

## ORIGINAL RESEARCH ARTICLE

# Effect of 1.25(OH)<sub>2</sub>D<sub>3</sub> on experimental autoimmune neuritis and its mechanism

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### ABSTRACT

**Objective:** To study the potential therapeutic effects of active vitamin D<sub>3</sub> (1.25(OH)<sub>2</sub>D<sub>3</sub>) in the experimental autoimmune neuritis (EAN). **Methods:** The EAN model was established by actively immunizing Lewis rats with synthetic PO<sub>180-199</sub> peptide and Freund's complete adjuvant. 1.25(OH)<sub>2</sub>D<sub>3</sub> treatment was given, weight change of rats and clinical score were analyzed. HE staining was used to detect the inflammatory cell infiltration of sciatic nerves and demyelination of sciatic nerves was observed by transmission electron microscope (TEM) at the same time. The expressions of inflammatory cytokines IL-17, IL-10, TGF-β, IFN-γ were detected by ELISA, and the expressions of Th17, Treg were examined by RT-PCR. **Results:** 1.25(OH)<sub>2</sub>D<sub>3</sub> ameliorated body weight loss and myelin lesions. It decreased expressions of inflammatory cytokines IL-17, IFN-γ and ROR $\gamma$ t while those of IL-10, TGF-β and FoxP3 were increased. **Conclusions:** 1.25(OH)<sub>2</sub>D<sub>3</sub> can improve the clinical pathological changes of EAN rats, and the mechanism may be related to the changes of inflammatory cytokines. 1.25(OH)<sub>2</sub>D<sub>3</sub> is expected to become a new strategy for the clinical treatment of GBS/EAN.

**Keywords:** Experimental Autoimmune Neuritis; 1.25(OH)<sub>2</sub>D<sub>3</sub>; Inflammation Cytokines; T-lymphocytes

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## 1. Introduction

Guillain-Barré syndrome (GBS), also known as acute inflammatory demyelinating polyneuropathy, is an autoimmune disease characterized by demyelination of peripheral nerves and nerve roots and infiltration of small vascular inflammatory cells. Due to the unknown etiology, symptomatic supportive therapy and specific immunotherapy, such as plasma exchange and intravenous immunoglobulin, are mainly used, but the effects of the two treatments are not satisfactory. At present, there is no measure to block the progress of the disease. EAN is a classic animal model of GBS. It has the pathological characteristics of peripheral nerve demyelination and inflammatory cell infiltration. Its clinical manifestations and neurophysiological changes are very similar to human GBS. It is widely used in the basic research of the pathogenesis and treatment of GBS. 1.25(OH)<sub>2</sub>D<sub>3</sub> is the active form of vitamin D. After binding with vitamin D receptor (VDR), it forms a dimer with retinoid X receptor (RXR) and binds to vitamin D response elements (VDRE) on the DNA sequence of downstream genes, so as to control the transcription of downstream genes. It has long been believed that vitamin D mainly plays a role in calcium and phosphorus metabolism and bone metabolism. However, VDR is expressed in almost all human

tissue types, including immune cells<sup>[1]</sup>. Recent studies have shown that vitamin D plays a role in innate and specific immunity by regulating the activation of T lymphocytes, B lymphocytes and macrophages. Epidemiological studies show that vitamin D deficiency is associated with a variety of autoimmune diseases<sup>[2-5]</sup>. Spanier *et al.*<sup>[6]</sup> found that supplementation of vitamin D<sub>3</sub> can reduce the incidence rate and severity of experimental autoimmune encephalomyelitis (EAE). However, Meehan *et al.*<sup>[7]</sup> proposed that it's hypercalcemia caused by 1.25(OH)<sub>2</sub>D<sub>3</sub> which can improve EAE, rather than the therapeutic effect of 1.25(OH)<sub>2</sub>D<sub>3</sub> itself. However, the effect of vitamin D on EAN is not clear. Therefore, this study established an EAN rat model, observed the behavioral manifestations, pathological changes and inflammatory cytokines of EAN rats after 1.25(OH)<sub>2</sub>D<sub>3</sub> treatment, and comprehensively evaluated the effect of 1.25(OH)<sub>2</sub>D<sub>3</sub> on EAN and its mechanism.

## 2. Materials and methods

### 2.1 Materials

#### 2.1.1 Experimental animals

Healthy male Lewis rats, 6–8 w, weighing 160–180 g, were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd.

#### 2.2.2 Main reagents

Peripheral nerve myelin antigen PO<sub>180-199</sub> was purchased from GL Biochem (Shanghai) Ltd.; Freund's incomplete adjuvant and active vitamin D<sub>3</sub> were purchased from Sigma company of the United States; mycobacterium tuberculosis H37Ra was purchased from Difco company; Trizol was purchased from Invitrogen; ELISA kits for IL-17, IL-10, TGF-β, and IFN-γ were purchased from ABclonal.

### 2.2 Methods

#### 2.2.1 Establishment of the EAN model and treatment with 1.25(OH)<sub>2</sub>D<sub>3</sub>

25 Lewis rats were randomly divided into the normal control group (Control), model group (EAN), low-dose vitamin D group (VDI), medium-dose vitamin D group (VDM) and high-dose

vitamin D group (VDh), with 5 rats in each group. 250 μg PO<sub>180-199</sub> was emulsified in the same amount of complete Freund's adjuvant (containing 10 mg·mL<sup>-1</sup> mycobacterium tuberculosis H37Ra) as the sensitizers. The rats in the model group and vitamin D treatment groups were injected with sensitizers at multiple points on the soles of both feet of the hind limbs for a total of 100 μL/rat. From the 5<sup>th</sup> day after immunization, VDI, VDM, and VDh groups were given 0.25 μg·kg<sup>-1</sup>, 1 μg·kg<sup>-1</sup>, 4 μg·kg<sup>-1</sup> 1.25(OH)<sub>2</sub>D<sub>3</sub> by gavage administration once a day for 7 days. The control and EAN groups were given 0.2 mL peanut oil by gavage administration every day for 7 days.

#### 2.2.2 Clinical score of nervous system signs

From the 0 day of immunization, two experimenters weighed, observed and scored the rats at the same time every day. The scoring criteria are as follows: 0 point: normal; 1 point: the tension of rat tail muscle decreases and the tail tip turns up; 2 points: caudal paralysis, and loss of righting reflex; 3 points: loss of righting reflex; 4 points: gait disorder and abnormal posture; 5 points: hemiplegia of hind limbs; 6 points: moderate paralysis of hind limbs; 7 points: severe paralysis of hind limbs; 8 points: quadriplegia; 9 points: on the verge of death; 10 points: death.

#### 2.2.3 Evaluation of action potential of sciatic nerves

The action potential conduction velocity and latency of rat sciatic nerves were measured by a biological signal recorder. The methods are as follows: at the peak of onset, lay the rats in a prone position on a constant temperature operating table under 10% chloral hydrate anesthesia, shave the hair of the left leg, cut off the skin of the left hip after routine disinfection, cut off the blunt separation along the fascia, free and expose the sciatic nerves, and put them in the stimulating electrode recording electrode, set the nerve stimulation indexes as: 1 Hz, 5 mA, 0.1 ms, and evoke compound muscle action potential (CAMP). The CAMP amplitude, latency and motor nerve conduction velocity (MNCV) were detected and recorded by a four-channel biological signal collector. The nerve

conduction of each rat was recorded 3 times. During the measurement, the nerve stem was kept moist with tabletop liquid.

#### **2.2.4 Morphological evaluation of sciatic nerves**

On the 15<sup>th</sup> day after immunization, the sciatic nerves were immediately separated under 10% chloral hydrate anesthesia, fixed in 4% paraformaldehyde, dehydrated with gradient alcohol, transparentized by xylene, and then embedded in paraffin, and sectioned. The infiltration of lymphocytes and macrophages between sciatic nerve bundles and around small vessels was observed under HE staining light microscope.

#### **2.2.5 Ultra-structural observation of sciatic nerves**

On the 15<sup>th</sup> day after immunization, the sciatic nerves were immediately isolated under 10% chloral hydrate anesthesia, and fixed in 0.2 mol·L<sup>-1</sup> glutaraldehyde. The demyelination of sciatic nerve axons was observed by a transmission electron microscope after routine dehydration, embedding, ultrathin section and staining.

#### **2.2.6 Detection of IL-17, IL-10, TGF- $\beta$ and IFN- $\gamma$ levels by ELISA**

On the 15<sup>th</sup> day after immunization, blood was collected from the retroorbital venous plexus, centrifuged at 3,000 rpm for 5 min, and the supernatant was retained. Add 100  $\mu$ L standard substances or samples to be tested into the detection hole of the enzyme plate according to the requirements of the manual and incubate at 37 °C for 2 h. Add 100  $\mu$ L antibodies to each hole after plate washing and incubate at 37 °C for 1 h. After plate washing, add 100  $\mu$ L enzyme binding substrate respectively and incubate at 37 °C for 30 min. Discard the liquid in the hole and add 100  $\mu$ L TMB after plate washing. Add 100  $\mu$ L termination solution after 15 min. Set the wavelength at 450 nm, zero the blank hole, and immediately detect the OD value with an enzyme labeling instrument. The concentration of IL-17, IL-10 TGF- $\beta$  and IFN- $\gamma$  in serum was calculated according to the standard curve.

#### **2.2.7 Detection of IL-17, IFN- $\gamma$ , RORrt,**

#### **FoxP3 mRNA levels by RT-PCR**

On the 15<sup>th</sup> day after immunization, the rats were anesthetized with 10% chloral hydrate and routinely disinfected, and the spleen was taken out in a sterile environment. Grind the spleen with Trizol in liquid nitrogen, and extract total RNA. The RNA concentration was measured by ultraviolet spectrophotometer and then reverse transcribed into cDNA for RT-PCR to detect the level change of IL-17, IFN- $\gamma$ , RORrt, FoxP3 mRNA. The primer design was as follows: FoxP3 F: 5' CCT ACC CAC TGC TGG CAA ACG 3', R: 5' ACT TCT CTC TGG AGG AGG CAC TG 3'; RORrt F: 5' AGG TAT GAC CGA TGC TCT TA 3', R: 5' TAT TTT CGG ATA AGT CTA GG 3'; IL-17 F: 5' TGG ACT CTG AGC CGC ATT GA 3', R: 5' GAC GCA TGG CGG ACA ATA GA 3'; IFN- $\gamma$  F: 5' AAA GAC AAC CAG GCC ATC AG 3', R: 5' CTT TTC CGC TTC CTT AGG CT 3'; GAPDH F: 5' TCG TGG AGT CTA CTG GCG TCT T 3', R: 5' CAT TGC TGA CAA TCT TGA GGG AG 3.

#### **2.3 Statistical methods**

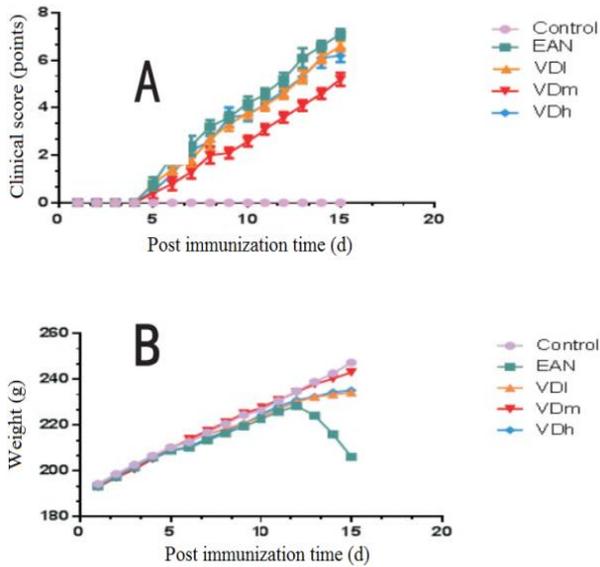
SPSS 16.0 statistical software was used to analyze the data. The measurement data were expressed as  $\bar{x} \pm s$ . The measurement data between groups were compared by analysis of variance. The pairwise comparison between multiple samples was carried out by Mann-Whitney test. The difference  $P < 0.05$  is statistically significant.

### **3. Results**

#### **3.1 Weight change and clinical score of rats**

The rats in the EAN group began to appear symptoms on the 5<sup>th</sup> day after immunization, which reached the peak on the 15<sup>th</sup> day. The clinical scores of the VDI group ( $6.6 \pm 0.23$ ), the VDM group ( $5.2 \pm 0.28$ ) and the VDh group ( $6.2 \pm 0.33$ ) were lower than those of the EAN group ( $7.1 \pm 0.35$ ). The effect in the VDM group was more obvious, but there was no significant difference between groups ( $P > 0.05$ ). The weight gain rate of rats in the EAN group decreased from the onset of clinical symptoms, and the weight decreased rapidly at the peak of onset on the 13<sup>th</sup>–15<sup>th</sup> day. However, the weight gain rate of rats in the 1.25(OH)<sub>2</sub>D<sub>3</sub> intervention

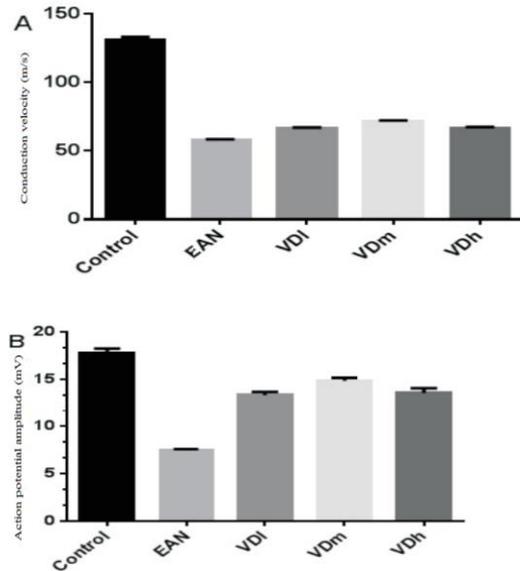
group decreased from the onset of clinical symptoms, but there was no significant weight loss. The weight loss of the VDI, VDM and VDh groups was lower than that of the EAN group, but there was no significant difference compared with the EAN group ( $P > 0.05$ ). See **Figure 1**.



**Figure 1.** Effects of  $1.25(\text{OH})_2\text{D}_3$  treatment on clinical scores (A) and weight (B) of EAN rats.

### 3.2 Neurophysiological changes in EAN rats

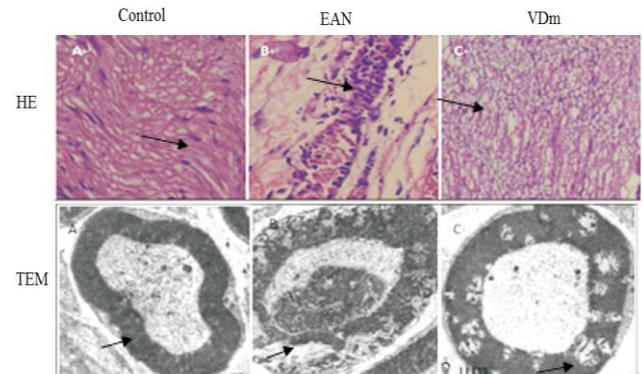
The sciatic nerve conduction velocity in the EAN group was  $(57.7 \pm 0.78) \text{ m}\cdot\text{s}^{-1}$ , which was significantly lower than that in the control group  $(133.3 \pm 0.28) \text{ m}\cdot\text{s}^{-1}$  ( $P < 0.05$ ); the conduction velocity of sciatic nerves in the VDI group, VDM group and VDh group were  $(66.7 \pm 0.45) \text{ m}\cdot\text{s}^{-1}$ ,  $(71.8 \pm 0.56) \text{ m}\cdot\text{s}^{-1}$  and  $(66.4 \pm 1.15) \text{ m}\cdot\text{s}^{-1}$  respectively, which were significantly improved compared with the EAN group, especially in the VDM group ( $P < 0.05$ ). The amplitude of action potential of sciatic nerves in the EAN group was  $(7.49 \pm 0.13) \text{ m}\cdot\text{s}^{-1}$ , which was lower than that in the control group  $(17.8 \pm 0.44) \text{ m}\cdot\text{s}^{-1}$  ( $P < 0.05$ ); the action potential amplitudes of sciatic nerves in the VDI group, VDM group and VDh group were  $(13.3 \pm 0.36) \text{ m}\cdot\text{s}^{-1}$ ,  $(14.8 \pm 0.31) \text{ m}\cdot\text{s}^{-1}$  and  $(13.6 \pm 0.47) \text{ m}\cdot\text{s}^{-1}$ , respectively, which were significantly higher than those in the EAN group. In conclusion,  $1.25(\text{OH})_2\text{D}_3$  treatment reduced peripheral nerve injury in EAN rats, and the VDM group had the best effect. See **Figure 2**.



**Figure 2.** Effects of  $1.25(\text{OH})_2\text{D}_3$  treatment on sciatic nerve conduction velocity (A) and action potential amplitude (B) in EAN rats.

### 3.3 Sciatic nerve inflammatory cell infiltration and demyelination in EAN rats

At the peak of the disease, the sciatic nerves were examined by HE staining and a transmission electron microscope.



**Figure 3.** Inflammatory cell infiltration and demyelination of sciatic nerves were detected by HE staining and a transmission electron microscope.

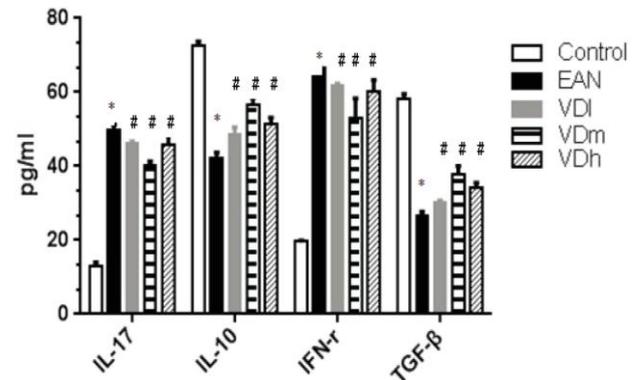
The results of HE staining showed that compared with the control group, a large number of inflammatory cells infiltrated around the small vessels of sciatic nerves in the EAN group, while the inflammatory cell infiltrated around the vessels in the VDM group was significantly reduced (**Figure 3** HE - A, B, C). The results of the transmission electron microscope showed that the axonal structure of myelinated nerve fibers in the control group was normal, and the myelin lamina was regularly arranged around the center of the circle. In the EAN group, there were swelling of myelin lamina, non-

eycomb changes and peeling of myelin inner layer and axon. After treatment with  $1.25(\text{OH})_2\text{D}_3$ , the changes of myelin swelling and cavity formation in the VDM group were less than those in the EAN group (Figure 3 TEM - A, B, C).

### 3.4 Detection of IL-17, IL-10, TGF- $\beta$ and IFN- $\gamma$ in rats' serum by ELISA

Existing theories believe that inflammatory cytokines are one of the factors aggravating the disease of EAN. Therefore, we used ELISA to detect IL-17, IL-10, TGF- $\beta$  and IFN- $\gamma$  level in peripheral blood of rats in each group. The results showed that compared with the control group, the serum inflammatory cytokines IL-17 and IFN- $\gamma$  in the EAN group increased obviously. The levels of IL-10 and TGF- $\beta$  decreased significantly. The difference ( $P < 0.05$ ) is of statistical significant. Compared with the EAN group, serum IL-17 and IFN- $\gamma$  in the VDI, VDM and VDh groups after  $1.25(\text{OH})_2\text{D}_3$  treatment decreased significantly. The levels of IL-10 and TGF- $\beta$  increased significantly ( $P < 0.05$ ). There were significant differences in the pairwise comparison between the VDI group vs the VDM group and the VDM group vs the VDh group

( $P < 0.05$ ), but there was no significant difference between the VDI group vs the VDh group ( $P > 0.05$ ). See Figure 4.

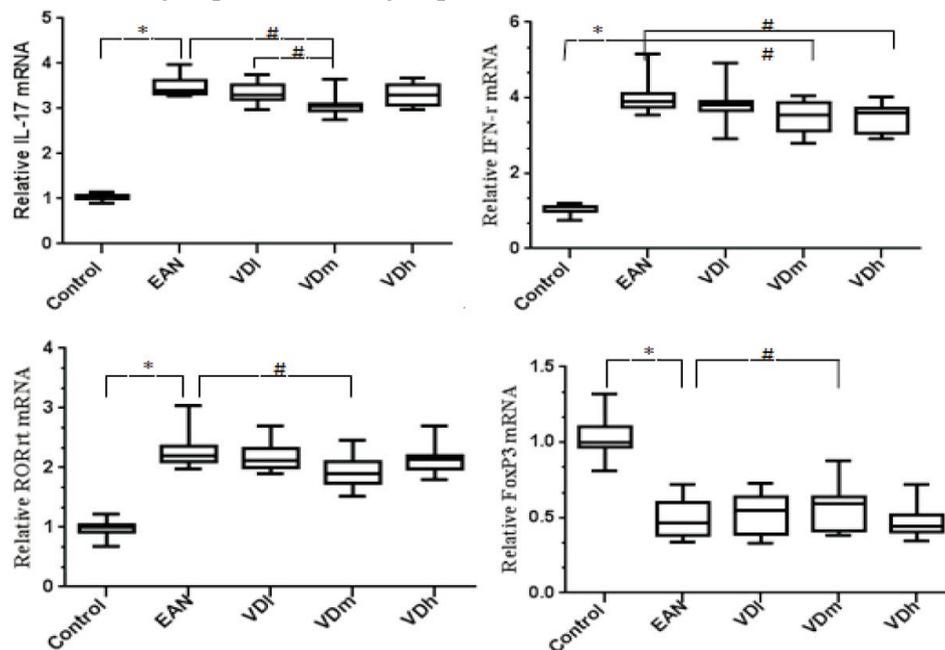


**Figure 4.** The level of inflammatory cytokines in peripheral blood ( $\bar{x} \pm s$ ).

Note: \* compared with the control group,  $P < 0.05$ ; # compared with the EAN group,  $P < 0.05$ .

### 3.5 Detecting of the expression level of inflammatory cytokine mRNA in rat spleen by RT-PCR

At the peak of onset, the inflammatory cytokine mRNA in the spleen of rats in each group was detected by RT-PCR.



**Figure 5.** Expression levels of inflammatory cytokines and T lymphocyte mRNA in spleens ( $2^{-\Delta\Delta CT}$ ,  $\bar{x} \pm s$ ).

Note: \* compared with the control group,  $P < 0.05$ ; # compared with the EAN group,  $P < 0.05$ .

The results showed that compared with the control group, the expression level of IL-17 and IFN- $\gamma$ , RORrt mRNA in the EAN group increased significantly and the level of FoxP3 mRNA de-

creased significantly. The difference was of statistical significance ( $P < 0.05$ ). Compared with EAN group, the expression level of IL-17 and IFN- $\gamma$ , RORrt mRNA in VDM decreased significantly, and

the level of FoxP3 mRNA increased significantly ( $P < 0.05$ ). Compared with the EAN group, the VDh group showed obvious decrease only in the IFN- $\gamma$  level. The level of IL-17 in the VDM group was significantly lower than that in the VDI group ( $P < 0.05$ ). The above experimental results showed that the expression level of inflammation related cytokines decreased after 1.25(OH) $_2$ D $_3$  intervention treatment, and the therapeutic effect of medium-dose 1.25(OH) $_2$ D $_3$  was significantly better than that of the low- and high-dose groups. See **Figure 5**.

#### 4. Discussion

At present, it is considered that GBS is an autoimmune disease mediated by T cells. The main clinical symptoms are limb weakness and symmetrical delayed paralysis of limbs. The main pathological features are demyelination of peripheral nerves and nerve root and infiltration of small vasculitis cells. EAN is a classic animal model of GBS, very similar to GBS in pathological changes, clinical manifestations and neuroelectrophysiological changes, and is widely used in the study of the pathogenesis and treatment of GBS<sup>[8]</sup>. Many studies have shown that the destruction of immune homeostasis is the basis of GBS. Th17, Treg cells and their effectors constitute a complex network of downstream genes regulated by VDR and systemic lupus erythematosus and multiple sclerosis, which are involved in the occurrence, development and prognosis of GBS. Under physiological homeostasis, Th1/Th2 and Th17/Treg cells are in dynamic balance<sup>[9,10]</sup>. The characteristic cytokine IL-17 secreted by Th17 cells participates in the occurrence of autoimmune diseases by inducing the inflammatory cascade of target organs. Treg cells with high expression of transcription factor FoxP3 inhibit Th cell function, secrete inhibitory inflammatory cytokines, inhibit autoimmune response and maintain immune homeostasis through direct contact. When the body is stimulated by pathogens, Th1 and Th17 cells are activated to produce inflammatory response against pathogens. If the regulation of this process is unbalanced, it may lead to autoimmune diseases. In the early stage of EAN, IFN- $\gamma$  and IL-17 levels in peripheral blood increase signifi-

cantly; after the disease enters the recovery period, IFN- $\gamma$  and IL-17 levels decrease gradually<sup>[11]</sup>. The content of IL-17A in lymphocytes of EAE rat model increases significantly<sup>[12]</sup>, Th1 cells in peripheral blood of patients with GBS increases significantly, and Treg cells decreases significantly. Our previous study found that the expression of Th1/Th2 and Th17/Treg in peripheral blood of GBS patients is unbalanced. Gastrointestinal perfusion of bifido-bacterium infantis in ENA rats can improve the Th17/Treg imbalance and autoimmune inflammatory response<sup>[13,14]</sup>.

Vitamin D has no biological activity. It is transformed into the active form of 1.25(OH) $_2$ D $_3$  and plays a role in combination with VDR. VDR widely exists in immune cells and the central nervous system, such as hypothalamus, hippocampus and cortical neurons. Recent studies have shown that vitamin D plays a role in innate and specific immunity by regulating T lymphocytes; at the same time, studies have shown that vitamin D can not only affect the proliferation and maturation of T cells, but also reduce the expression of IL-17, promote the proliferation of Treg cells and enhance their immune tolerance<sup>[15,16]</sup>. Kevin *et al.*<sup>[17]</sup>, Tedeschi *et al.*<sup>[18]</sup> and Volt *et al.*<sup>[19]</sup> detected that the downstream genes regulated by VDR are related to systemic lupus erythematosus and multiple sclerosis. Studies of Hau *et al.*<sup>[20]</sup>, and Jeffery *et al.*<sup>[21]</sup> showed that the therapeutic effect of 1.25(OH) $_2$ D $_3$  in rheumatoid arthritis and psoriasis was related to IL-17A.

In this study, 0.25  $\mu\text{g}\cdot\text{kg}^{-1}$ , 1  $\mu\text{g}\cdot\text{kg}^{-1}$ , 4  $\mu\text{g}\cdot\text{kg}^{-1}$  1.25(OH) $_2$ D $_3$  were used to intragastric treatment of EAN rats. The results showed that compared with the P0 peptid-induced EAN model group, 1.25(OH) $_2$ D $_3$  reduced the clinical symptoms, clinical scores and weight loss in EAN rats. The sciatic nerve conduction velocity was significantly improved. From the perspective of morphology, the method effectively reduced the sciatic neuritis cell infiltration and myelin sheath loss, among which the 1  $\mu\text{g}\cdot\text{kg}^{-1}$  VDM group had the best effect. Research of Mohammadi *et al.*<sup>[22]</sup> showed that vitamin D reduces infiltration and demyelination of inflammatory cells into the central nervous system, which is consistent with our results.

The inflammatory cytokines in peripheral blo-

od of EAN rats were detected at the peak of the disease.  $1.25(\text{OH})_2\text{D}_3$  decreased the expression of pro-inflammatory cytokines IL-17 and IFN- $\gamma$ , and increased the expression of anti-inflammatory cytokines IL-10 and TGF- $\beta$  in peripheral blood of EAN rats. The moderate-dose group (VDm) had a worse effect, while the high-dose group had a better effect. IFN- $\gamma$ , on the one hand, promotes the pathogenesis of EAN by activating macrophages to produce NO and reactive oxygen intermediates, and on the other hand, down-regulates IL-17 by inhibiting the amplification of Th17 cells in the IL-23 pathway, which plays a bidirectional role in EAN. Speck *et al.*<sup>[23]</sup> confirmed that high expression of TGF- $\beta$  and IL-10 is related to the recovery and remission of EAE, and neutralizing or inhibiting TGF- $\beta$  and IL-10 can aggravate the severity of EAE. However, Treg adoptive transfer can reduce the levels of TGF- $\beta$ , IL-10 and IL-4, thereby alleviating EAE. The results of this study suggest that  $1.25(\text{OH})_2\text{D}_3$  changed the balance of pro-inflammatory cytokines and anti-inflammatory cytokines in the EAN rat model, alleviated EAN clinical symptoms, and the therapeutic effect was dose-dependent. Further detection of Th17 and Treg cell transcription factors in the spleen of EAN rats at the peak of the disease showed that compared with the control group, the expression levels of IL-17, IFN- $\gamma$  and RORrt mRNA in EAN rats increased, while FoxP3 mRNA level decreased.  $1.25(\text{OH})_2\text{D}_3$  decreased the expression levels of IL-17, IFN- $\gamma$  and RORrt mRNA in EAN rats, and up-regulated the level of Foxp3 mRNA. Recombinant IL-17 aggravates sciatic inflammatory cell infiltration and demyelination in EAN rats at acute stage. Reinfusion of amplified Treg cells *in vitro* can alleviate neuralgia and peripheral neuroinflammatory demyelination changes after peripheral nerve injury in EAN rats<sup>[24]</sup>. In this study, treatment of  $1.25(\text{OH})_2\text{D}_3$  and up-regulation of FoxP3 were followed by down-regulation of Th17 and other transcription factors, indicating that the distribution of Th17/Treg cells and inflammatory cytokines in EAN rats changed, and the imbalance of Th17/Treg was corrected. The treatment of  $1.25(\text{OH})_2\text{D}_3$  may be related to Th17/Treg re-balance. Ahangar-Parvin *et al.*<sup>[25]</sup> and Haqhmorad *et al.*<sup>[26]</sup> found that vitamin D could prevent the onset

of EAE and change the proportion of Th1, Th17, Th2 and Treg cells in EAE, which is consistent with our research results.

VDR, as the nuclear receptor of  $1.25(\text{OH})_2\text{D}_3$ , can play a role in regulating gene transcription by binding to the VDRE sequence of the downstream gene promoter region. Tone *et al.*<sup>[27]</sup> found VDRE sequence in the highly preserved non-coding sequence of FoxP3 gene in rats.  $1.25(\text{OH})_2\text{D}_3$  stimulates FoxP3 expression in CD4<sup>+</sup> CD25<sup>-</sup>T cells in human and rats<sup>[26]</sup>. FoxP3 has the ability to directly inhibit IL-17 and IFN- $\gamma$ , FoxP3, RORrt and Runx1 interact to inhibit IL-17 production, and VDR can block the expression of Runx1 and inhibit RORrt transfer viability<sup>[28,29]</sup>. These studies show that  $1.25(\text{OH})_2\text{D}_3$  may stimulate FoxP3 expression by directly binding to the VDRE sequence of FoxP3 gene intron to promote FoxP3 promoter activity, thereby regulating the expression of RORrt, inhibiting the pro-inflammatory Th17 response in the autoimmune process and alleviating EAN inflammation.

To sum up,  $1.25(\text{OH})_2\text{D}_3$  may mediate the protective effect on EAN by influencing the number and function of T cells, inducing the differentiation of Treg cells, reducing the production of pro-inflammatory cytokines and increasing the expression of anti-inflammatory cytokines. Therefore supplementing  $1.25(\text{OH})_2\text{D}_3$  may be an adjunct therapy for GBS, but the disease progression of the EAN rat model is self-limited. During the limited treatment period of the EAN model, whether the immunomodulatory effects of  $1.25(\text{OH})_2\text{D}_3$  can be reproduced in patients with GBS requires further study. The results of this study expound the mechanism of  $1.25(\text{OH})_2\text{D}_3$  treatment for EAN, which provides experimental basis for its future application in the prevention and treatment of GBS.

## Conflict of interest

The authors declare no potential conflicts of interest.

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