Effect of Vitamin D_3 On the Expression of Antimicrobial Peptide Gene in Experimental Pneumonia in Calf

Parisa Asgharpour
Azad University: Islamic Azad University
Zohre Eftekhari
Pasteur Institute of Iran
Mohammad Goli Nadealian
University of Tehran Faculty of Veterinary Medicine
Gholam Reza Nikbakht Borojeni
University of Tehran Faculty of Veterinary Medicine
Mohammad Reza Mokhber Dezfouli (mokhberd@ut.ac.ir)
University of Tehran Faculty of Veterinary Medicine

Research Article

Keywords: Vitamin D3, Antimicrobial peptides, Pneumonia, Pasteurella multocida.

Posted Date: March 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-263112/v1

Abstract

Background

Vitamin D_3 has been identified as an immunomodulatory agent that confronts the pathogens via stimulating antimicrobial peptides (AMPs).

Objective

The effects of vitamin D_3 on the expression of AMPs was assessed in experimental pasteurellosis in calves.

Methods

10 Holstein crossbred male calves (2–4 months) were chosen and randomly divided into the two groups. *Pasteurella multocida* was prepared (3×10^9 CFU/mL) and inoculated in the trachea. Vitamin D_3 was injected to the treatment group after confirming the pneumonia. Blood samples were obtained from both groups at different time intervals and the peripheral blood mononuclear cells (PBMCs) were isolated. Clinical symptoms were recorded. Broncho-alveolar lavage was performed to evaluate the lung cell content. On the other hand, 10^{-6}, 10^{-7}, and 10^{-8} molar (M) of vitamin D3, was used to evaluate the expression of CD4, BMAP34, and BNBD4 genes using PBMCs under the in vitro conditions.

Results

The prescription of vitamin D3 to the treatment group caused a decline in clinical signs. Following the vitamin D_3 injections the treatment groups under the in vivo conditions, significant increase was observed in the expression level of Defensin (BNBD4), and CD4. Evaluation of bronchoalveolar lavage fluid (BALF) revealed that the amount of neutrophils decreased after vitamin D injection. In vitro, increased expression of Catalicidin (BMAP34), Defensin (BNBD4), and CD4 was observed at a concentration of 10^{-6} M of vitamin D_3.

Conclusion

The present study indicated that vitamin D_3, exerts immunomodulatory effects on many infectious diseases via activation of VDR pathways and stimulation of AMP production.

Introduction

Calf pneumonia is a major problem in dairy and beef herds and can be caused by different etiologies; therefore it is termed a ‘multifactorial disease’. Various factors including viruses (respiratory syncytial virus (RSV), bovine viral diarrhea (BVD), infectious bovine rhinotracheitis (IBR)), bacteria (*Pasteurella multocida*, *Mannheimia haemolytica*), inadequate colostrum intake, poor nutritional status and hygiene in pens, insufficient ventilation, over crowdedness, and stress lead to pneumonia (Dabo et al. 2008; Smith 2015).

The outbreak of cattle pneumonia in different regions of the world has been reported between 10–70% (Gibbs 2001; Mohammadi et al. 2006). The mortality rate due to respiratory diseases in dairy cattle has also been reported between 2.2–9.4%. Approximately 3% of born calves have been died due to pneumonia in the first 12 weeks of their lives (Geraert 2006; Mohammadi et al. 2007; Constable et al. 2017).

Pasteurellosis is one of the most important contagious diseases in the world. *Pasteurella multocida* and *Mannheimia haemolytica* are considered as the commensal gram-negative bacteria in the upper respiratory tract of animals. Under stresses including environmental, managemental, or infectious factors, those agents can cause growth and extension of bacteria to the lower respiratory tract and often produce mild to severe clinical signs (Smith 2015; Mohammadi et al. 2007; Selvaraj et al. 2009; Karimkhani et al. 2011).

The clinical manifestations include a rise in temperature, respiratory distress with nasal discharge, and then in severe cases, recumbence and death may be the result (Smith 2015; Constable et al. 2017).

Vitamin D_3 not only plays a significant role in calcium homeostasis and bone metabolism, but also modulates innate and adaptive immunity in the respiratory and cardiovascular systems (Bikle 2009; Guillot et al. 2010). Recently, the role of vitamin D_3 signaling in the modulation of innate immunity has been indicated. As immune system stimulation due to bacterial infection can cause change 25D into 1, 25 D. Furthermore, produced 1,25D induces the expression of genes encoding antimicrobial peptides (AMPs), specifically Cathelicidin antimicrobial peptide (CAMP) (White 2010). An efficient immune system requires adequate vitamin D_3 levels for the expression of AMPs in some epithelial cells (Anderson and Rings 2008). Moreover, vitamin D_3 exert broad anti-inflammatory effects on the adaptive immune system and can increase the number and/or function of T regulatory cells (Tregs). Researchers have highlighted the correlation of low levels of vitamin D_3 with an increased risk of infections of respiratory system (Hewison 2011; Ozkanlar et al. 2012; Pincikova et al. 2007). Considering the role of vitamin D_3 as an immunomodulator in the activation of host resistance against different infections in humans and some animals, its use as adjunctive therapy together with other therapeutic agents, can cause a decrease in antibiotics resistance in livestock (DiRosa et al. 2011).

AMPs are an important part of the intrinsic and acquired immunity of most living organisms against pathogens. AMPs are small molecular weight proteins which reveal broad-spectrum activity against microorganisms such as gram-positive and gram-negative bacteria, viruses, yeasts, protozoa, and fungi (Reddy et al. 2006; Beisswenger and Bals 2005). A variety of cells including epithelial and phagocytic cells such as macrophages, neutrophils, and natural killer cells synthesize and secrete AMPs. The mechanism of action of AMPs includes the involvement, damage, and rupture of the outer cell membrane of the organism (Beisswenger and Bals 2005).

The Cathelicidin family of AMPs have been isolated from several different types of mammals and hence are considered as important AMPs with linear α helical domains (Reddy et al. 2004). They play a significant role in mammalian innate immune defense against invasive bacterial and viral infections. In cattle, different Cathelicidin were named Bac5, Bac7, BMAP27, BMAP28, and BMAP34 (Zantetti 2004). Additionally, Defensins are identified as a member of AMP families' and are structurally divided into α, β, and θ-Defensins. In cattle, β Defensin is generally found in neutrophils, alveolar macrophages, trachea, colon, small intestine, lung and spleen this type of Defensin varies in sites of expression, structure, and biological activities (Tecle et al. 2010; Hazlett and Minhao 2011). Defensins act by disrupting the structure of bacterial cell membranes. They also play a major role in defense against pathogens in many organs. These peptides have a wide range of antimicrobial activities and are

recognized as novel therapeutic agents because they enhance immune system activity as immune modulators. Their best feature is recognized as their ability to eliminate invasive agents such as gram-negative and gram-positive bacteria, fungi and viruses. However, nowadays it has been determined that these peptides are able to attract the inflammatory cells and activate adaptive and innate immune responses (Tecle et al. 2010; Hazlett and Minhao 2011).

In vitro studies showed that vitamin D_3 has therapeutic and anti-inflammatory effects via modulating the innate and adaptive immune system. So that, vitamin D_3 can be up-regulating the expression of T cells such as CD4 and AMPs.

Due to the high prevalence of respiratory diseases in human societies, the application of vitamin D_3 as an auxiliary treatment for activating the AMPs interacting with innate and adaptive immune receptors deems useful. Therefore, the intake of vitamin D_3 in therapeutic and prophylactic doses is recommended, and in this regard, in the present study, we aimed to evaluate the immunomodulatory effect of vitamin D_3 on the mRNA expression of AMPs in experimental pneumonia in calves.

Materials And Methods

Experimental study design

For this study, based on accepted criteria by Iran National Research Ethics Committee, ten Holstein male crossbred calves (2-4 months of ages) for experimental study were chosen. The calves were selected from an industrial farm of Tehran suburb and transferred to the Research Veterinary Hospital after initial clinical examinations and proved their healthy conditions by veteran. The criteria for entrance into the study were normal rectal temperature, respiratory sounds, heart rates and lack of nasal and eyes discharge. Subsequently, calves were randomly divided into the treatment and control groups by veterinary hospital staffs, numbered and adaptation period carried out about 6 days after entrance day. The calves were kept individually in a closed and isolated pens designed for large livestock, with appropriate flooring, temperature, food, and water. The experimental study sites were thoroughly isolated and investigated during the study to completely prevent the risk of any other infection. The blood samples (10 mL) were obtained from the jugular vein before the beginning of the study, at the challenge time (bacterial inoculation or 0 hours of the study), 24, 48, 72, 96, and 120 hours of study, and poured into the sterile tubes containing lithium heparin anticoagulant for the isolation of peripheral blood mononuclear cells (PBMCs). In the beginning of the study, the calves were restrained and the tracheal tube was gently inserted into the trachea and fixed. Then lavage catheter was inserted and the prepared *Pasteurella multocida* (PMC66) was inoculated by the final volume 10 mL (3×10^9 CFU / mL).

Isolation and preparation of the bacteria

Pasteurella multocida isolated from the lung of a calf with pneumonia by Razi Vaccine and Serum Research Institute, was grown in phenol red-free RPMI 1640 medium supplemented with 200 mM L-glutamine and diluted in sterile phosphate buffered saline (PBS), pH 7.4. To confirm the species of bacteria, bacterium-specific primers were designed by ALLELE ID software and Polymerase Chain Reaction (PCR) was performed accordingly. The *toxA* gene of the *Pasteurella multocida* was amplified using two standard forward and reverse primers (Table1) (Abed et al. 2020; Algammal et al. 2020). PCR was performed in a final reaction volume of 20 µL containing 10 µL of 2× PCR Master Mix, 1µL of each primer (both from SinaClon BioScience, Iran), 2 µL template DNA and 6 µL

double-distilled water. Then PCR amplification was performed as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 10 s, annealing at 52°C for 20 s, and extension at 72°C for 60s, and final extension at 72°C for 5 min. Finally, PCR products were analyzed using 1% agarose gel electrophoresis.

Table 1 PCR primers designed by ALLELE ID.

	Primer sequence (5'-3')	Length	Tm	GC (%)
Forward primerReverse primer	CTTAGATGAGCGACAAGGGAATGCCACACCTCTATAG	1819	52.2052.19	50.0047.37

Inoculation of bacteria

According to previous and pilot study, appropriate dilution of bacteria (3×10^9 CFU / mL) was prepared. Before launching study, the calves were restrained. The tracheal tube was inserted gently into the trachea and the tube cuff was fixed. Then quickly lavage catheter was inserted through the endotracheal tube which inoculated the prepared *Pasteurella multocida* (PMC66) for experimental pneumonia induction by the final volume of 10 mL.

Clinical signs evaluations

The clinical signs such as rectal temperature, ocular and nasal discharge, cough, abnormal breathing and auscultation of abnormal lung sounds were evaluated by the practitioner in the calves before the intervention, after inoculation, and every 24 hours to correctly classify the calf and identify required therapeutic interventions. Calves with this infection has at least 2 clinical signs of respiratory infection, and therefore were considered as pneumonia cases (Constable et al. 2017; DeRosa et al. 2000).

Injection of Vitamin D_3

After diagnosis of calves with pneumonia by clinical examination, 10 mL of vitamin D_3 (Daropakhsh® Co.) was injected intramuscularly to the treatment group.

Considering ethical issues, at the end study, all of the animals were intramuscularly injected with appropriate antibiotics according to antibiogram susceptibility for three days (Z-Oxyject 20% LA (Rooyan Darou Co., Oxytetracycline 20% LA, 20 mg/kg); Tylomax 20 (RooyanDarou. Co., Tylosin 20%, 10 mg/kg)).

Total blood cell count

The blood samples were collected in sterile tubes containing the EDTA and transferred to the laboratory to be analyzed by auto cell counter. To differentiate white blood cells, smears were prepared, fixated by alcohol and stained by Wright Giemsa (1: 5 ratio).

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) was performed in the calves anesthetized with Propofol (Fresenius Kabi, U.S.A.) (Diprivan®5mg/kg) using a sterilized and flexible catheter with a 3-5 cc balloon cuff (Supa Co, Iran) at the challenge time (start time (0h) of study or bacterial inoculation) , 24 h after bacterial inoculation, 48 h bacterial

after inoculation, and in the end of the study (120 h). The BAL catheter was inserted into the trachea using a tracheal tube, and its positioning was confirmed by repeated coughing. Then, the balloon cuff, inflated with 3 mL of air and subsequently, 5 aliquots of 200-300 mL pre-warmed sterile saline solution (37^{o}C), was infused, and immediately after infusion, the lavage fluid was aspirated applying negative pressure. The lavage fluid was mixed and pooled in a sterile tube maintained on ice and immediately transferred to the biochemistry laboratory. The calves were under critical care after the BAL procedure to prevent any bronchial complications.

To obtain a cell-free supernatant, BALF was centrifuged at 450 g for 15 min. For cytological evaluation of BALF, the cells were precipitated by centrifugation and stained using Wright-Giemsa staining and direct smear preparation.

Blood sampling and isolation of peripheral blood mononuclear cells (PBMCs)

The collected blood samples were diluted by sterile PBS (Sigma-Aldrich) added gently and slowly to Ficoll Histopaque (5 mL) (Biowest Co, Germany). The whitish buffy coat (PBMCs) which was formed in the inter-phase between histopaque and the medium was aspirated. PBMCs isolated from the whole blood were re-suspended in RPMI1640 (Roswell Park Memorial Institute Medium 1640) (BIO-IDEA Co.) (Containing 20 % fetal bovine serum (FBS) (Gibco®)) and antibiotics (Pen/Strep 0.01 *%; Sigma* Aldrich) with a concentration of 10^6 cell/mL. Then, 0.5 mL of cell suspension was added to each well of a 24-well cell culture plate.

Real time PCR for gene expression assay

RNA was extracted from the PBMCs using the RNX-plus Cinna Gen kit (SinaClon BioScience Co.), according to the manufacturer's protocol. Quality and quantity of the extracted total RNA was evaluated by spectrophotometer Nano Drop 2000.

Reverse transcription PCR was conducted by Cinna-Gen First Strand cDNA synthesis kit (Cinna-Gen, Iran) using random hexamer primers. The absence of RNase H enhances the synthesis of long cDNA because the RNA strand does not degrade in DNA-RNA hybrid form during the first strand cDNA synthesis.

Primers were designed by ALLELE ID software. Then, primers were verified by primer-BLAST program in NCBI databases (Table2). The accession number of selected genes include: Bos taurus CD4 molecule (CD4), mRNA GenBank accession No. NM_001103225 and XM_588150; Bos taurus defensin, beta 4A (DEFB4A), mRNA GenBank accession No. NM_174775; Bos taurus cathelicidin antimicrobial peptide (CAMP), mRNA GenBank accession No. NM_174831; Bos taurus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA GenBank accession No. NM_001034034 and XM_598051. Finally, Real-time PCR was used for gene expression analysis and cytokine quantification.

Real-time PCR was performed with Light Cycler Fast Start DNA Master Syber Green Kit using cDNA dilution (in DEPC water). After each cycle, the temperature was increased to the primers' respective annealing temperature, and fluorescence of SYBR green bound to double-stranded DNA was measured at 530 nm (Light Cycler fluorescence channel F1). A melting curve analysis was performed to evaluate the possibility of non-specific amplification or primer-dimer formation.

Table 2 Primers designed by the ALLELE ID software.

No.	Gene name	Primer sequence (5'-3')	MW	OD (1000 µL)	Tm	GC (%)	Length (bp)
1	*CD4 F*	TCCCACTCACCTTCGAGTAT	5988	4	57.30	50	20
2	*CD4 R*	CACCTTCACCTCTCTGTTCTTC	6523	4	60.25	50	22
3	*BMAP34 F*	CCAACTGGGAACTGACATAGAG	6777	4.5	60.25	50	22
4	*BMAP34 R*	CCCTGGAGGCTTTGTGAAA	5844	4	56.67	52.63	19
5	*BNBD4 F*	CATGAGACAGATTGGCACCT	6126	4.5	57.30	50	20
6	*BNBD4 R*	GCAGTTTCTGACTCCGCATT	6059	4.9	57.30	50	20
7	*GAPDH F*	TGAGATCAAGAAGGTGGTGAAG	6913	5	58.39	45.45	22
8	*GAPDH R*	GCATCGAAGGTAGAAGAGTGAG	6898	4.5	60.25	50	22

The genes used in this study included Cathalicidin (BMAP34), Definsin (BNBD4), and Cluster of Differentiation 4 (CD4). The reference gene was GAPDH.

Real time -PCR for the analysis of antimicrobial peptide mRNA (in vitro assay)

To increase the accuracy and reliability of the study, the in vitro expression of the target genes was evaluated. To this end, 80 mL blood samples were collected in sterile heparinized tubes to isolate PBMC and the expression levels of the target genes were assessed after inoculation of vitamin D_3. Three different concentrations of vitamin D_3 (Daropakhsh® Co.), namely, 10^{-6}, 10^{-7}, and 10^{-8} M were used to evaluate the gene expression of CD4, BMAP34 and BNBD4.

Ethics approval and consent to participate

All experimental protocols were approved by Iran National Research Ethics Committee (Ethical Code No: IR.IAU.SRB.REC.1397.137). Based on the code of ethics received by the relevant committee, all testing steps were performed in accordance with the relevant instructions and regulations.

Statistical analysis

Data were tested for normal distribution of the parameters with Kolmogorov-Smirnov test. The comparisons of different time intervals of the study were performed using repeated measurement ANOVA. Moreover, the results of this study were analyzed using the independent T test and post hoc Tukey test. Additionally, for the analysis of data before and after vitamin D_3 injections, we used paired t test. Differences with *P* values of less than 0.05 were considered statistically significant.

Results

Confirmation of the isolated bacteria

According to the primers designed, PCR was performed to confirm the species of bacterium. The images obtained on the gel electrophoresis revealed the *Pasteurella multocida* with a molecular weight of 865 bp (Fig. 1) (Abed et al. 2020; Algammal et al. 2020).

Assessment of clinical signs

Clinical signs of pneumonia were monitored during the first 12 hours after bacteria inoculation so that elevated rectal temperature, nasal and in some cases eyes discharge, coughing, and abnormal respiratory sounds were recorded in both groups. In the treatment group, after vitamin D_3 injection, the overall clinical symptoms decreased in comparison with the control group, along with a complete recovery which was observed in some calves after 120 hours (Fig. 2).

Hematological and Biochemical appearance

After bacterial inoculation, significant increases were observed in total white blood cell (WBC), total proteins, lymphocytes, and eosinophils within 24 hours of the study. However, WBC count, total protein, lymphocyte, and eosinophil count decreased after the administration of vitamin D_3 in the treatment group in comparison with the control group. These findings, with clinical signs, indicated an improvement in the symptoms of pneumonia in the treatment group compared to the control group ($P < 0.05$).

According to the hematological findings, no significant differences were observed between the two groups in terms of blood components ($P>0.05$), which confirmed the calves' health conditions before inoculation of bacteria. Fibrinogen levels in two groups increased after bacterial inoculation and in the control group remained in high level compared to the calves received vitamin D_3 ($P < 0.05$).

Evaluation of bronchoalveolar lavage fluids

There was a significant difference between the control and treatment groups in terms of cell contents evaluated in the BAL (P=0.0001). The number of BALF neutrophils at the challenge time and on the last day of the study (120 hours) were significantly different in comparison to their number before challenge (P=0.0001) (Fig. 3). Furthermore, quantitative analysis of mast cell counts at different hours showed that the number of cells at the time of vitamin D_3 injection was significantly different from their number at other study hours (P=0.0001) (Fig. 4). Additionally, analysis of BALF lymphocytes at different hours of the study showed that the number of cells at challenge time was significantly different from their content at the time of vitamin D_3 injection and at 48 hours of the study (P=0.0001) (Fig. 5). Moreover, the evaluation of BALF macrophages revealed that the number of cells were significantly different at 24 and 72 hours of study in comparison to that at other time points (P=0.0001) (Fig. 6). However, the eosinophil numbers revealed no significant differences between the two mentioned groups.

Gene expression of antimicrobial peptides in infected animals

Significant differences were observed between the control and treatment groups (P = 0.04) in terms of the level of Defensin however differences were not significant considering the time points (Fig. 7).

BMAP34 gene was considered for the expression of the Cathalicidine AMP. No significant changes were identified between the control and treatment groups in the BMAP34 gene expression at different hours of the study.

Nonetheless, a significant difference was observed in the CD4 gene expression between the control and treatment groups (P=0.0001) to such a degree that CD4 gene expression changes between the control and treatment groups at 48, 72, and 120 hours after the challenge time were significantly different (P <0.05) (Fig. 8).

Gene expression of antimicrobial peptides in isolated cells in vitro condition

In vitro evaluation was performed to compare the results with the in vivo conditions. The concentration of vitamin D_3 applied in this study were 10^{-6}, 10^{-7}, and 10^{-8} M according to various studies. The highest concentration of vitamin D_3 used in this study, via 10^{-6} M, significantly increased the expression of Cathelicidin (BMAP34), β-Defensin (BNBD4) and CD4 genes. Moreover, evaluation of the results of vitamin D_3 treatment showed a significant difference in the expression levels of BNBD4, BMAP34 and CD4 between the control and treatment groups in 10^{-6} M of vitamin D_3 (P<0.05).

A concentration of 10^{-8} M of vitamin D_3 revealed a significant difference in the expression levels of CD4 gene between the two groups. However, no significant changes were observed for BNBD4 and BMAP34 mRNA levels (P<0.05). Moreover, the expression levels of BNBD4, BMAP34 and CD4 in the treated cells did not change following the addition of 10^{-7} M of vitamin D_3 (Figs. 9-11).

Discussion

Calf pneumonia is caused by bacteria, viruses and mycoplasmas in addition to the environmental factors which cause many economic impacts on the herds (Mohammadi et al. 2004). The presence of antibiotic residues in dairy products has led scientists to apply appropriate therapeutic alternatives to antibiotics (Harder and Schroder 2007). The immune system and some of the epithelial cells that are exposed to the pathogens, have an ability to produced and secreted antimicrobial peptides (AMPs). Because of the AMP can participate in defense mechanism, we can use as an alternative to antibiotic consumption (Harder and Schroder 2007). According to human studies, vitamin D_3 as an appropriate immunomodulator promotes host resistance against intracellular infections (Selvaraj et al. 2009; Hewison 2011). Recently, due to the outbreak of respiratory infections, the positive effects of vitamin D_3 on the respiratory immune system has been confirmed making it an especially interesting compound that affects the expression levels of lung AMPs (Alva-Murillo et al. 2014). Considering the availability and cost-effectiveness of vitamin D3 in the cattle breeding industry, the modulatory effects of vitamin D_3 was evaluated in this study.

According to the human viral respiratory infections study, it was proved that tracheal and bronchial epithelial cells can cause the expression of a high level of vitamin D-regulated genes and fortunately this vitamin can play a protective role against viral infections (Telcian et al. 2016).

Infectious agents (for example: bacteria, virus, fungi and so on), environmental bad situation, weakness or dysfunction of the immune system and so on, can cause pneumonia (Mohammadi et al. 2004; DeRosa et al. 2000). In this study, we produced pneumonia by the native strain of *Pasteurella multocida* that was previously isolated from the lungs of calves with pneumonia by the Razi Vaccine & Serum Institute, Iran (Mokhber Dezfouli et al. 2017). In the present study, *Pasteurella multocida* alone can produce clinical symptoms caused by a variety of factors such as fever, mucosa, and sometimes mucopurulent discharge, cough, increased respiratory rate, and sound.

The expression levels of bovine β-Defensin 3 (BNBD3), BNBD4, BNBD6, BNBD7 and BNBD10 after using the vitamin D_3 was increased in the bovine monocyte cultures (Merriman et al. 2015). The effect of vitamin D_3 in the regulation of multiple β-Defensin expression in cattle, has been proved. In human studies, has the anti-inflammatory effects of Defensins and their contribution to lung inflammatory responses have also been verified (Tecle et al. 2010).

After bacterial inoculation in the present study, a significant enhancement was reported in the concentration of Defensine and CD4 in calves with experimental pneumonia. Our results corroborated the general pattern of AMPs changes reported in previous studies (Merriman et al. 2015; Hayes et al. 2015).

Based on the obtained results, the in vitro expression levels of pulmonary AMPs and CD4 was completely dependent on the dose of vitamin D_3, as gene expressions increased following an increase of dose of vitamin D_3. While in the in-vivo conditions, the expression of Defensin and CD4 increased in the treatment group even with low levels of vitamin D_3 and infection was ameliorated though the expression of Cathalicidin perhaps required a higher dose of vitamin D_3.

Vitamin D_3 provision at acceptable costs to the cattle industry, its significant role in livestock production, besides the modulatory effects which were evaluated in experimental pneumonia in the calves introduce this vitamin as an important nutritional substance for decreasing the severity of infectious diseases (Smith 2015; Afsal et al. 2014). Hence in the present study, the use of appropriate doses of vitamin D_3 as a therapeutic supplement to improve the function of the immune system was confirmed.

In agreement with the present study, the protective effects of vitamin D_3 supplementation on respiratory infections were demonstrated via regulation of AMPs genes which play a significant role against viral and bacterial infections (Telcian et al. 2016). In patients with tuberculosis and cystic fibrosis, therapeutic effects of vitamins and their immunomodulatory effects have been investigated and the effects on the improvement of clinical symptoms have been verified (Hewison 2011; Afsal et al. 2014). Furthermore, the positive impacts of vitamin D_3 in stimulating various cytokines have been accepted and proved in previous studies, however its consistent influence on replication or clearance of influenza virus, respiratory syncytial virus or corona virus in human respiratory epithelial cell culture is still unclear (Guillot et al. 2010; Asgharpour et al. 2020).

The positive effects of vitamin D_3 on the recovery from infectious diseases such as tuberculosis, chest and wound infections, influenza, eye infections, and wound healing have been demonstrated (Hazlett and Minhao 2011). Hence, supplementation with 1, 25 dihydroxycholecalciferol increased the expression of Cathelicidin, and restricted the replication of *Mycobacterium tuberculosis* (Mtb) in human macrophages (Afsal et al. 2014).

An experimental study in sheep showed that the bacterial load decreased in the pulmonary tissues and BALF following the injection of 0.5 mg Cathelicidin (Izadpanah et al. 2005). In the present study, Vitamin D_3 could increase the in vivo expression of Defensin and CD4 with a dose of 3000000 IU, as well as increasing the expression of Defensin, Cathelicidin, and CD4 in cell culture with a higher dose.

Administration of different doses of vitamin D_3 may improve the immune system function, and decrease the severity of bacterial and viral infections in livestock. On the other hand, the vitamin D_3 deficiency may act as a risk factor in the dysfunction of defensive system especially respiratory organs.

Some blood parameters such as white blood cells, lymphocyte, eosinophil, and fibrinogen increased after the inoculation of bacteria.

The results obtained from BALF assessment showed that the level of neutrophils increased following the experimental pneumonia, while it decreased after vitamin D_3 injection. The macrophage contents also increased after vitamin D_3 injection in the treatment group. The cell contents and BALF cell patterns for the expressions of

antimicrobial genes confirmed the immunomodulatory and antimicrobial effects of vitamin D_3 in experimental pneumonia.

Bronchoalveolar lavage (BAL), as a standard diagnostic procedure for evaluating the inflammatory and immune processes of the lung infectious diseases, reveals the process of lung diseases and efficacy of treatment after lower respiratory tract infections (Smith 2015; Costable and Guzman 2001).

Macrophages and neutrophils, as dominant immune responders in the defense system of ruminants, especially cattle exert protective effects against various infections. Following the experimental pneumonia, neutrophil levels increased, as determined by BALF assessment. While after vitamin D_3 injection, the number of macrophages increased and that of neutrophils decreased in comparison to the control group.

The correlations between reduced pulmonary inflammation as determined by BALF cell counts and increased expression of Defensin and CD4 genes subsequent to vitamin D_3 intake were proved in this study.

Considering the leading roles of Cathalicidin and Defensin in pulmonary disease and the correlation between their expression and vitamin D_3 level, it could be suggested that vitamin D_3 deficiency may exacerbates lung problems. In other words, increased vitamin D_3 level improves the expression of AMPs, especially Defensin, as well as improving lung function during pneumonia.

Consequently, it can be assumed that during the corona virus outbreak, vitamin D_3 intake may exert and positive effects on immune system through the expression of AMPs and regulation of cellular functionalities in the lung; thereby, improving the lung function and reducing the pulmonary impairment.

In both animal models and human cell lines, vitamin D_3 affected lung immunity and revealed protective effects on experimental interstitial pneumonia in this study. Numerous *in vitro* studies have indicated the considerable contribution of vitamin D_3 to local “respiratory homeostasis” one or the other by stimulating the expression of AMPs or by directly interfering with the replication of respiratory infectious agents (Lippolis et al. 2011; Zdrenghea et al. 2017; Tsujini et al. 2019).

During the pneumonia outbreak, it seems deficiency of this vitamin can reduced the expression of AMPs, and subsequently lead to the increment in the rate of lung dysfunction. Therefore, sufficient levels of this vitamin can remarkably reduce the severity of pulmonary symptoms caused by various bacteria.

Conclusion

Important functions of vitamin D_3 have been understood in calcium metabolism and phosphate homeostasis, activation of VDR pathways, and production of antimicrobial peptides (AMPs) for supporting the immune system. According to the results of this study, the immunomodulatory effects of vitamin D_3 and accordingly the significant improvement was observed in the clinical symptoms and increment of expression of AMPs especially lung AMPs in the in vitro and in vivo situations were proved. Therefore, given the major role of vitamin D_3 in improving the function of AMPs as well as inflammatory and pro-inflammatory cytokines, it is recommended that this vitamin should be used along with common antibiotic treatments to improve the healing process and decrease the frequency and severity of respiratory diseases.

Abbreviations

AMPS: Antimicrobial Peptides; PBMCs: Peripheral Blood Mononuclear Cells; M: Molar; BALF: Broncho Alveolar Lavage Fluid; RSV: Respiratory Syncytial Virus; BVD: Bovine Viral Diarrhea; IBR: Infectious Bovine Rhinotracheitis; CAMP: Cathelicidin Antimicrobial Peptide; RDS: Respiratory Distress Syndrome; PBS: Phosphate Bufferd Saline; PCR: Polymerase Chain Reaction.

Declarations

Acknowledgment. I would like to express my gratitude to the research team of the Institute of Biomedical Research, University of Tehran and Islamic Azad University, Science and Research Branch.

Authors' Contributions. All authors contributed to the study conception and design. case selection, data collection, data analysis and manuscript preparation were performed by Eftekhari Z, Asgharpour P, under the supervision of Mokhber Dezfouli MR. All authors edited, read, and approved the final manuscript.

Funding. The authors of this article did not receive any specific funding for this project.

Availability of data and material. All data generated and analyzed during this study are included in this published article and its supplementary information files.

Compliance with ethical standards. For this study, ten Holstein male crossbred calves (2-4 months of ages) for experimental study were chosen from an industrial farm of Tehran suburb and transferred to the Research Veterinary Hospital after initial clinical examinations and proved their healthy conditions by veteran. The criteria for entrance into the study were normal rectal temperature, respiratory sounds, heart rates and lack of nasal and eyes discharge. Subsequently, calves were randomly divided into the treatment and control groups by veterinary hospital staffs, numbered and adaptation period carried out about 6 days after entrance day. The calves were kept individually in a closed and isolated pens designed for large livestock, with appropriate flooring, temperature, food, and water. The experimental study sites were thoroughly isolated and investigated during the study to completely prevent the risk of any other infection. The blood samples (10 mL) were obtained and isolation of peripheral blood mononuclear cells (PBMCs), and the expression of selected gene was performed in the in vitro and in vivo conditions. All experimental processes were performed according to the approved protocol by Iran National Research Ethics Committee (Ethical Code No: IR.IAU.SRB.REC.1397.137).

Consent to participation. Authors participated voluntarily in the research.

Consent for publication. All authors read and approved the final manuscript and give consent for publication.

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

References

1. Abed AH, El-Seedy FR, Hassan HM, Nabih AM, Khalifa E, Salem SE, Wareth G, Menshawy AMS (2020) Serotyping, genotyping and virulence gene characterization of *Pasteurella multocida* and *Mannheimia haemolytica* isolates recovered from pneumonic cattle calves in North Upper Egypt. Vet Sci 7:174.

2. Afsal K, Hatishankar M, Banurekha VV, Meenakshi N, Parthasarathy RT, Selvaraj P (2014) Effect of 1,25-dihydroxy vitamin D3 on cathelicidin expression in patients with and without cavitary tuberculosis. Tuberculosis 94:599-605.
3. Algammal AAM, El-Sayed ME, Youssef FM, Saad SA, Elhaig MM, Batiha GE, Hozzein WN, Ghobashy MOI (2020) Prevalence, the antibiogram and the frequency of virulence genes of the most predominant bacterial pathogens incriminated in calf pneumonia. AMB Express 10:99.
4. Alva-Murillo N, Téllez-Pérez AD, Medina-Estrada I, Alvarez-Aguilar C, Ochoa-Zarzosa A, López-Meza JE (2014) Modulation of the inflammatory response of bovine mammary epithelial cells by cholecalciferol (vitamin D) during Staphylococcus aureus internalization. Microb Pathog 77:24-30.
5. Anderson DE, Rings M (2008) Current veterinary therapy: food animal practice. Elsevier Health Sciences.
6. Asgharpour P, Mokhber Dezfouli MR, Nadealian MG, Eftekhari Z, Nikbakht Borojeni GR (2020) Effects of 1, 25-dihydroxy vitamin D3 on clinical symptoms, pro-inflammatory and inflammatory cytokines in calves with experimental pneumonia. Research in Veterinary Science 132:186-193.
7. Beisswenger C, Bals R (2005) Antimicrobial Peptides in Lung Inflammation. Chem Immunol Allergy 86:55-71.
8. Bikle DD (2009) Vitamin D and immune function: understanding common pathways. Curr Osteoporos Rep 7:58-63.
9. Constable PD, Hinchcliff KW, Done SH, Grunberg W (2017) Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. Elsevier Health Sciences.
10. Costabel U, Guzman J (2001) Bronchoalveolar lavage in interstitial lung disease. J Curr Opin Pulm Med 7:255-61.
11. Dabo SM, Taylor JD, Confer AW (2008) Pasteurella multocida and bovine respiratory disease. Anim Health Res Rev 8:129-150.
12. DeRosa D, Mechor G, Staats J, Chengappa M, Shryock T (2000) Comparison of Pasteurella spp. simultaneously isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. J Clin Microb 38:327-32.
13. DiRosa M, Malaguarnera M, Nicoletti F, Malaguarena L (2011) Vitamin D3: a helpful immune-modulator. Immunology 134:123-139.
14. Geraert D (2006) The importance of mycoplasma bovis in bovine respiratory Disease. Tijdschr Diergeneskd 131:124-126.
15. Gibbs A (2001) Practical approach to the control of pneumonia in housed calves. In Practice 23:32-9.
16. Guillot X, Semerano L, Saidenberg-Kermanac'h N, Falgarone G, Boissier MC (2010) Vitamin D and inflammation. Joint Bone Spine 77:552–557.
17. Harder J, Schroder JM (2007) Antimicrobial Peptides Role in Human Health and Disease. Springer International Publishing.
18. Hayes CE, Hubler SL, Moore JR, Barta LE, Praska C, Nashold FE (2015) Vitamin D Actions on $CD4^+$ T Cells in Autoimmune Disease. Front Immunol 6:100.
19. Hazlett L, Minhao WU (2011) Defensins in innate immunity. Cell and Tissue Research 343:175–188.
20. Hewison M (2011) Antibacterial effects of vitamin D. Nat Rev Endocrinol 7:337–345.
21. Izadpanah A, Gallo RL (2005) Antimicrobial peptides. J Am Acad Dermatol 52:381-390.

22. Karimkhani H, Zahraie salehi T, Sadeghi zali MH, Karimkhani M, Lameyi R (2011) Isolation of pasteurella multocida from cows and buffaloes in Urmia's Slaughter House. Archives of Razi Institute 66:37-41.
23. Lippolis JD, Reinhardt TA, Sacco RA, Nonnecke BJ, Nelson CD (2011) Treatment of an intramammary bacterial infection with 25-hydroxyvitamin D. PLoS One 6:e25479.
24. Merriman KE, Kweh MF, Powell JL, Lippolis JD, Nelson CD (2015) Multiple b-defensin genes are upregulated by the vitamin D pathway in cattle. Journal of Steroid Biochemistry & Molecular Biology 154:120–129.
25. Mohammadi GR, Ghazvini K, Panah HA (2006) Antimicrobial susceptibility testing of *Mannheimia haemolytica* and *Pasteurella multocida* isolated from calves with dairy calf pneumonia. Archives of Razi Institute 61:91-96.
26. Mohammadi GR, Nazifi S, Rezakhani A, Esmailnejad Z (2007) Effect of transportation stress on blood and bronchoalveolar lavage fluid components in calves. Comparative Clinical Pathology 16:85-95.
27. Mohammadi GR, Sharifi K, Sepahi S (2004) Comparison of the therapeutic effects of florfenicol Tilmicosin in enzootic calf bronchopneumonia. Journal of the Faculty of Veterinary Medicine University of Tehran 59:277-282.
28. Mokhber Dezfouli MR, Dousti M, Lotfollahzadeh S, Eftekhari Z, Nikbakht Boroujeni GR (2017) Detection of annexin I and annexin II in serum of calves affected by experimental pneumonia with *Pasteurella multocida*. Journal of Veterinary Research 72:261-268.
29. Ozkanlar Y, Aktas MS, Kaynar O, Ozkanlar S, Kirecci E, Yildiz Y (2012) Bovine respiratory disease in naturally infected calves: clinical signs, blood gases and cytokine response. Revue de Medicine Veterinaire 163:123-130.
30. Pincikova T, Paquin-Proulx D, Sandberg JK, Flodström-Tullberg M, Hjelte M (2007) Vitamin D treatment modulates immune activation in cystic fibrosis. Clin Exp Immunol 189:359–371.
31. Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. International Journal of Antimicrobial Agents 24:536–547.
32. Selvaraj P, Prabhu Anand S, Harishnkar M, Alagarasu K (2009) Plasma 1,25 dihydroxy vitamin D3 level and expression of vitamin D receptor and Cathelicidin in pulmonary tuberculosis. J Clin Immunol 29:470-8.
33. Smith BP (2015) Large Animal Internal Medicine. 5th edition. Elsevier Health Sciences.
34. Tecle T, Tripathi SH, Hartshorn KL (2010) Defensins and cathelicidins in lung immunity. Innate Immunity 16:151–159.
35. Telcian AG, Zdrenghea MT, Edwards MR, Stanca VL, Mallia P, Johnston SL, Stanciu LA (2016) Vitamin D increases the antiviral activity of bronchial epithelial cells in vitro. Antiviral Research 137:93-101.
36. Tsujini R, Ushikoshi-Nakayama T, Yamazaki N, Matsumoto I (2019) Pulmonary activation of vitamin D3 and preventive effect against interstitial pneumonia. J Clin Biochem Nutr 65:245-251.
37. White JH (2010) Vitamin D as an inducer of cathelicidin antimicrobial peptide expression: past, present and future. J Steroid Biochem Mol Biol 121:234-8.
38. Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol 75:39–48.
39. Zdrenghea MT, Makrinioti H, Bagacean C, Bush A, Johnston SL, Stanciu LA (2017) Vitamin D modulation of innate immune responses to respiratory viral infections. Rev Med Virol 27.

Figures

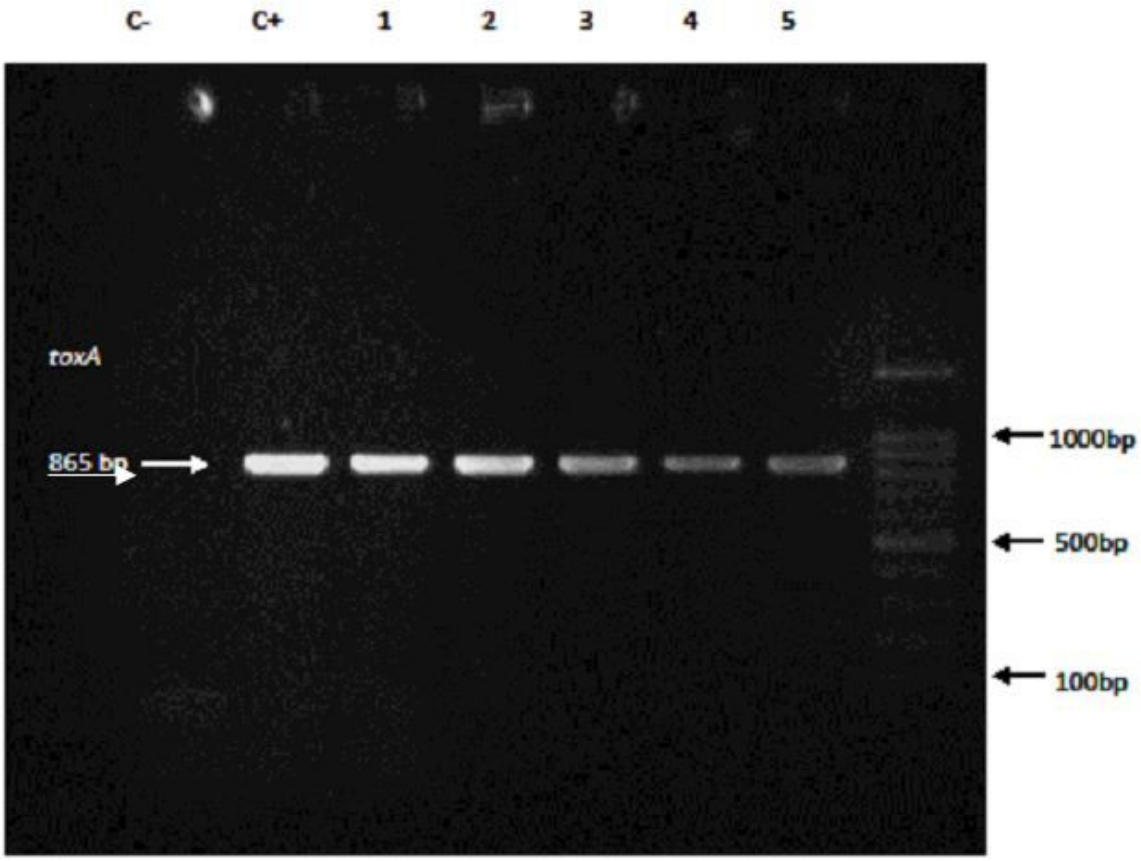

Figure 1

PCR product loaded with Pasteurella multocida on agarose gel, for definitive confirmation of the bacterial species.

Figure 2

Clinical symptoms in calves with experimental pneumonia. 1 & 2. Eye discharge. 3 & 4. Nasal discharge. 5. Auscultation to the respiratory sounds. 6. Rectal temperature.

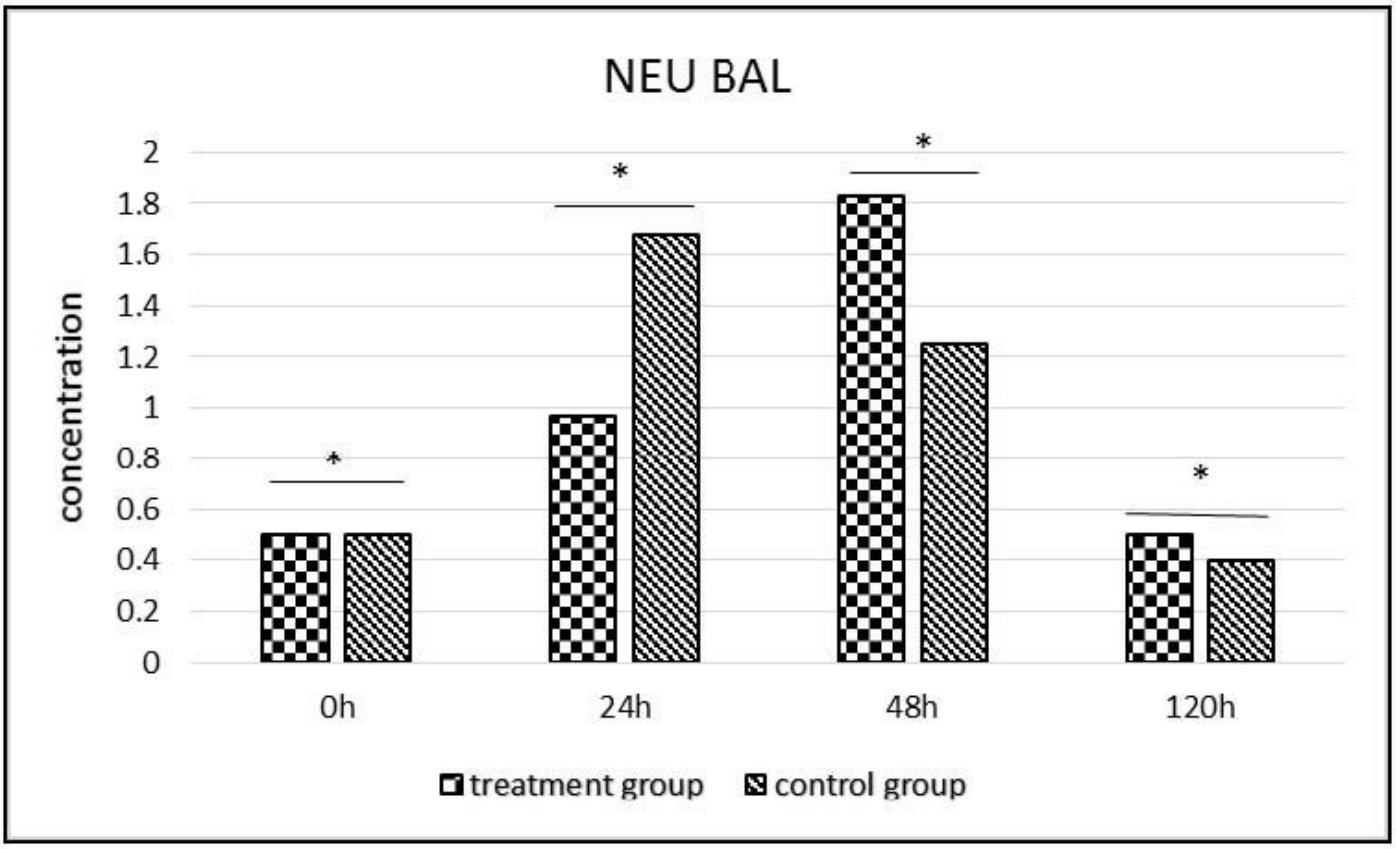

Figure 3

Comparison of BALF neutrophils counts between the control and treatment groups at different time points. .* Significant difference at P <0.05.

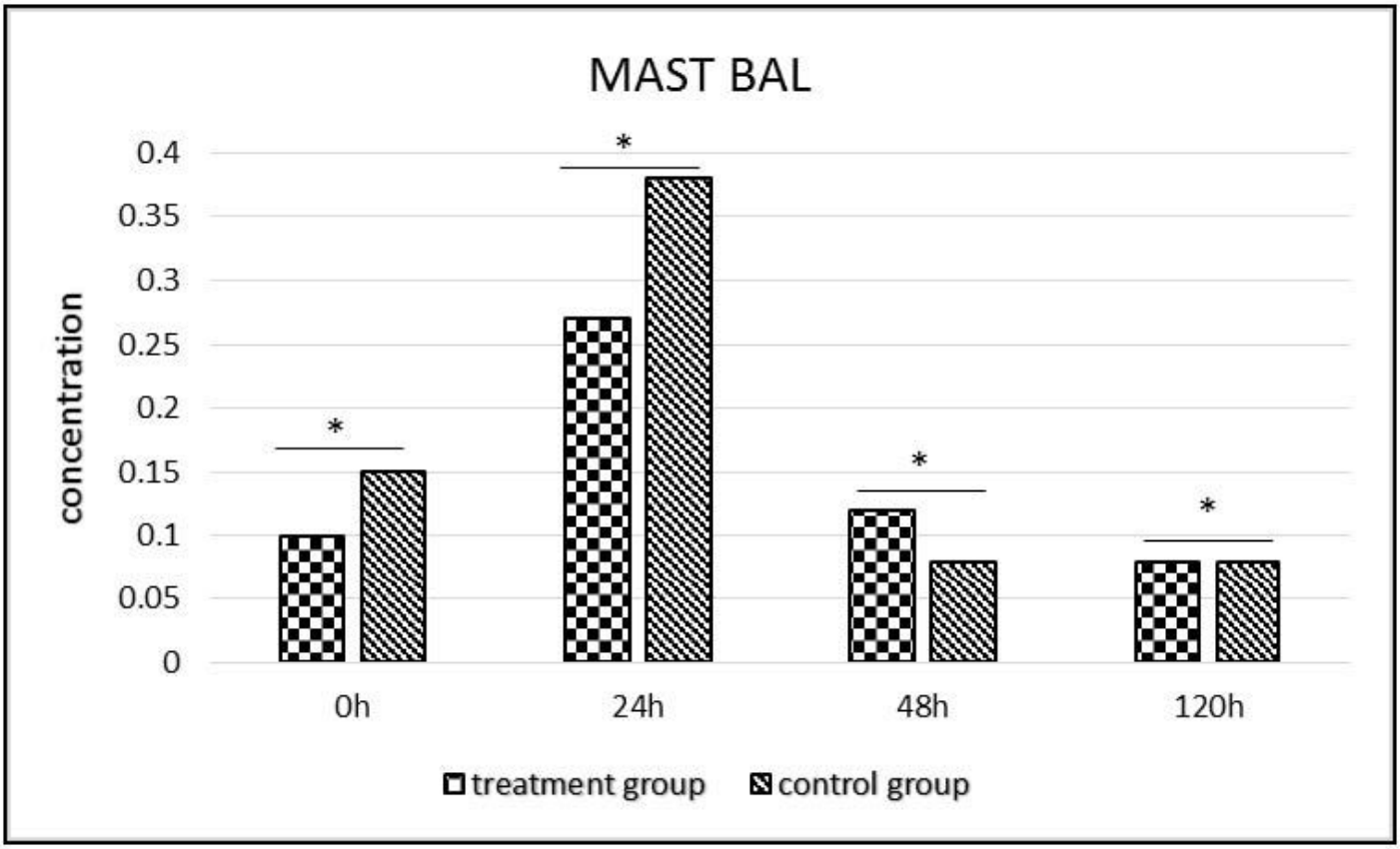

Figure 4

Differences in BALF mast cells between the control and treatment groups at different time points. .* Significant difference at P <0.05.

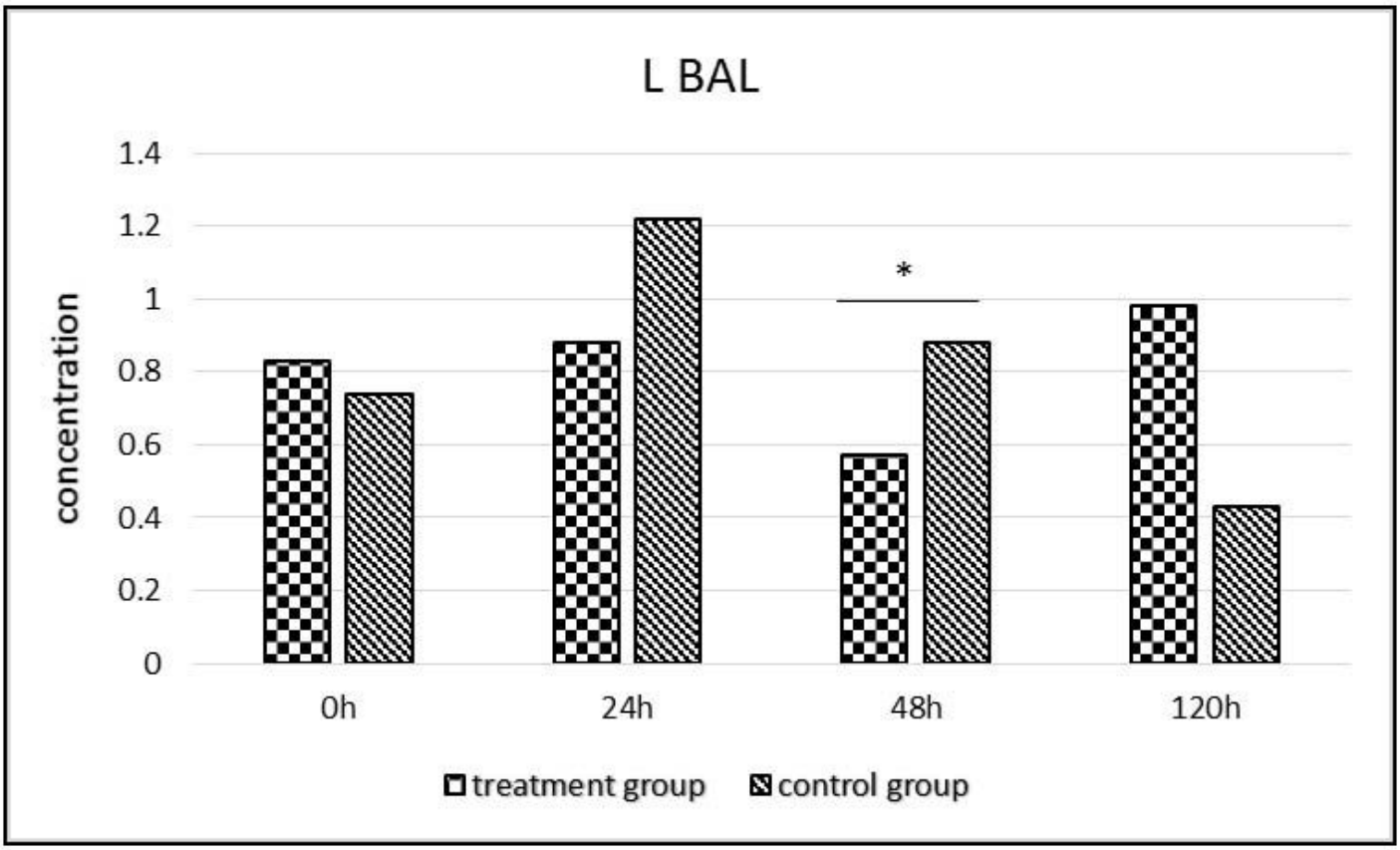

Figure 5

Differences in BALF lymphocytes between the control and treatment groups at different time points. .* Significant difference at P <0.05.

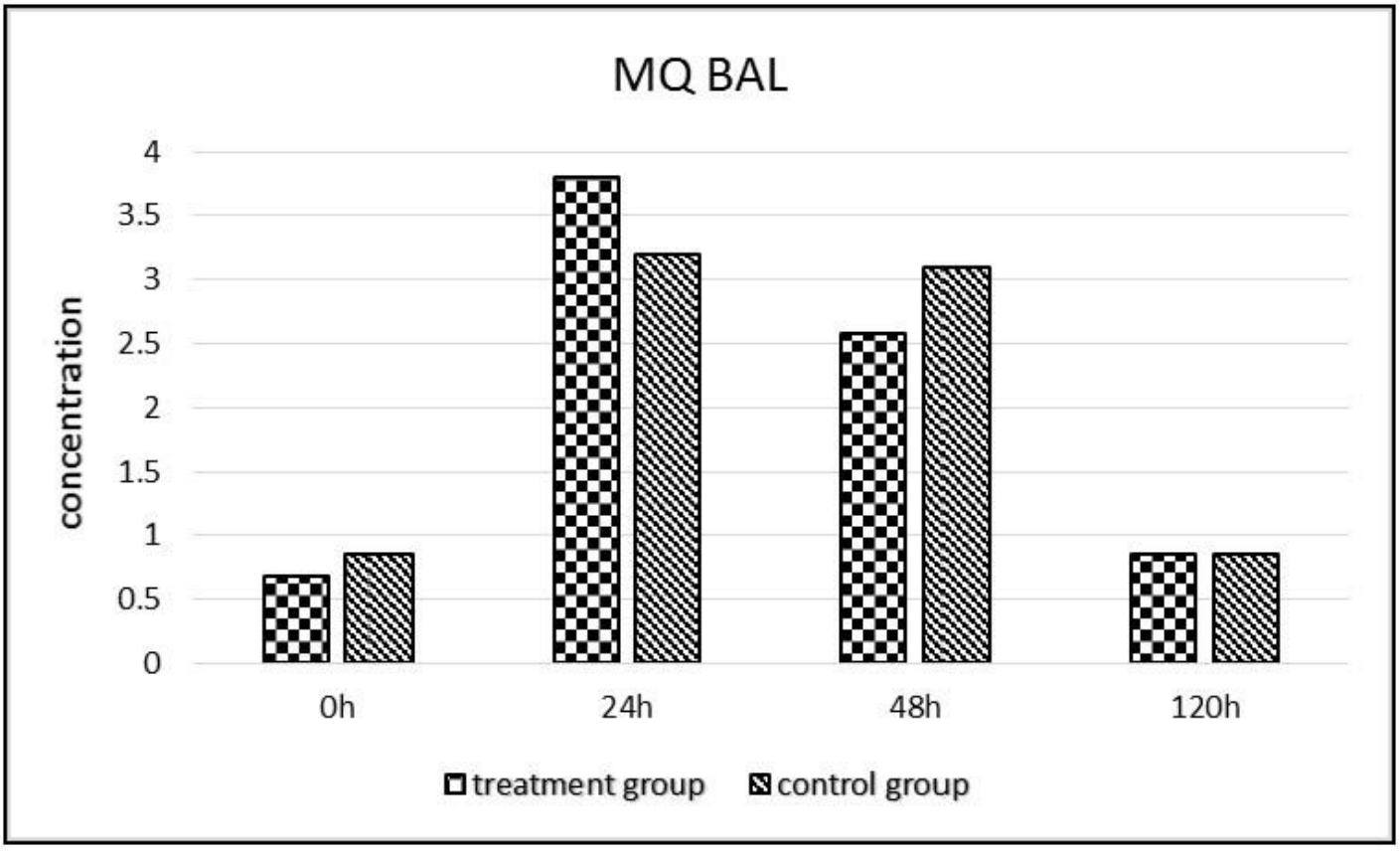

Figure 6

Differences in BALF macrophages between the control and treatment groups at different time points.

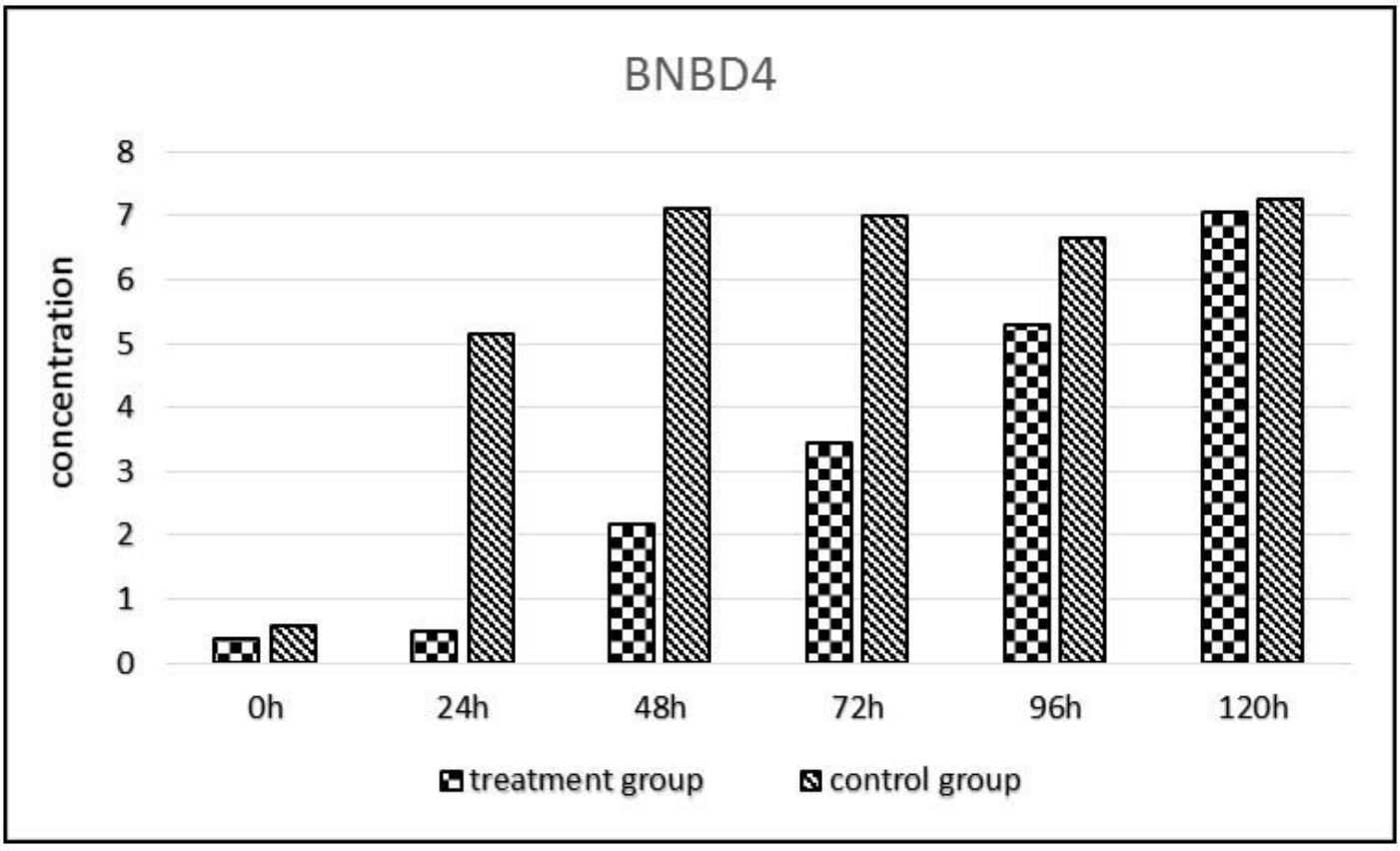

Figure 7

β-Defensin (BNBD4) gene expressions in control and treatment groups in vitro.

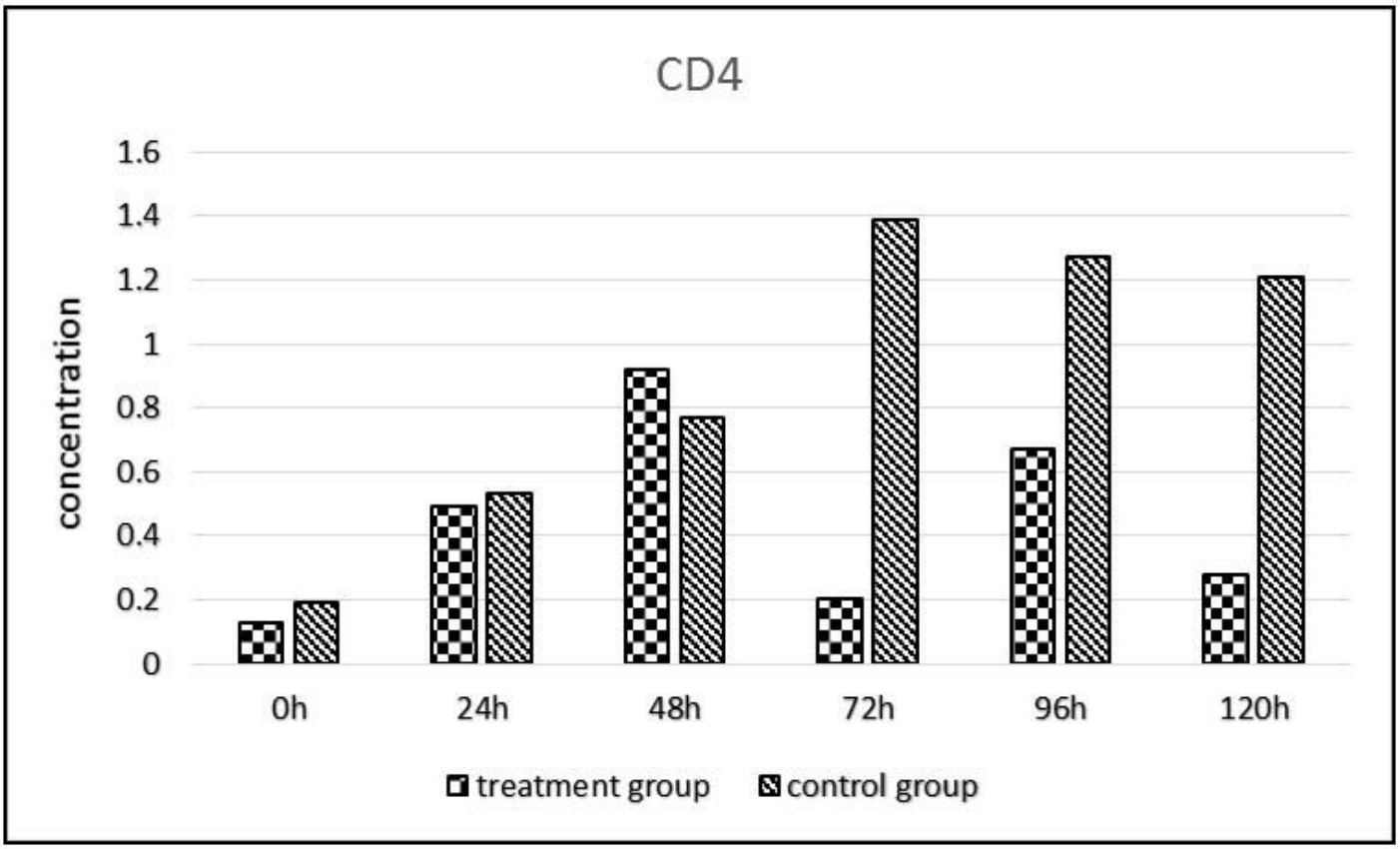

Figure 8

CD4 gene expressions in control and treatment groups in vitro, according to LSD post hoc test, at 48, 72 and 120 hours after the challenge time.

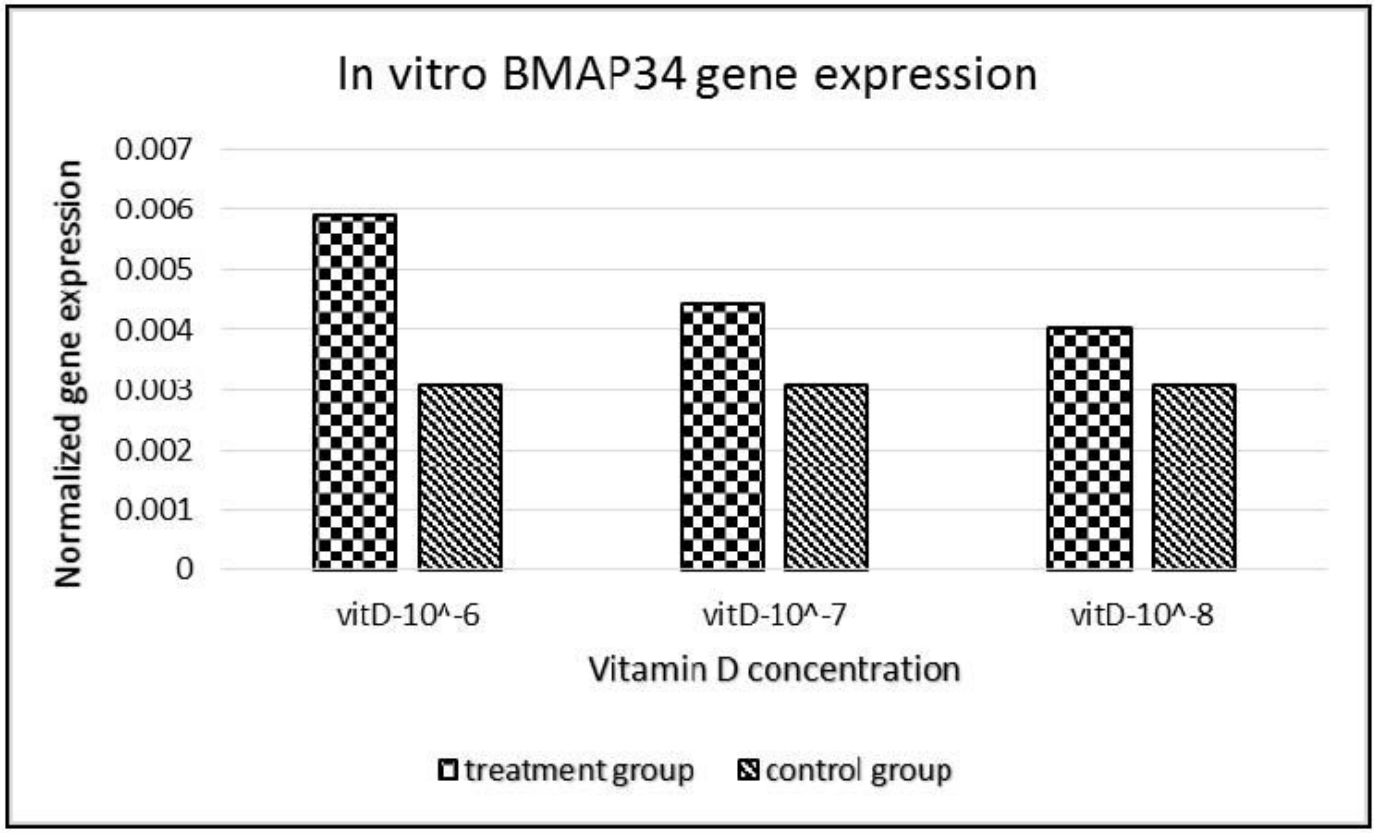

Figure 9

The comparative expression of Cathelicidin gene (BMAP34) between the study groups treated with different vitamin D3 concentrations in in-vitro conditions.

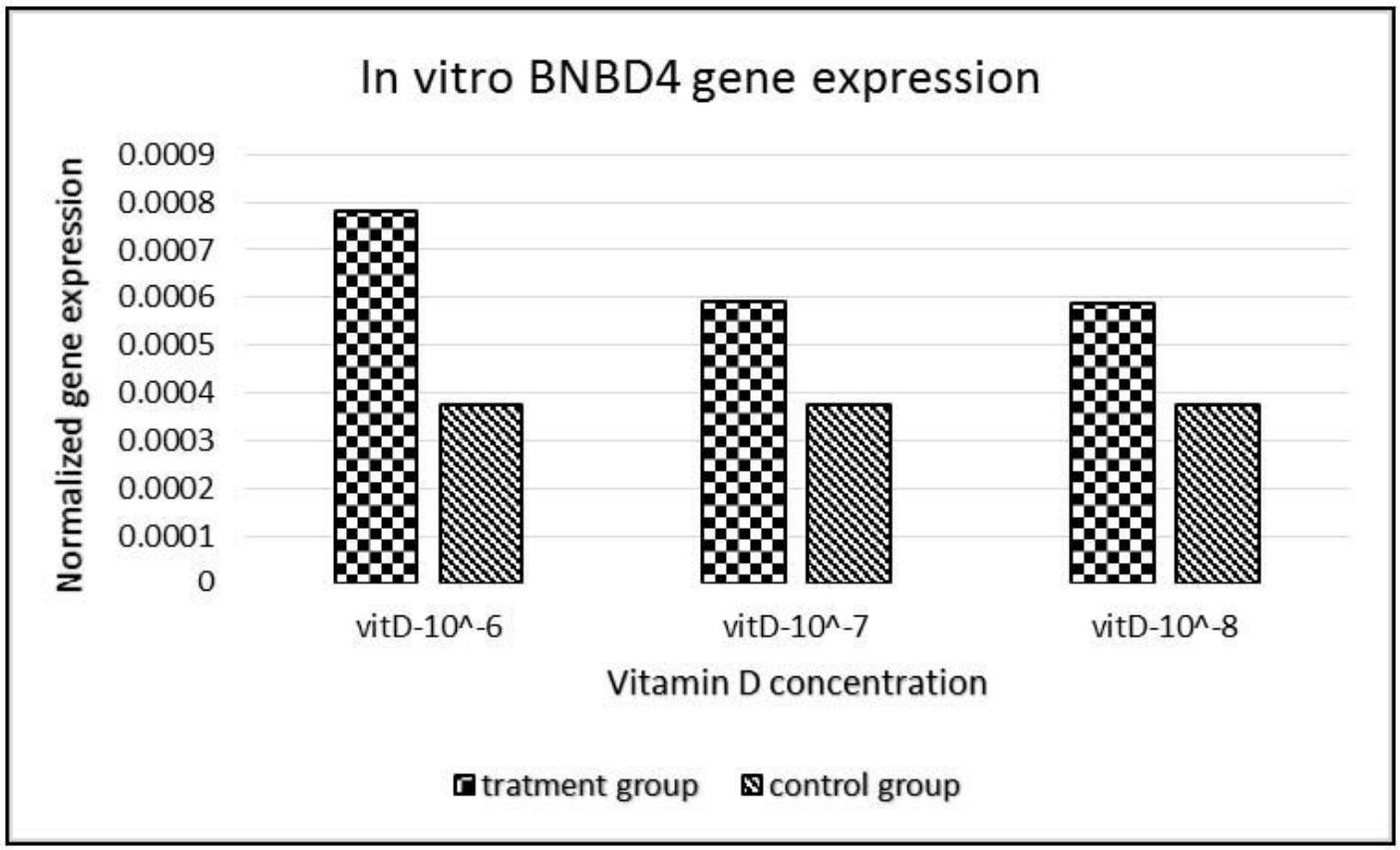

Figure 10

The comparative expression of Definsin gene (BNBD4) in in-vitro conditions between the study groups treated with different vitamin D3 concentrations.

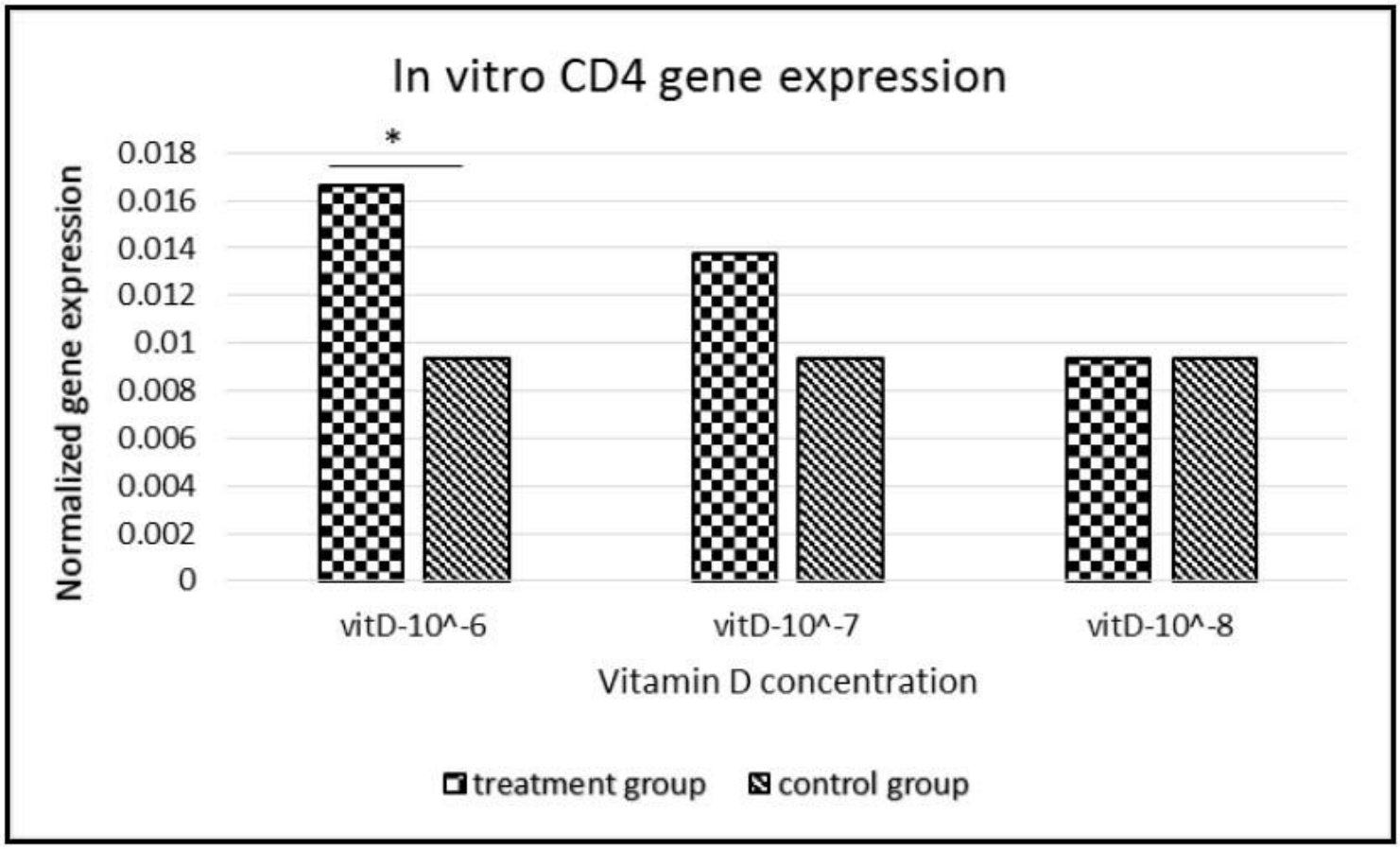

Figure 11

The comparative expression of CD4 in in-vitro conditions between the study groups treated with different vitamin D3 concentrations.