IMPROVEMENT EFFECT OF LONG-TERM VITAMIN D SUPPLEMENTATION ON SKELETAL MUSCLE FATIGUE IN OVERTRAINING RATS

Fu-Lin Lu[1], Ling-Heng Kong[2, *]
[1]The Athletic Department of Xi'an Medical University, Xi'an - [2]Department of Basic Medicine, Xi'an Medical College, Xi'an, China

ABSTRACT

Introduction: *To investigate the improvement effect of long-term vitamin D supplementation on skeletal muscle fatigue in rats and related mechanism.*

Materials and methods: *The skeletal muscle fatigue rats were prepared and divided into four groups according to whether vitamin D was supplemented and whether there was skeletal muscle fatigue, namely control group (Con), exercise group (Exe), control + vitamin D supplementation group (Con+VD) and Exercise + vitamin D supplementation group (Exe + VD). The levels of vitamin D, serum IL-1β, TNF-α and IL-6 in rats were detected by Elisa; the exercise ability of rats was observed by open field test and rotating rod fatigue test. The levels of IL-1β, TNF-α and IL-6 in rat gastrocnemius muscle were detected by RT-PCR. The expressions of NLRP3 and Caspase-1 in the gastrocnemius muscle of rats were detected by Western Blot.*

Results: *The vitamin D level decreased significantly in the Exe group, but increased significantly in Con+VD and Exe+VD; the exercise ability decreased significantly in the Exe group, but increased significantly in Exe+VD; the inflammation level of the rats was significantly higher in the Exe group than in the other three groups. Skeletal muscle IL-1β was significantly negatively correlated with 25(OH)D3, 1,25(OH)2D3, walking distance, rod time, and rod rotation speed. The expression of NLRP3 and Caspase-1 was significantly increased in the Exe group, but decreased significantly after vitamin D supplementation.*

Conclusion: *Vitamin D supplementation can alleviate the muscle fatigue state and improve the exercise ability of overtraining rats by regulating the inflammation level in the body.*

Keywords: *Vitamin D, Skeletal muscle fatigue, Inflammation level, NLRP3.*

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Forewords

Moderate physical exercise can effectively improve the health level of the population, but some special occupations, such as athletes and soldiers, require long-term physical exercise and are thus prone to overtraining. About 20%~30% athletes develop hyperkinetic syndrome due to overtraining and insufficient rest, which seriously harms their physical and mental health[(1)]. The most common symptom of overtraining is skeletal muscle fatigue, which will seriously affect the daily life and health of athletes. Studies have shown that overtraining can increase the body's inflammatory response, which is also manifested in skeletal muscle[(2)]. Reducing the body's inflammatory response can effectively alleviate the fatigue of skeletal muscle, and improve the exercise and coordination ability of skeletal muscle. Vitamin D is a multifunctional vitamin required for the human body. Current studies have shown that vitamin D can prevent falling among the elderly because it can effectively improve muscle strength, content, and muscle coordination[(3, 4)]. At the same time, some research report that vitamin D is significantly related to the body's inflammatory response[(5, 6)]. Therefore, in this study, through long-

term supplementation of vitamin D to overtraining rats, we observe its improvement effect on blood and skeletal muscle inflammatory response in the rats and its alleviation of skeletal muscle fatigue to preliminarily explore its mechanism.

Materials and methods

Materials

Animals

In this study, 24 3-month-old Sprague Dawley (SD) male rats (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.) weighing 180g~220g were selected. According to the follow-up experimental arrangement, we randomly divided the animals into 4 groups for cage culture, and the rearing conditions were SPF level.

Experimental reagents

Rat 25 hydroxyvitamin D3 (25 (OH) D3) 1,25 dihydroxyvitamin D3 (1,25(OH)2D3) detection kits were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd. IL-1β, TNF-α and IL-6 ELISA kits were purchased from Shanghai Jianglai Biotechnology Co., Ltd. Anti-caspase-1 and Anti-NLRP3 antibodies were purchased from Thermo Scientific. VD drops were purchased from Qingdao Double Whale Pharmaceutical Co., Ltd. PCR reaction kit was purchased from TaKaRa, Japan. Caspase-1 activity detection kit was purchased from Solar.

Research methods

Modeling and grouping

The rat overtraining model was prepared by using an exercise roller. Rats exercised at a roller speed of 25m/min, 60min/time, 6 days/week. With the extension of the rat culture time and the adaptation to the training, rats were trained twice a day in 1 week after the start of modeling, 3 times a day in 4th~8th week, 4 times a day in 8th~12th week, 5 times a day in 12th ~16th week. The untrained rats were normally housed in the rearing cage. According to whether training was given and whether vitamin D was supplemented, we divided experimental animals into 4 groups: control group (Con), exercise group (Exe), control+vitamin D supplementation group (Con+VD) and exercise+vitamin D supplementation group (Exe+VD). Vitamin D was supplemented by gavage at a dose of 3.0 μg/(kgxd).

Serum vitamin D detection

Blood collection of the orbital venous plexus in rats before modeling (0 week), 8 weeks after modeling (8 weeks), at 12th week and 16th week. Blood samples were detected using Rat 25 hydroxyvitamin D3 and 1,25 dihydroxyvitamin D3 ELISA kits, and operation steps strictly followed the kit's operating instructions.

Open field test

The rats were placed in a square wooden box of 100cmx100cm, a camera was connected at 2m from the top of the wooden box to record the movement trajectory of the rat in the wooden box, and then ANY-maze software was used for analysis. The time point of each test was 18:00-19:00. The test environment should be quiet and the light should not be too strong.

The test lasted for 5 minutes. This experiment aims to verify the activity level and exploration ability of rats in the resting state.

Detection of inflammation levels

Blood collection of the rats orbital venous plexus in 16 weeks after modeling. Blood samples were detected using IL-1β, TNF-α and IL-6 ELISA detection kits, and the operation steps strictly followed the kit's operating instructions. The rats were then sacrificed, the rat gastrocnemius muscle was removed, and the tissue specimens were fixed in 4% paraformaldehyde overnight.

The gastrocnemius muscle was then dissected in 4°C normal saline. Total RNA was extracted from gastrocnemius muscle after weighing. Reverse transcription to cDNA and Real-time PCR reactions according to the procedures of specification. The primer sequences of IL-1β, TNF-α, IL-6 and GAPDH are shown in Table 1.

qPCR primer	sequences (5'-3')
IL-1β (forward)	AACTGTGAAATAGCAGCTTTCG
IL-1β (reverse)	CTGTGAGATTTGAAGCTGGATG
TNF-α (forward)	GCATGATCCGAGATGTGGAACTGG
TNF-α (reverse)	CGCCACGAGCAGGAATGAGAAG
IL-6 (forward)	TGCACTGTCAGAAAACAATCTG
IL-6 (reverse)	CCAGAGCAGATTTTCAATAGGC
GAPDH (forward)	GACAACTTTGGCATCGTGGA
GAPDH (reverse)	ATGCAGGGATGATGTTCTGG

Table 1: Primers sequences.

Western Blot detection

Total protein was extracted from muscle tissue in 1.2.5. Protein extraction of cells were using RIPA protein extraction buffer containing protease inhibitors. The protein concentration detection were using the BCA protein assay kit. SDS-PAGE gel separates proteins based on molecular weight and then proteins were transferred to PVDF membranes. NLRP3 and Caspase-1 primary antibodies were used to incubate membrane, next is to HRP-conjugated secondary antibodies incubation to visualization protein content.

Caspase-1 activity detection

Total protein was extracted from muscle tissue in 1.2.5. The activity of Caspase-1 detected according to the procedures of specification.

Statistical analysis

All data were statistically analyzed by T-test, analysis of variance and Pearson's correlation analysis in SPSS 17.0 statistical software. P<0.05 as statistically different.

Results

Changes in vitamin D expression

By collecting blood samples from rats and measuring the content of 25(OH)D3 and 1,25(OH)2D3, it was found that the baseline values of 25(OH)D3 and 1,25(OH)2D3 did not statistically different among the four groups (P>0.05).

At 8th week, the content of 25(OH)D3 did not change significantly in the four groups (P>0.05), but the content in the Exe+VD group was significantly increased than the content of 1,25(OH)2D3 in the Exe group (P<0.05). Con+VD and Exe+VD groups had significantly higher 25(OH)D3 content than Con group and Exe group at 12th week (P<0.05), but only Exe+VD group had higher content of 1,25(OH)2D3 than Exe group (P<0.05). At 16th week, the contents of 25(OH)D3 and 1,25(OH)2D3 were significantly higher in the Exe+VD group than in the Con group and Exe group (P>0.05). Also, the contents of 25(OH) D3 and 1,25(OH)2D3 were higher in Con+VD group than in Exe group (P<0.05), as shown in Table 2 .

With the change of time, the content of 25(OH) D3 and 1,25(OH)2D3 did not change significantly in the Con group (P>0.05); while the content of 1,25(OH)2D3 in the Exe group was significantly lower at 16th week than at 0 week (P>0.05). The contents of 25(OH)D3 and 1,25(OH)2D3 in the Con+VD group increased with time, but there was no significant difference (P> 0.05). The content of 25(OH)D3 in Exe+VD group was significantly higher at 12th week and 16th week than at 0week (P<0.05), and the content of 1,25(OH)2D3 was significantly higher at 16th week than at 0week (P<0.05), as shown in Table 2.

	Con	Exe	Con+VD	Exe+VD
$25(OH)D_3$ (ng/ml)				
0week	35.27±4.64	34.14±5.23	35.97±3.56	34.99±4.82
8week	36.42±4.79	33.26±4.55	37.31±5.77	38.98±6.52
12week	34.27±5.03	31.38±4.96	41.42±4.63^{bc}	42.79±5.88^{abc}
16week	33.38±6.67	29.43±4.68	40.97±5.13^{e}	45.53±4.49^{ad}e
$1,25(OH)_2D_3$ (pg/ml)				
0week	118.31±12.44	116.62±11.37	119.51±13.31	118.69±12.51
8week	117.69±13.48	109.62±14.49	124.37±14.08	128.96±14.28^{g}
12week	119.59±18.27	102.51±16.63	127.73±16.65	131.38±17.85^{c}
16week	117.88±13.38	99.93±13.31^{f}	131.38±13.2^{b}e	139.64±12.21^{ade}

Table 2: Changes in vitamin D expression.

Note: compared to Exe+VD (0week), $^{a}P<0.05$; compared to Con (12week), $^{b}P<0.05$; compared to Exe (12week), $^{c}P<0.05$; compared to Con (16week), $^{d}P<0.05$; compared to Exe (16week), $^{e}P<0.05$; compared to Exe (0week), $^{f}P<0.05$; compared to Exe (8week), $^{g}P<0.05$.

Changes in exercise ability

Rats' mobility was assessed by the open field test. No significant difference among the four groups at the baseline level of mobility (P>0.05). At 8th week, the rats' walking distance was significantly higher in the Con+VD group than in the Exe group (P>0.05), but it didn't show diversity between the Con group, Exe+VD group and the Exe group (P<0.05). It is no significant difference in walking distance between Con group, Con+VD group and Exe+VD group at 12th week and 16th week (P>0.05), but had significantly longer walking distance than Exe group (P<0.05). (Figure 1A)

The muscle coordination ability of the rats was assessed by the rotating rod test. It was no significant change in rod exercise time among the four groups of rats at 0 and 8th week (P>0.05). There was no significant difference either in rod exercise time between the Con group, the Con+VD group and the Exe+VD group At 12th week and 16th week (P>0.05), but with a value significantly higher than the Exe group (P<0.05). (Figure 1B) The rod rotation speed did not change significantly at 0 week (P>0.05). At 8th week, 12th week and 16th weeks, the rod rotation

speed was significantly higher in the Con group, the Con+VD group and the Exe+VD group than in the Exe group (P<0.05).

At 12th week and 16th week, the rod rotation speed was significantly higher in the Exe+VD group than in the Con group (P<0.05), which was also significantly higher in Exe+VD than in the Con+VD group at 16th week (P<0.05). (Figure 1C).

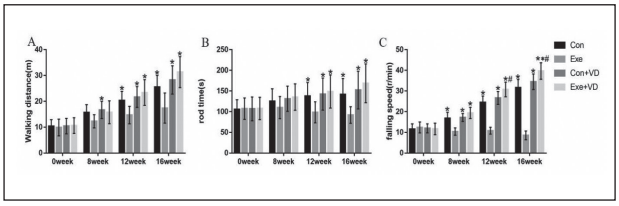

Figure 1: Exercise ability of rats
*A: Comparison of walking distance of rats in different groups; B: Comparison of rod exercise time of rats in different groups; C: Comparison of rod rotation speed of rats in different groups. Note: compared to Con+VD, *P<0.05; compared to Con, #P<0.05; compared to Exe, *P<0.05.*

Changes in inflammation level

Through the detection of serum inflammatory-related factors in rats, it was found that in the Con group, Con+VD group and Exe+VD group ,there was no significant changes in levels of IL-1β, TNF-α and IL-6 in serum, (P>0.05), but compared to Exe group,all were significantly lower (P<0.05).

(Figure2 A~C) Through further detection of inflammatory response in skeletal muscle, we found that the trend was similar to that in serum, that is, there was no significant change between the Con group, Con+VD group and Exe+VD group (P>0.05), but all were significantly lower compared to Exe group (P<0.05). (Figure 2 D~E).

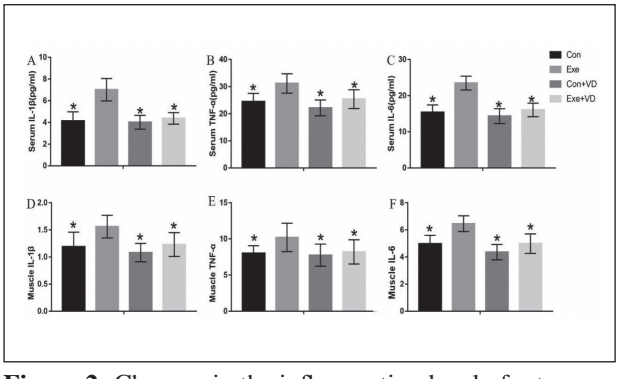

Figure 2: Changes in the inflammation level of rats
*A: the IL-1β content in the serum of rats in different groups; B: the TNF-α content in the serum of rats in different groups; C: the IL-6 content in the serum of rats in different groups; D: the relative expression of IL-1β gene in rat muscle of different groups; E: the relative expression of TNF-α gene in serum of rats in different groups; F: the relative expression of IL-6 gene in serum of rats in different groups. Note: compared with Exe, *P<0.05.*

Correlation comparison

We selected IL-1β in skeletal muscle as the object, and judged the correlation between IL-1β and other factors by Pearson correlation analysis. It was found that IL-1β showed a significant negative correlation with 25(OH)D3, 1,25(OH)2D3, walking distance, rod time and rod rotation speed (P<0.05), as shown in Table 3.

Related factor	Muscle IL-β	
	r	P
$25(OH)D_3$	-0.157	0.001
$1,25(OH)_2D_3$	-0.132	0.005
Walking distance	-0.105	0.036
Rod time	-0.149	0.003
Falling speed	-0.186	0.000

Table 3: Correlation analysis.

The effect of vitamin D on NLRP3/Caspase-1/IL-1β axis of skeletal muscle

The exercise rats were divided into groups based on whether they were given vitamin D supplementation, namely the Exe group and the Exe+VD group.

By WB detection, we found that NLRP3 expression of the skeletal muscle was significantly reduced in the Exe+VD group (Figure 3A).

Then, the downstream Caspase-1 was detected by WB detection and activity detection, and it was found that the content and activity of Caspase-1 also decreased significantly (P<0.05) (Figure 3A, B).

By supplementing rats with MCC950 (NLRP3 inhibitor), we found that, compared to the Exe group, the levels of IL-1β in rat skeletal muscle was significantly lower (P<0.05) (Figure 3C).

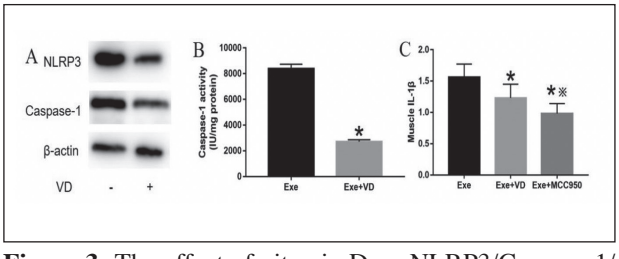

Figure 3: The effect of vitamin D on NLRP3/Caspase-1/IL-1β axis of skeletal muscle
*A: WB detection of the expression of NLRP3 and Caspase-1 protein in the muscle of rats in different groups. B: Caspase-1 activity detection in muscle of rats in different groups; C: Relative expression of IL-1β gene in muscle of rats in different groups. Note: Compared with Exe, *P<0.05; compared with Exe+VD, ※P<0.05.*

Discussion

Athletes, soldiers, or some sports addicts can be susceptible to skeletal muscle fatigue due to long-term high-intensity sports training, which will seriously affect the patient's daily life and even cause sports injuries. This study aims to evaluate the improvement effect on fatigue that long-term vitamin D intake in overtraining rats and the possible mechanisms involved. Those studies indicated that long-term vitamin D supplementation to overtraining rats can effectively improve the free activity level of the rats in the resting state, and strengthen the balance ability and coordination ability of the rats. In this study, it was also found that the level of inflammation was significantly increased in the body of overtraining rats, but was reduced after vitamin D supplementation, which may be related to the NLRP3/Caspase-1/IL-1β signaling pathway.

Vitamin D, as an essential vitamin for the human body, exerts a significant impact on human health. Vitamin D can increase calcium and phosphorus absorption to promote the growth and development of bone tissue[7]. Vitamin D also has close relation with tumors[8], immune system diseases[9], cardiovascular diseases[10], diabetes[11] and obesity[12]. So far, more and more researches have also shown that vitamin D improves muscle function significantly and enhance muscle strength and balance[3, 4]. Therefore, this study investigates its effect on skeletal muscle of long-term fatigued rats by supplementing vitamin D. It was found that the content of 25(OH)D3 and 1,25(OH)2D3 decreased in long-term exercise rats without additional vitamin D supplementation. We speculate that overtraining puts the rats in a hypermetabolic state, and the vitamin D content of ordinary diet is insufficient to meet the needs of overtraining rats. After adequate supplementation, we found that the vitamin D content presented a significantly upward trend in the Exe+VD group compared to the Con+VD group. We speculate that exercise can promote the absorption of vitamin D content. Hence, we believe that if vitamin D is not supplemented in time, long-term exercise rats will be short of vitamin D, and their absorption capacity is stronger compared to non-exercise rats after sufficient supplementation.

At the same time, we tested the exercise ability of rats in different groups through open field test and rotating rod test in this study, finding that after long-term vitamin D supplementation, rats showed better activity ability in the resting state, while long-term exercise without vitamin D supplementation significantly decreased its activity. Studies have shown that vitamin D have the ability to maintain muscle function and muscle content in people of different ages, thereby improving their exercise capacity[13, 14]. The results of this study are consistent with previous studies. However, most of previous studies were aimed at normal adolescents or the elderly, which did not consider people with muscle fatigue caused by long-term exercise.

Studies have shown that overtraining can increase the body's inflammatory response, also increasing inflammatory factors in the blood and skeletal muscle. This will cause damage to muscle cells and inhibit muscle tissue regeneration. In this study, it is remarkable that overtraining rats aggravated inflammatory response in the gastrocnemius muscle and blood, with the expression levels of IL-1β, TNF-α and IL-6 in the Exe group is significantly higher than in the Con group. At the same time, through correlation analysis, we found that the higher the level of muscle tissue inflammation, the weaker the exercise ability of the rats. This suggests that reduced activity in overtraining rats may be due to high levels of inflammation in muscle tissue. At the same time, the inflammation level in muscle tissue was also negatively correlated with the body's vitamin D content. In this study, the expression levels of IL-1β, TNF-α and IL-6 are reduced to normal after vitamin D supplementation.Shengqiao Wu et al. found that vitamin D supplemented with budesonide can more effectively reduce the inflammatory response of the airway in children with asthma[14]. At the same time, some scholars have found that vitamin D can effectively relieve or improve the symptoms of acute colitis[15]. Therefore, we believe that vitamin D can effectively reduce the inflammatory response of the body, including muscle tissue[16].

NLRP3 is widely involved in a variety of inflammatory-related diseases in the body[16], and its activation leads to up-regulated Caspase-1 expression and increased activity, followed by the up-regulation of inflammation-related factors such as IL-1β[17, 19]. In this study, we found that activation of NLRP3 is reduced effectively by vitamin D, resulting in the down-regulation of its downstream Caspase-1 and IL-1β. Therefore, we believe that vitamin D inhibits the inflammatory response of muscles caused by overtraining through the NLRP3/Caspase-1/IL-1β signaling pathway, thereby improving the exercise capacity of rats[20]. In summary, overtraining can increase inflammation in muscle tissue and reduce

exercise ability, while vitamin D supplementation can effectively alleviate this problem. Therefore, according to the results of this study, we suggest that long-term overtraining people should supplement vitamin D appropriately to relieve muscle fatigue and reduce sports injuries[21].

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Corresponding Author:
Ling-Heng Kong
Department of basic medicine, Xi'an Medical College, Xi'an, 710021, China
Email: 359476087@qq.com
(China)