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Association of D-Dimer-Fibrinogen Ratio, Platelet-Lymphocyte Ratio, and Vitamin D Levels with Pneumonia in Calves: Insights into Inflammation and Coagulation Mechanisms

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THE objective of the proposed study was to establish a correlation between the levels of D-dimer-fibrinogen ratio (DFR), platelet-lymphocyte ratio (PLR), and vitamin D in patients with calf pneumonia. Three groups were established for this purpose. Group I consisted of healthy controls (n=7), while Group II (n=7) and Group III (n=7) were comprised of animals with pneumonia who received different treatments. Hemogram, coagulation parameters, and vitamin D levels were measured in all three groups of animals. Additionally, PLR and DFR were calculated and subsequently analyzed. The DFR measurements on Day 0 for calves with pneumonia exhibited a notable difference compared to those of the healthy group. Meanwhile, the Day 0 vitamin D measurements in healthy calves were significantly lower from those in calves with pneumonia. However, there was no significant difference observed in the PLR levels between healthy calves and calves with pneumonia who received treatment. The study's results suggest that the levels of vitamin D have an effect on the inflammatory process and coagulation parameters in calves with pneumonia.

Keywords: Calf, D-dimer, Fibrinogen, Pneumonia, Platelet-lymphocyte ratio, Vitamin D

Introduction

Bovine respiratory disease complex (BRDC) is a significant issue in cattle husbandry, characterized by high morbidity, mortality, and substantial economic costs [1]. Respiratory diseases, particularly in calves older than 2 mounts of age, are the primary cause of mortality [2]. Various factors, including environmental stressors, can cause commensal bacteria to transform into opportunistic pathogens, leading to secondary respiratory infections [3]. Pneumonia can be caused by a range of factors, such as viruses (Respiratory Syncytial Virus, Bovine Viral Diarrhea, Infectious Bovine Rhinotracheitis), bacteria (*Pasteurella multocida, Mannheimia haemolytica*), insufficient intake of colostrum, malnutrition, poor hygiene, inadequate ventilation, and stress. The clinical symptoms of pneumonia include fever, dyspnea, nasal discharge, depression and anorexia, and in severe cases, even death [4]. Gastrointestinal and respiratory infections play a crucial role in the development of sepsis, particularly in neonatal and weaning calves. Sepsis is a systemic inflammatory response triggered by infections that can cause septic shock and multiple organ failure syndrome [5]. Patients with sepsis experience imbalances in coagulation and fibrinolysis as part of their body's defense mechanisms in response to the development of organ dysfunction. Sepsis-induced coagulopathy (SIC) and disseminated intravascular coagulopathy (DIC) are primarily caused by coagulation, platelet activation, inflammation of cells such as neutrophils and lymphocytes, and damage to vascular endothelial cells [6].

Vitamin D, a steroid hormone that occurs naturally in two forms, vitamin D2 and D3,

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regulates numerous biological functions, including skeletal health and calcium-phosphorus homeostasis. Vitamin D metabolites are involved in regulating various prothrombotic and antithrombotic factors in the coagulation cascade, which can be beneficial as an anticoagulant [7]. Several studies have demonstrated a correlation between low levels of 25(OH)D and idiopathic deep vein thrombosis [8]. Furthermore, vitamin D levels have been suggested as a predictive factor for deep venous thromboembolism in patients with ischemia [9]. Platelets play a vital role not only in hemostasis but also in regulating the body's antimicrobial host defense system, and they contribute significantly to the process of inflammation and tissue repair through their ability to induce inflammation, as well as other mechanisms [10].The platelet-to-lymphocyte ratio (PLR), which is calculated by dividing the absolute number of platelets by the absolute number of lymphocytes, has been suggested as a useful biomarker to determine inflammatory status, provide a differential diagnosis, and assist in prognostic evaluation in systemic inflammatory response, similar to the neutrophil-to-lymphocyte ratio (NLR) [11].

In various diseases with pulmonary thromboembolism, elevated levels of D-dimer are commonly observed. The role of D-dimer in causing vascular complications is still debated; however, fibrinogen is considered an acute-phase reactant and an inflammation marker. In a study conducted by Kucher et al. [12], lower levels of fibrinogen were observed in patients with acute pulmonary embolism [12]. Furthermore, as the level of pulmonary occlusion increased, fibrinogen levels decreased. These results suggest that the D-dimer-fibrinogen ratio could be a highly specific biomarker for pulmonary embolism.

The primary objective of this research was to examine deeper into the possible interrelationships among the D-dimer to fibrinogen ratio, the platelet to lymphocyte ratio, and the concentration levels of Vitamin D, within the context of bovine pneumonia. We intended to explore these parameters in calves diagnosed with pneumonia, aiming to identify potential associations or patterns that may improve our understanding of the disease's underlying pathophysiology.

Material and Methods

Ethical approve

This study has been approved by the Local Animal Experiments Ethics Committee of Aydın Adnan Menderes University under the number 2020/049, Aydın, Türkiye.

Animal material

The animal material utilized in this study was sourced from patients who presented to the Aydın Adnan Menderes University Faculty of Veterinary Medicine, Department of Internal Medicine for diagnosis and treatment, as well as from calves residing in nearby dairy cattle farms.

Design of groups

Both male and female Holstein breed calves at the weaning age (45-70 days) were included in the study. The animals were divided into two main groups: healthy and sick with pneumonia. The healthy group (Group I) comprised seven calves that were selected based on their age, breed, and sex distributions, which were similar to those of the sick group. Their health was assessed by reviewing their medical history, clinical findings, and laboratory data. The group with pneumonia (n=14) was divided into two subgroups (Group II and III) based on differences in clinical practices. The sick calves in Group II (n=7) and Group III (n=7) were randomly assigned to treatment protocols. Figure 1 provides a visual representation of the calf grouping procedures used in the study.

Clinical procedure

Calves were assessed based on heart rate, respiratory rate, body temperature, ocular discharge, nasal discharge, and ear score to determine the severity of pneumonia and the degree of impact [13]. Briefly as characterized in aforementioned study [13], clinical signs including rectal temperature, coughing, nasal discharge and eye or ear scores were ranged from 0 to 3 as clinical signs progress from normal (0), to mildly abnormal (1), moderately abnormal (2), and severely abnormal (3). Calves with a total respiratory score≥5 or that have two or more clinical signs with score 2 or 3 are noted to have pneumonia.

Calves in Groups II and III, which were selected and diagnosed with pneumonia based on clinical and laboratory findings, were administered with tilmicosin (Rosin, Sanovel, Türkiye) at a dosage of 10 mg/kg and meloxicam (Maxicam, Sanovel, Türkiye) at a dosage of 0.5 mg/kg subcutaneously. The calves were monitored on days 0 and 3 after the administration of antibiotics and non-steroidal anti-inflammatory drugs, which were given

twice at 48-hour intervals. Calves in Group II with pneumonia were treated with subcutaneous injection of tilmicosin at a dosage of 10 mg/kg and intramuscular injection of Vitamin D3 (D vit ampoule, Deva, Türkiye) at a dosage of 300,000 IU, both given once at 48-hour intervals. Calves in Group III with pneumonia were treated with subcutaneous injection of tilmicosin at a dosage of 10 mg/kg, subcutaneous injection of meloxicam at a dosage of 0.5 mg/kg, and intramuscular injection of Vitamin D3 at a dosage of 300.000 IU, all given once at 48-hour intervals. Sampling procedures were conducted during the monitoring on days 0 and 3.

The blood samples were collected from both the healthy control group and the pneumonia group by venipuncture of the *Vena jugularis*, using tubes containing both lithium heparin and citrate. The healthy group had their blood drawn only once, while the calves in the pneumonia group had their blood drawn twice, on day 0 and day 3 prior to any interventions being administered.

Blood samples taken in citrated tubes were centrifuged (Hettich, Germany) at 3000 rpm for 15 min after fibrinogen and D-dimer measurements were performed immediately after blood collection.

Plasma samples obtained from citrated blood samples were used to measure fibrinogen and D-dimer levels using a coagulometer (Healvet, China). These measurements were performed using commercial test kits as described by the manufacturer. A brief description of the test procedures for D-dimer and fibrinogen is provided below. For the D-dimer analysis, the analyzer was switched on and the ID Chip Numbers suitable for D-Dimer test kits were matched with the device. Then, 15 mL of serum sample were taken using an automatic pipette and mixed with buffer solution to minimize vigorous mixing and foam formation. Thereafter, 75 mL of the resulting mixture were carefully dropped into the test kit without foaming, and the results were analyzed using an automatic analyzer. For the fibrinogen analysis, the coagulation analyzer was switched on and the ID Chip numbers compatible with Fibrinogen test kits were matched with the device. Once the device automatically reached the required temperature for measurement, 20 mL of whole blood sample were carefully dropped into the test kit, and the results were recorded.

The levels of vitamin D (25-OH-D3) in plasma samples obtained from lithium heparin blood samples were measured using an immunochromatographic test device (Savant-100, China). For these measurements, commercial test kits (Savant 25-OH-D_3 test kit, Beijing China) that were specifically produced for the device by the manufacturer were used. Briefly, for the vitamin D analysis, the device was first switched on then, for calibrating the device the ID Chip numbers that were defined for each test box were matched with the device. In an Eppendorf tube, 30 µL of plasma sample and 30 µL of diluent liquid were mixed, and the resulting 60 µl of mixture was allowed to stand at room temperature for 15 minutes. Afterward, the mixture was carefully dropped into the test kit, and immunochromatographic measurements were performed.

Following coagulation measurements, D-dimer and fibrinogen results were obtained, and the D-dimer results, expressed in ng/mL, were converted to g/mL. Subsequently, D-Dimer-Fibrinogen Ratio (DFR) results were calculated by dividing the D-dimer count by the fibrinogen count in g/mL. In addition, platelet counts, which were obtained using the same method, were divided by the total lymphocyte count to obtain the Platelet-Lymphocyte Ratio (PLR). These ratios were calculated for each calves at both Day 0 and Day 3.

Statistical analyses

The collected data was subjected to transformation procedures after assessing the normality of the distributions. The normal distribution of the data of the groups in treatment days were evaluated using visual methods (histogram graph and Q-Q graph) and the Shapiro-Wilk test. As the distributions were found to be non-normal, non-parametric tests such as the Mann-Whitney U test for group comparisons and Wilcoxon test for within-group comparisons were employed in the statistical analysis. The SPSS 24.0 (IBM, USA) program was utilized for statistical analysis and a p-value less than 0.05 was considered statistically significant.

Results

Clinical findings

The evaluation helped to discern the calves most affected by the disease. The control group had an average score of 2, while the sick groups, which received different treatments, had an average score of 5.5. Although no statistically significant differences were observed in the

mean scores of the sick groups, their scores were significantly higher ($p<0.05$) than those of the control group.

Vitamin D, coagulation and inflammation markers findings

Changes in vitamin D, DFR and PLR on the sampling days of calves distributed to pneumonia and healthy control groups are presented in Table 1.

Significant differences (P = 0.05) were observed in the Vitamin D levels of the healthy control group, group II, and group III during the day 0 measurements of the randomly assigned calf groups. The Vitamin D levels in Group II and Group III were 19.67±1.32 ng/mL and 19.78±0.82 ng/mL, respectively, three days after Vitamin D administration. Moreover, a statistically significant difference (P = 0.05) was observed in the Vitamin D measurements between day 0 and day 3 for both Group II and Group III, as depicted in Figure 2.

In the healthy control group, the DFR measurements on Day 0 were found to be 2.71±0.34, whereas in Group II, the DFR values were 1.67±0.16. The DFR values of Groups II and III, were significantly lower (P = 0.029) than those of healthy calves. On the 3rd day after the treatment, the DFR values of Group II and Group III were 2.07±0.47 and 2.51±1.07, respectively (P = 0.029). Although a decrease was observed in the values of Group III compared to the Day 0 data, the result was not statistically significant (P = >0.05) (Figure 3).

At day 0, the PLR were 128.43±21.55 in the Group I, 133.05±18.39 in Group II, and 206.12±30.61 in Group III. There was no significant difference found between the PLR values of the healthy group and Group II and III. On Day 3, the PLR values of Group II and Group III were 150.44±4.37 and 173.92±24.17, respectively. While there was a significant decrease noted in Group III, no significant difference was found between the Day 0 and Day 3 PLR measurements (Figure 4).

Discussion

Pneumonia has been observed to affect a significant proportion, up to 80-90%, of the calves on cattle farms, leading to an outbreak. Despite this, the mortality rate associated with these infections is generally around 5% [14,15]. These outbreaks are typically observed over a period of 2-5 weeks, involving various etiological agents and predisposing factors, particularly during the calving period [16-18]. Low levels of mucosal immunity in the nasal secretions of 2–4-year-old calves, specifically of IgG1, IgG2, and IgA, increase the risk of morbidity from pneumonia, in addition to other predisposing factors [19,20]. In our study, we observed that the age ranges of the calves evaluated were consistent with those described in the literature, and pandemic-level outbreaks were present in almost every farm from which the calves originated. Similar to the SARS-CoV-2 outbreak, previous study have indicated that calves with pneumonia exhibit inflammatory changes in their white blood cell counts and cytokine levels, as well as alterations in their chemokines and antimicrobial peptide levels [21]. In cases of pneumonia, the presence and effect of pathogens are known to regulate the excretion of many inflammatory mediators [22]. Additionally, vitamin D levels have been identified as a factor that affects immunity, with the number of vitamin D receptors on Th1 and Th17 cells playing a role in this process [23,24]. Vitamin D levels are low in calves with diarrhea symptoms due to different etiological agents, indicating that vitamin D may be involved in regulating the immune response in calves [25,26].

In our study, plasma vitamin D levels were lower in sick calves with clinical signs of pneumonia compared to healthy calves. This finding is consistent with previous studies that have shown that vitamin D levels are decreased in many infectious diseases [25,26]. Furthermore, the preventive effects of vitamin D administration before infection have been demonstrated in a calf model experimentally induced with pneumonia [4]. In children with pneumonia between the ages of 6 months and 5 years have shown that a single oral administration of 100,000 IU of vitamin D reduces clinical findings. However, this treatment has no effect on the recurrence of pneumonia or on the duration of hospitalization of patients [27]. In adult humans, low levels of vitamin D have been linked to increased mortality in those infected with COVID-19 [28,29]. Vitamin D supplementation, in addition to conventional treatments, may have a positive effect on the survival of COVID-19 patients [30].

Our investigation revealed that the administration of vitamin D, in conjunction with standard therapeutic interventions, resulted in a noteworthy elevation of the circulating levels of

vitamin D in individuals displaying symptomatic evidence of pneumonia. When evaluating the clinical changes, we observed that the calves in the vitamin D and antibiotic group recovered more slowly than the vitamin D + antibiotic + NSAID (Nonsteroidal anti-inflamatory drug) group. The vitamin D levels of the calves in the healthy group were also found to be lower than those in the treatment groups. Although the calves in this study were of similar age, the inability to control for the varying vitamin D levels in their diets, as well as the fact that they were raised in different farms, limited the scope of the investigation in these aspects. Nevertheless, the importance of vitamin D in pneumonia patients, which has become increasingly prominent since the onset of COVID-19, cannot be overstated, as infections have been linked to thromboembolic processes involving platelet activation, aggregation, and fibrinolysis due to coagulopathy and vitamin D deficiency feedback mechanisms. In cases of vitamin D deficiency, platelet activation and aggregation are impaired while fibrinolysis and thrombosis are increased. Furthermore, vitamin D plays a crucial role in inflammation, endothelial dysfunction, and immune response [31]. NLR and PLR are simple and cost-effective biomarkers used to evaluate inflammation in patients. Additionally, biomarkers such as PLR can be used to provide prognostic information for various malignancies, cardiovascular diseases, and renal patients [32-34]. In a study examining the correlation between vitamin D and PLR levels, PLR measurements were found to be an independent biomarker of vitamin D levels, along with parathyroid hormone, calcium, sex, and creatinine [35]. Numerous studies have shown a negative correlation between vitamin D and inflammatory biomarkers in various inflammatory conditions [36]. In our study, we examined calves with pneumonia in terms of the PLR and found statistical changes in the direction of increased vitamin D levels after application compared to healthy animals, but similar increases in PLR levels were not statistically significant. We suspect that differences in the changes were due to the calves not being selected from a sample area, and the etiological factor in the occurrence of pneumonia could not be revealed. Furthermore, we found that the higher PLR levels in Group 3 calves compared to Group I and Group II were likely due to the anti-aggregant effect of the non-steroidal anti-inflammatory agent used. Previous studies have reported that the activation of Vitamin D receptors inhibits platelet aggregation, leading to increased susceptibility to thrombosis formation in atherosclerotic vascular diseases [37]. Moreover, changes in toll-like receptor levels due to vitamin D administration can help control the inflammatory state by responding to pathogen-associated molecular patterns and damage-associated molecular patterns caused by infection, thus reducing sepsis levels [38]. Additionally, vitamin D has been reported to decrease CD40 levels in blood cells, triggering angiopoietin 2 and angiopoietin 1 activations, increasing the stability of cell endothelium, and reducing inflammation [39-41]. In vitro studies have also shown that vitamin D has decreasing effects on tissue factors and adhesion molecules in activated (inflammatory) tissue cell lines, leading to a reduction in thrombin formation [42,43]. In our research, the decline in thrombin production observed in the Group 3 bovine subjects can plausibly be attributed to the suppression of the inflammatory response facilitated by both the administration of vitamin D and the NSAID agent. This suppression appears to lead to a reduction in the levels of fibrinogen and an elevation in the levels of DFR.

Conclusion

In contrast to human medicine, veterinarians rely on readily measurable biomarkers such as PLR and DFR for the diagnosis and prognosis of infectious diseases that may result in sepsis. However, it is worth noting that the current study has primarily focused on the veterinary medicine field, indicating a limited scope of research in this area. To obtain more comprehensive insights, future studies should consider larger sample sizes involving diverse animal groups and explore various infection conditions. Conducting such studies would contribute to a broader understanding of the subject matter and enhance the applicability of findings in veterinary medicine.

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Conflicts of interest

The authors declared no competing interests.

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TABLE 1.Vitamin D, DFR and PLR findings.

Group		Healthy (Group 1)	VitD + Antibiotic (Group 2)	VitD + Antibiotic + NSAID (Group 3)	P value
			$\bar{X}$± SE		
Vitamin D ng/ml	Day 0	14.31±0.86^{a}	16.68± 0.61^{bx}	16.35±0.61^{bx}	**0.05**
	Day 3	-	19.67± 1.32^{y}	19.78±0.82^{y}	
DFR g/ml	Day 0	2.71±0.34^{a}	1.68± 0.16^{b}	3.25±0.45^{c}	**0.029**
	Day 3	-	2.07± 0.47	2.51±1.07	
PLR 10^9/L	Day 0	128.43±21.55	133.05± 18.39	206.12±30.61	0.105
	Day 3	-	150.44± 4.37	173.92±24.17	

^{a,b}: Values shown with different letters in the same row are statistically significant

^{x,y}: Values shown with different letters in the same column are statistically significant

DFR: D-Dimer-Fibrinogen Ratio, PLR: Platelet-Lymphocyte Ratio, NSAID: Nonsteroidal Antiinflammatory Drugs

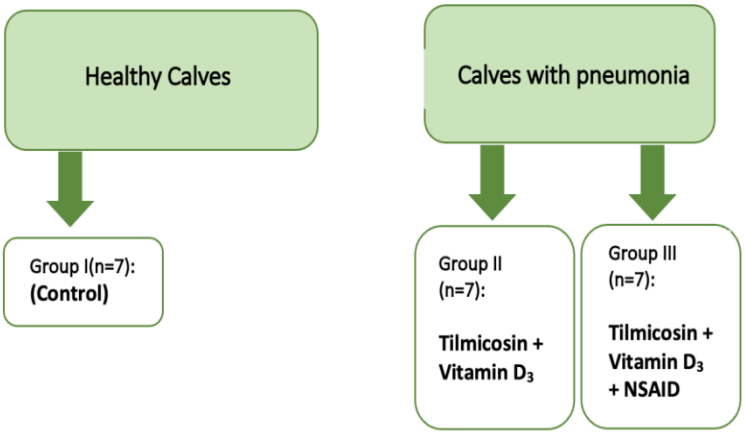

Fig.1. Classification of calves used in the study.

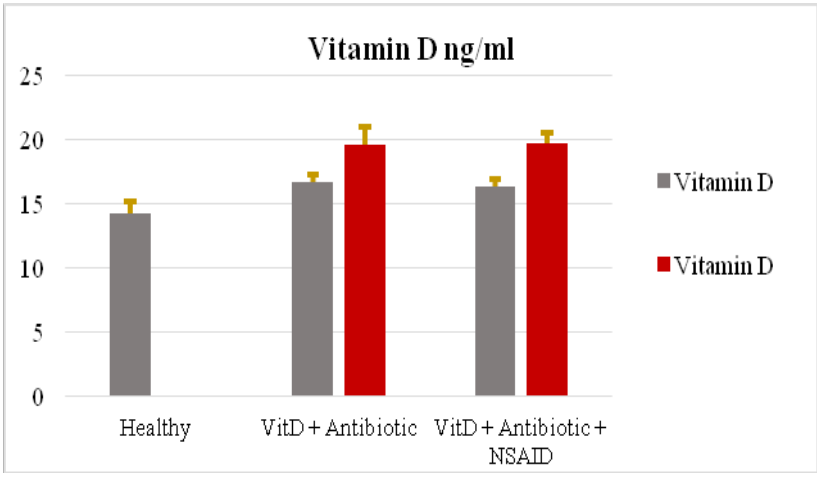

Fig.2. Changes in vitamin D concentrations.

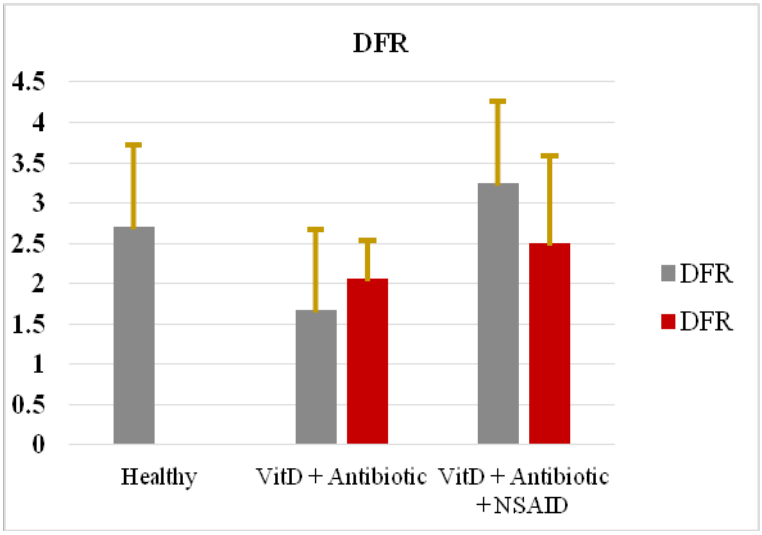

Fig. 3. Changes in DFR levels.

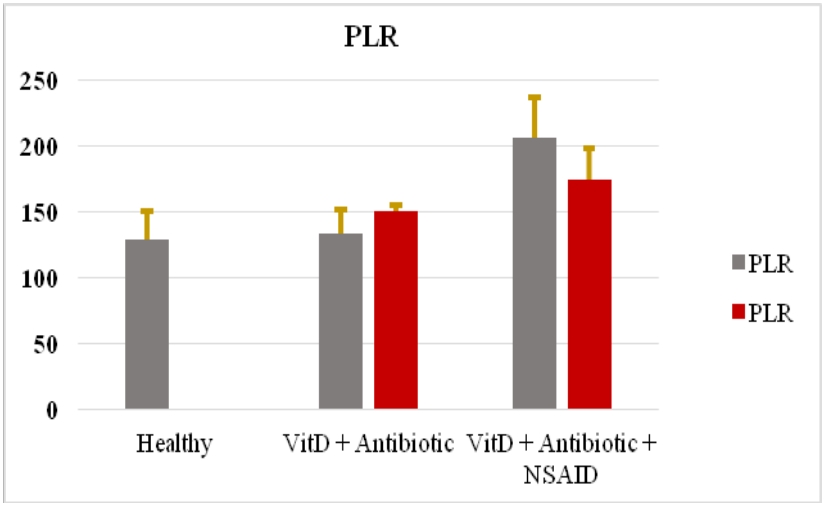

Fig.4. Changes in PLR levels.

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