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Original Article

# The Effect of Vitamin D on the Humoral Immune Response of Calves to the Sheeppox Virus Vaccine

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## ABSTRACT

**Introduction:** Although the efficacy of the vaccine is influenced by various factors, including vitamin D levels, studies in this field have yielded different results. Vaccination with the attenuated sheeppox virus vaccine (RM/65) remains a preventive strategy in countries where lumpy skin disease (LSD) is prevalent, but there is no evidence from experimental research on the effect of vitamin D on the immune response to this vaccine for the prevention of LSD.

**Materials & Methods:** Twenty calves were selected and divided into four groups. To ensure that calves had different baseline levels of vitamin D, they were divided into two groups. One group was exposed to sunlight, while the other two groups were kept in the shade. Each of these groups was further divided into two treatment groups: One receiving vitamin D (cholecalciferol) injections and the other a control group. All groups received the attenuated sheeppox virus vaccine (RM/65), while only two groups received vitamin D simultaneously with the vaccine. Blood samples were collected from each calf weekly for six weeks. Sheeppox virus antibodies were measured according to the World Organization for Animal Health (WOAH) protocol, with a neutralization index (NI) titer of ≥1.5 international units considered protective.

**Results:** Statistical analysis revealed a significant increase in sheeppox virus antibody levels within individual groups after day 21 ($P \leq 0.01$). However, no significant differences were observed between the four groups beyond this time point. Consequently, antibody levels in the groups receiving vitamin D did not differ from those in the other groups.

**Conclusion:** These findings are consistent with previous studies, indicating that vitamin D supplementation does not affect the efficacy of the vaccine.

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## 1. Introduction

Lumpy skin disease (LSD) is a highly prevalent disease caused by a Capripoxvirus within the Poxviridae family. It primarily affects cattle in Africa and was first reported in the Middle East in 1989 [1]. LSD is a significant concern as it can lead to decreased production and create opportunities for concurrent diseases in cattle [2]. Vaccination is the primary method for controlling the disease in endemic regions [1]. Moreover, due to cross-reactivity, immunity against one member of the *Capripoxvirus* genus can provide protection against other members. This has led to the use of the attenuated sheeppox vaccine (RM/65) as a means to combat LSD [1]. Numerous factors contribute to the variability in vaccine response. Research has shown that the status of minerals and vitamins can significantly impact the efficacy of vaccination [3]. Specifically, vitamin D metabolites play a crucial role as modulators in the immune system [4]. However, the influence of these metabolites on the immune system and vaccination remains complex and not fully understood [5-7]. Complicating matters further, there is a lack of consensus between in vivo and in vitro studies [8-15]. Moreover, it is believed that the effect of vitamin D on the immune system may vary depending on the specific pathogen or vaccine and their respective pathogenesis pathways [16]. Based on the aforementioned, each pathogen or vaccine activates a specific pathway, which likely involves the activation of different receptors and the release of certain mediators in the immune system [16]. This, in turn, leads to specific effects on vitamin D metabolism within the immune system and the reciprocal impact of vitamin D on immune function [17]. As mentioned earlier, vitamin D has the ability to modulate the immune system in direct proportion to the level of stimulation it receives. However, there has been a lack of experimental studies investigating the specific effects of vitamin D3 on the immune response to the attenuated sheeppox vaccine. Therefore, the purpose of this study is to address this gap and achieve the following objective: To investigate the influence of co-administration of an intermediate dose of cholecalciferol injection with the attenuated sheeppox vaccine on vaccination outcomes.

## 2. Material and Methods

### 2.1. Animals

In this study, twenty calves were selected from a semi-industrial farm in Qazvin Province, in the northwest of Iran. The calves were between three and four months old. Their diet consisted of 80% straw and 20% alfalfa, without any vitamin or mineral supplements. This setup allowed the researchers to investigate the effects of sunlight exposure or cholecalciferol injection on the immune system and the metabolism of 25D3 with minimal interference from the digestive system.

The calves were randomly divided into four groups: Groups A and B were housed in a semi-roofed area where they had the freedom to move around and seek sunlight exposure. Groups C and D, on the other hand, were confined to a barn with brick walls and a roof that blocked sunlight completely, resulting in a dark environment.

This experimental design enabled the researchers to examine the impact of sunlight exposure and cholecalciferol injection on the calves' immune system and the metabolism of 25D3 under different environmental conditions.

### 2.2. Blood sampling

Blood samples were collected from each calf weekly for a period of six weeks, starting from day 0. On the days of vitamin and vaccine injections, blood samples were taken before the injections.The blood samples were obtained from the jugular vein using gel activator vacuum tubes in an aseptic manner. To maintain the integrity of the samples, they were immediately placed in a cool box near the ice pack. The collected blood was centrifuged, and sera were stored at -20 ˚C until examination.

### 2.3. Injection of vitamin D

At the start of the third week (the experiment's 21st day), the calves in groups A and C were administered intramuscular (IM) injection of cholecalciferol (11,000 IU/kg) [1].

### 2.4. Vaccination

All calves were vaccinated on day 21 with the attenuated sheeppox vaccine (sheeppox virus strain RM-65, Razi Vaccine and Serum Research Institute, Iran), subcutaneously in the neck area at 10 times the recommended dose for sheep [18].

### 2.5. Assessment of sheeppox virus antibodies

The titer of antibody against sheeppox vaccine was evaluated based on the neutralization index (NI) [19], at the Poxvirus Reference Laboratory of Razi Vaccine and Serum Research Institute in Iran. For this purpose, sera were diluted at a ratio of 1:5 in Eagle's culture medium contain-

ing HEPES (Eagle's/HEPES) and inactivated at 56 ˚C. After inactivation, 50 µL of each serum sample was added to all wells (A to H) of two columns in a cell culture microplate. A positive control serum was used in columns 7 and 8, and a negative control serum in columns 9 and 10. In columns 11 and 12, only culture medium was added as a cell control. Then, in wells A to G of each column (except columns 11 and 12), 50 µL of serial dilutions of 105, 104, 103.5, 103, 102.5, 102 and 101.5 TCID50/mL of a standard strain of sheeppox virus were added, respectively. In row H of each column (as a control of serum toxicity to cells), 50 µL of culture medium was added. After one hour of incubation at 37 ˚C, 100 µL of cell suspension (MDBK cells) was added to each well. The microplates were incubated for nine days at 37 ˚C. According to World Organisation for Animal Health (WOAH) instructions [19], starting from the fourth day of incubation, the wells were observed daily using an inverted microscope to detect cytophatic effects of the viruus. The NI for each serum is equal to the difference between the logarithm of the virus titers in the columns containing the negative serum and the tested serum sample. Indexes equal to or greater than 1.5 (≥1.5) were considered positive.

### 2.6. Statistical analysis

The collected data were analyzed using IBM SPSS Statistics for Windows, version 22.0 (Armonk, NY: IBM Corp). In this study, a two-way repeated measures analysis of variance (ANOVA) was performed to evaluate the calves in the treatment groups. LSD post hoc analysis was used to determine the main effects of treatment, time, and the interaction between treatment and time. Prior to conducting the ANOVA, the normality of the data distribution was assessed using the Shapiro-Wilk test. The results of the test indicated that the data followed a normal distribution (P>0.05).

For all statistical analyses, a significance level of P<0.05 was considered statistically significant.

## 3. Results

The experiment was conducted over a period of 42 days (six weeks). On day 21, all groups underwent vaccination, and members of groups A and C also received cholecalciferol injections, as described in the methodology section. To facilitate a more accurate and meaningful comparison, the results were analyzed separately for two time periods: Before treatment (from the start of the experiment to the 21st day) and after treatment (from the 21st to the 42nd day), corresponding to the administration of cholecalciferol and vaccine. In order to examine the effect of cholecalciferol injections, groups B and D, which did not receive the injections, were considered control groups, while groups A and C were regarded as treatment groups. The data on 25D3 variations among the different groups and the effect of sunlight exposure have been published in Mousavi Rad et al. (2023). For further details, please refer to that article [20].

According to the WOAH, a titer of 1.5 IU or greater in the NI is considered a protective antibody level. The case of calf number 318 from group A, which had a titer of 2 IU NI against the sheeppox virus on day 0 of the experiment, was therefore excluded from the statistical analysis.

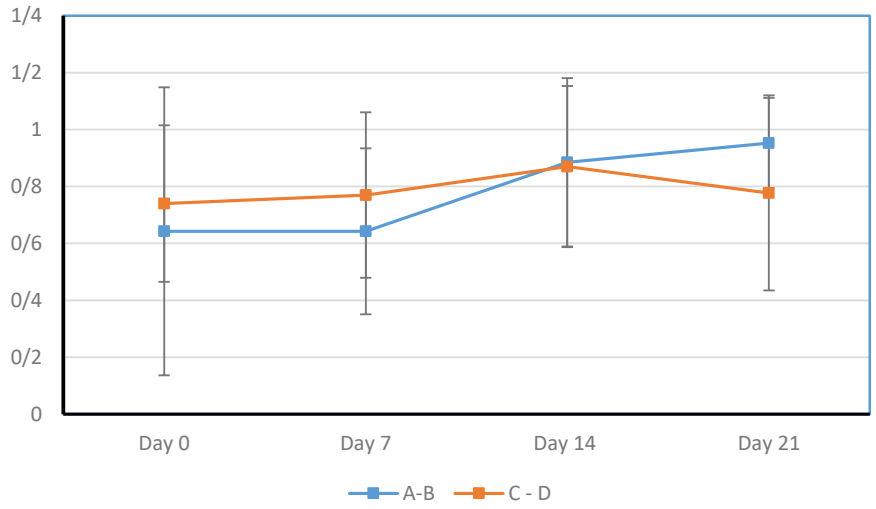


**Figure 1.** Variation of sheeppox antibody levels (IU of NI) in groups A-B and C-D during the first three weeks of the experiment

Note: It can be observed that the variations in both groups were not statistically significant (P>0.05).

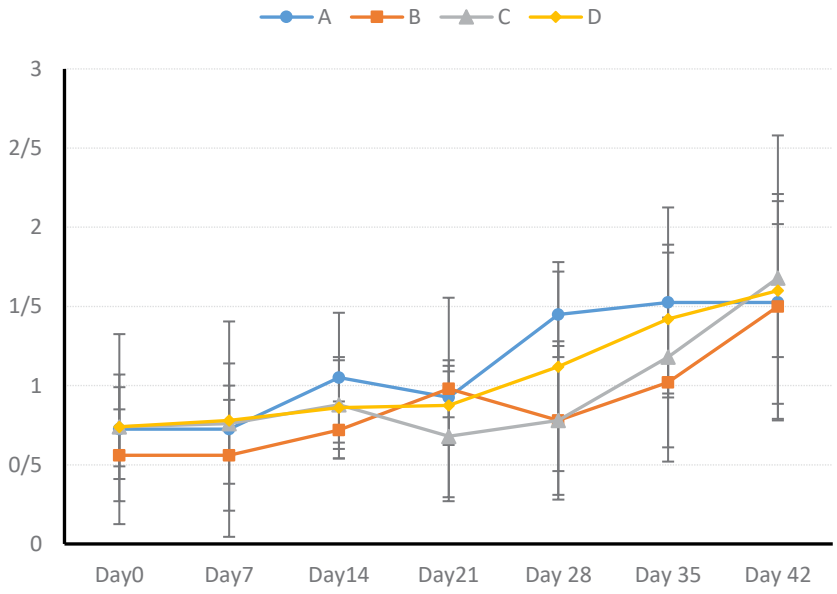


**Figure 2.** Variation of sheeppox antibody levels (IU of of NI) in groups A, B, C, and D over the course of seven weeks in the experiment

Note: The factor of time was found to have a significant effect in all groups after the 21st day (P≤0.01).

The statistical analysis indicated that there was no significant difference between the sun-exposed group (A and B) and the non-exposed group (C and D) until day 21. During the initial three weeks, the variation in anti-sheeppox antibody levels between both groups was not found to be statistically significant (Figure 1, Table 1).

The statistical analysis indicated that there was a significant difference in sheeppox antibody levels within each group after the 21st day day (P≤0.01); however, no significant differences were observed between the four groups after this time point (Figure 2).

## 4. Discussion

The previous article [20] extensively discussed the changes in 25D3 levels across different groups and the interplay between baseline 25D3 and injected cholecalciferol. Briefly, it was highlighted that depriving ruminant calves of vitamin D-rich meals and sunlight leads to a rapid and significant decline in 25D3 concentration within three weeks. In groups C and D, the 25D3 levels dropped to less than 10 ng/mL, consistent with earlier findings [21, 22]. Conversely, in groups A and B, where calves had the freedom to choose between light and shade throughout the day, the 25D3 concentration eventually increased to above 30 ng/mL. On day 21 of the experiment, we administered IM injections of 11,000 IU/kg cholecalciferol to groups A and C. Interestingly, there was no noticeable difference in 25D3 levels between groups A and B, both of which were exposed to sunlight. 25D3 serves as a marker for the presence of regulatory mechanisms, as its levels did not increase further after injection in group A. This regulatory mechanism has also been observed in cows receiving adequate supplemental cholecalciferol and sufficient exposure to sunlight [21]. This mechanism prevents an excessive increase in 25D3 concentration. It seems that when plasma concentrations of baseline 25D3 are sufficient, the activity of the liver's 25-hydroxylase enzyme will be inhibited [23]. We could assume that this feedback mechanism was activated in group A due to a sufficient concentration of 25D3 produced by sun exposure, which prevented a further increase in 25D3 levels after cholecalciferol injection. Additionally, the findings from group C support the hypothesis that a low baseline 25D3 concentration at the time of cholecalciferol injection allows for the continued activity of the 25-hydroxylase enzyme. This could be attributed to the fact that the plasma 25D3 concentration was still low on injection day. Furthermore, the observed phenomenon may be related to the calves' preference for sunlight exposure. It is possible that the calves in group A had less exposure to UV light compared to the control group B after the vitamin D injection. Similar behavior has been observed in panther chameleons following cholecalciferol supplementation in their diet [24].

**Table 1.** Details of variation in antibodies titers (IU of NI) against sheeppox virus

| Group | Case Number | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 | Day 35 | Day 42 |
|---|---|---|---|---|---|---|---|---|
| A | 318 | 2 | 2.2 | 1.7 | 2.3 | 1.2 | 2.5 | 2.5 |
| | 304 | 0.7 | 0.7 | 1 | 1 | 1.2 | 1.2 | 1.2 |
| | 308 | 0.7 | 0.7 | 1.5 | 1 | 1.7 | 2.2 | 2.2 |
| | 341 | 0.5 | 0.5 | 0.7 | 0.7 | 1.2 | 1.2 | 1.7 |
| | 310 | 1 | 1 | 1 | 1 | 1.7 | 1.5 | 1 |
| | Mean±SD | 0.72±0.6 | 0.72±0.68 | 1.05±0.41 | 0.92±0.63 | 1.45±0.27 | 1.52±0.6 | 1.52±0.64 |
| B | 314 | 0.2 | 0.2 | 0.5 | 1 | 1 | 1.2 | 2.5 |
| | 315 | 0.8 | 1 | 1 | 0.7 | 1 | 1.5 | 1.5 |
| | 349 | 0.7 | 0.7 | 0.7 | 1.2 | 1.2 | 1.2 | 1.5 |
| | 338 | 0.2 | 0.2 | 0.7 | 1 | 0.7 | 0.7 | 1.5 |
| | 312 | 0.7 | 0.7 | 0.7 | 1 | 0 | 0.5 | 0.5 |
| | Mean±SD | 0.52±0.29 | 0.56±0.35 | 0.72±0.18 | 0.98±0.18 | 0.78±0.47 | 1.02±0.41 | 1.5±0.71 |
| C | 300 | 0.7 | 0.7 | 1.2 | 1 | 1.5 | 1.5 | 2.2 |
| | 302 | 0.7 | 0.7 | 0.7 | 0.5 | 0.7 | 1 | 1.5 |
| | 303 | 0.2 | 0.2 | 0.5 | 0.2 | 0.2 | 1.2 | 2.5 |
| | 326 | 1 | 1 | 1 | 0.5 | 0.5 | 0.2 | 0.2 |
| | 342 | 1 | 1.2 | 1 | 1.2 | 1 | 2 | 2 |
| | Mean±SD | 0.72±0.33 | 0.76±0.38 | 0.88±0.28 | 0.68±0.41 | 0.78±0.5 | 1.18±0.66 | 1.68±0.9 |
| D | 313 | 0.7 | 0.7 | 1.2 | 1 | 0.5 | 1.5 | 1.5 |
| | 348 | 0.5 | 0.7 | 1.2 | 0/5 | 0.7 | 1.2 | 1 |
| | 309 | 0.5 | 0.5 | 0.5 | 1 | 2.2 | 2.2 | 2 |
| | 299 | 1 | 1 | 0.7 | 0.5 | 1.2 | 1 | 2 |
| | 301 | 1 | 1 | 0.7 | 1 | 1 | 1.2 | 1.5 |
| | Mean±SD | 0.74±0.25 | 0.78±0.22 | 0.86±0.32 | 0.88±0.25 | 1.12±0.66 | 1.42±0.47 | 1.6±0.42 |

The results related to the effect of vitamin D on the immune response to the sheeppox virus vaccine in calves align with several other studies [9, 11, 25]. It seems that understanding the relationships between vitamin D metabolites is crucial in explaining the effects of vitamin D in various tissues and immune microenvironments. This topic was discussed in detail as follows.

Although the age of the studied calves was between 90 to 120 days, they did not have maternal antibodies that protect them against LSD before being vaccinated with the sheeppox virus vaccine (RM/65). With the exception of calf number 318, that had protective antibodies on day 0 of the experiment. It is supposed that this calf may have been exposed to a wild strain of LSD prior to the start of the experiment. Administration of the sheeppox vaccine (RM/65) increased antibody titers in all groups when compared to themselves. The antibody titer against the vaccine reached nearly two after 21 days, indicating its effectiveness as an immunizer [2]. Hence, the utilization of the sheeppox vaccine (RM/65) at the recommended dose from the Razi Vaccine and Serum Production Research Institute, which is ten times higher than that used in sheep, can effectively provide immunity against LSD in calves.

As mentioned earlier, there was a noticeable variation in antibody titers among groups C and D, which both had a significant deficiency in vitamin D concentration at the time of vaccination. These two groups did not show any significant differences compared to groups A and B, which had sufficient levels of 25D3 at the time of vaccination. Understanding the reason behind this lack of difference among the four groups is challenging due to the diverse observations reported in previous studies [26, 27]. According to this research, it was expected that at least group D would exhibit a better response to the vaccine compared to groups A and B, because they observed that low baseline levels of 25D were accompanied by an increase in antibody production. This was based on in vitro observations of the relationship between vitamin D deficiency and the prevalence of multiple sclerosis [9, 27]. These studies concluded that vitamin D induces apoptosis in plasma cells, leading to lower antibody levels in in vitro experiments. Therefore, a deficiency in vitamin D can potentially reverse these results and lead to an increase in antibody concentration by promoting the proliferation of plasma cells. However, when focusing on the findings of the present study and several other in vivo studies, it becomes apparent that these conclusions may be flawed and cannot fully support the observed in vivo results.

The aforementioned studies [7, 28] have reported consistent evidence indicating that the presence of vitamin D is associated with better immunogenicity. For example, Zitt et al. found that vaccination against hepatitis B was more effective in patients with chronic kidney disease when their 25D3 concentrations were within the normal range [29]. Accordingly, it was expected that group B in our study would have shown a better response, at least compared to groups C and D. In our study and some other studies [7, 9, 28], it appears that solely measuring levels of 25D3 or 1.25D3 may not be sufficient to fully understand the effect of vitamin D on B cells. It may be more informative to also measure the levels of 1.25D3 and 24.25D3 in addition to 25D3 in order to gain a better understanding of the mechanisms involved in vitamin D's impact on humoral immunity. It is likely that these two metabolites, 1.25D3 and 24.25D3, closely interact with each other, particularly in cells and tissues that do not rely on the parathyroid hormone (PTH) axis [4] or in specific microenvironments [27]. In fact, within the immune system, different vitamin D metabolites are known to play regulatory roles in immune function [27]. It is possible that thermodynamic principles govern the effects of vitamin D metabolites on target cells in tissues and cells that are independent of the PTH axis [30]. According to some studies, elevated concentrations of 25D3 or 1.25D3 in the body activate the 24-hydroxylase enzyme, resulting in the formation of an inactive metabolite called 24.25D3. However, the reverse process of this activation takes longer to occur based on thermodynamic principles [30]. In simpler terms, when the concentrations of 25D3 or 1.25D3 decrease, the production of 1-hydroxylase, which is responsible for increasing 1.25D3 levels, cannot happen as quickly as the rapid activation of 24-hydroxylase. Consequently, this activation period requires more time [30]. It is unfortunate that the present study only measured the concentration of 25D3 and did not investigate other variables. However, Kashi et al. (2019) [4] observed that when the concentration of 25D3 reached a sufficient level, the concentration of 24.25D3 also began to rise. This increase in 24.25D3 caused the ratios of 1.25D3:24.25D3 and 25D3:24.25D3 to decrease, with a greater impact seen in the 1.25D3:24.25D3 ratio. Essentially, the increase in 24.25D3 led to a reduction in the effects of 1.25D3 and prevented it from interacting with the immune system effectively. It is possible that in groups A and B, the increase in 25D3 was accompanied by a rise in 24.25D3 levels. This increase in 24.25D3 may have altered the proportion of active metabolites and diminished the impact of 1.25D3 in the immune microenvironment. Conversely, this effect could have occurred in groups C and

D, where the lower concentration of 24.25D3 may have resulted in an increase in the effect of free 1.25D3 in the immune microenvironment. Therefore, it is possible that the final effect of vitamin D in all four groups was approximately similar to a certain extent. Another possible explanation for the lack of difference between the four groups or the similar effects of vitamin D could be attributed to the synthesis of 24-hydroxylase in B lymphocytes. It appears that B cells rely on a paracrine source of 1.25D3, although they express the CYP24A gene (which codes for 24-hydroxylase) strongly when stimulated with 1.25D3 [27]. This suggests that the effectiveness of 24.25D3 may be more pronounced in B cells. Consequently, we believe that the 24-hydroxylase produced in B cells may exert immediate and potent intracrine effects on these cells, inhibiting the impact of 1.25D3 in groups A and B.

In this study, only humoral immunity was measured, and cellular immunity was not assessed. In all honesty, we consider the interplay between vitamin D, its metabolites, and B cells in microenvironments to be a complex subject that requires further investigation. Furthermore, in order to address the question of why there was no difference between groups with adequate baseline 25D levels and the opposing groups, it is important to consider the type of plasma cell involved. Plasma cells can be classified into two types based on their lifespan and antigen responses, as indicated by previous studies [27, 31]. These types include plasma cells with a short lifespan and plasma cells with a long lifespan. Plasma cells with a long lifespan are generated within secondary germinal centers (GCs) that are associated with survival niches [32]. In addition to their long lifespan and production of highly specific antibodies, plasma cells also play a crucial role in immunogenicity processes. In some cases, the antibody titer may decrease after a short duration. This could be attributed to the presence of plasma cells with short lifespans that did not receive the necessary survival signals from GCs. As a result, these plasma cells undergo apoptosis, leading to a decrease in antibody concentration [27]. According to Rolf et al. (2016) [27], it has been observed that vitamin D is not capable of inducing apoptosis in plasma cells with a long lifespan, rendering it ineffective in these cells. This finding could potentially explain the observations made by Décard [33] and the present study. In Décard's study, no difference in MS-specific IgG levels was observed after 12 weeks of vitamin D therapy, despite a decrease in the concentration of antibodies to Epstein-Barr virus (anti-EBNA-1) in the cerebrospinal fluid of these patients [33]. Additionally, data from Décard et al. suggests that serum 25D3 levels decline in the two to three years preceding the onset of MS, while anti-EBNA-1 IgG titers increase [33]. Rolf et al. (2016) hypothesizes that the presence of anti-EBNA-1 IgG in the cerebrospinal fluid is likely associated with plasma cells with a short lifespan, and the fluctuation in vitamin D levels may influence the production of these cells [27]. Moreover, Thorsen's [25] study, which included vitamin D therapy for patients with type 1 diabetes, found no difference in antibody titers against pancreatic β-cells. He considered another aspect of the effect of 1.25D3 on the plasma cells. He and colleagues assumed that non-observable results are most likely caused by the effect of 1.25D3 on plasma cells. They mentioned that 1.25D3 inhibits plasma cells formation from B cells and does not affect existing plasma cells [25]. Thus, vitamin D therapy does not reduce antibodies against pancreatic β-cells in a short duration. Considering that the primary source of the most specific IgG (s) in plasma is plasma cells with a long lifespan, as established by research [27], it is reasonable to surmise that Rolf's hypothesis [27] regarding Decard's study [33] also pertains to our observation. It has been confirmed that the ability of attenuated vaccines to induce GCs [34] is responsible for their capacity to confer resilient immunity. Therefore, it can be hypothesized that the establishment of GCs and plasma cells with a prolonged lifespan, induced by an attenuated vaccine, may have hindered or negated the apoptosis signal triggered by vitamin D in plasma cells. This assumption is supported by our study as well as previous studies that utilized the attenuated vaccine as a reference. It is plausible that the presence of sufficient levels of vitamin D, along with the prolonged lifespan of plasma cells, allowed for the release of IgG against the sheeppox vaccine without triggering apoptosis, resulting in a lack of a decrease in antibody levels. Consequently, in our current study, there was no significant difference observed between the four groups. It is likely that the persistence of suitable concentrations of 25D in case number 318 did not lead to a change in antibody titer due to the presence of GCs and long-lived plasma cells that were generated by a wild strain of the virus. In order to address why we did not observe a difference among the four groups, Thorsen's hypothesis [25] may be worth considering. Animal studies have shown that stress must occur immediately before the initial antigen exposure in order to affect in vivo immune induction [35]. Further research has demonstrated that stress experienced after this time has no impact on the immune response [35]. Human studies have also confirmed that stress close to an immunological challenge has an immune-modulating effect, while stress near a recall or repeat of the challenge has a significantly diminished effect [4]. Therefore, attempting to resolve stress or improve 25D levels after the

vaccination would be ineffective. According to Thorsen's logic [25], the authors of the present study expected to observe different effects in groups A and B compared to other groups, particularly in group B, as it did not experience stress (insufficient concentration of 25D) at the time of vaccine injection. However, it is important to consider that the active form of vitamin D (1.25D3) and its proportion with other vitamin D metabolites may play a more decisive role in the microenvironment between immune cells, rather than just the sufficiency of 25D.

Taking all of the above into account, it is believed that paying attention to the timing of the challenge (vaccination day in the present study), the presence of stress (25D insufficiency), and their interaction effects on the immune system, specifically in relation to the proportion of vitamin D metabolites, is crucial. It is hypothesized that if groups C and D were deprived of more sunlight, similar to Kashi's experiment [4], the effects of 25D3 deficiency on the ratios of 25D3/24.25D3 and 1.25D3/24.25D3 would become more apparent, potentially leading to a weaker humoral system response. It is evident from Figure 2 that the injection of cholecalciferol did not result in a change in antibody titer in groups A and C. This raises the question of why the injection of cholecalciferol did not impact the humoral function.

In group A, the negative feedback loop was triggered by an adequate amount of basic 25D3. This feedback likely restricted the precursor of 1.25D3 (25D3) and prevented an increase in its levels. As a result, the effect of vitamin D on the humoral system was not observed [36].

On the other hand, in group C, this phenomenon could be attributed to the high dosage of injected vitamin D and the physiological time lag required to convert cholecalciferol to 25D3, followed by subsequent effective events [37].

## 5. Conclusion

In conclusion, the results of the present study indicate that co-administration of cholecalciferol did not have any impact on humoral immunity following vaccination with RM/65. Based on these findings, it is suggested to better evaluate the effect of vitamin D on immune response in vaccination; both humoral and cellular immunity will be assessed. In addition, 25D3 alone may not be a suitable parameter for determining the effect of vitamin D on the humoral immune system. It is recommended to also assess the levels of active metabolites of vitamin D and their proportion in order to gain a better understanding of vitamin D's role in immune function.

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## Compliance with ethical guidelines

This study was approved by the Ethics Committee of Shahid Chamran University of Ahvaz, Ahvaz, Iran (Code: EE/1401.2.24.140941/scu.ac.ir). All experiments were performed in accordance with the proposal approved by this committee.

## Data availability

All data analyzed during this study are included in this article.

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This study was extracted from the PhD dissertation of Seyed Ali Mousavi Rad, approved by the Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran. This study was supported by the Shahid Chamran University of Ahvaz, Ahvaz, Iran (Grant No.: SCU. VC98.132).

## Authors' contributions

Conceptualization, study design, project administration, and supervision: Mohammad Rahim Haji Hajikolaei and Mohammad Nouri; Data acquisition: Mohammad Rahim Haji Hajikolaei, Seyed Ali Mousavi Rad, and Mohammad Nouri; Data interpretation and analysis: Masoud Reza Seyfi Abad Shapouri, Masoud Ghorbanpour, and Mohammad Hassn Ebrahimi Jam; Statistical analysis: Masoud Ghorbanpour; Writing the original draft: Mohammad Rahim Haji Hajikolaei and Seyed Ali Mousavi Rad; Review and editing: Mohammad Nouri and Mohammad Hassn Ebrahimi Jam.

## Conflict of interest

The authors declared no conflict of interest.

## References

[1] Constable PD, Hinchcliff KW, Done SH, Grünberg W. Veterinary medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. Amsterdam: Elsevier Health Sciences; 2016. [Link]

[2] Al-Salihi K. Lumpy skin disease: Review of literature. Mirror of research in veterinary sciences and animals. 2014;3(3):6-23.

[3] Tuppurainen ESM, Venter EH, Shisler JL, Gari G, Mekonnen GA, Juleff N, et al. Review: Capripoxvirus Diseases: Current Status and Opportunities for Control. Transbound Emerg Dis. 2017; 64(3):729-45. [DOI:10.1111/tbed.12444] [PMID]

[4] Kashi DS. The influence of Vitamin D on in vivo immunity [PhD dissertation]. Wales: Bangor University (United Kingdom); 2019. [Link]

[5] Aoun B, Dourthe ME, Davourie Salandre A, Souberbielle JC, Ulinski T. Do vitamin D plasma levels impact vaccine response in children with idiopathic nephrotic syndrome? Pediatr Nephrol. 2012; 27(11):2161-2. [DOI:10.1007/s00467-012-2273-y] [PMID]

[6] Chirumbolo S, Bjørklund G, Sboarina A, Vella A. The role of vitamin D in the immune system as a pro-survival molecule. Clin Ther. 2017; 39(5):894-916. [DOI:10.1016/j.clinthera.2017.03.021] [PMID]

[7] Abdelgawad AA, Saoud HAEA, Mohamed AG, Fathi MA, Mokhtar ER. Evaluation of BCG Vaccine Immunogenicity in Relation to Vitamin D Status in a Group of Egyptian Children. Open J Pediatr. 2020; 10(2):320-31. [DOI:10.4236/ojped.2020.102033]

[8] Yip KH, Kolesnikoff N, Yu C, Hauschild N, Taing H, Biggs L, et al. Mechanisms of vitamin D3 metabolite repression of IgE-dependent mast cell activation. J Allergy Clin Immunol. 2014; 133(5):1356-64, 1364.e1-14. [DOI:10.1016/j.jaci.2013.11.030] [PMID]

[9] Sadarangani SP, Whitaker JA, Poland GA. "Let there be light": The role of vitamin D in the immune response to vaccines. Expert Rev Vaccines. 2015; 14(11):1427-40. [DOI:10.1586/14760584.2015.1082426] [PMID]

[10] Zimmerman RK, Lin CJ, Raviotta JM, Nowalk MP. Do vitamin D levels affect antibody titers produced in response to HPV vaccine? Hum Vaccin Immunother. 2015; 11(10):2345-9. [DOI:10.1080/21645515.2015.1062955] [PMID]

[11] Viard JP, Assuied A, Lévy Y, Souberbielle JC, Thiébaut R, Carrat F, et al. No positive association between vitamin d level and immune responses to hepatitis B and Streptococcus pneumoniae Vaccination in HIV-Infected Adults. PLoS One. 2016; 11(12):e0168640. [DOI:10.1371/journal.pone.0168640] [PMID]

[12] Calton EK, Keane KN, Raizel R, Rowlands J, Soares MJ, Newsholme P. Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells. Redox Biol. 2017; 12:814-20. [DOI:10.1016/j.redox.2017.04.009] [PMID]

[13] Clarke J. Regulation of immune system homeostasis by vitamin d signaling: implications for the central nervous system [PhD dissertation]. Quebec: McGill University; 2021. [Link]

[14] L Bishop E, Ismailova A, Dimeloe S, Hewison M, White JH. Vitamin D and immune regulation: Antibacterial, antiviral, anti-inflammatory. JBMR Plus. 2020; 5(1):e10405. [DOI:10.1002/jbm4.10405] [PMID]

[15] Pirdel L, Pirdel M. A Differential Immune Modulating Role of Vitamin D in Urinary Tract Infection. Immunol Invest. 2022; 51(3):531-45. [DOI:10.1080/08820139.2020.1845723] [PMID]

[16] Enioutina EY, Visic D, McGee ZA, Daynes RA. The induction of systemic and mucosal immune responses following the subcutaneous immunization of mature adult mice: Characterization of the antibodies in mucosal secretions of animals immunized with antigen formulations containing a vitamin D3 adjuvant. Vaccine. 1999; 17(23-24):3050-64. [DOI:10.1016/S0264-410X(99)00147-4] [PMID]

[17] Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, Ida H. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. Am J Clin Nutr. 2010; 91(5):1255-60. [DOI:10.3945/ajcn.2009.29094] [PMID]

[18] Ben-Gera J, Klement E, Khinich E, Stram Y, Shpigel NY. Comparison of the efficacy of Neethling lumpy skin disease virus and x10RM65 sheep-pox live attenuated vaccines for the prevention of lumpy skin disease-The results of a randomized controlled field study. Vaccine. 2015; 33(38):4837-42. [DOI:10.1016/j.vaccine.2015.07.071] [PMID]

[19] World Organisation for Animal Health (WOAH). Sheep pox and goat pox. Paris: WOAH terrestrial manual; 2024. [Link]

[20] Mousavi Rad SA, Haji Hajikolaei MR, Nouri M, Seyfi Abad Shapouri MR, Ghorbanpoor M. Determination of the fate of Cholecalciferol injected by the basis of 25D3 plasma concentration. Sci Rep. 2023; 13(1):4094. [DOI:10.1038/s41598-023-29870-9] [PMID]

[21] Hymøller L, Jensen SK, Lindqvist H, Johansson B, Nielsen MO, Nadeau E. Supplementing dairy steers and organically managed dairy cows with synthetic vitamin D3 is unnecessary at pasture during exposure to summer sunlight. J Dairy Res. 2009; 76(3):372-8. [DOI:10.1017/S0022029909004130] [PMID]

[22] Hymøller L, Jensen S. Vitamin D3 synthesis in the entire skin surface of dairy cows despite hair coverage. J Dairy Sci. 2010; 93(5):2025-9. [DOI:10.3168/jds.2009-2991] [PMID]

[23] Bogh MK, Schmedes AV, Philipsen PA, Thieden E, Wulf HC. A small suberythemal ultraviolet B dose every second week is sufficient to maintain summer vitamin D levels: A randomized controlled trial. Br J Dermatol. 2012; 166(2):430-3. [DOI:10.1111/j.1365-2133.2011.10697.x] [PMID]

[24] Ferguson GW, Gehrmann WH, Karsten KB, Hammack SH, McRae M, Chen TC, et al. Do panther chameleons bask to regulate endogenous vitamin D3 production? Physiol Biochem Zool. 2003; 76(1):52-9. [DOI:10.1086/374276] [PMID]

[25] Thorsen SU, Pipper CB, Johannesen J, Mortensen HB, Pociot F, Svensson J. 25-Hydroxyvitamin D, Autoantigenic and Total Antibody Concentrations: Results from a Danish Case-control Study of Newly Diagnosed Patients with Childhood Type 1 Diabetes and their Healthy Siblings. Scand J Immunol. 2018; 87(1):46-53. [DOI:10.1111/sji.12632] [PMID]

[26] Leete P, Willcox A, Krogvold L, Dahl-Jørgensen K, Foulis AK, Richardson SJ, et al. Differential insulitic profiles determine the extent of β-cell destruction and the age at onset of type 1 diabetes. Diabetes. 2016; 65(5):1362-9. [DOI:10.2337/db15-1615] [PMID]

[27] Rolf L, Muris AH, Hupperts R, Damoiseaux J. Illuminating vitamin D effects on B cells-the multiple sclerosis perspective. Immunology. 2016; 147(3):275-84. [DOI:10.1111/imm.12572] [PMID]

[28] Patel N, Penkert RR, Jones BG, Sealy RE, Surman SL, Sun Y, et al. Baseline serum vitamin A and D levels determine benefit of oral vitamin A&D supplements to humoral immune responses following pediatric influenza vaccination. Viruses. 2019; 11(10):907. [DOI:10.3390/v11100907] [PMID]

[29] Zitt E, Sprenger-Mähr H, Knoll F, Neyer U, Lhotta K. Vitamin D deficiency is associated with poor response to active hepatitis B immunisation in patients with chronic kidney disease. Vaccine. 2012; 30(5):931-5. [DOI:10.1016/j.vaccine.2011.11.086] [PMID]

[30] Vieth R. How to optimize vitamin D supplementation to prevent cancer, based on cellular adaptation and hydroxylase enzymology. Anticancer Res. 2009; 29(9):3675-84. [PMID]

[31] Stebegg M, Kumar SD, Silva-Cayetano A, Fonseca VR, Linterman MA, Graca L. Regulation of the germinal center response. Front Immunol. 2018; 9:2469. [DOI:10.3389/fimmu.2018.02469] [PMID]

[32] Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology E-book: cellular and molecular immunology E-Book. Amsterdam: Elsevier Health Sciences; 2014. [Link]

[33] Décard BF, von Ahsen N, Grunwald T, Streit F, Stroet A, Niggemeier P, et al. Low vitamin D and elevated immunoreactivity against Epstein-Barr virus before first clinical manifestation of multiple sclerosis. J Neurol Neurosurg Psychiatry. 2012; 83(12):1170-3. [DOI:10.1136/jnnp-2012-303068] [PMID]

[34] Tizard IR. Vaccines for Veterinarians E-Book.Amsterdam: Elsevier Health Sciences; 2019. [Link]

[35] Fleshner M, Watkins LR, Lockwood LL, Bellgrau D, Laudenslager ML, Maier SF. Specific changes in lymphocyte subpopulations: a potential mechanism for stress-induced immunomodulation. J Neuroimmunol. 1992; 41(2):131-42. [DOI:10.1016/0165-5728(92)90063-Q] [PMID]

[36] Casas E, Lippolis J, Kuehn L, Reinhardt T. Seasonal variation in vitamin D status of beef cattle reared in the central United States. Domest Anim Endocrinol. 2015; 52:71-4. [DOI:10.1016/j.domaniend.2015.03.003] [PMID]

[37] Krueger LA, Beitz DC, Onda K, Osman M, O'Neil MR, Lei S, et al. Effects of d-α-tocopherol and dietary energy on growth and health of preruminant dairy calves. J Dairy Sci. 2014; 97(6):3715-27. [DOI:10.3168/jds.2013-7315] [PMID]