Archives of Razi Institute, Vol. 76, No. 5 (2021) 1509-1517

DOI: 10.22092/ari.2020.352209.1552

Original Article

Effects of Parenteral Vitamin D3 Supplementation on Hematological Parameters of Healthy Holstein Bulls

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Received 13 October 2020; Accepted 22 November 2020
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Abstract

Vitamin D has been shown to play physiological functions beyond calcium and phosphorus homeostasis and control bone metabolism in the body since its cellular receptors are present in numerous tissues. A total of 20 healthy bulls were divided into four groups to evaluate the effect of different doses of vitamin D3 on the number of bovine blood cells. Groups A, B, C, and D received 11,000, 22,000, 33,000, and 44,000 units/kg of vitamin D3, respectively. The control group was injected with 10 ml of physiological saline intramuscularly. Blood samples were taken before the injection, as well as 2, 4, and 6 days after the injection; furthermore, the white blood cell counts (including granulocytes and lymphocytes), hematocrit, haemoglobin, and platelets were examined by a cell counter. The results showed that vitamin D could cause leukopenia (e.g., neutropenia and lymphopenia), thrombocytopenia, as well as an increase in hematocrit and hemoglobin levels in the blood. Although the mentioned increase or decrease is largely dose- and time-dependent, the first and best group to indicate this is group B. To find the second group, the investigation of the long-term effects of injections, especially in high doses, and evaluation of different tests are required with larger groups over a longer period.

Keywords: Vitamin D3, White blood cell, Hematocrit, Hemoglobin, Platelet, Cattle

1. Introduction

The function of vitamin D is classically known to be a factor in regulating calcium metabolism and bone health. However, different physiological roles of vitamin D have been shown in recent years, and it has been observed that in different cell types, genes related to vitamin D receptors are expressed (1). Vitamin D is a steroid-like hormone that works by binding to its receptors. The calcium function of vitamin D is related to calcium metabolism; however, its non-calcium function is related to the role of vitamin D in diseases, such as hypertension, diabetes mellitus, cardiovascular disease, cancer, and autoimmune diseases. These roles are very broad because vitamin D plays a role in the widespread expression of many genes in cells. The role of vitamin D in regulating the function of immune cells, as well as its role in the proliferation, differentiation, and differentiation of hematopoietic cells, is one of those non-calcium roles of vitamin D. A good example is that vitamin D plays a role in the treatment of blood malignancies (2).

Vitamin D is involved in the regulation and proliferation of many cells in the body, such as the normal growth of breast cells and liver cells, as well as the maturation of pneumocytes (2). As mentioned earlier, the immunomodulatory function of vitamin D has been demonstrated in numerous studies (3). This role, which affects the function of the immune system,

can be due to the effect of vitamin D on innate immunity. Macrophages and monocytes play a key role in initiating nonspecific responses to pathogens or tissue damage, and vitamin D can differentiate monocyte precursors into adult macrophages (4). Since vitamin D is a potent regulator of monocyte activity and following a tissue transplant, inflammatory responses can themselves cause severe tissue damage, vitamin D can reduce the severe tissue damage associated with inflammation (5). In addition, vitamin D inhibits the maturation of monocyte dendritic cells derived from monocytes and suppresses their ability to present antigens to T cells (6). Increased expression of vitamin D receptors also occurs with activation of T cells. It is possible that vitamin D exerts its effects on inflammation and autoimmune diseases through its regulatory effect on T helper 17 (Th17) cell and ultimately affects regulatory T cells (7). As can be observed in T cells, activated B cells also increase the expression of Vitamin D Receptor (VDR) genes; accordingly, the activated B cells can metabolize vitamin D and respond to vitamin D (8).

Other studies show that vitamin D is also effective in erythropoiesis because VDR is expressed in the bone marrow by certain cellular subtypes, such as stromal and accessory cells (9). Elevated haemoglobin (HGB) and hematocrit (HCT) levels are observed following vitamin D treatment (10). Although many studies have examined the relationship between serum vitamin D concentrations and inflammation, there are not many studies that have examined the relationship between serum vitamin D concentrations and leukocyte populations in humans or animals. In a study conducted on smokers, an inverse relationship was revealed between vitamin D concentration and white blood cell count (WBC) (11). Another study by Aronson found a negative correlation between vitamin D content and WBC counts, and vitamin D was also negatively correlated with HGB (12). Another study on cats in a veterinary hospital (Hospital for Small Animals, Royal Dick School of Veterinary Studies) found that cats with neutrophilia had lower levels of vitamin D than cats with lower neutrophils in the normal range (1).

Furthermore, no study has been performed on the relationship of vitamin D with the count and indices of blood cells in cattle. The present study aimed to investigate the effect of different doses of vitamin D on changes in WBC (lymphocytes and granulocytes), HCT, HGB, and platelets (PLT) in cattle, along with its relationship with these variables.

As in previous studies, it has been concluded that the expression of genes affected by vitamin D is dose- and time-dependent (13). This study aimed at understanding the changes in the count of blood cells, following the changes in the dose of vitamin D supplements. Moreover, it attempted to investigate whether the changes were dose-or time-dependent.

Although the results of the effect of sex hormones on the function and number of blood cells are different, the results of studies based on a meta-analysis showed that testosterone has a moderate immune-suppressant effect on immune function. Testosterone suppresses immune function, and the effect of estrogen varies depending on the measurement of immunity used. On the other hand, the effect of estrogen depends on the level of immunity used. Estrogen suppresses cell-mediated immune function while reducing the parasitic load. These results suggest that these correlation studies have a limited value for studying the effects of sex hormones on immune function. Finally, it can be concluded that the use of bulls due to less stress than cows (including milking and pregnancy) and the possibility of homogeneity of experimental groups, is a good model for generalizing the results to Bovine (14).

2. Materials and Methods

A total of 20 Holstein bulls (15) weighing approximately 300 kg were randomly divided into five groups. The health of all cows was ensured, and the bulls were fed with a TMR diet consisting of 1 kg of hay, 1 kg of wheat straw, 6 kg of corn silage, and 4.5 kg of grain concentrate (per head). Group A was the

group that received 3,300,000 units of vitamin D3 injected intramuscularly (with the same therapeutic dose for cows) (16). In group B, 6,600,000 units (twice the therapeutic dose), in group C, 9,900,000 units (three times the therapeutic dose), and in group D, 132,000,000 units (four times the therapeutic dose) were injected intramuscularly into cows. The other group is the control group and received 10 ml of physiological saline by intramuscular injection. Blood samples were taken intravenously, just before the vitamin D and physiological seruminjection, as well as 2,4,and 6days after the injection. This study was conducted by the Faculty of Veterinary Medicine, Semnan University, Semnan, Iran, at Deimeh Farm (50 km from Garmsar-Semnan road) in the fall, 2018.

Whole blood samples were taken immediately using the Kinhoden Model Cell Counter (Made in Japan: The device is made for human and animal purposes and can be used for different animal species by setting up the device), and the values of WBC, HGB, HCT, PLT, lymphocytes, and neutrophils were measured. These tests were performed for all groups after each blood sampling. To assess the normal distribution of data, the *Shapiro-Wilk* test was used, and most parts of the data were non-parametric. Moreover, due to the dearth of data in each group, non-parametric analysis of data was considered. Friedman test was also employed to compare the differences between measured values over time. In order to make a comparison between different groups at each time point, the Kruskal-Wallis test was implemented. Statistical analysis of the samples was performed in SPSS software (version 20).

3. Results

There was no statistically significant difference between groups A and C in the WBC counts at different times ($P>0.05$); however, there was a significant difference between groups D and B ($P<0.05$). According to the results of the WBC count shown in figure 1, the trend of decreasing the amount of WBC from day 0 (exactly the moment before the injection of vitamin D) to the sixth day after the injection is quite evident, and this change is observed in all groups. However, this decrease was the lowest in group A and the highest in group D.

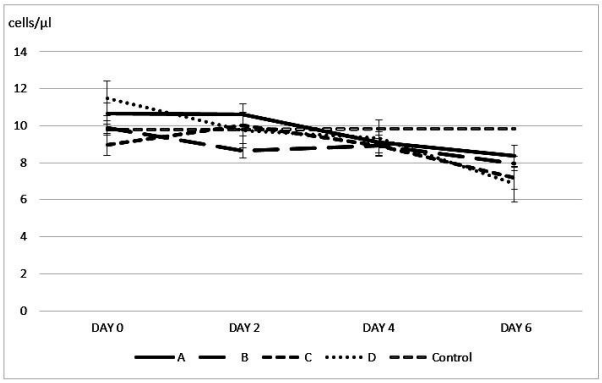

Figure 1. White blood cell counts on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

There was a statistically significant difference among groups A, B, and D regarding HCT values at different times. On the other hand, no significant difference was observed in group C. As can be noted from the results for HCT (Figure 2), the trend of increasing HCT values from day 0 to day 6 after injection is evident, and this change is observed in all groups; however, this increase was the lowest in group A and the highest in group D.

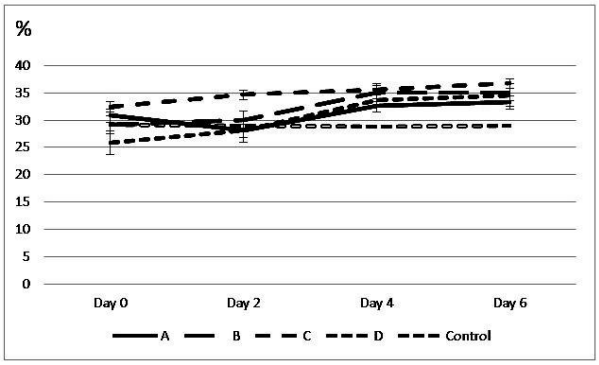

Figure 2. Hematocrit percents on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

Although a significant difference was detected in terms of the mean corpuscular volume (*MCV*) and

mean corpuscular hemoglobin (*MCH*) values in just group B over time, *MCV*, *MCH*, and mean corpuscular hemoglobin concentration (*MCHC*) values showed no significant differences over time in all groups (Table 1).

Table 1. Red blood cell indices, including mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin (MCH) (pg), and mean corpuscular hemoglobin concentration (MCHC) (g/dL) in different groups during days 0 to 6

	MCV0	MCV2	MCV4	MCV6	MCH0	MCH2	MCH4	MCH6	MCHC0	MCHC2	MCHC4	MCHC6
A	34.12	32.40	33.20	33.20	10.32	10.75	9.87	12.57	28.87	29.90	29.97	30.07
B	31.25	31.92	35.10	37.30	9.70	11.90	11.80	11.42	30.20	29.35	30.60	31.40
C	33.52	35.52	37.07	39.02	10.27	12.80	12.02	12.40	28.47	30.10	30.52	31.42
D	30.62	30.95	35.00	36.12	9.37	9.55	12.40	11.7	28.05	27.95	29.92	30.35
Control	30.40	33.20	29.10	35.40	10.68	12.10	10.50	10.35	29.85	30.05	29.90	28.70

There was a statistically significant difference in all groups regarding the levels of HGB at different times ($P<0.05$).The HGB levels increased in different groups with a slight slope during the days after vitamin D administration; however, this upward trend peaks on the sixth day. The growth rate of HGB during these six days is lower in group A, compared to that in other groups. In contrast, it is higher in group D, compared to that in other groups (figure 3).

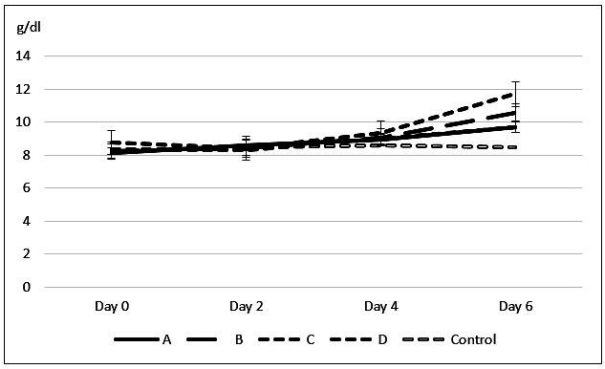

Figure 3. Hemoglobin values on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

There was a significant difference among groups A, B, and C in terms of PLT values at different times; however, no significant difference was found in group D. The measurement of PLTs during six days after vitamin D administration is shown in figure 4. As can be observed, the values decrease in all groups, and on the sixth day, they are less than those on day 0 in the same group. Nonetheless, the severity of the decrease in group D is not higher than that in other groups. Therefore, the changes in PLT count in group C show the greatest decrease, and group B stands in the next rank. In groups A and D, the PLT count decreases until the sixth day, and this decrease in groups A and D goes through a similar process. Regarding platelet distribution width, no significant differences were detected among all groups from day 0 to day 6 (Table 2).

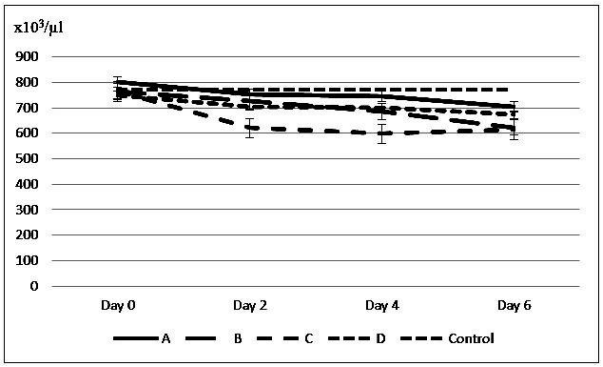

Figure 4. Platelet levels on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

There was a significant difference between groups A and C regarding the counts of lymphocytes at different times; however, no significant difference was found in groups D and B. There was a significant difference in all groups in terms of the counts of granulocytes at different times. As mentioned in the case of decreasing

changes in WBC values, there is a similar pattern in the number of lymphocytes and granulocytes. As shown in figures 5 and 6, the lymphocyte and granulocyte counts decrease over 6 days after the injection of vitamin D, and just similar to the WBC graph, the reduction is the lowest and highest in groups A and D, respectively.

Table 2. Platelet distribution width (PDW) (%) and platelet (PLT) levels (10^9/L) on day 0 and days 2, 4, and 6 after vitamin D administration in groups A, B, C, D, and control

Group	PLT0	PDW0	PLT2	PDW2	PLT4	PDW4	PLT6	PDW6
A	802.5	17.70	735.75	14.70	745.00	14.17	702.5	14.05
B	765.5	14.20	725.00	14.20	685.00	14.10	622.5	13.85
C	763.75	13.92	620.00	13.92	597.50	13.95	615.00	13.87
D	748.75	14.27	705.00	14.27	700.00	13.85	672.5	14.02
Control	770.85	13.80	770.55	12.80	770.85	13.25	770.75	13.90

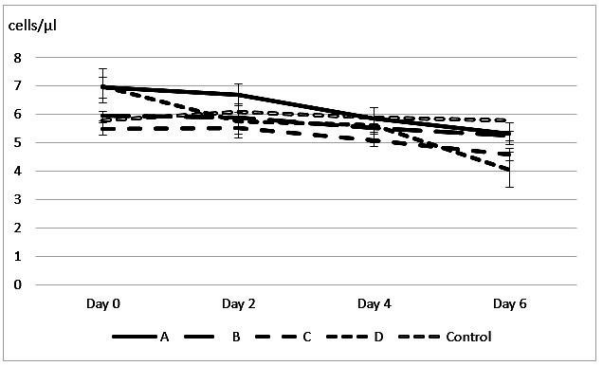

Figure 5. Lymphocyte counts on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

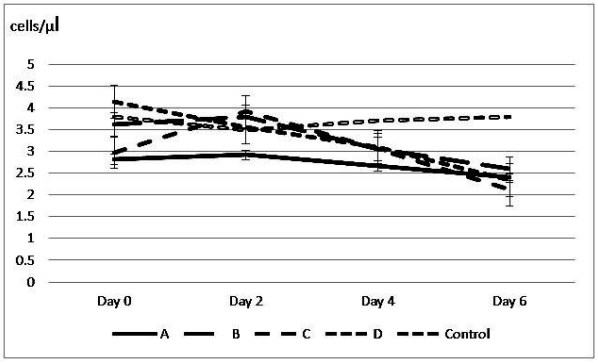

Figure 6. Granulocyte counts on day 0 and days 2, 4, and 6after vitamin D administration in groups A, B, C, D, and control

4. Discussion

The results of the present study show that vitamin D cannot reduce the WBC counts in therapeutic doses; moreover, the increasing dose of vitamin D supplement in cattle to four times the usual dose causes a sharp decrease in the WBC counts (Leucopenia). Although there are overall declining changes in WBC values in both groups A and C, these changes are not statistically significant. The WBC counts were within the normal range of Bovine species (4900-12,000 cells/µl) (16) both before and up to 6 days after vitamin D injection. As Vanham showed in 1988, vitamin D had distinct antiproliferative effects (17). These results may confirm the results of our study; however, Icardi in 2013 showed that vitamin D can regulate systemic cytokines and increase WBC (18). Despite these conflicting results with our findings, it is accepted that vitamin D plays a very important role in the immunomodulation of the immune system (3).In many cases, serum vitamin D levels can be changed following inflammation, and even vitamin D can act as a negative acute-phase protein (19); in addition, hypovitaminosis D may be a consequence of chronic inflammatory disease rather than the cause of it. This means that vitamin D supplementation can be a beneficial treatment for many inflammatory diseases and in many

inflammatory diseases, such as tissue transplant complications, a reduction in the number of WBC helps to prevent the progression of the disease (20).

Evidence suggests that hypovitaminosis D may predispose to an acute inflammatory response (21). To confirm the observations confirming the anti-proliferative effects of vitamin D, studies have been conducted on the effectiveness of vitamin D in the form of a chemotherapy drug for cancer (22), and it has been proven that vitamin D deficiency in chronic leukaemia has been associated with poor prognosis and decreased patient survival (23).

However, the results showed that vitamin D reduced the WBC. The question raised here is "Does an increase in vitamin D dosage further reduce the counts of WBC?" The answer is yes. Soliman, Eldabbagh (24) showed that the administration of a mega dose of vitamin D in children led to an increase in WBC counts; however, this increase was not statistically significant. Titmarsh hypothesized in 2016 that there might be a negative correlation between vitamin D levels and WBC counts (1).

The change in the leukocyte counts is more affected by the changes in lymphocytes and neutrophils. An increase in the expression of vitamin D receptors by activating T cells makes it possible for vitamin D to exert its effects on inflammation and autoimmune diseases through its regulatory effect on Th17 cells (25).

As can be seen in T cells, active B cells increase the expression of VDR genes. Therefore, with the activation of cells, the expression of mRNA genes related to VDR increases. Some studies suggest that VDR expression in leukocyte subsets can increase leukocyte migration from tissue to blood (26). In another study in 1984, the results showed that vitamin D had an inhibitory effect on lymphocyte proliferation (27). Regarding the effect of vitamin D on neutrophil populations, a study by Titmarsh, Cartwright (1) found that cats with neutrophils had lower serum vitamin D, compared to cats with normal neutrophil populations. A human study in 2011 showed a negative relationship between neutrophil count and vitamin D (28). Surveys in 2013 have confirmed such results (29).

The results of our study also showed that the levels of lymphocytes and neutrophils decreased (neutropenia and lymphopenia) within six days after vitamin D injection, and this decrease followed a steep slope in group D. Decreases in lymphocyte and granulocyte counts (lymphopenia) were not observed in all groups. However, in groups B and D related to lymphocytes, this decrease was not statistically significant. This negative relationship between vitamin D and granulocytes has been in line with the results of previous studies. Studies have been performed on the relationship of vitamin D with HCT and HGB, and these findings indicate that vitamin D affects erythropoiesis (9); moreover, an increase in HCT and HGB occurs after treatment with vitamin D (10).

Vitamin D can stimulate erythrocyte precursor cell receptors, which enhance the maturation and proliferation of congenital erythroid cells. Furthermore, it may increase the production of erythropoietin (18). Another study found that the administration of vitamin D to patients with inflammation could improve anaemia (30).

Marwah, Walls (31) showed a positive association of vitamin D with HGB and red blood cell (RBC) counts. On the other hand, Doudin, Becker (32) revealed that vitamin D had an inhibitory role in regulating hematopoiesis in adolescents, and there was an indirect relationship among HGB parameters, including HGB, RBC, and serum levels of vitamin D. The author attributes this indirect link to the possible role of this circulating steroid hormone (vitamin D) in the formation of RBC from homocytoblasts (32). The results of our study show that vitamin D at higher doses (four times the usual dose) can increase the concentration of HGB on days 4 to 6 after injection. At normal doses, vitamin D injections do not affect increasing or decreasing HGB levels (the changes were statistically significant in all groups). As the results of the present study show, increasing the dose of vitamin D can lead to an increase in HCT in all groups that

received vitamin D; however, this increase in group C does not show a statistically significant difference. Among these, the highest increase in HCT is observed in group D (the group that received the most vitamin D). Accordingly, it can be said that vitamin D has a direct and dose-dependent relationship with the amount of HCT and HGB.

Another blood cell the effectiveness of which has been studied in injecting vitamin D is platelet count. PLT are active cell structures that do not have a nucleus; however, they are made up of megakaryocytes in the bone marrow and play an important role in blood flow, as well as inflammation (33). Akbas, Becker (34) showed a negative relationship between vitamin D deficiency and PLT count. Similarly, Park, Kim (35) showed a negative relationship between vitamin D levels and blood PLT count. Yildirim, Solmaz (22) showed that vitamin D deficiency may play a role in increasing mean platelet volume as an indicator of PLT size. In the same vein, Kara and Soylu (36) showed a clear negative relationship between serum levels of vitamin D and PLT counts (36). According to studies, vitamin D has anti-thrombogenic, anti-inflammatory, and anticoagulant activity. Aihara, Azuma (37) showed that the vitamin D-VDR system may play an important role in anti-thrombogenic function among *in vivo* samples. The vitamin D-VDR system appears to increase the expression of anti-thrombogenic factor genes and inhibit the expression of thrombogenic genes (37).

The effect of vitamin D administration on reducing PLT count was investigated in this study. PLT counts decreased in all groups, except for the control group, from the day of injection until the sixth day after injection; however, these changes were not statistically significant in group D. According to all information extracted from the present study, it can be said that vitamin D can increase the WBC count by increasing the dose. It also reduces PLT counts. An increase in the amount of HGB and HCT also results from an increase in the dose of vitamin D.

To answer the question of how much an increase in vitamin D3 dose intensify these reductions or increases, it may be said that an increase or decrease caused by group B is more specific than that in other groups and is statistically significant, except for the lymphocyte count test. Due to the risk of poisoning at high doses of vitamin D3, if favorable changes are desired in blood cells, it may be reasonable to say that increasing the dose of vitamin D to twice the usual dose (recommended for best calcemic effects) is reasonable.

In conclusion, the results of our study show that the use of vitamin D causes certain effects on blood cells, which show their effects better with increasing dose and over time. Since it was impossible to follow the cows for blood sampling and examination in the following weeks and months, further studies over longer periods may show better and more accurate results, especially regarding the effect of high doses of vitamin D on blood cell counts. The expression of VDR-dependent genes in leukocyte subsets can increase leukocyte migration from tissue to blood (26). Erythroid progenitor cells in the bone marrow undergo a series of regulated processes related to cell proliferation and differentiation. Given the longer lifespan of RBCs, compared to WBCs (which are more susceptible to removal and isolation by the spleen) (32), it is probable that changes in RBCs due to vitamin D administration may require a longer time.

Authors' Contribution

Study concept and design: M. K.
Acquisition of data: M. A.
Analysis and interpretation of data: A. J. J.
Drafting of the manuscript: F. R. Z.
Critical revision of the manuscript for important intellectual content: M. K.
Statistical analysis: M. A.
Administrative, technical, and material support: M. K.

Ethics

All experiments were carried out according to ethical rules for the care and use of laboratory animals and were approved by the Experimental Animals Committee of Semnan University, Semnan, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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