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Relation between hepcidin levels and hematologic parameters in cattle with theileriosis[1)]

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Summary

This study aimed to determine the relationship between hepcidine levels and some hematological and biochemical parameters after anemia in natural theileriosis cows. This research was conducted with a total of 25 cattle, including 10 healthy controls and 15 cattle with natural theileriosis. 1098 bp positivity was determined according to the PCR result. Leukocyte (WBC), erythrocyte (RBC), hematocrit (HCT) and hemoglobin (HGB) levels were decreased. However, red blood cell distribution width (RDW), Platelet (PLT) and hepcidin levels were increase determined in natural theileriosis cows according to the control group. In addition, a positive correlation was detected between hepsin and iron parameters although there is no statistical significance between them and a negative correlation was determined between hepcidine and HCT, HGB and RDW parameters. As a result, it was determined that erythrocyte parameters decrease and increase in iron parameters and heptidine levels in cattle with theileriosis. It can be concluded that the diagnostic importance.

Keywords: cattle, hepcidin, *Theileria*

Theileriosis is an acute or subacute protozoal disease commonly observed in tropical and subtropical countries in which the *Theileria* species are spread to ruminants like cattle, sheep, goat and buffalo by vector ticks from the family *Ixodidae*. Turkey is also affected by this disease, which leads to substantial economic losses in cattle breeding (2, 9, 16, 28).

Animals with theileriosis show clinical symptoms that include impaired general health, increased cardiac and respiratory frequency, loss of appetite, and reduced or ceased rumen motility and rumination (26). Examination of visible mucosae and conjunctiva may show petechias and bruises (3, 36). Similar bleeding may be found in the sclera, palate, and the tip of the tongue. Conjunctival edema and ulcerations may occur as the disease advances. Lung auscultation reveal scrackles due to the edema. The animal has a mild intermittent cough (26). In most patients, unilateral or bilateral superficial lymph nodes (Lnn. cervicalis superficialis and Lnn. subiliaci) will be enlarged to various extents. Although marked unilateral enlargement of one of these lymph nodes is a typical finding for the disease, Lnn. subparaditicus and Lnn. inguinalis may also be enlarged (2, 19, 25).

Hepcidin is an antibacterial peptide hormone produced in the hepatocytes, composed of 25 amino acids highly protected by disulphide bonds, and which plays a key role in iron metabolism. Hepcidin is defined as the hemostatic regulator of iron absorption in the intestines, iron turnover inmacrophages, and iron release from the hepatic stores (7, 8, 10, 11, 21, 22). Hepcidin regulates the cellular release of iron by interacting with ferroportin, a transmembrane protein. Hepatic production of hepcidin increases when iron stores are sufficient and elevated. Thus, the pathway that carries iron from the enterocytes to the plasma in the small intestines is blocked. The production of hepcidin is decreased in hypoxia and anemia, when iron storage is low (29). In addition to hepatocytes, hepcidin is also found in the stomach, kidneys, lungs, skeletal muscles, adipose tissue of the brain and cardiac tissue as well as in the urine, cerebrospinal fluid, bile and pleural fluid (5, 6, 8).

Hepcidin synthesis is stimulated by increased levels of tissue and plasma iron leading to its interaction with the transmembrane protein ferroportin, which results in reduced iron release from macrophages and hepatocytes and the reduced enteric absorption of iron, thereby preventing iron damage and reactive characteristics in the organism. In addition to preventing damage

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to the body, another reason to control iron metabolism is the accelerated bacterial proliferation in the presence of increased iron concentrations, making patients more vulnerable to pathogens after iron overload (5, 6, 8). Hepcidin production is decreased in hypoxia and in anemia with low iron stores (1, 8).

Hepcidin synthesis is increased by iron loading while it is decreased in anemia and hypoxia. Hepcidin synthesis is stimulated during inflammation, leading to the intake of iron by macrophages, reduced plasma iron levels, and inflammation anemia (5, 6, 10). The discovery of hepcidin and its role in iron metabolism may provide new opportunities for the treatment of inflammation anemia and hemochromatosis (8).

The objective of this study was to determine the levels of hepcidin, a hormone discovered in recent years and defined as a homeostatic regulator of iron absorption in the intestines, iron turnover in macrophages and iron release from hepatic stores, in cattle with theileriosis and to demonstrate its potential relationship with hematologic parameters.

Material and methods

This research was approved by the Animal Research Ethics Committee of Yüzüncü Yıl University in Van, Turkey (Protocol No: 01.12.2014 and 27552122-608). The animals in this study consisted of 25 cattle in total, 15 of which were naturally infected with theileriosis and 10 were healthy, as diagnosed by clinical and laboratory findings. The animals were first clinically examined at the place of business where the animal owners reported symptoms of the disease. Blood samples were drawn with proper methods into one hematology and one biochemistry test tube from the vena jugularis of each animal suspected to have theileriosis. Treatment was immediately prescribed to the animals with microscopically confirmed disease. The sera of the animals was collected in eppendorf tubes and stored in a deep freeze (–20°C) if the pathogen agent was positive. When 15 sera with the disease agent were reached, hepcidin levels were studied by the ELISA (Bovine Hepcidin-25 [Hepc25] ELISA Kit, Cusabio®) method and the agent was identified by polymerase chain reaction (PCR). DNA was extracted using a DNA isolation kit (Termo) in line with the manufacturer's instructions. DNA was extracted using a DNA isolation kit (Thermo) according to the manufacturer's instructions. Extracted DNA was stored at 4°C in a refrigerator. The primer sets used in this study were forward 5'-AGTTTC TGACCT ATCAG-3' reverse 5'-TTGCCT TAAACT TCCTTG-3' (4). For the amplification of the Theileria genus, 989F and 990R primers were used to amplify a 1098-bp fragment of the SSU rRNA gene. Polymerase Chain Reaction (PCR) was performed for 30 µl of total reaction volume containing 13.5 µl of Promega DEPC water, 1.5 µl of $MgCl_2$ (25 mM/ml), 2.0 µl of Promega dNTP, 2.5 µl of 10 × Taq buffer, 0.5 µl of 50 u/µl Promega Taq polymerase, and 1.5 µl of each primer and 2 µl of template DNA. The reaction for the Theileria genus was repeated for 30 cycles under the following conditions: 5 minutes at 94°C, 30 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 72°C, and 7 minutes at 72°C. Agarose gel electrophoresis was used for the analysis of amplified DNA, and the results were visualized using a UV transilluminator.

In addition, routine hematologic parameters such as WBC, RBC, HGB, HTC, MCV, MCH, MCHC, RDW, and PLT were measured (Veterinary Blood Counter, MS4-s) in the blood samples and biochemical parameters including Fe, UIBC (unsaturated iron binding capacity) and TIBC (total iron binding capacity) were measured (Chemistry Analyzer, BS-120, Mindray) in the sera samples.

The group Student's t test was used for statistical analysis of the data. SPSS statistical package software was used for calculations. For correlation Spearman test was used.

Results and discussion

Clinical findings. Inspection of the healthy controls revealed no abnormal clinical findings. Respiratory and cardiac frequencies and body temperature measurements were within normal ranges. Whereas in the patient group the general health condition was impaired and average body temperature was 39.9°C with increased respiratory and cardiac frequency, loss of appetite, and decreased rumen motility and rumination. Conjunctivas and mucosa were either hyperemic or anemic with petechiae and echymoses. Some of the cases had crackles in lung auscultation due to edema. In most patients, unilateral or bilateral superficial lymph nodes (Lnn. cervicalis superficialis and Lnn. subiliaci) were enlarged.

Hematologic and biochemical findings. Hematologic and biochemical findings showed in Table 1. WBC, RBC, HGB, and HTC levels of animals with theileriosis and the controls showed a statistically significant ($p < 0.001$) decrease, while RDW, hepcidin ($p < 0.001$), and PLT ($p < 0.01$) levels showed a significant increase. No correlation exists for hepcidin (Tab. 2).

Tab. 1. Hematologic and biochemical parameters among patient and controls groups ($\bar{x} \pm SD$)

Parameter	Control group ($\bar{x} \pm SD$; n = 10)	Patient group ($\bar{x} \pm SD$; n = 15)
WBC (10^9/l)	10.15 ± 0.52	4.02 ± 0.40[b]
RBC (10^{12}/l)	7.26 ± 0.13	4.18 ± 0.11[b]
HGB (g/dl)	10.15 ± 0.34	8.64 ± 0.19[b]
HCT (%)	30.73 ± 1.09	25.75 ± 0.48[b]
MCV (fl)	34.51 ± 0.71	36.73 ± 0.71
MCH (pg)	13.05 ± 0.16	13.68 ± 0.22
MCHC (g/dl)	36.21 ± 0.35	37.35 ± 0.50
RDW	15.43 ± 0.72	26.79 ± 0.48[b]
PLT (10^9/l)	507.01 ± 27.84	857.37 ± 91.12[a]
Fe (µg/dl)	100.65 ± 8.98	92.75 ± 8.48
Fe-TIBC (µg/dl)	266.88 ± 18.85	274.06 ± 11.36
Fe-UIBC (µg/dl)	152.89 ± 22.29	182.92 ± 10.35
Hepcidin (ng/ml)	19.264 ± 6.11	32.204 ± 7.95[b]

Explanation: a, b means with different superscripts are significantly different a – $p < 0{,}01$; b – $p < 0.001$

Tab. 2. Correlation data of important hematologic and biochemical parameters among patient groups

Parameter	Hepcidin (ng/ml)	RBC (10^{12}/l)	HGB (g/dl)	HCT (%)	RDW	Fe (µg/dl)	Fe-TIBC (µg/dl)	Fe-UIBC (µg/dl)
Hepcidin (ng/ml)	1							
RBC (10^{12}/l)	0.39	1						
HGB (g/dl)	–0.037	0.779[x]	1					
HCT (%)	–0.055	0.823[x]	0.957[x]	1				
RDW	–0.041	0.177	0.362	0.35	1			
Fe (µg/dl)	0.035	–0.311	–0.136	–0.173	–0.342	1		
Fe-TIBC (µg/dl)	0.424	–0.035	0.211	0.166	0.209	0.471	1	
Fe-UIBC (µg/dl)	0.487	0.156	0.326	0.281	0.408	–0.262	0.742[x]	1

Explanation: x – $p < 0.01$

Molecular analyse findings. The microscopic examination of the Giemsa-stained blood smears of infected cattle revealed the presence of free and intracellular forms (Fig. 1). All samples positive by thin blood smears were also positive by PCR (Fig. 2).

Many investigators (3, 24, 30, 36) note that the clinical findings of theileriosis are related toprogressive anemia, and its complications are seen during the course of the disease. Additionally, symptoms of pseudo-pericarditis may be seen (15).

In this study, the inspection of healthy controls revealed no abnormal clinical findings. Respiratory and cardiac frequencies and body temperature measurements were within normal ranges. This was similar to the findings in healthy cattle, as pointed out by investigators (3, 17, 36). Clinical findings in ill animals included high fever, swelling of lymph nodes, tachypnea, tachycardia, paleness or hyperemia of conjunctival mucosae with petechial hemorrhages, dyspnea and coughing. Some of the animals had petechial hemorrhages on the bald portions of the skin. In addition, decreased rumen motility and rumination and loss of appetite were found. These findings were similar to the clinical findings in cattle with theileriosis, as expressed by many investigators (3, 27, 36).

The most typical hematologic finding in theileriosis is the progressive anemia seen in the course of the disease (15, 16, 24, 26, 31, 33). Therefore, parameters related to anemia such as RBC and/or HTC and HGB are commonly used in the evaluation of anemia as seen in the disease (3, 17, 39). A study (27) grouped animals with theileriosis according to the levels of HCT as mild (23.99-20.0), moderate (19.99-12) and severe (< 11.99) anemia and reported that levels of HCT, HGB, RBC and MCHC were decreased with the severity of anemia while the MCV level was increased.There was no difference between groups with regard to the levels of WBC, lymphocytes, neutrophils or MCH. In this study, levels of the hematologic parameters RBC, HGB, HCT, MCV, MCH, MCHC and RDW in control animals (Tab. 1) were within the reference ranges established for healthy cattle (3, 17, 36, 38).

Compared to healthy cattle, the animals infected by *Theileria* showed lower values of RBC, HGB, HCT, MCHC and PLT and a higher MCV level, while leukocytosis arose initially in leukopenic cases with no significant change in basophil and monocyte counts (3, 24, 33, 36). Indeed, it has been reported that leucopenia (14, 21, 23, 30) or leukocytosis (14, 30, 34) may be observed in theileriosis.

Investigators (14, 18, 30) have reported that leukopenia may be seen in the terminal or latent phases of the disease following the leukocytosis observed in

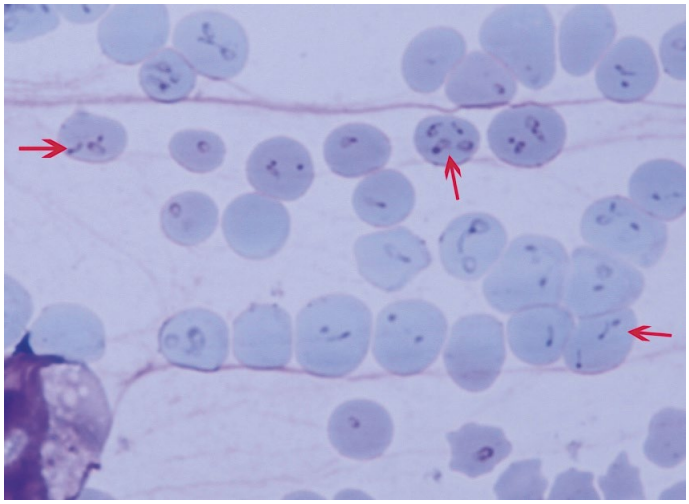

Fig. 1. Blood smear from a cow with theileriosis showing approximately 40 per cent of erythrocytes infested with *Theileria* parasites (arrows) (Giemsa)

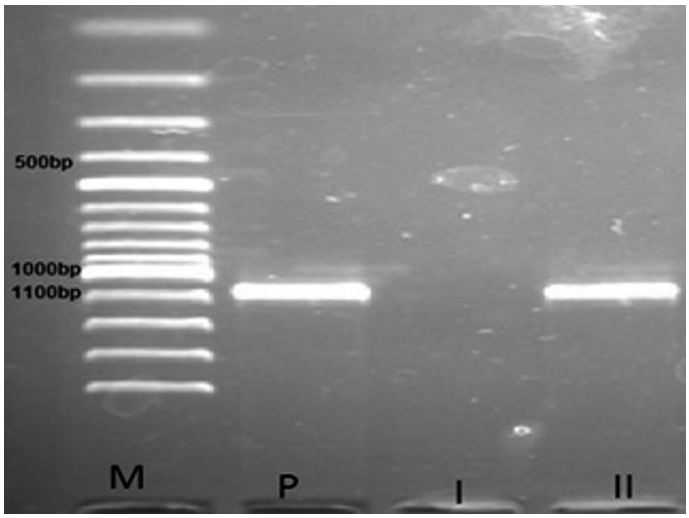

Fig. 2. Gene sequence image of *Theileria* gene
Explanation: lane M: molecular size marker (Qiagen, Germany); lane P: positive control; lane I: negative control; lanes II: positive PCR products of *Theileria* spp.

the acute or initial phase. According to these data, the present study showed that WBC levels are substantially decreased ($p < 0.001$) in animals with theileriosis (Tab. 1). These findings parallel those of the investigators (14, 18, 30). This may indicate that lymphocyte proliferation against invading protozoons is compromised as the disease progresses.

Although the most typical hematologic change in tropical theileriosis caused by *Theileria annulata* is anemia, its mechanism has not been clarified (24, 40). Some investigations (19, 36) report the anemia as normocytic, normochromic, and non-regenerative, while Omer et al. (24) points out a macrocytic hypochromic anemia and Alan and Helbert (1) state that it is hypochromic macrocytic initially, which becomes normocytic hypochromic in later phases. Ramin et al. (27) classified cattle with theileriosis according to the severity of anemia as normocytic normochromic in mild anemia, normocytic hyperchromic in moderate anemia, and macrocytic hypochromic in severe anemia.

In the present study levels of RBC, HGB, HCT, MCH, MCHC, RDW and PLT among hematologic parameters were within reference ranges established for healthy cattle by investigators (3, 17, 36, 38).

RBC, HGB, and HCT levels in the patient group were significantly decreased ($p < 0.001$) while the RDW level was increased ($p < 0.001$) compared to the controls. In addition, no statistically significant change was observed in the levels of MCV, MCH and MCHC. These findings indicate a decrease in the levels of RBC, HGB and HCT without a statistical difference in the levels of MCV, MCH and MCHC, andall values being within the reference range (26, 38) suggests a normochromic anemia. The normochromic anemia found in all animals with theileriosis in this study is consistent with the findings of the investigators (19, 36) but different from that of Omer et al. (24). The increase in RDW, an indicator of diversity in red blood cell width, and MCV within normal ranges indicates hemolytic anemia (37, 38). In this study, data from the control group was found to be within ranges established for healthy cattle by investigators. The increase in the level of RDW in animals with the disease compared to the control group indicates the release of microcytic red blood cells into the circulation to buffer the anemia as a reaction to hemolytic anemia.

The progressive anemia observed in this study is the result of morphological changes in membranous glycolipid and protein components on the surface of the red blood cells doubled by increased osmotic fragility, abnormal accelerationin the clearance of red blood cells as a result of the phagocytosis of damaged red blood cells due to IgG, increased oxidative damage, the presence of hemolytic activity in animals with a high parasitic load, cellular immune response, and increased clearance of bovine red blood cells both with and without parasites in the peripheral blood (12, 20, 28, 32, 33, 41, 42).

The increase in platelet levels is generally secondary to the increased production of factors such as IL-1, IL-3, IL-6 and IL-11. Secondary thrombocytosis occurs during or following a hemorrhage, in particular when the hemorrhage results in iron deficiency. Hemolytic anemia may sometimes occur after various chronic inflammations. Rapid adhesion, deformation, secretion and aggregation of the platelets occur when they are exposed to vascular wall damage or a foreign surface (38). Theileriosis is among the diseases that lead to coagulopathy in cattle (35).

Singh et al. (34) suggested that megakaryocytes may be affected due to the suppression of bone marrow by parasites and/or byproducts after parasitemia and that the degranulation of platelets in peripheral blood that occurs after thrombocytorexis and thrombocytolysis results in thrombocytopenia. This study showed that platelet levels were increased compared to the controls ($p < 0.01$). This is unlike what is suggested by the investigators (19, 24, 34, 36), while similar to the findings by Turgut (38) and Şentürk (35). This difference is thought to originate from individual PLT increases in the animals in the patient group as a response of the body to anemia and petechial hemorrhages.

Iron levels were decreased in animals with theileriosis compared to the controls while levels of Fe-TIBC and Fe-UIBC were increased. However, these fluctuations are not statistically meaningful (Tab. 1). Hence, the increases in the levels of RDW, MCV and PLT as a response to these findings support eventual anemia. Hepcidin is an antibacterial peptide hormone produced in the hepatocytes, composed of 25 amino acids highly protected by disulphide bonds and plays a key role in iron metabolism (7, 10, 11, 21, 22). The decrease in RBC levels and the increase in Fe-TIBC and Fe-UIBC may be a defense against some secondary opportunistic bacteria and fungi (13, 21).

According to Başol et al. (8), hepcidin is defined as the hemostatic regulator of iron absorption in the intestines, iron turnover in macrophages, and iron release from the hepatic stores. Hepcidin regulates the cellular release of iron by interacting with ferroportin, a transmembrane protein. Hepatic production of hepcidin is increased when iron stores are sufficient and elevated. The production of hepcidin is decreased in hypoxia and anemia with low iron storage (29). The cysteine content of hepcidin is higher compared to other antimicrobial peptides known to be rich in cysteine such as snakin, defensin, tachyplesin, and protegrin (13). A study demonstrated that hepcidin is more active against *Staphylococcus aureus*, *Staphylococcus epidermidis*, group *B Streptococci* and *Candida albicans* (13). Hepcidin exerts its antimicrobial effect by destroying the membrane of the microorganism as well as the reduction of serum iron in inflammation resulting in an inappropriate medium for the microorganisms (21, 22). The level of hepcidin was 19.264 ± 6.11 ng/mL in the controls and 32.204 ± 7.95ng/mL in animals with

theileriosis. This increase may be in order to regulate blood levels of iron as expressed by the investigators (13, 21, 22). This is consistent with Rossi's statement as mentioned before (29).

Despite the increase in iron parameters and the levels of hepcidin in the animals with theileriosis compared to the controls in this study, correlation analyses revealed no statistical significance. Further studies including more animal samples and also defining the species of theileriosis may give significant results.

This study is the first study to determine hepcidine levels in cattle with theileriosis. It was concluded that the determination of hepcidin levels in cattle with theileriosis may be beneficial in the diagnosis, treatment decision and prognosis, while further investigations should be carried out on this subject.

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