/d and 10ug/kg/d VD respectively. It lasted for 21 days. Markers of serum biochemistry and oxidative stress were evaluated. And hepatic morphology was also examined. The liver indexes of rats in VD-M group and VD-H group decreased compared to those in model group. Compared to rats in model group, rats in VD-H group had significantly lower serum levels of AST, ALP and DBIL, and significantly lower serum levels of ALP and DBIL in VD-M group. And compared with model group, rats in VD groups (VD-M, VD-H), the arrangement of hepatic cells tended to be normal, the degeneration and necrosis of hepatocytes and the infiltration of inflammatory cells were improved. Also, rats in VD-H group showed significant increase in GSH-PX and decrease in MDA. The levels of MDA also decreased in VD-L and VD-M groups. VD could inhibit the elevation of AST, ALP and DBIL in serum of rats induced by INH combined RFP and improved the pathological changes of liver tissues. And VD can inhibit oxidative stress by increasing the activity of antioxidant enzymes and reduce the damage of liver cells caused by lipid peroxidation.**

Keywords: Vitamin D; Anti-tuberculosis drugs induced liver injury; Oxidative stress

Received 2 April 2018, Revised 21 May 2018, Accepted 25 May 2018

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1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by Mycobacterium tuberculosis. In 2017, there were 10.4 million new TB cases and 1.3 million deaths from TB worldwide. China is one of the countries with high incidence of TB, which ranks third in the world after India and Indonesia[1]. Directly Observed Therapy short course (DOTs) is the standard TB therapy recommended by the World Health Organization (WHO), mainly including a combination of first-line anti tuberculosis drugs, such as Isoniazid (INH) and Rifampicin (RFP)[2]. However, first-line anti tuberculosis drugs have potential liver toxicity, which can easily cause liver injury. The occurrence of liver injury in the course of anti-TB treatment can lead to the interruption of treatment, which is easy to induce drug-resistant TB or the recurrence of TB, increasing the difficulty and burden of anti-TB treatment[3,4].

At present, anti-TB drugs induced liver injury (ATLI) is still a major challenge in anti-TB treatment. There are many studies on the hepatic toxicity of INH, RFP or INH combined with RFP[5-7], but the mechanism of its action is still unclear. It is generally believed that INH is oxidized by CYP450 in vivo to generate oxygen free radicals and other active intermediates. These active products deplete the antioxidant substances such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), then attack the unsaturated fatty acids on the membrane, lead to lipid peroxidation, and cause liver cell damage[8,9]. In liver, RFP deacetylation provides acetyl for acetylation of INH and accelerates the metabolism of INH. INH and RFP are more toxic to liver than isoniazid or rifampicin alone[5].

Clinically, there is no effective treatment for ATLI. The general therapy is to withdraw one or more antituberculotic, and strengthen supportive therapy to accelerate drug excretion, so that liver injury can gradually recover[3,10]. But drug-resistant TB can be emerged if the withdrawal of drugs is too long or drugs used irregularly. In general, hepatoprotective is used in the course of anti-TB treatment. Now, there are many kinds of hepatoprotective drugs, which can be divided into anti-inflammation, choledochal, detoxification, promoting liver cell recovery, enzyme lowering, liver cell membrane protection and so on. It is reported that for mild or moderate hepatocyte or mixed liver injury, bicyclic alcohol or glycyrrhizic acid is more effective when inflammation is severe, and silymarin has better effect when inflammation is mild[11]. Ursodeoxycholic acid has a good effect on liver injury caused by hepatic juice accumulation[12]. But, the exact efficacy of these drugs is still controversial. Choice hepatoprotective drugs carefully for those drugs are metabolized in liver. Abusing of hepatoprotective drugs just cannot protect liver but increase the burden of the liver.

Vitamin D (VD) is an essential nutrient in human

body. In addition to regulating calcium and phosphorus metabolism and bone health, VD also participates in a series of physiological and pathological processes such as defense, inflammation, immune regulation, repair and so on[13-15]. 1,25(OH)2D3 is the bioactive form of VD in vivo, combining with vitamin D receptor (VDR) in vivo to play its unique role. Studies have confirmed that VDR is widely distributed in a variety of cells (monocytes, macrophages, T cells, islet cells, etc.)[16]. VD plays a role in the treatment of various diseases[17-19]. VD may participate in the treatment of diabetes by regulating insulin secretion, β cell survival, intracellular calcium flux and insulin sensitivity[20]. VD is related to the treatment of TB. VD can reduce the activity of Mycobacterium tuberculosis by increasing the fusion of phagocytosis and lysosome of the phagocytic cells infected with Mycobacterium tuberculosis, and enhance the bactericidal ability of the body by promoting the synthesis of antimicrobial peptides[3,21]. In the treatment of non-alcoholic fatty liver, VD can improve liver injury by reducing the level of oxidative stress and inhibiting inflammatory reaction[22]. However, the study on the protective effect of VD on ATLI has not been reported.

The main purpose of this study was to observe the effects of VD on serum biochemistry, pathology and oxidative stress in rats with liver injury induced by isoniazid and rifampicin. To explore the liver protection effect of VD and whether VD can be used as a nutritional supplement to assist the treatment of ATLI, to provide a new idea for the improvement of ATLI in clinic in the future.

2. Material and methods

2.1. Chemicals and drugs

INH was purchased from Shanghai Pharmaceutical Co., Ltd. Xinyi Pharmaceutical General Factory and RFP from Leshan Three Pharmaceutical Co., Ltd. Long march. VD is provided by Qingdao Double Whale Medicine Co., Ltd. Glutathione peroxidase (GSH-px), superoxide dismutase (SOD) and malondialdehyde (MDA) kits were purchased in Nanjing Jiancheng Bioengineering Institute, China.

2.2. Animals

The experiments were carried out with male SD rats (180-220g) obtained from Qingdao Experimental Animal and Animal Experimental Center. The rats were raised in the Experimental Animal Center of the Medical College of Qingdao University, with the ambient temperature at 23-25℃ and the relative humidity 55-65%. And rats were fed with common feed, drinking water freely, and with a standard 12h/12h light/dark cycle. All animal procedures were reviewed and approved by the Animal Ethical Committee of Laboratory Animals of Qingdao.

2.3. Design of the study

After one week of adaptive feeding, 50 rats were randomly divided into 5 groups according to their body weight: Normal control group (control), Model group (model), Vitamin D low dose (VD-L) group, Vitamin D medium dose (VD-M) group, Vitamin D high dose (VD-H) group, and 10 rats in each group. All rats were given intragastric administration twice a day: firstly, control group was given 0.5% sodium carboxymethyl cellulose (CMC-Na); model, VD-L, VD-M and VD-H groups were given isoniazid (100mg/kg/d) + rifampicin (150mg/kg/d). Secondly, control and model groups were fed with soybean oil, VD-L, VD-M and VD-H groups were fed with 1ug/kg/d, 5ug/kg/d and 10ug/kg/d VD respectively. It lasted for 21 days.

2.4. Sample collection

After the day 21 of intervention, the rats were fasted for 12h. Weighing, abdominal anaesthesia by pentobarbital natrium, and blood collection from the abdominal aorta. The blood samples were centrifuged and then stored at –80℃ until use. Rat liver was picked and weighed. One portion of liver tissue was fixed in 10% formalin for hematoxylin and eosin (H + E) staining and immunohistochemical studies. 10% homogenate of liver tissue was prepared from 1g of rat liver tissue. Residual liver tissue and homogenate were stored at -80℃.

2.5. Biochemical analysis

The biochemical indexes of serum alanine aminotransferase (ALT), alanine aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL) and direct bilirubin (DBIL) were detected by automatic biochemical analyzer of Laboratory Department of Qingdao Affiliated Hospital (GE Hitachi).

2.6. Histopathological examination

The 10% formalin-fixed liver samples were embedded in paraffin and cut into 5-μm thick sections, and then stained with H&E. Hepatic histopathological changes were observed by Olympus BX60 microscope (Japan Olympus Corp).

2.7. Activities of SOD and GSH-PX in liver tissue

Activities of SOD and GSH-PX were detected by visible spectrophotometer (Shanghai Instrument Electric Analysis Instrument Co., Ltd). And SOD was determined by hydroxylamine assay method.

2.8. MDA concentration in liver tissue

Lipid peroxidation was determined by measurement of MDA formation, which was determined by thiobarbituric acid reactive substance

and detected by visible spectrophotometer (Shanghai Instrument Electric Analysis Instrument Co., Ltd).

2.9. Statistical Analysis

Data were analyzed by SPSS17.0 (SPSS Inc, Chicago, IL, USA). Results were expressed as mean ±SD. One-way ANOVA was carried out for multigroup comparisons and LSD was used for the comparisons between two groups.

Table 1. Changes in body weight of rats in each group (g)

Group	0 d	1 d	7 d	14 d	21 d
Control	262.71±28.32	272.08±29.99	309.93±28.60	332.48±29.94	338.56±35.45
Model	255.80±31.00	269.95±29.57	279.09±31.12#	296.95±30.23#	307.75±27.47#
VD-L	255.33±32.18	269.32±31.94	287.27±28.80#	303.74±29.00#	279.20±30.05#
VD-M	255.00±36.13	277.17±28.32	290.46±25.79#	311.58±28.18#	281.45±22.28#
VD-H	256.11±31.23	270.19±31.80	291.76±26.43#	300.65±34.51#	289.26±41.52#

Note: Control, normal control group; Model, model group; VD-L, vitamin D low dose group; VD-M, vitamin D medium dose group; VD-H, vitamin D high dose group. Values are the mean±SD. #, P<0.05 vs control group; *, P<0.05 vs model group.

Table 2 Levels of serum liver functions in rats

Group	ALT (U/L)	AST (U/L)	TBIL (umol/L)	DBIL (umol/L)	ALP (U/L)
Control	47.20±8.31	114.50±23.06*	2.42±1.04*	0.95±0.46*	203.10±62.42*
Model	52.50±17.80	138.30±18.05#	15.21±6.27#	12.51±6.28#	264.30±55.06#
VD-L	50.50±14.64	138.44±14.59#	13.45±3.45#	8.67±2.38#	237.90±86.81
VD-M	44.20±11.53	119.70±18.28*	10.48±3.71#	7.98±3.22#*	216.60±55.85
VD-H	39.00±9.02*	118.14±17.73*	10.14±4.04#	7.48±2.78#*	171.40±45.85*

Note: Control, normal control group; Model, model group; VD-L, vitamin D low dose group; VD-M, vitamin D medium dose group; VD-H, vitamin D high dose group. Values are the mean±SD. #, P<0.05 vs control group; *, P<0.05 vs model group.

3. Results

3.1. Body mass

As shown in Table 1, body mass was measured on days 7, 14 and 21 after intervention. In the first two weeks of intervention, a gradual increase in body mass gain in all groups was observed (P<0.05). The body mass of control group was higher than that of other groups after 7 days of intervention (P<0.05). In addition, it is showed a trend of weight loss in all groups after 14 days of intervention.

3.2. Liver index

There was a significant difference in liver index of control group (2.81±0.20) compared with model group (3.14±0.25)(P<0.05). Compared with model group, liver index of VD-M (2.86±0.22) and VD-H (2.85±0.29) groups decreased (P<0.05) (Figure 1).

3.3. Serum biochemical markers

Compared with control group, the serum levels of AST, ALP, DBIL, and TBIL in model group are significantly higher (P<0.05). There is no significant difference in the levels of AST between control and model group. The levels of serum AST, DILI and ALP in VD-H group are higher than those in model group (P<0.05). Compared with model group, the levels of serum AST and DBIL in VD-M group were decreased (P<0.05) (Table 2).

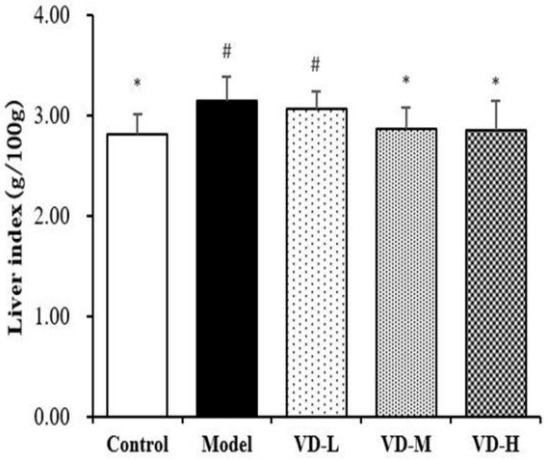

Figure 1. Liver indexes of rats in each group.

3.4. Liver Histopathological Changes under Light Microscopy

Histological assessments were performed to further evaluate the hepatotoxicity. As shown in Figure 2, in control group, the hepatic lobule structure was normal, and the hepatocyte cord was arranged in a radial pattern centered on the central

vein. The morphology of hepatocytes was normal and the size was homogeneous. In model group, there was a disorder of liver tissue and inflammatory infiltration of liver cells, accompanied by slight vacuolar degeneration and necrosis. In VD groups (VD-M, VD-H), the arrangement of hepatic cells tended to be normal, the degeneration and necrosis of hepatocytes and the infiltration of inflammatory cells were improved.

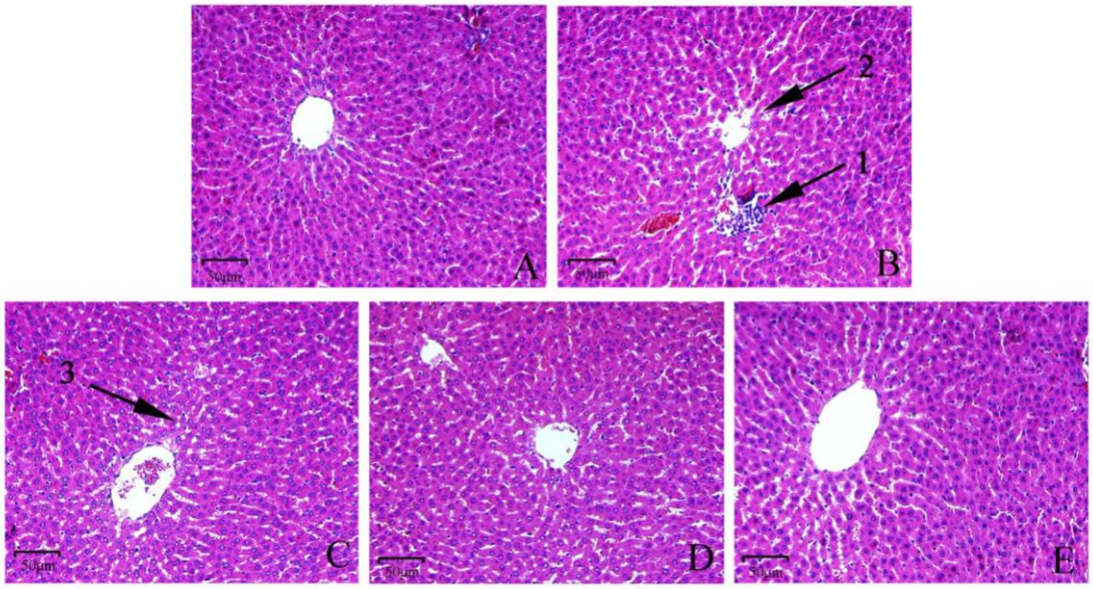

Figure 2. Photomicrography of liver sections from rat in the normal control group (A), model group (B), Vitamin D low dose (VD-L) group (C), Vitamin D medium dose (VD-M) group (D), and Vitamin D high dose (VD-H) group (E). 1, inflammatory cell infiltration; 2, mild degeneration and necrosis of hepatocytes; 3, vacuolization (hematoxylin and eosin stain, original magnification ×200).

Table 3 Activities of SOD, GSH, GSH-PX and MDA concentration in rats liver

Group	SOD (U/mgprot)	GSH-PX (U)	MDA (nmol/mgprot)
Control	289.19±32.16*	237.44±20.08*	7.70±1.55*
Model	226.96±22.86#	187.75±17.30#	10.77±1.88#
VD-L	251.31±54.07#	194.72±5.74	8.52±1.25*
VD-M	261.68±33.86	219.06±58.82	7.74±1.10*
VD-H	254.57±28.99	225.03±26.65*	6.84±1.89*

Note: Control, normal control group; Model, model group; VD-L, vitamin D low dose group; VD-M, vitamin D medium dose group; VD-H, vitamin D high dose group. Values are the mean±SD. #, P<0.05 vs control group; *, P<0.05 vs model group.

3.5. Activities of SOD and GSH-PX and MDA concentration

As shown in Table 3, compared with control group, the activities of SOD and GSH-PX decreased significantly and the level of MDA increased in liver tissue of model group (P<0.05). Compared with model group, the GSH-PX activity in VD-H group was about 1.2 times higher than that in model group. And compared with model group, the MDA levels in VD-L, VD-M and VD-H groups decreased by 20.89%, 28.13% and 36.49% respectively.

4. Discussion

Our results suggest that VD can be used as a nutritional supplement for the adjuvant treatment of ATLI. Isoniazid combined with rifampicin could induce liver injury in rats. VD intervention can effectively improve liver function and histopathological changes in rats with liver injury. VD inhibited oxidative stress by increasing antioxidant enzymes activities and decreased lipid peroxidation injury in rat hepatocytes.

Serum ALT, AST, ALP, TBIL and DBIL are commonly used in clinical evaluation of liver function, their levels can reflect the degree of liver damage[23]. ALT and ALP mainly exist in the cytoplasm of hepatocytes, and AST mainly in hepatocyte mitochondria. ALP is mainly excreted by the liver, and the synthesis of ALP is increased when

the liver cells are damaged, which leads to the increase of ALP in the blood[24]. AST is the main index for detecting liver injury in clinic. It increases slowly and lasts for a long time, so it is often used in the detection of drug induced liver injury[25]. The elevation of TBIL or DBIL could also indicate liver dysfunction. In present study, INH combined with RFP resulted in the increase of serum AST, ALP, TBIL and DBIL in rats. The intervention of VD effectively inhibited the increase of AST, ALP and DBI, suggesting that VD can inhibit the damage caused by anti-tuberculosis drugs on the membrane of hepatocytes and mitochondria. These results are further assured by the histopathological examination of liver tissue in our study. VD intervention significantly ameliorated the pathological changes of hepatocyte inflammation, vacuolar degeneration and necrosis in rats. In addition, the changes of liver indexes in this study also suggest that VD has a protective effect on the liver.

Oxidative stress refers to the imbalance between oxidation and antioxidation in vivo. A large number of oxidation intermediates attack the unsaturated fatty acids on the membrane resulting in lipid peroxidation and damaging to liver cells. Studies have shown that oxidative stress and lipid peroxidation play an important role in the pathogenesis of ATLI[26,27]. Hydrazine and acetyl hydrazine produced by INH in the metabolic process are oxidized to generate active intermediates such as ROS. These active products will deplete the antioxidants such as SOD and GSH-PX in the body, resulting the oxidative antioxidant system out of balance and causing liver injury[6, 28]. The results of our study show that VD inhibited the decrease of GSH-PX activity and the accumulation of MDA in liver tissue induced by INH combined with RFP in rats, and further to alleviate the injury of lipid peroxidation to the liver of rats. Our results were supported by Seif and Abdelwahad[29], in their study, the intervention of VD significantly increased the activity of GSH-PX and decreased the level of MDA in the liver after ischemia-reperfusion injury in rats. The inhibitory effect of VD on oxidative stress has been further confirmed in the study Wang[30]. Their studies have highlighted the effect of calcitriol on oxidative stress in cardiac injury induced by isoproterenol. Some studies have explored the mechanism of vitamin D inhibiting oxidative stress. The study performed by Nikooyeh and Neyestani suggested that the inhibition of vitamin D on oxidative stress is due to the antioxidant activity of the subunits of VD (7-dehydrocholesterol, VD3, VD2, calcitriol), which reduces the level of lipid peroxidation[31]. And some other studies pointed that VD up-regulates the catalytic subunit of reduced glutathione (GCLC), increases the level of reduced glutathione (GSH), enhances the antioxidant ability of the body, and then inhibits oxidative stress[31-34].

5. Conclusion

In conclusion, in our study we found that VD may probably be used as a nutritional supplement for adjuvant treatment of ATLI. On the one hand, VD could inhibit the elevation of AST, ALP and DBIL in serum of rats induced by INH combined RFP and improved the pathological changes of liver tissues. On the other hand, VD can inhibit oxidative stress by increasing the activity of antioxidant enzymes and reduce the damage of liver cells caused by lipid peroxidation.

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