



1,25-Dihydroxyvitamin D3 attenuates disease severity and induces synoviocyte apoptosis in a concentration-dependent manner in rats with adjuvant-induced arthritis by inactivating the NF- κ B signaling pathway

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Received: 19 March 2018 / Accepted: 17 July 2018 / Published online: 10 August 2018
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Abstract

An aggressive proliferation of synoviocytes is the hallmark of rheumatoid arthritis (RA). Emerging evidence shows that inhibiting the NF- κ B signaling pathway with 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] may be a therapeutic approach for controlling inflammatory diseases. In this study, we demonstrated the protective effects of three different 1,25(OH)2D3 concentration on adjuvant-induced arthritis (AA) rats through the NF- κ B signaling pathway and their pro-apoptotic roles in cultured adjuvant-induced arthritis synoviocytes (AIASs). AA rats were prepared by injecting complete Freund's adjuvant and independently given daily intraperitoneal injection of 1,25(OH)2D3 at concentrations of 50, 100, and 300 ng/day/kg. Subsequently, AIASs were isolated from the inflamed joints of AA rats to test the effects of 1,25(OH)2D3 on AIASs in vitro. Intraperitoneal injection of 1,25-(OH)2D3 was found to induce a concentration- and time-dependent improvement in relieving the symptoms of AA. We found an increased paw withdrawal thermal latency (PWTl) in the affected paw of AA rats as the concentration of 1,25-(OH)2D3 increased. 1,25-(OH)2D3 treatment reduced levels of inflammatory factors in synovial tissues of AA rats. In the case of cultured AIASs, 1,25-(OH)2D3 was shown to inhibit cell proliferation and induce cell apoptosis in a concentration-dependent manner. Additionally, 1,25-(OH)2D3 inhibited the activation of the NF- κ B signaling pathway. In conclusion, our study provides evidence emphasizing that 1,25(OH)2D3 has the potential to attenuate disease severity in RA potentially due to its contributory role in synoviocyte proliferation and apoptosis. The protective role of 1,25(OH)2D3 against RA depends on the NF- κ B signaling pathway.

Keywords 1,25(OH)2D3 · NF- κ B signaling pathway · Rheumatoid arthritis · Synoviocyte · Adjuvant-induced arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterized by chronic inflammation in joints associated with synovial hyperplasia and infiltration of inflammatory cells, ultimately causing bone and cartilage destruction [1, 2]. RA affects approximately 1% of the worldwide population; physical disability and joint damage are the major contributors associated with the reduction in life quality and significant mortality [3, 4]. The managements of RA are primarily performed with the use of disease-modifying anti-rheumatic drugs [5], which can reduce or reverse symptoms and progression of joint damage [6]. In addition to these conventional agents, some biologic agents were developed for targeting specific inflammatory mediators, but these require

subcutaneous or intravenous administration, have toxicities, or are accompanied by many side effects [7]. Therefore, it is urgent to clarify the potential analgesic mechanisms to alleviate RA and establish more efficient therapeutic strategies.

The inhibition of production of inflammatory factors, synovial hyperplasia, and ascendant apoptosis of synoviocytes are considered important targets of therapy for RA [8]. Most symptoms of RA are presumed results from the activation of some transcription factors and signals such as activator protein-1 (AP-1) and NF- κ B [9]. In clinical patients, some other effective RA drugs exhibit their roles by inhibiting NF- κ B [10]. Moreover, NF- κ B activation accelerates synovial hyperplasia by promoting cell proliferation and inhibiting apoptosis of synoviocytes [11]. In that case, NF- κ B seems to activate RA by regulating cell growth and cell apoptosis in synoviocytes. Gonzalez-Pardo et al. has proved that 1,25(OH)2D3 regulates the NF- κ B signaling pathway by decreasing NF- κ B translocation to the nucleus [12]. Recent data have provided evidence that 1,25(OH)2D3 triggered inhibition of CD⁴⁺ T cells, thereby suppressing the immune response of RA [13]. Herein, in this study, we speculate that 1,25(OH)2D3 is involved in the ablation of RA development through its inhibitory roles in the activation of the NF- κ B signaling pathway and apoptosis of synoviocytes in adjuvant-induced arthritis (AA) rats, an animal model of RA.

Materials and methods

Ethics statement

The study was conducted with the approval of the Animal Ethics Committee of Shandong Provincial Qianfoshan Hospital, Shandong University. All experimental procedures were performed on laboratory animals in accordance with institutional guidelines for the care and use of laboratory animals.

Study subjects

Fifty adult male Sprague–Dawley (SD) rats, weighing 180–220 g, provided by the animal center, were housed under specific pathogen-free (SPF) conditions, at temperature of around 22 °C and with humidity of 49.7% under a 12/12-h light/dark cycle. All rats were treated with 1-week adaptive feeding before experiment.

AA rat model establishment

Rats were anesthetized with 2–4% isoflurane. The left rear toe of rats was disinfected with 75% alcohol. Then rats were induced by subcutaneous injection of 100 μ L complete

Freund's adjuvant (CFA) containing 10 mg of Bacillus Calmette–Guerin (BCG) on the lateral ankle of left hind limb using a disposable, sterile syringe. After model establishment for half an hour, the ankle and toes on the left hind limb were induced with a local inflammatory response of swelling. Red or swollen joints can be observed about 10 days after immunization.

Animal grouping and treatment

Ten from 50 SD rats administrated subcutaneous injection of 0.1 mL glacial acetic acid (0.01 mol/L) on the right hind toe to exclude the sensitization effect of solvents in CFA were included into a normal group. The remaining forty rats were processed with the above model establishment procedure. Ten days later, the forty rats were randomly divided into the AA group ($n = 10$, PBS, i.p) and 1,25(OH)2D3-treated groups, including high concentration of 1,25(OH)2D3 group (H group, $n = 10$, 300 ng/day/kg 1,25(OH)2D3, i.p), medium concentration of 1,25(OH)2D3 group (M group, $n = 10$, 100 ng/day/kg 1,25(OH)2D3, i.p) and low concentration of 1,25(OH)2D3 group (L group, $n = 10$, 50 ng/day/kg 1,25(OH)2D3, i.p). The 1,25(OH)2D3 was dissolved in PBS. Rats in 1,25(OH)2D3-treated groups were provided calcium (20 mg/100 g) by drinking water. Rats in the normal group and 1,25(OH)2D3-treated groups were treated with the same food. On the 26th day after immunization, the rats were killed via intraperitoneal injection of pentobarbital (45 mg/kg). The joints were preserved for the following experiment.

Arthritis index (AI) observation and radiological scoring

The swelling on right limb was observed every 4 days from the day of administration to the day when rats were killed, for 16 consecutive days. The volume difference of the limb before and after inflammation was defined as the swelling degree. The joint lesions of rats were observed and recorded on the 10th, 14th, 18th, 22nd and 26th day after inflammation, evaluated by the Score Method [14]. AI was determined by the cumulative scores of the lesion of the normal limbs, in which 0 point referred to no swelling, 1 point referred to mild toe joint swelling, 2 points referred to toe joint and metatarsal swelling, 3 points referred to swelling of foot below the ankle, 4 points referred to swelling of the foot and ankle. Except the AI of the immunized limb, the total AI (the highest: 12 points) of the other three limbs was recorded. Meanwhile, the swelling score of each rat was obtained by the sum of the score of each joint.

X-ray films of rat joint in each group were taken 26 days after inflammation, with the score of X-ray film of the normal group as control. X-ray films were scored in accordance with the criteria from Sharp method [15] using a scale from

0 (no damage) to 5 (severe damage: extensive bone erosion or bone loss) for bone erosion and a scale from 0 to 4 points for joint space narrowing: 0 points for no narrowing, 1 point for local joint narrowing, 2 points for diffuse narrowing < 50% of the original joint space, 3 points for diffuse narrowing > 50% of the original joint space and 4 points for ankylosis. The sum of the two scores was the radiological score of X-ray film.

Measurement of paw withdrawal thermal latency (PWTL)

The rats were loosely restrained under clear acrylic boxes placed on a 3-mm glass plate. PWTL was measured by the Thermal Paw Stimulation System (BME-410C, Bo Bang Chemical Co., Ltd., Shenzhen, China; 10 V, 30 W, spot diameter 0.5 cm). Radiant heat stimulation was applied by directing a beam of light at the mid-plantar surface of the paw of the rat through the glass plate. To avoid tissue damage, the cut-off time for the heat stimulation was 30 s. Thermal stimulation was applied three times for each rat at an interval of 6–8 min. Time from the beginning of the thermal stimulation to the brisk withdrawal of the hind paw was recorded. The average of the three times was defined as PWTL. The PWTL of the normal group was used as pain threshold baseline. The PWTL was measured 2 h after 1,25(OH)₂D₃ treatment on the 10th day after inflammation. Then PWTL was measured every 5 days.

Tissue extraction and hematoxylin–eosin (HE) staining

Synovial tissues were extracted from the joints of rats in each group, cut into small pieces, and incubated with 2 mg/mL collagenase I at a temperature of 37 °C for 4 h. Then the tissue suspension after incubation was filtered by a nylon membrane (70 μm) and centrifuged (400 g) for 10 min. Next, synoviocytes were collected for subsequent experimentation.

Rats were killed by femoral artery bloodletting 26 days after immunization. The ankle of immunized limb from rats in each group was fixed in formalin, decalcified with nitric acid and washed with tap water overnight. And tissues were then embedded in paraffin after dehydration, sliced into sections, stained with HE, and finally observed under an optical microscope with images collected.

Immunofluorescence

Synoviocytes of AA rats with and without 1,25-(OH)₂D₃ treatment were seeded in a six-well plate (1×10^5 cells/well) for culturing. After the synoviocytes covered the plate, they were fixed in 4% paraformaldehyde for 15 min, washed with PBS three times and treated with 0.5% Triton X-100 for

10 min. Next, the synoviocytes were washed with PBS three times and then blocked by 5% goat serum at room temperature for 1 h. The primary antibody p65 (ab16502, Abcam, USA) diluted at 1:500 was incubated at 4 °C overnight. The following day, the primary antibody was washed with PBS three times. The secondary antibody was incubated at room temperature for 1 h, devoid of light. Following three PBS washes, 4',6-diamidino-2-phenylindole (DAPI) was used to dye the nuclei for 5 min with the fluorescence observed.

5-Ethynyl-2-deoxyuridine (EdU) assay

Synoviocytes in each group were inoculated into a 96-well plate with three duplicated wells set for each group. A complete medium containing 50 μM EdU was added to the synoviocytes on the following day for a 2-h culturing at 37 °C with 5% CO₂. After washing with PBS three times, 4% paraformaldehyde was used to fix the synoviocytes and 0.5% Triton X-100 was used to rupture the cell membrane for 5 min, followed by three PBS washes. Apollo dye liquor was added to the synoviocytes for 30-min incubation, devoid of light, and 0.5% Triton X-100 and methanol were, respectively, applied to clean the synoviocytes, three times each. Then DAPI was used to stain the nuclei, followed by cell counting and photographing. The experiment was repeated three times.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Paw tissue was extracted and added with 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was extracted from the tissues according to the instructions of the TRIzol kit. The purity and concentration of RNA in the tissues and cells were detected using an ultraviolet spectrophotometry (UV1901, Aucybest, Shanghai, China). PrimeScript™ RT reagent kit (RR047A, Beijing Zhijiefangyuan Technology Co., Ltd., Beijing, China) was used for RT-qPCR, which was performed in an ABI 7900HT real-time qPCR instrument (ABI 7900, Shanghai Pu Di Bio Technology Co., Ltd., Shanghai, China) with the two-step method. Three duplicated wells were set for each gene in each sample, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the internal reference. The specific sequences were as follows: (1) mTNF-α: forward: 5'-AAT GGC CTC CCT CTC ATC AGT-3'; reverse: 5'-GCT ACA GGCTTG TCA CTC GAA TT-3'; (2) mIL-6: forward: 5'-TCC AAT GCT CTC CTA ACA GAT AAG-3'; reverse: 5'-CAA GAT GAA TTG GAT GGT CTT G-3'; (3) mIL-1β: forward: 5'-TGG TAC ATC AGC ACC TCA CA-3'; reverse: 5'-TTA TGT CCT GAC CAC TGT TGT TT-3'.

Terminal uridine nick-end labeling (TUNEL) assay

The synoviocytes of rats in the normal group and 1,25(OH)2D3-treated groups were seeded into a six-well plate at a density of 1×10^5 cells/well and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Then the apoptosis of synoviocytes was detected in compliance with the instructions of In Situ Cell Death Detection Kit [Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. The synoviocytes of rats were fixed in 4% paraformaldehyde at room temperature for 1 h and penetrated with 0.1% Triton X-100. Then the cells were incubated with the addition of TUNEL reaction complex for 1 h and labeled with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The cells were labeled by TUNEL assay and observed under a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan). All synoviocytes were labeled by the DAPI. The TUNEL-positive cells (weak positive) were considered apoptotic cells.

Western blot analysis

Synovial tissues from AA rats in the control and 1,25-(OH)2D3-treated groups were preserved in liquid nitrogen. After grinding, the tissues were lysed in lysis buffer [0 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3 , 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mg/mL aprotinin and 100 mg/mL phenylmethanesulfonyl fluoride (PMSF)]. Protein concentration was determined using an ultraviolet-visible (UV-Vis) spectrophotometer. Then the extracted protein samples were separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature, incubated overnight at 4 °C with the addition of anti-p65 (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA). After that, they were washed three times in TBST, 10 min each time, and then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:5000; Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China). The protein bands were developed by enhanced chemiluminescent.

Statistical analysis

All data analyses were conducted using SPSS 18.0 software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as the mean \pm standard deviation. Comparisons of data obeying normal distribution between two groups were performed with unpaired two-tailed *t* tests while

comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA). A $p < 0.05$ was considered to be statistically significant.

Results

1,25-(OH)2D3 treatment induces a concentration- and time-dependent improvement in relieving the symptoms of AA

At first, we observed the value of AI on the 10th, 14th, 18th, 22nd and 26th day from AA rats without or with 1,25(OH)2D3 treatment of different concentration, so as to identify the effects of 1,25-(OH)2D3 on the symptoms of RA. As shown in Fig. 1, the AI in AA rats was decreased after 1,25(OH)2D3 treatment and the decrease was much more obvious with the increase of 1,25(OH)2D3 concentration ($p < 0.05$). Taken together, 1,25-(OH)2D3 treatment could induce a concentration- and time-dependent improvement in relieving the symptoms of AA.

1,25-(OH)2D3 treatment decreases radiological score of AA rats in a concentration-dependent manner

AI was considered an index to reflect the severity of RA. To further evaluate the influence of 1,25(OH)2D3 on RA, the radiological score of X-ray films was evaluated, the results of which among the normal rats, AA rats without or with 1,25(OH)2D3 treatment of different concentration are shown in Fig. 2. The radiographic score in the AA rats without 1,25(OH)2D3 treatment was increased significantly compared with that in the normal rats, revealing the success of model establishment ($p < 0.05$). The radiographic score was decreased after 1,25(OH)2D3 treatment and the decrease was much more evident with the increase of 1,25(OH)2D3

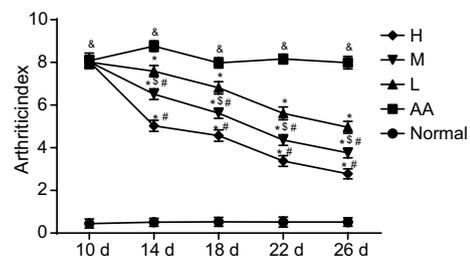


Fig. 1 The arthritic index in AA rats is decreased after intraperitoneal injection of 1,25-(OH)2D3 at concentrations of 50, 100, and 300 ng/day/kg, from 10th day to 26th day. & $p < 0.05$, vs. the normal rats; * $p < 0.05$, vs. the AA group; # $p < 0.05$, M, H groups vs. L group; \$ $p < 0.05$, M group vs. H group. One-way ANOVA was used to perform statistical analysis. AA adjuvant-induced arthritis

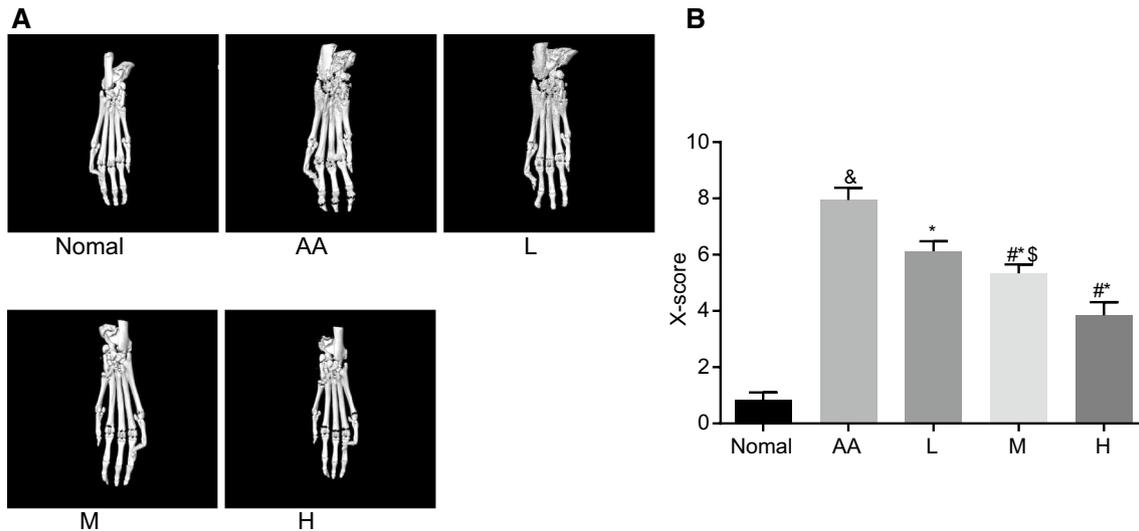


Fig. 2 The radiographic score is lowered after intraperitoneal injection of 1,25-(OH)₂D₃ at concentrations of 50, 100, and 300 ng/day/kg; **a** X-ray films for each group; **b** statistical histogram of radiographic score in each group. & $p < 0.05$, vs. the normal rats; * $p < 0.05$,

vs. the AA group; # $p < 0.05$, M, H groups vs. L group; \$ $p < 0.05$, M group vs. H group. One-way ANOVA was used to perform statistical analysis. AA adjuvant-induced arthritis

concentration ($p < 0.05$). Therefore, it was suggested that 1,25-(OH)₂D₃ treatment could decrease radiological score of AA rats in a concentration-dependent manner.

1,25-(OH)₂D₃ treatment delays PWTL of affected paw in AA rats in a concentration-dependent manner

Arthritis causes more sensitivity to heat pain. Subsequently, the impacts of 1,25(OH)₂D₃ on thermal pain and

PWTL were detected. Compared with the AA rats without 1,25(OH)₂D₃ treatment, the PWTL was increased after 1,25(OH)₂D₃ treatment ($p < 0.05$), together with remission of sensitivity to heat pain caused by arthritis. The remission was much more obvious with the increase of 1,25(OH)₂D₃ concentration ($p < 0.05$) (Fig. 3a). There was no statistical difference in the PWTL among each group ($p > 0.05$) (Fig. 3b). The above findings indicated that 1,25-(OH)₂D₃ could prolong PWTL in RA rats and attenuate the sensitivity to heat pain.

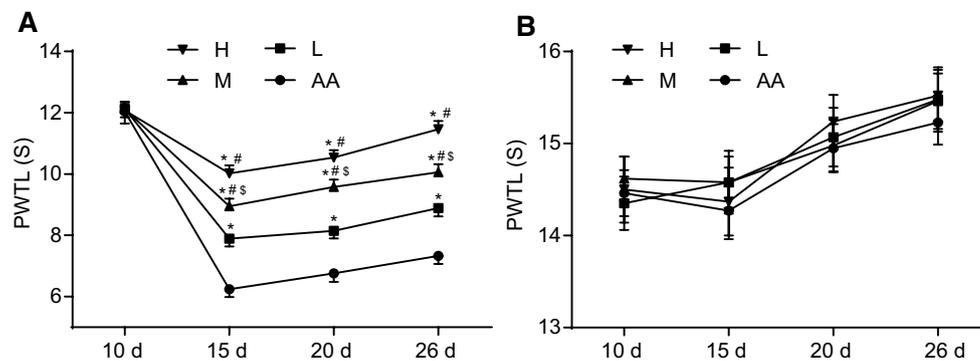


Fig. 3 1,25-(OH)₂D₃ treatment delays PWTL of affected in AA rats in a concentration-dependent manner. **a** Significant changes of PWTL of affected paw in AA rats and 1,25(OH)₂D₃-treated rats from 10th day to 26th day; **b** no remarkable changes of PWTL of normal paw in AA rats and 1,25(OH)₂D₃-treated rats from 10th day to 26th day;

& $p < 0.05$, vs. the normal rats; * $p < 0.05$, vs. the AA group; # $p < 0.05$, M, H groups vs. L group; \$ $p < 0.05$, M group vs. H group. One-way ANOVA was used to perform statistical analysis. AA adjuvant-induced arthritis, PWTL paw withdrawal thermal latency

1,25-(OH)2D3 treatment prevents inflammatory cell infiltration and synovium hyperplasia in synovial tissues in AA rats

Next, HE staining was utilized to observe the histopathological changes of synovial tissues with RA following 1,25(OH)2D3 treatment. As shown in Fig. 4, the synovial tissues in the normal rats showed a small amount of inflammatory cell infiltration and no synovial hyperplasia. The synovial tissues in the AA rats showed a large amount of inflammatory cell infiltration and synovial hyperplasia. The inflammatory cell infiltration and synovial hyperplasia were decreased significantly after 1,25(OH)2D3 treatment and the decrease was more significant with the increase of 1,25(OH)2D3 concentration. Therefore, 1,25(OH)2D3 treatment was conducive to the alleviation of RA-induced histopathological deterioration in synovial tissues.

The ankle joints of the normal rats exhibited intact cartilage, bone, and synovium. According to the histologic evaluation of the ankle joints in the AA group, adipose

marrow mesenchymal cells were found to be with embedded inflammatory cells, synovium with multiple giant cells, massive periosteal proliferation, periarticular inflammation, as well as pannus formation. Rats receiving 1,25-(OH)2D3 at different dose had notably decreased degree of arthritis, indicating a significant decrease of synovial inflammatory cell infiltration, synovial lining hyperplasia, as well as bone destruction (Fig. 4).

1,25-(OH)2D3 treatment downregulates the expression of NF- κ B in synoviocytes of AA rats

Subsequently, we performed immunofluorescence to investigate whether 1,25-(OH)2D3 could affect the expression of NF- κ B. The results revealed that the expression of NF- κ B was significantly downregulated in synoviocytes of AA rats after treatment with 1,25-(OH)2D3 of different concentrations. The immunofluorescent staining was used to detect NF- κ B p65 localization, and the results showed that the high dose of 1,25-(OH)2D3 blocked the activation of

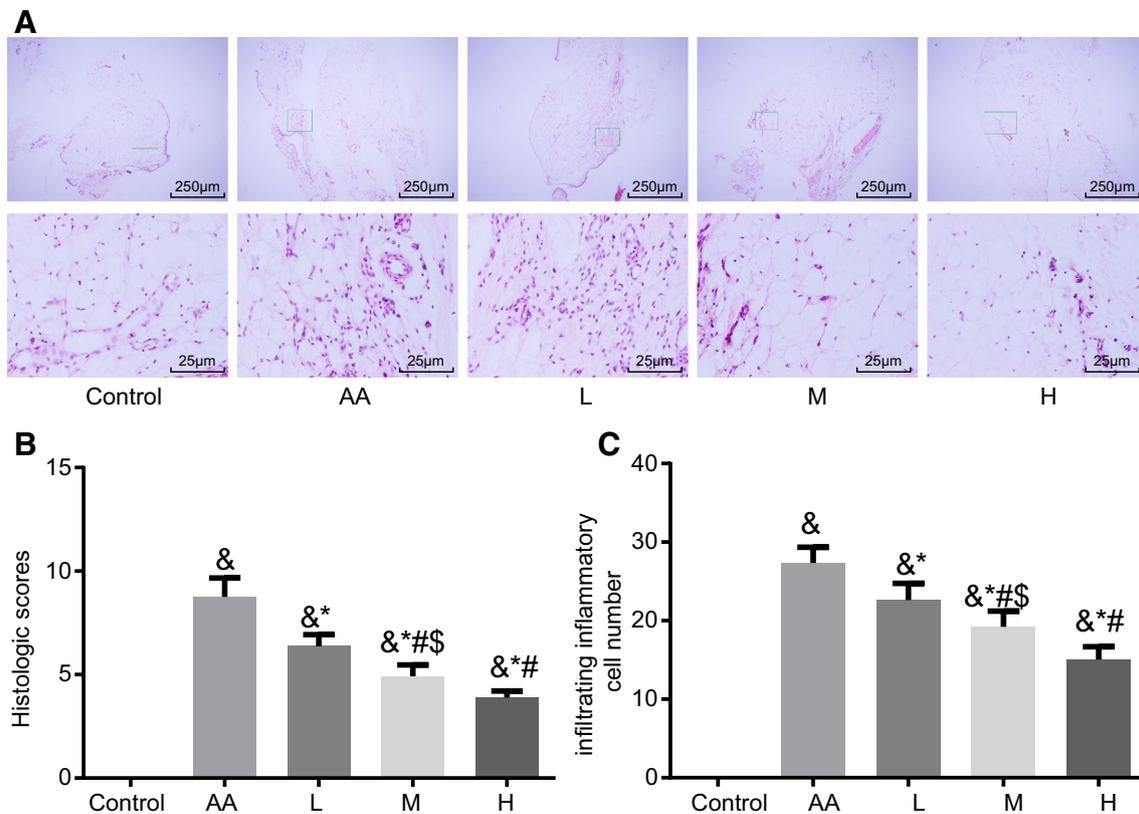


Fig. 4 HE staining shows that 1,25-(OH)2D3 treatment prevents inflammatory cell infiltration and synovium hyperplasia in synovial tissues in AA rats, observed under the optical microscope ($\times 200$). **a** HE staining showing histological comparison of rats in each group. Lower panels indicate outlined boxes from upper panel at $\times 5$ original magnification. Scale bars = 500 μ m (upper panel) and 100 μ m (lower

panel); **b** histologic scores of the joint for rats in each group. Data are expressed as mean \pm standard deviation from three animals in each group; **c** statistical chart of number of infiltrating inflammatory cells in rats in each group. & $p < 0.05$, vs. the normal rats; * $p < 0.05$, vs. the AA group; # $p < 0.05$, M, H groups vs. L group; \\$ $p < 0.05$, M group vs. H group. AA adjuvant-induced arthritis, HE hematoxylin–eosin

Fig. 5 1,25-(OH)₂D₃ treatment downregulates the expression of NF-κB in synoviocytes of AA rats, determined by immunofluorescent staining (scale bar = 50 μm). [&] $p < 0.05$, vs. the normal rats; ^{*} $p < 0.05$, vs. the AA group; [#] $p < 0.05$, M, H groups vs. L group; [§] $p < 0.05$, M group vs. H group. One-way ANOVA was used to perform statistical analysis. AA adjuvant-induced arthritis, DAPI 4',6-diamidino-2-phenylindole

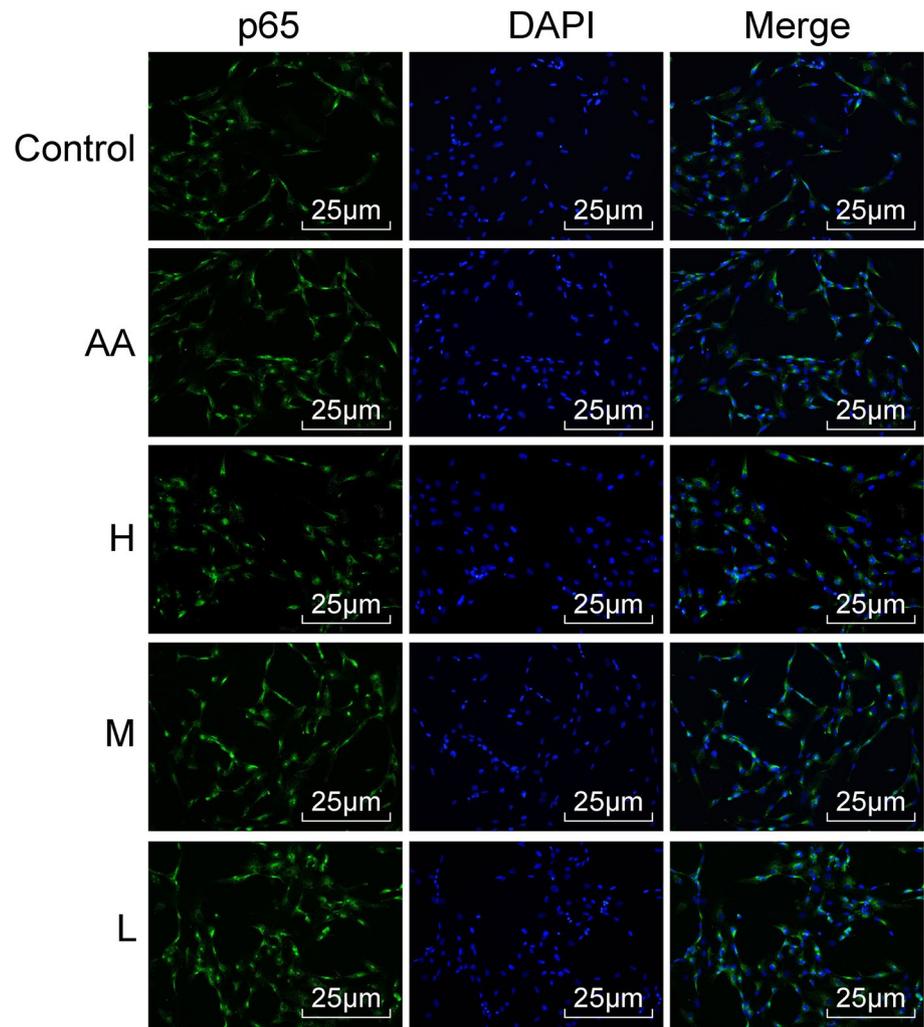
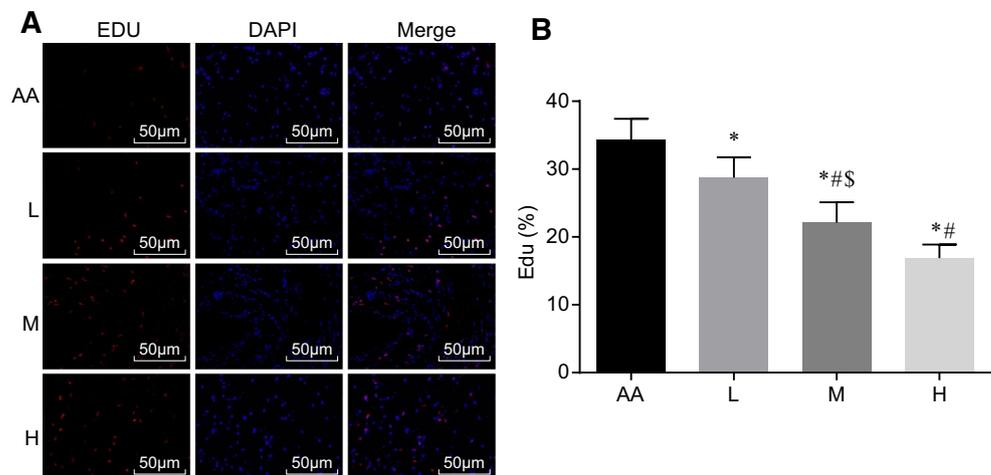


Fig. 6 EdU cell proliferation assay demonstrates that 1,25-(OH)₂D₃ treatment inhibits proliferation in cultured AIASs. **a** Statistical histogram of EdU cell proliferation assay for each group; **b** experimental results of EdU cell proliferation assay in each group. $n = 10$; scale bar = 200 μm



NF-κB (Fig. 5). The above findings demonstrated that 1,25-(OH)2D3 treatment could downregulate the expression of NF-κB in synoviocytes of AA rats.

1,25-(OH)2D3 treatment inhibits proliferation in cultured AIASs

To determine the effects of 1,25-(OH)2D3 on proliferation of synoviocytes, EdU cell proliferation assay was employed, results of which are presented in Fig. 6. The proliferation of synoviocytes in AA rats after 1,25(OH)2D3 treatment was significantly inhibited ($p < 0.05$). The proliferation proportion was notably decreased in cultured AIASs as the dose of 1,25(OH)2D3 increased ($p < 0.001$). These findings suggested that 1,25-(OH)2D3 might inhibit the proliferation of synoviocytes in RA rats.

1,25-(OH)2D3 treatment reduces levels of inflammatory factors in synovial tissues of AA rats

RT-qPCR was performed to determine the mRNA expression of inflammatory factors in synovial tissues of AA rats, as shown in Fig. 7. The mRNA expression of TNF-α, IL-1β and IL-6 in synovial tissues of AA rats after 1,25(OH)2D3 treatment was decreased significantly ($p < 0.05$), suggesting that 1,25(OH)2D3 could ameliorate the inflammatory response of AA rats, and this effect was enhanced with the increase of the 1,25(OH)2D3 concentration ($p < 0.001$). These findings confirmed that 1,25-(OH)2D3 might ameliorate the inflammatory response of AA rats in a concentration-dependent manner.

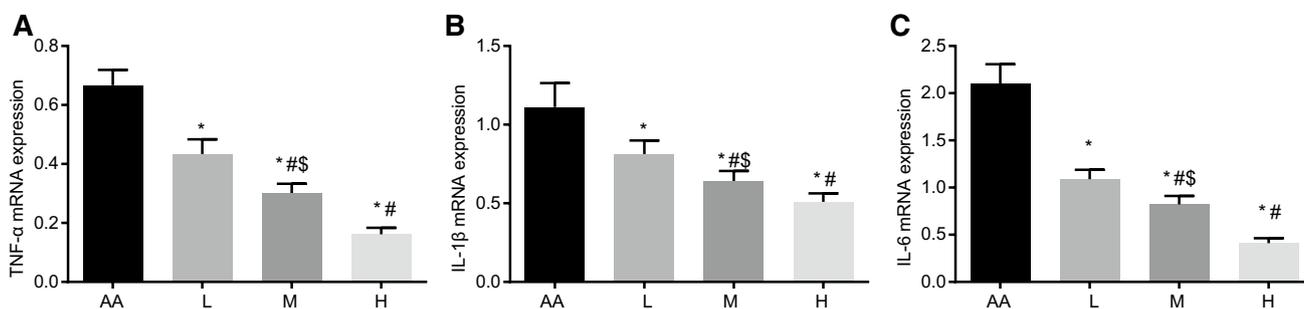


Fig. 7 1,25-(OH)2D3 treatment reduces levels of inflammatory factors in synovial tissues of AA rats, detected by RT-qPCR. ³p < 0.05, vs. the normal rats; *p < 0.05, vs. the AA group; #p < 0.05; M, H groups vs. L group; \$p < 0.05, M group vs. H group. AA adjuvant-

induced arthritis, RT-qPCR reverse-transcription quantitative polymerase chain reaction, TNF-α tumor necrosis factor-α, IL-1β interleukin-1β, IL-6

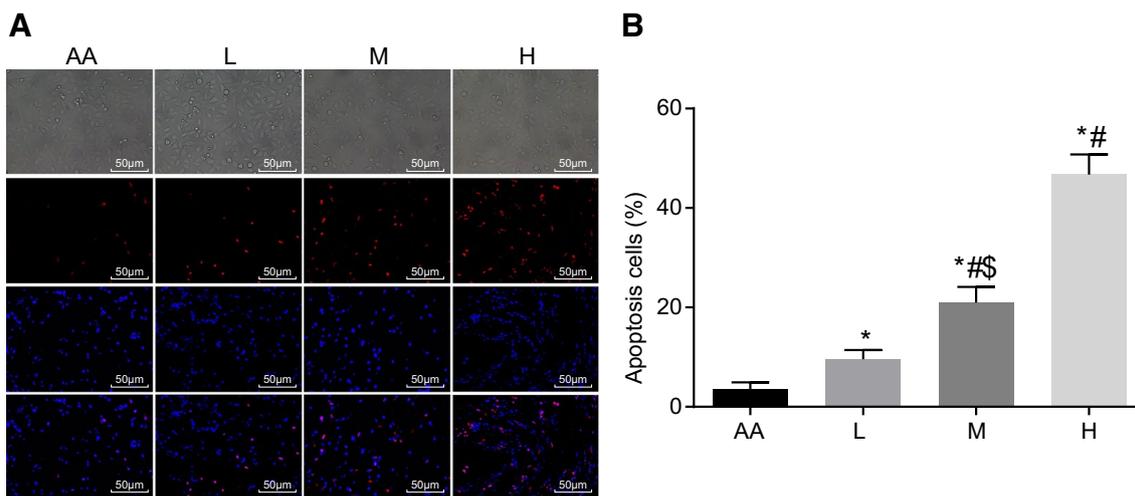


Fig. 8 1,25-(OH)2D3 treatment induces apoptosis in cultured AIASs, detected by TUNEL assay. a TUNEL stains in AIASs (×400, TUNEL-positive cells were red stained, with blue-stained nuclei); b the percentage of TUNEL-positive cells. ³p < 0.05, vs. the nor-

mal rats; *p < 0.05, vs. the AA group; #p < 0.05; M, H groups vs. L group; \$p < 0.05, M group vs. H group. One-way ANOVA was used to perform statistical analysis. AA adjuvant-induced arthritis, AIASs adjuvant-induced arthritis synoviocytes

1,25-(OH)2D3 treatment induces apoptosis in cultured AIASs

In the following experiment, to determine the effects of 1,25-(OH)2D3 on apoptosis of synoviocytes, TUNEL method was employed, results of which are presented in Fig. 8. The apoptosis of synoviocytes in AA rats after 1,25(OH)2D3 treatment was increased significantly ($p < 0.05$). The TUNEL-positive cells were increased in AA rats after 1,25(OH)2D3 treatment, suggesting that 1,25(OH)2D3 could induce the apoptosis of synoviocytes. The higher the 1,25(OH)2D3 concentration was, the higher the apoptosis rate of synoviocytes observed in AA rats ($p < 0.001$). These findings confirmed that 1,25-(OH)2D3 might accelerate the apoptosis of synoviocytes in RA rats.

1,25-(OH)2D3 treatment inhibits the NF- κ B signaling pathway in synovial tissues of AA rats

Emerging evidence shows that inhibiting the NF- κ B signaling pathway with 1,25(OH)2D3 may be a therapeutic approach for controlling inflammatory diseases. To confirm whether the regulatory mechanism of 1,25(OH)2D3 in preventing RA development is dependent on the NF- κ B signaling pathway, we examined the protein expressions of p-IKK β , p65 and p-I κ B α in synovial tissues of AA rats following 1,25-(OH)2D3 treatment by western blot analysis. The detailed data are presented in Fig. 9. The protein levels of p-IKK β , p65 and p-I κ B α were decreased after 1,25(OH)2D3 treatment, which was more significant as 1,25(OH)2D3 concentration increased ($p < 0.05$). It was suggested that 1,25(OH)2D3 could inactivate the NF- κ B signaling pathway in synovial tissues.

Discussion

Adjuvant-induced arthritis in rats is a model of chronic systemic inflammation and a model for research on the pathogenesis of RA which has similar features as that of human being [16]. In this study, we investigated the effects of 1,25(OH)2D3, with different concentration, on the symptoms of RA, proliferation and apoptosis of synoviocytes with the involvement of the NF- κ B signaling pathway. It is suggested that 1,25(OH)2D3 could suppress proliferation and increase apoptosis of synoviocytes and alleviates the symptoms of RA via the inactivation of the NF- κ B signaling pathway in a concentration-dependent manner.

Initially, our results demonstrated that AA rats with 1,25(OH)2D3 treatment showed decreased AI, radiological score, inflammatory cell infiltration and synovial hyperplasia, in addition to reduced levels of inflammatory factors. Generally, 1,25(OH)2D3, the most active form of vitamin D, is a secosteroid hormone that plays key roles in regulating calcium homeostasis, bone metabolism and immunomodulatory properties [17, 18]. The synovial hyperplasia causes transformed cells and destroys periarticular bone and cartilage [19]. Moreover, suppressed inflammation, reduced synovial hyperplasia, and induced synoviocyte apoptosis are considered important targets of therapy for RA [8]. Similarly, 1,25(OH)2D3 has been found to be capable of reducing intimal hyperplasia [20]. Moreover, in line with our study, 1,25(OH)2D3, combining TNF blockade, could reduce the levels of inflammatory factors IL-6 and IL-8, thereby controlling synovial inflammation [21].

Another important finding was that 1,25(OH)2D3 treatment could inhibit proliferation and promote apoptosis of synoviocytes. In addition, the expression of NF- κ B and protein levels of p-IKK β , p65 and p-I κ B α of the synovial tissues

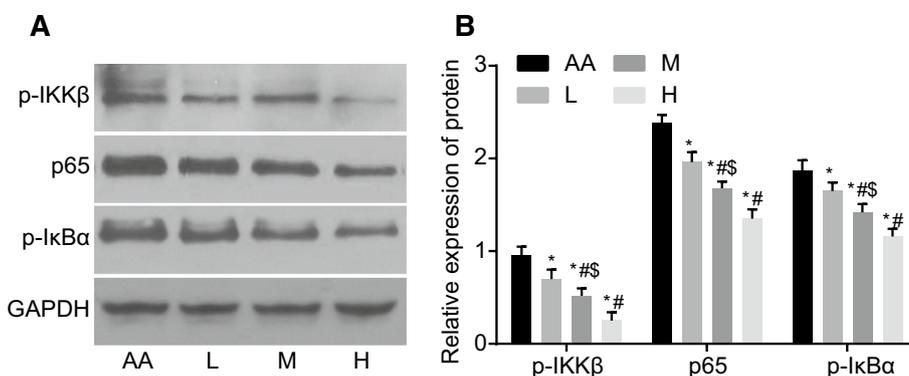


Fig. 9 1,25-(OH)2D3 treatment inhibits the NF- κ B signaling pathway in synovial tissues in AA rats, detected by western blot assay. **a** Gray value of p-IKK β , p65 and p-I κ B α bands, determined by western blot analysis; **b** the protein levels of p-IKK β , p65 and p-I κ B α , determined

by western blot analysis; $\&p < 0.05$, vs. the normal rats; $*p < 0.05$, vs. the AA group; $\#p < 0.05$, M, H groups vs. L group; $\$p < 0.05$, M group vs. H group. AA adjuvant-induced arthritis

in AA rats were found to be decreased by 1,25(OH)₂D₃. Synoviocytes were considered to be key effector cells in RA [22]. Actually, synoviocytes actively participate in the invasive processes of RA which affects tissue destruction [23]. Since synoviocytes proliferate abnormally, they produce high expressions of destructive cytokines and enzymes to decrease the susceptibility to spontaneous or induced cell apoptosis [24]. NF-κB is identified to be activated in some chronic autoimmune diseases with its regulatory role in the immune and inflammatory responses, especially RA [25, 26]. NF-κB activation accelerates synovial hyperplasia by promoting cell proliferation and inhibiting apoptosis of synoviocytes [27]. Thus, NF-κB seems to be an inhibitor of cell apoptosis and a positive regulator of cell growth in synoviocytes. According to evidence from previous experiment by Fu et al., the activation of the NF-κB signaling pathway could be identified with detection of the protein levels of p-IKKβ, p65 and p-IκBα [28]. Consistent with our results, another study reported that 1,25(OH)₂D₃ could inhibit the NF-κB signaling pathway in endothelial cells transformed by Kaposi sarcoma-associated herpes virus G protein-coupled receptor [12]. Some data have reported that 1,25(OH)₂D₃ directly regulates the NF-κB signaling pathway by decreasing NF-κB translocation to the nucleus and increasing IκBα protein expression in macrophages [29]. Therefore, we speculated that 1,25(OH)₂D₃ treatment could inhibit proliferation and promote apoptosis of synoviocytes by inactivating the NF-κB signaling pathway.

In conclusion, the key findings of the study revealed that 1,25(OH)₂D₃ could inhibit the activation of the NF-κB signaling pathway, thereby inhibiting the proliferation and promoting the apoptosis of synoviocytes, which could ameliorate RA. Therefore, 1,25(OH)₂D₃ may have potential for treatment of RA and is a novel promising therapeutic agent for autoimmune diseases. However, though in vitro data are promising, it is not adequate to attribute to vitamin D in immune diseases pathogenesis and treatment in vivo yet. Nevertheless, obtaining a normal vitamin D status is paramount in preventing RA-related osteoporosis; therefore, the correction of a deficient vitamin D status should be suggested to each rheumatic patient. Currently, there is no consensus about the best regimen for these patients and further studies are required to better address this important area of medical problems. Furthermore, more research on the postulated role in immune modulation might prove to be more relevant than expected. In the near future, further insights into vitamin D physiology could lead the way to new therapeutic uses of this old, but still promising molecule.

Acknowledgements This study was supported by Key Research and Development Program of Shandong Province (no. 2016GSF201232) and the Natural Science Foundation of Shandong Province (no. ZR2017LH028). We would like to give our sincere appreciation to the reviewers for their helpful comments on this article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The study was conducted with the approval of the Animal Ethics Committee of Shandong Provincial Qianfoshan Hospital, Shandong University. All experimental procedures were performed on laboratory animals in accordance with institutional guidelines for the care and use of laboratory animals.

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