The effect of baseline 25D3 plasma concentration on complement system activity against RM/65 vaccination

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Abstract

Introduction: This study aimed to investigate the effects of basic plasma levels of 25D3 and Cholecalciferol administered concurrently with vaccination on the complement system of calves following RM/65 vaccination.

Methods: To achieve this goal, 20 calves aged between three and four months, were selected and randomly assigned to four groups. Groups A and B were housed in a partially covered. In contrast, Groups C and D were confined to enclosed spaces. After three weeks, all groups received the vaccine. However, Groups A and C were also co-administered 11,000 IU/kg of cholecalciferol in conjunction with the vaccination. After vaccination, no significant variations in the hemolytic activity of the complement system were observed in Groups A and B, which had sufficient baseline levels of 25D3. Based on the physiological links between vitamin D, heparin, and zinc, the authors hypothesized a complex relationship between these host elements and attenuated virus pathways.

Results: The present findings indicate that if the baseline concentration of 25D3 falls outside the optimal range, the humoral component of the complement system may be impaired, even following the administration of an attenuated sheep pox virus vaccine. This impairment in the alternative pathway (AP 50) heightens susceptibility to infections, especially viral infections. Maintaining adequate levels of 25D3 during the challenge period supports tissue homeostasis, mineral balance, and immune function, which enhance the performance of the complement system and may reduce viral virulence.

Conclusion: Therefore, monitoring 25D3 levels is essential for assessing the vulnerability of the body to infection after vaccination.

Keywords: Basic plasma Vit D concentration, Cholecalciferol injection, Complement system, Sheep pox, vaccine

1. Introduction

The Poxviridae family, particularly the genus Capripox virus, is responsible for a significant disease known as lumpy skin disease (LSD). It is highly prevalent in countries across the Middle East [1]. Research has shown that members of the Poxviridae family can alter immune system parameters, including the complement system, leading to overall attenuation of the immune response [2]. Vaccination is the most critical strategy for controlling disease in endemic areas [1]. Immunity to one member of the Capri pox virus genus can confer cross-protection against other members, allowing for the effective management of lumpy skin disease (LSD) using the sheep pox attenuated vaccine or RM/65 strain [1]. One of the effects of Poxviridae on the immune system is suppression of the host's complement system [2]. The RM/65 strain is a member of the Poxviridae family [2]. Given the essential role of the complement system in overall resistance to infectious diseases [3], Therefore, investigating its effects on the host complement system is crucial. Several studies have sought to determine the effect of vitamin D on the activity of the complement system or its components [4-6]. However, to the best of our knowledge, no study has investigated the effect of basic 25D3 on complement system activity following exposure to the RM/65 strain. In a previous article, the authors discussed in detail the influence of basic 25D3 concentration on the response to cholecalciferol injection [7]. In this study, we aimed to evaluate the effects of co-administered basic 25D3 and cholecalciferol as a vaccine on the activity of the complement system in subjects exposed to the RM/65 strain.

2. Material and methods

2.1. Animal housing

A semi-industrial farm in the Qazvin region of Iran was selected to house 20 calves aged three–four months. The calves were randomly divided into four groups. Groups A and B were housed in a partially covered building, allowing the animals to move freely and access sunlight whenever desired. In contrast, Groups C and D were kept in confined spaces, where the barn walls and roof shielded them from sunlight, resulting in a dark and poorly lit environment.

2.2. Study length and injection time design

The experiment lasted for six weeks, from August 5 to September 16, 2020. Two distinct time periods were considered in this study. The first period spanned from the start of the trial to the third or 21st day of the trial. During this phase, the calves were divided into two primary groups to assess different concentrations of 25D3. On the 21st day, all four

groups (A, B, C, and D) received an injection of the sheep pox vaccine at a dosage ten times the recommended amount for sheep. Additionally, groups A and C were co-administered 11,000 IU/kg of cholecalciferol alongside the vaccine. Consequently, the second period extended from the 21st day to the conclusion of the experiment.

2.3. Plasma storage and blood sample collection

Blood samples were collected weekly for six weeks using a gel activator vacuum tube, which was aseptically filled from the jugular vein. A cool box was used to ensure that the samples remained at 4°C during the collection process. Following collection, a centrifuge was used to separate the plasma, which was subsequently stored at -20°C until analysis.

2.4. Vitamin D measurement

Starting on the first day of the experiment, the 25D3 levels were measured weekly using a commercial ELISA kit (Ideal Tashkis, Iran). This kit can accurately estimate the concentration of 25D3 in the serum or plasma within the range of 2–120 ng/mL, with a precision of 10%. To perform the assay, 25 µL of the samples, calibrators, and controls was added to separate duplicate wells. Next, 100 µL of the vitamin D-releasing reagent was added to each well, and the plate was incubated at room temperature for 30 min. After incubation, 100 µL of vitamin D HRP conjugate was added to each well and allowed to react for an additional 30 min at room temperature. The wells were then washed and 100 µL of chromogen substrate (TMB + H2O2) was added, allowing the reaction to proceed for approximately 20 min. The process was halted by adding 50 µL of the stop solution. Optical density (OD) measurements were obtained using an Accureader ELISA reader (Taiwan) at a wavelength of 450 nm. A 4-parameter logistic (4PL) program was employed to calculate vitamin D concentration based on the standards provided.

2.5. Assessment the alternative pathway of complement system

In the initial step, blood samples collected from rabbits were centrifuged to separate cellular components from the plasma. Following centrifugation, the supernatant was carefully removed and the cell pellet was washed three times with phosphate-buffered saline (PBS). Next, the diluted serum (1:15) was combined with 75 µL of a 0.1% red blood cell (RBC) suspension in each well of the plate. The plates were then incubated for 24 h at 4°C. Serum that had undergone heat inactivation of the complement system (56°C for 30 min) served as the negative control, whereas fresh

cow serum was used as the positive control. After incubation, the optical absorption of the supernatant from each well was measured using a spectrophotometer at 490 nm.

The difference between the mean optical absorption of the test serums and that of the negative controls was considered the hemolytic benchmark for activation via the alternative pathway.

3. Results

Individuals in Groups A and C received cholecalciferol injections on day 21, coinciding with the date when all groups were vaccinated against Sheep pox. To facilitate a more accurate comparison, we analyzed two distinct time periods: before and after cholecalciferol and vaccine injections, specifically from day 21 to day 42, which marks the end of the experiment. Based on the evaluation of various group outcomes, Groups B and D were presumed to be the two groups that did not receive cholecalciferol injections. After six weeks of the experiment, Groups B and D were effectively compared as treatment and control groups, allowing for a clearer assessment of the impact of cholecalciferol on the subjects.

3.1. Plasma concentrations of 25D3 vary as follows

A sufficient concentration of 25D3 was achieved in Groups A and B after three weeks of treatment ($P < 0.001$). In contrast, Groups C and D, which were not exposed to sunlight, experienced a significant decline in plasma concentrations of 25D3 over the same three-week period ($P < 0.001$). This indicated a substantial relationship between 25D3 concentration and both time and group treatment ($P < 0.001$). Moreover, the effectiveness of the various treatment groups and timeframes exhibited significant variation ($P < 0.01$). Notably, Groups B and D, which did not receive injectable vitamin D, demonstrated significantly different 25D3 concentrations throughout the six-week duration of the experiment ($P \leq 0.05$). Specifically, during the first week, Group D's concentration of 25D3 was significantly different from that observed in the subsequent weeks ($P \leq 0.05$).

3.2. Alternative hemolytic pathway assessment of complement system

As illustrated in Figure 1, the hemolytic activity of the alternative pathway of the complement system showed a significant increase in Groups A and B after three weeks ($P \leq 0.01$). Conversely, the activity of the complement system significantly decreased in the sun-deprived Groups C and D until three week of the experiment ($P \leq 0.001$), particularly

from August 5 to August 12 ($P \leq 0.001$). After three weeks, the mean hemolytic activity in Group A was measured at 34.4 U/mL, while Group B recorded a mean activity of 33.5 U/mL. The difference between the two groups was not statistically significant. Similar results were observed for Groups C and D, which had mean activities of 11.7 U/mL and 13.05 U/mL, respectively, with no significant differences between them. After August 26, variations in hemolytic activity were not significant in Groups A and B. However, significant changes were observed in Groups C and D ($P \leq 0.001$).

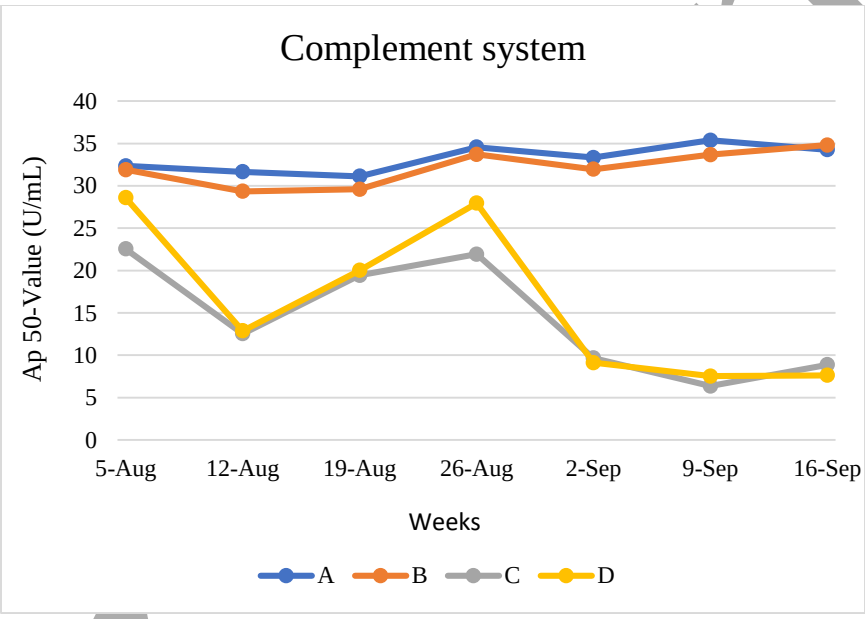

Figure.1: The Complement system AP 50 (U/mL) measurement. The variation in Ap 50 was significant in groups A-B ($P \leq 0.01$) and C-D ($P \leq 0.001$) during the first four weeks (26-Aug). after 26-Aug the variations in groups C and D were significant ($P \leq 0.001$).

4. Discussion

4.1. 25D3 variations

In a previous study [7], the authors extensively discussed the variability of 25D3 levels and how they are influenced by the underlying concentrations of basic 25D3 in the blood. However, this topic is only briefly mentioned here, demonstrating that depriving calves of vitamin D-rich meals and exposing them to natural light over a 21-day period led to a rapid and significant reduction in 25D3 concentrations. In groups C and D, the 25D3 levels fell below 10 ng/mL, which aligns with and reinforces previous findings [8,9]. Our findings from groups A and B revealed that when calves are allowed to choose freely between light and shade in an environment that offers both throughout the day, their 25D3 concentrations can increase to approximately 30 ng/mL. To our knowledge, the authors of this study are the first to report the significance of allowing calves to choose between sunlight and shade.

The 21st day of the trial, 11000 IU/kg cholecalciferol intramuscular injections were administered to groups A and C. It is important to note that groups A and B, both of which were sun-exposed groups, showed no significant 25D3 difference. Since 25D3 stopped developing after injection in group A, it served as a marker for the presence of regulatory systems. Additionally, this regulatory mechanism is observed in cows that receive sufficient supplementary cholecalciferol and sunlight exposure [10]. This mechanism likely prevents the concentration of 25D3 from increasing too rapidly According to [11], the 25-hydroxylase enzyme in the liver is likely to be inhibited, resulting in a reduction in its activity when the baseline concentration of 25D3 is sufficient. We can infer that the activation of this feedback mechanism in Group A result of adequate 25D3 production from sun exposure, which prevented the levels of 25D3 from rising significantly after cholecalciferol injection.

This idea is further supported by observations in Group C, which exhibited insufficient baseline levels of 25D3 at the time of cholecalciferol injection. Due to the low plasma 25D3 levels on the day of injection, it is possible that 25-hydroxylase remained active and continued its enzymatic function. For more details on the variations in 25D3 across different groups, please refer to a previous study conducted by the present authors [7].

The effects of basic 25D3 and cholecalciferol on the alternative pathway (AP 50) of complement system C3 convertases play crucial roles in viral immunity by initiating alternative pathways. They spontaneously cleave C3, leading to the formation of a dense layer of C3b on viral particles. This coating enhances recognition of the virus by the immune system, making it easier for phagocytes to identify and engulf the virus [2]. Consequently, the present authors assessed the alternative pathway (AP 50) as a primary method of the complement system in combating viruses.

Impairment of complement system activity can lead to a significant increase in susceptibility to infections [12]. Certain viruses, such as those in the Poxviridae family, can attenuate the complement system [12]. Such an occurrence was observed in Groups C and D, both of which exhibited insufficient baseline levels of 25D3. One week after the injection of the vaccine-attenuated strain of Sheep pox (RM/65), the alternative pathway (AP 50) in these two groups experienced a significant decline ($P \leq 0.001$). This sudden decrease is likely related to the action of viral regulators of Complement Activation (vRCA). Poxviridae RCAs specifically target C3 convertases, thereby inhibiting viral opsonization, which is essential for viral neutralization and phagocytosis. Additionally, they block the generation of C3a and C5a, which alters immune system priming and prevents the formation of the membrane attack complex (MAC), ultimately preserving viral integrity [2]. It is well established that viral complement regulators interact with glycosaminoglycans (GAGs), particularly heparin [13]. Unfortunately, there is currently no information available regarding how capripox viruses, specifically the RCA (recombinant capripox viruses), interact with glycosaminoglycans (GAGs). However, it is generally understood that the interaction between Poxviridae recombinant capripox viruses (RCAs) and glycosaminoglycans (GAGs) is influenced by the electrostatic charge of specific regions within the RCA, such as the complement control protein (CCP) compartment or the linker region between two CCPs. This interaction is primarily driven by the negative charge of GAGs, which facilitates their binding to positively charged sites within the RCA [12]. Investigations have revealed how and where vaccinia virus complement control protein (VCP) interacts with heparin. This intermittent and ionic reversible binding may enhance VCP's efficacy of VCP against the complement system, reduce its cellular toxicity, and extend its residence time within host cells [12].

An important question arises: why did the levels of AP 50 in groups A and B not significantly decrease following vaccine administration, despite the previously discussed viral mechanisms? We believe that this observation may have several underlying reasons, some of which will be discussed later. One possible explanation is the complex interplay between the basic 25D3, zinc, and heparin (GAG) concentrations. Unfortunately, the authors could not measure these important parameters; however, it seems necessary to consider this important issue, at least as a hypothesis. Evidence suggests that zinc is essential for maintaining the integrity and proper functioning of cell membranes [14], playing a critical role in the membrane antioxidant system [15] and in the formation of certain protein channels [16]. Additionally, it has been documented those infections, such as camel pox, as well as viral diseases, such as infectious bursal disease, Newcastle disease, and human immunodeficiency virus, are associated with decreased zinc

concentrations [17]. This phenomenon may occur because zinc is sequestered into cytosolic antioxidant enzymes as a protective mechanism against oxidative stress [17]. Furthermore, based on the findings in the Shams document [18], which indicate that plasma zinc levels in the hypovitaminosis D group were significantly lower than those in the control group, the authors proposed that zinc concentrations in groups A and B were likely more adequate than those in groups C and D, attributing this difference to sufficient levels of 25D3 following vaccination. Moreover, adequate concentrations of free zinc in plasma are essential for maintaining the integrity of the plasma membranes of all blood cells, including mast cells and eosinophils, which are common sources of heparin [19]. However, zinc deficiency may lead to an increase in endogenous heparin concentrations, likely resulting from the degranulation of adipocytes and subsequent release of heparin [20]. The authors of the present study hypothesized that low zinc concentrations could compromise the integrity of the plasma membranes of mast cells and adipose tissue, leading to leakage of mast cell granules and heparin from adipose tissues into the plasma in groups C and D. This may explain why the appropriate concentrations of C3 convertase and the subsequent effective function of the alternative pathway following attenuated vaccine injection were not influenced by the availability of heparin for viral RCAs in groups A and B.

It has been observed in both human and murine mast cells, 25D3 and 1,25D3 can reduce the production of pro-inflammatory and vasodilatory mediators driven by IgE-mediated activation of mast cells [21]. A similar phenomenon may have occurred in groups A and B following vaccine injection, leading to the removal of heparin from the reach of virus complement control proteins (RCAs). As mentioned earlier, C3 is a critical component of the alternative pathway, and its cleavage into its derivatives initiates this pathway [5]. Evidence suggests that the vitamin D concentration is directly related to the C3 component of the complement system [22]. Based on Pirdel et al [22], this implies that while elevated C3 levels may not directly contribute to an increase in AP 50, they may play a crucial role in sustaining the supportive function of the complement system by utilizing the alternative pathway in groups A and B. Another important aspect is the role of vitamin D in the physiological inhibition of the complement system. One of the key immunomodulatory functions of vitamin D is its ability to decrease interferon (IFN γ) levels [23], and in vitro studies have demonstrated a direct relationship between the concentration of interferon-gamma (IFN-γ) and the synthesis of factor H in fibroblasts, specifically L cells [23]. It is possible that an appropriate concentration of 25D3 led to a reduction in IFN-γ levels, resulting in decreased factor H levels in Groups A and B. This may have enhanced the efficacy of the complement system, making it less inhibited by factor H than in groups C and D.

The authors of the present study proposed that the outcome of virus or attenuated vaccine pathophysiology is influenced by host-specific physiological conditions, particularly the basal concentration of 25D3 at the time of the poxvirus challenge. As illustrated in Figure 1, the only difference between groups B and D was the baseline concentration of 25D3 at the time of injection. We anticipated a decrease in AP 50 following the injection of the attenuated strain of sheep pox (RM/65) in group D. However, this effect was not significant in the calves from group B. In fact, 25D3 appears to mitigate the impact of the attenuated virus on the host complement system by sustaining an appropriate immune and physiological status. Another notable observation pertains to groups D and C. Both groups experienced a significant decrease in vitamin D concentration, which coincided with a decline in AP 50 during the third week of the experiment. This decrease was particularly pronounced in the first week. Despite the continuing drop in 25D3 concentration after seven days, the hemolytic activity of the complement system began to increase, with a significant increase observed in group D. This increase likely occurred independently of the 25D3 levels. This phenomenon can be explored by considering the following three key points. The first is the physiological aspect of complement component synthesis, which is produced by various organs and cells, including immune cells [24]. The second point concerns the 1,25D3. It has been established that the active form of vitamin D exerts a significant influence on the synthesis of complement system components [22]. The third and final point refers to a recent observation involving free 1,25D3 concentration in a woman with a complete genetic absence of Vitamin D Binding protein (DBP). In this case, the concentration of 25D3 was exceedingly low; however, no clinical manifestations related to bone health or other factors were observed. Remarkably, free 1,25D3 concentrations in various tissues remained within the normal range. Similar phenomena have also been observed in mice lacking DBP [25]. In the present study, although the concentration of 25D3 continued to decline, the hemolytic activity of the complement system (measured by AP 50) began to increase after the second week. This observation suggests that the active form of 1,25D3 may have started to rise independently of the parathyroid hormone (PTH) axis, potentially via autocrine or paracrine signaling pathways in the cells involved in the synthesis of complement system components. According to (Bouillon et al., 2020) [25], 1,25D3 is likely restored to normal levels in organs and tissues that interact with various components of the complement system. This restoration may explain the observed increase in AP 50 days after treatment.

As mentioned earlier, in groups C and D, AP 50 experienced a severe and sudden decline following the introduction of the attenuated virus into the body. This observation likely relates to the influence of 1,25D3 on bodily and immune

system homeostasis, as previously discussed in response to the question, “Why did AP 50 in groups A and B not decrease after vaccine injection?” It is likely that, in groups C and D, there was an insufficient precursor of 1,25D3 to achieve the necessary concentration for maintaining immune homeostasis at the time of challenge. Moreover, it is important to note that the compensatory mechanism for converting 25D3 to 1,25D3, relying on the paracrine and intracrine pathways, is generally slower than the PTH-dependent mechanism for increasing 1,25D3 levels [7]. Thus, it can be inferred that the physiological lag time required to convert 25D3 to 1,25D3 was longer than the time frame required to observe the effects of 1,25D3 on the complement system immediately following the challenge. As noted in the Methods section, we administered 11,000 IU/kg of cholecalciferol to groups A and C on the 21st day of the experiment, three weeks into the study. No significant differences were observed when comparing these groups with their counterparts B and D. This finding suggests that cholecalciferol injections at this intermediate dose are not an effective strategy for maintaining the complement system activity during the challenge phase. The physiological lag time required to convert cholecalciferol to either 25D3 or 1,25D3 is likely a critical factor contributing to the ineffectiveness of cholecalciferol injections in this context [26]. In the present study, Group C served as the most illustrative example of this physiological lag time. It is important to emphasize that in Group C, the 25D3 concentration did not exhibit a significant increase immediately following cholecalciferol injection; instead, it took approximately two weeks for a noticeable rise to occur. This delayed response can be explained by Hollis et al. ’s (1977) theory, which suggests that [you may continue with the specific explanation or implications of Hollis et al.'s theory. Hollis et al. (1977) administered a double dose of injectable cholecalciferol (22,000 IU/kg) to adult dairy cows and observed that there was no significant increase in 25D3 concentration during the first week following the injection. They proposed that a physiological response initiates a reduction in the activity of P450 (25-hydroxylase) enzymes when a high dose of cholecalciferol is introduced into the body. This feedback mechanism likely contributed to the minimal increase in plasma 25D3 levels observed during the initial week post-injection in Group C in the present study. In addition, Krueger et al. (2014) [27], support the idea that the physiological time required for cholecalciferol conversion is dose-dependent. In their study, they administered 800 IU/kg cholecalciferol to newborn calves, and unlike the findings of Hollis et al [28] and those of the current investigation, they observed a significant doubling of 25D3 levels within the first week following the injection. Notably, the amount of cholecalciferol administered in the Krueger study was comparable to that used in the Hollis study, highlighting the variability in response based on dosage [28]. Thus, it can be inferred that an intermediate dose of cholecalciferol triggers a physiological negative feedback mechanism

that reduces the activity of 25-hydroxylase. This reduction in enzymatic activity may result in the precursor 1,25D (25D3) becoming less accessible to 1α-hydroxylase. Consequently, this disruption in the metabolic pathway prolongs the time required to achieve stable concentrations of 1,25D in tissues, ultimately impacting its effectiveness in the complement system during the challenge phase.

The authors of the present study assumed the ineffectiveness of cholecalciferol injection on the complement system activity in group A is the result of a different pathophysiological pathway compared with group C. This is probably due to the regulatory mechanism that depends on a sufficient concentration of baseline 25D3 in group A. Consequently, injected cholecalciferol did not increase 25D3 levels [9]. It is probable that a sufficient concentration of 25D3 in the plasma causes negative regulatory feedback on the 25-hydroxylase enzyme, preventing the saturation of 25D3 synthesis [7]. This feedback ensures that 25D3 concentrations remain within the optimal physiological range, without negatively affecting the active form of vitamin D (1.25D3). Consequently, in this group, the sufficiency of 25D3 inhibited 25-hydroxylase activity, maintaining the 25D3 concentration. This event likely inhibits the effect of 1.25D3 on the complement system function.

5. Conclusion

Monitoring the 25D3 concentration is crucial for predicting the body's susceptibility to infections post-vaccination. We confirmed that if the baseline concentration of 25D3 was not within a suitable range, even after introducing the attenuated sheep pox virus as a vaccine, the humoral part of the complement system would become dysfunctional. This dysfunction in AP 50 increases the susceptibility to infections, particularly viral infections. A suitable concentration of 25D3 during the challenge period ensures hemostasis of tissues, minerals, and the immune system, resulting in effective complement functionality and/or reduced viral virulence.

Ethics approval

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.

Conflict of interest

The authors declare that there is no conflict of interest.

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Data Availability

The data sets generated during the present study are available from the corresponding author upon reasonable request.

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