

# Influence of vitamin D deficiency on T cell subsets and related indices during spinal tuberculosis

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**Abstract.** The present study investigated the effects of vitamin D deficiency on T cell subsets in patients with spinal tuberculosis. In addition, the influence of vitamin D deficiency was investigated on the expression of cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in intervertebral disc lesions of patients. One hundred and seventeen patients with spinal tuberculosis who received operative treatment in the Department of Orthopedics in Wuhan City Third Hospital from March 2012 to March 2015 were collected. The patients were divided depending upon vitamin D content into the control group (64 cases, vitamin D content <25 nmol/l) and experimental group (53 cases, vitamin D content >50 nmol/l). Immunofluorescence method was applied to determine the content of T cell subsets in both groups of patients. Intervertebral disc lesion tissues of two groups of patients were obtained during surgery then treated with HE staining and immunohistochemical staining. The values of average optical density obtained under light microscope were observed as the expression quantities of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , to explore the relationship between vitamin D and the expression of cytokines. When vitamin D is lacking, the expression of T lymphocyte subsets in patients with spinal tuberculosis significantly decreased. Compared with experimental group, the difference was statistically significant ( $P<0.05$ ). Further, the expression of cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in intervertebral disc lesion tissues of patients with spinal tuberculosis were significantly higher than those of patients with spinal tuberculosis whose vitamin D content was normal ( $P<0.05$ ). In the control group, vitamin D content was negatively correlated with the expression of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . The expression of T lymphocyte subsets in patients with vitamin D deficiency was significantly reduced, and the immune function decreased. The expression of IL-1 $\beta$ , IL-6

and TNF- $\alpha$  in lesions were significantly higher than those of patients with normal vitamin D content. In addition, the lower the content of vitamin D was, the more active the expression of inflammatory factors were, which was not conducive to the recovery of tuberculosis lesions.

## Introduction

Tuberculosis is an infectious disease caused by the pathogenic bacterium *Mycobacterium tuberculosis*. Besides, the development of tuberculosis vaccine and anti-tuberculosis drugs, tuberculosis epidemic situation around the world is quite severe due to existence of drug-resistant strains. According to the statistical data from global patients with tuberculosis, the number of infected patients in Chinese ranked the second in the world (1). Further, the mortality rate of patients from tuberculosis is approximately 130,000 every year, taking the first place in death toll of various infectious diseases (2). Osteoarticular tuberculosis has the highest morbidity, among which there are 50% of patients with spinal tuberculosis. The morbidity of osteoarticular tuberculosis accounts for 3-7% of total tuberculosis morbidity (3). The decreasing immunity of susceptible population is the prime cause of the rising trend. In terms of current research results, vitamin D deficiency is also linked with the susceptibility to *Mycobacterium tuberculosis* (4). However, a specific mechanism behind this relation is not clear (5). Therefore, the present study evaluated the influence of vitamin D deficiency on T cell subsets in patients with spinal tuberculosis. Furthermore, the influence of vitamin D deficiency on the expression of cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in intervertebral disc lesion of patients with spinal tuberculosis were also studied.

## Patients and methods

**Case collection.** One hundred and seventeen patients with spinal tuberculosis who received operative treatment in the Department of Orthopedics in Wuhan City Third Hospital (Wuhan, China) from March 2012 to March 2015 were collected. These subjects included 62 males and 55 females, with average age of  $39.74\pm 8.97$  years. The contents of vitamin D in patients were measured by ELISA method and the subjects were divided into control group (64 cases, with average age of  $41.58\pm 7.27$  years, vitamin D content

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Table I. Comparison of T cell subsets between two groups of patients.

Groups	CD3 <sup>+</sup> (%)	CD4 <sup>+</sup> (%)	CD8 <sup>+</sup> (%)	CD4 <sup>+</sup> /CD8 <sup>+</sup>	Vitamin D (nmol/l)
Control group	65.89±10.14	34.75±6.13	32.51±5.79	1.04±0.41	23.07±1.56
Experimental group	74.57±9.46	44.88±7.47	37.82±6.08	1.69±0.52	55.63±4.82
P-value	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

<25 nmol/l) and experimental group (53 cases, with average age of 39.13±8.41 years, vitamin D content >50 nmol/l). After pathological examination, all cases were diagnosed as spinal tuberculosis for the first time. The nutritional states of all patients were normal. The patients with the medical history of liver and kidney disease, acquired immune deficiency syndrome (AIDS), neoplastic disease, rheumatism immunological disease and thyroid disease etc were excluded. The study was approved by the Ethics Committee of Wuhan City Third Hospital. Written informed consents were signed by the patients and/or guardians.

**Main devices and reagents.** DNYS8-VORTEX-5 vortex vibrator was from Beijing Chinese and Western Yuanda Scientific and Technical Corporation, Beijing, China. Ultra-cold storage freezer was from Thermo Fisher Scientific (Waltham, MA, USA). Centrifugal machine was from Eppendorf (Happauge, NY, USA), FACSCanto II flow cytometry was from Shanghai Zhiyan Scientific Instrument Co. Ltd. (Shanghai, China); and DK-S420 Electro-Thermostatic Water Cabinet was obtained from Hangzhou Aipu Equipment Co. Ltd. (Zhejiang, China). The microscope and image acquisition system and KH-Q300 paraffin slicing machine were obtained from Hubei Kuohai Medical Treatment Science and Technology Co. Ltd. (Hubei, China).

Vitamin D detection kit was from Shanghai Keshun Biotechnology Ltd. (Shanghai, China), CD3/CD4/CD8 T cell staining kit for human was obtained from Beijing Lvyuanbode Biotechnology Ltd. (Beijing, China) and the regulatory T cell staining kit for human was of Shanghai Yanhui Biotechnology Co. Ltd. (Shanghai, China). Further, Leica IL-1 $\beta$  and Leica TNF- $\alpha$  were from Beijing Bioss Biotechnology Co. Ltd. DAB color development kit was from Beijing Zhongshan Ltd. (both, Beijing, China).

**Treatment of operation lesion.** A part of a tuberculosis lesion was obtained during surgery. According to different parts of the lesion, intervertebral discs were collected respectively, including necrotic end plate, fibrous ring and nucleus pulposus. The specimens obtained were washed by sterile water for injection and were then put into 10% formalin for fixation.

**Determination of vitamin D levels.** Venous blood (4 ml) was collected from all selected cases on admission, followed by serum preparation. Sera were then sealed and saved at -80°C. Vitamin D detection kit (Immunodiagnostic Systems Ltd., Boldon, UK) was used to test vitamin D levels in serums of patients. Absorbance was read at 450 nm wavelength. The standard curve was drawn to find the corresponding concentration value of vitamin D.

**Determination of T lymphocyte subsets.** Before treatment, 4 ml fasting blood of all selected cases were collected on admission, and T lymphocyte subpopulation was detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), including CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>+</sup>.

**Hematoxylin and eosin (H&E) staining.** The sections were conducted with transparent disposal by xylene and gradient de-waxing by alcohol. After hematoxylin staining for 15 min, 0.5% eosin solution was applied for re-dyeing followed by dehydration and sealing of slides.

**Immunohistochemical staining.** Paraffin section was used for transparent disposal by xylene and gradient dewaxing by alcohol. At 37°C, 0.3% hydrogen peroxide formaldehyde solution was added after digestion of smear through pepsase. It was sealed after 15 min, washed by PBS solution 2 times and incubated for 15 min with bovine serum albumin. Rabbit anti-human IL-1 $\beta$ , IL-6 and TNF- $\alpha$  polyclonal antibodies (1:500; cat. nos. 16806-1-AP, 21865-1-AP, 17590-1-AP; Proteintech, Wuhan, China) were added and saved at 4°C. The next day, antibodies were wiped off and washed by PBS solution. Goat anti-rabbit polyclonal antibody (1:1,000; cat. no. SA00001-2; Proteintech) was added for incubation, and DAB was used for color development. Finally, the section was dyed by hematoxylin and dispose after washing with distilled water and gradient de-waxing by alcohol.

**Observation and counting.** The dyed sections were magnified and recorded with image capture. Five erosion areas were selected randomly from intervertebral disc tissue. IPP software was used to measure the average optical value of cytokines, and the average of these five areas were taken as the expression quantity of cytokines.

**Statistical analysis.** SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistic analyses of the results. Measurement data were expressed as mean  $\pm$  standard deviation. Independent sample t-test was used to detect the comparison among groups. Pearson's correlation analysis was applied to the correlative analysis,  $\alpha=0.05$  as test level. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Comparison of T cell subsets.** The expression of T lymphocyte subsets in control group (short of vitamin D) was significantly lower than that of experimental group (vitamin D was normal) (P<0.05). At the same time, the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> in control group was significantly lower than that of experimental group (P<0.05) (Table I).

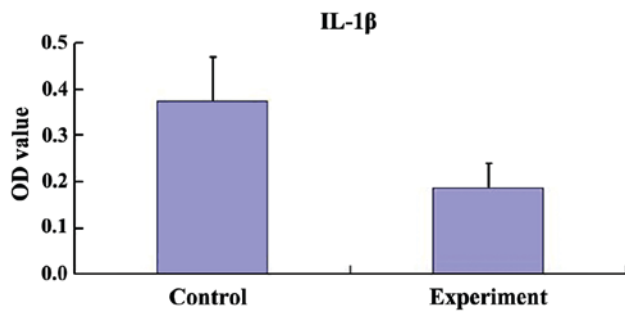


Figure 1. Comparison of IL-1 $\beta$  value in fibrous rings of two groups.

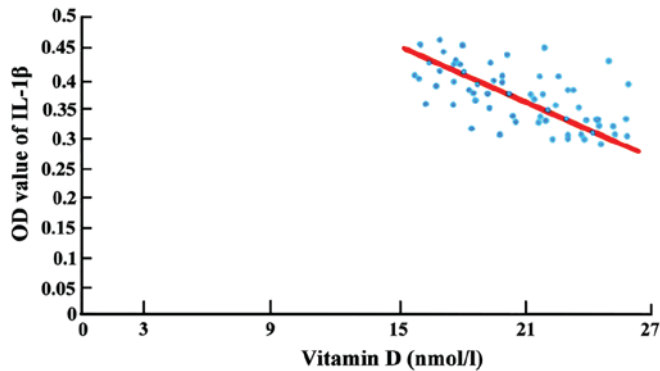


Figure 2. A negative correlation between vitamin D and IL-1 $\beta$  content in patients of control group ( $r = -0.742$ ,  $P < 0.05$ ).

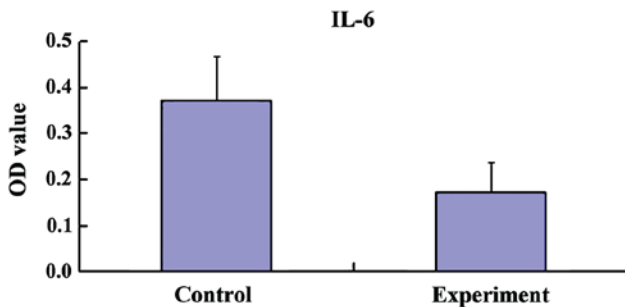


Figure 3. Comparison of IL-6 value in fibrous rings of two groups.

**Comparison of IL-1 $\beta$  value in fibrous rings.** The content of IL-1 $\beta$  of inflammatory factor in fibrous ring of control group was significantly higher than that of the experimental group,  $P < 0.05$  (Fig. 1). Furthermore, vitamin D of control group had a strong correlation with the content of IL-1 $\beta$  ( $r = -0.742$ ), with a negative correlation (Fig. 2).

**Comparison of IL-6 value in fibrous rings.** The content of IL-6 of inflammatory factor in fibrous ring of control group was significantly higher than that of the experimental group, and the difference had statistical significance,  $P < 0.05$  (Fig. 3). In addition, vitamin D of control group had a strong correlation with the content of IL-6 ( $r = -0.715$ ), with a negative correlation (Fig. 4).

**Comparison of TNF- $\alpha$  value in fibrous rings.** The content of tumor necrosis factor TNF- $\alpha$  in fibrous ring of control group was significantly higher than that of experimental group, and

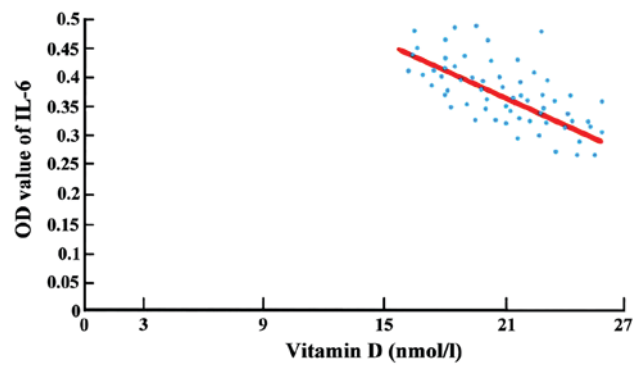


Figure 4. A negative correlation between vitamin D and IL-6 content in patients of the control group ( $r = -0.715$ ,  $P < 0.05$ ).

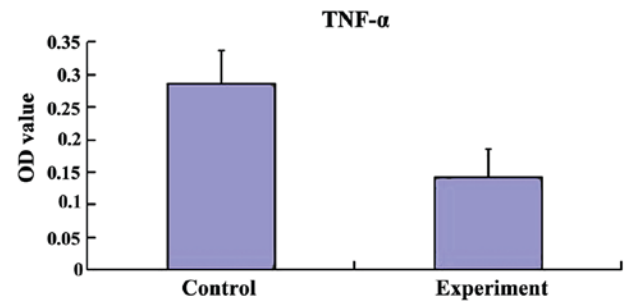


Figure 5. Comparison of TNF- $\alpha$  value in fibrous rings of two groups.

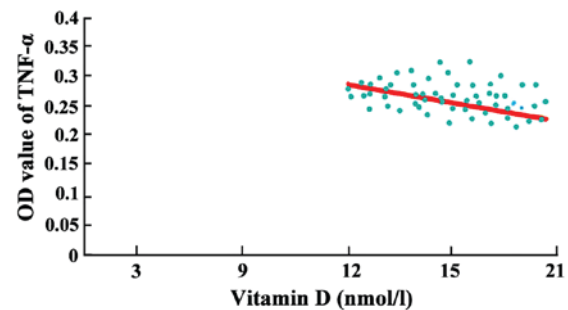


Figure 6. A negative correlation between vitamin D and TNF- $\alpha$  content in patients of the control group ( $r = -0.803$ ,  $P < 0.05$ ).

the difference had statistical significance,  $P < 0.05$  (Fig. 5). Moreover, vitamin D of the control group had a strong correlation with the content of TNF- $\alpha$  ( $r = -0.803$ ), with a negative correlation (Fig. 6).

## Discussion

At present, due to appearance of tuberculosis drug-resistance bacteria, the prevention and cure of tuberculosis is facing a serious situation (6). Therefore, exploring new treatment methods based on the existing medicines has a significant meaning. Recent research has revealed that vitamin D plays an important regulatory role in immune system of patients with tuberculosis. This in turn could improve the antibacterial ability of *Mycobacterium tuberculosis*. Thus, vitamin D could be taken as an adjuvant therapy for the patients with tuberculosis (7,8).

1, 25-(OH)<sub>2</sub>-D<sub>3</sub>, one of active ingredients of vitamin D, has a verified hormone effect in human body. This could adjust the immune function of organism to keep human immunity balance (9). T lymphocyte subset and monocytes are the target cells of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> immunoregulation, to enhance the immunity of organism by proliferation promotion (10). The results of the study showed that the expression of T lymphocyte subsets in patients with spinal tuberculosis (lacked vitamin D) were lower than those of patients (vitamin D content was normal). The level of T lymphocyte subsets *in vivo* could represent the immunity strength of cells in human body, as a crucial laboratory index reflecting cell function. When a person is infected with *Mycobacterium tuberculosis*, infection could inhibit the growth of immune cells and weaken normal immune cell function (11).

The ratio of CD4<sup>+</sup> and CD8<sup>+</sup> in T lymphocyte subsets reflects the immunocompetence of the organism. A high ratio indicates a good immunity, and decreasing ratio suggests a damaged immune-competence. It is also related to the severity of tuberculosis. The lower the ratio, the more serious the tuberculosis (12,13). The present study showed that the ratio between CD4<sup>+</sup>/CD8<sup>+</sup> of the patients with spinal tuberculosis (lack vitamin D) were lower than that of patients with spinal tuberculosis (vitamin D content was normal). The immunity of human body is regulatory and disequilibrium might result in an abnormal immunity (14).

Research of specific pathogenesis of spinal tuberculosis is not completely definite yet. It has been considered that the occurrence of spinal tuberculosis is related to the function of cytokines and abnormal immunity *in vivo*. A study found that IL-6 in spinal tuberculosis is one of the cytokines that led to osteoporosis (15). During the pathologic process, IL-6 could cause decreasing proteoglycan, so as to influence the compound of fibrocytes, which is one of the reasons that led to spine inflammatory lesion progress. In addition, IL-6 also influences the function of immune cells, leading to pathologic change of intervertebral disc among patients. A recent study showed that the contents of IL-6 in patients with spinal tuberculosis were higher than those of normal people (16). In the present study, the contents of IL-6 of patients with spinal tuberculosis (lacked vitamin D) were significantly higher than those of patients (normal vitamin D content). The expression of IL-6 had a negative correlation with Vitamin D. Thus, supplement of vitamin D might be helpful to decrease the generation of IL-6 in the inflammatory process and protect the intervertebral disc.

Tumor necrosis factor TNF- $\alpha$  has the ability to stimulate the production of cytokines Th1 and improve immunity of organism during the development of spinal tuberculosis (17). However, overexpression of TNF- $\alpha$  *in vivo* would lead to an immune injury process producing an undesirable effect on organism (18). In this study, TNF- $\alpha$  of intervertebral disc tuberculosis lesion collected from the patients with spinal tuberculosis was detected. It was observed that the contents of patients with vitamin D deficiency were higher than those of normal vitamin D group. In the same way, there was a negative correlation between them. Thus, keeping a proper vitamin D content *in vivo* is beneficial in prevention of overexpression of TNF- $\alpha$  in patients.

IL-1 $\beta$  is a proinflammatory cytokine responsible for a strong proinflammatory function against spinal tuberculosis

inflammation. During the process of stimulating organism to compound other cytokines, IL-1 $\beta$  also could activate monocytes and macrophages at the same time. This in turn leads to inflammatory infiltration of lesion in patients with spinal tuberculosis (19). Thus, IL-1 $\beta$  is an important factor causing inflammation and one of the reasons leading to abnormal expression of inflammatory cells in patients with spinal tuberculosis (20). The present study also found that the expression of IL-1 $\beta$  in vitamin D deficiency group was higher than that of normal vitamin D group as for the intervertebral disc lesions of patients with spinal tuberculosis, and this had a negative correlation with vitamin D content. Thus, it is suggested that vitamin D might be helpful to reduce the expression of IL-1 $\beta$  in intervertebral disc lesion of patients with spinal tuberculosis.

Collectively, the expression of T lymphocyte subsets in patients with spinal tuberculosis and vitamin D deficiency significantly reduced, and the immune function decreased as well. The expressions of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in lesion were significantly higher than those of patients with normal vitamin D content. In addition, the lower the content of vitamin D is, the more active the expression of inflammatory factors are, which is not conducive to the recovery of tuberculosis lesions. However, the number of study samples is not large enough, which may require further improvement in the following studies.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

SHZ drafted the manuscript. XW and SHZ treated the patients. MYF and HLL analyzed vitamin D levels. FB and TH helped with T lymphocyte subsets determination and hematoxylin and eosin staining. HYF contributed to immunohistochemical staining. All authors read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

The study was approved by the Ethics Committee of Wuhan City Third Hospital (Hubei, China). Written informed consents were signed by the patients and/or guardians.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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