Acta Veterinaria-Beograd 2024, 74 (1), 44-59
UDK: 636.32/.38:591.111
DOI: 10.2478/acve-2024-0004

Research article

INFLUENCE OF FREQUENT PHLEBOTHOMY ON BLOOD IRON CONCENTRATION, HAEMATOLOGICAL, METABOLIC AND ENDOCRINE PARAMETERS IN RAMS

Siniša GRUBAČ[1], Marko CINCOVIĆ[2]*, Miodrag RADINOVIĆ[2], Aleksandar POTKONJAK[2], Marina ŽEKIĆ[1], Jože STARIČ[3], Jasna PRODANOV RADULOVIĆ[1]

[1]Scientific Veterinary Institute Novi Sad, Rumenački put 20, Novi Sad, Serbia; [2]University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Trg Dositeja Obradovića 8, Novi Sad, Serbia; [3]Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia.

(Received 04 September 2023, Accepted 20 February 2024)

Frequent phlebotomies, even when only a small volume of blood is taken for diagnostic purposes, can lead to the development of iron deficiency with hematological and metabolic changes. The study aimed to determine the influence of frequent blood loss by phlebotomy on blood iron concentration (Fe), hematology, metabolic and endocrine parameters and their relationships. Blood samples were collected from 30 blood donor rams for 6 consecutive weeks, with approximately 10% of blood collected weekly. Such chronic blood loss resulted in a decrease in Fe. Indicators of iron transport in the bloodstream changed, so the value of total iron-binding capacity (TIBC) and unsaturated iron-binding capacity (UIBC) increased, while the value of transferrin saturation percentage (TS%) decreased. Hematological changes included a decrease in red blood cells, hemoglobin, mean red blood cell volume and hematocrit and a tendency for reticulocyte count and red cell distribution width to increase. Chronic blood loss resulted in a specific metabolic response that included the increase in glucose, cholesterol, triglycerides, aspartate-aminotransferase, and insulin resistance, while thyroxine, triiodothyronine and cortisol decreased and there was a tendency for lactate to increase and BHB to decrease. The mentioned blood parameters correlated with Fe and additionally showed greater changes when Fe was extrapolated to the level of clinical deficit (Fe=9μmol/L). These correlations suggest the need to monitor the metabolic and endocrine status during chronic blood loss, in addition to Fe and erythrocyte indices. Compared with previous results in other animal species, Fe may have a direct influence on metabolic processes in rams.

Keywords: phlebotomy, chronic blood loss, iron, complete blood count, metabolites, hormones.

*Corresponding author: e-mail: mcincovic@gmail.com

INTRODUCTION

Iron (Fe) is an essential micronutrient for ruminants. Fe deficiency rarely occurs in herbivores because it is present in sufficient quantities in forages that absorb it from the soil [1]. Fe deficiency can occur due to inadequate Fe intake or as a result of increased Fe loss, usually due to blood loss. Blood loss anemia can be acute when a larger volume of blood is lost in a short period or chronic when a smaller volume is lost over a short period, but blood loss is prolonged. When a large amount of blood is lost acutely, normocytic and normochromic anemia develops, accompanied by protein loss [2]. Chronic bleeding leads to anemia due to the loss of Fe with the development of microcytes [3]. Blood Fe also decreases in inflammatory anemia. However, this is due to increased Fe retention in the inflammatory cells, resulting in less Fe being delivered to the erythron and anemia [4].

The blood of rams is used for various technological processes (for example blood agar production) and blood donor rams can be subjected to frequent phlebotomy [5]. Frequent phlebotomy, even when only a small volume of blood is collected for diagnostic purposes, can lead to the development of anemia and Fe deficiency, which has numerous pathophysiological consequences [6,7].

Iron deficiency develops gradually through three stages: Fe depletion (decrease in Fe storage, no change in transport and functional Fe), Fe deficient erythropoiesis (decrease in Fe storage with changes in transport and functional Fe) and Fe deficiency (decrease in Fe storage with changes in transport and functional Fe and decrease in hemoglobin concentration) [8]. Fe is transported through the bloodstream via transferrin. The capacity of transferrin to bind Fe is referred as total iron-binding capacity (TIBC) and unsaturated iron-binding capacity (UIBC). It is known that these parameters increase in Fe deficiency and their value decreases in inflammation and liver diseases [9].

In one study, chronic phlebotomy in lambs was found to affect Fe and hemoprotein status, and available Fe was mainly directed into red blood cells [10]. Phlebotomy-induced anemia in sheep is a good experimental model to study anemia and red blood cell response [11,12] and thus Fe status. Iron status in cattle and sheep is mainly estimated by complete blood count [13]. In addition to the connection of Fe and hematological parameters, this microelement is directly related to the metabolic response and adaptation of the organism. Fe deficiency and indicators of its functional status have been found to correlate with the development of diabetes, insulin resistance, and changes in insulin secretion [14,15]. Fe is related to carbohydrate and lipid metabolism through the thyroid and adrenal glands by impairing lipid metabolism, ketogenesis, and thermoregulation, so the effect of iron is called "ferrocrinology" [16]. TIBC, UIBC and transferrin saturation percentage (TS%) also correlate with metabolic parameters [17].

The influence of controlled, chronic phlebotomy on the blood Fe level and its blood transport indicators and their relationship with hematological and metabolic parameters

has not yet been studied in rams. The objectives of this study were to investigate: a) the dynamic, weekly changes in blood Fe concentration, iron-binding capacity (TIBC, UIBC, %TS), and other hematological and metabolic parameters during 6 weeks of phlebotomy in rams to confirm that chronic blood loss causes changes in Fe values, indicators of Fe status, hematology values, and metabolic profile; b) the association of Fe, TIBC, UIBC, %TS values with hematology parameters and metabolic profile to determine whether the change in Fe value and iron binding capacity is related to metabolic and hematological adaptations; c) to determine the expected magnitude of changes in iron binding capacity and values of metabolic and hematological parameters when there is Fe deficiency in the blood.

MATERIALS AND METHODS

Animals and blood sampling

Thirty clinically healthy Bosanska pramenka rams, with negative flotation and sedimentation parasitological tests, were enrolled in the study. The rams were 1.5-2.5 years old and in optimal body condition (3-3.5 out of 5), weighing from 50 to 65 kg, housed in standard pens with a loose housing system. The study was conducted during the thermoneutral period of the year. Drinking water was available ad libitum. The ration for the rams consisted of 2 kg of quality hay; 1.5-2 kg of fodder carrot or sugar beets; 1.5 kg of a concentrated mixture containing 16% protein (45% corn meal, 20% oat meal, 15% barley meal, 17% soybean meal, 1% animal chalk, 2% vitamin supplement). The chemical composition of the rations was based on the recommendations of the National Research Council [18]. The concentrate mixture for rams contained at least 30 mg/kg Fe following the Serbian national regulations on the quality of animal feed ("Sl. glasnik RS", br. 4/2010).

Blood was sampled weekly from the jugular vein for 6 consecutive weeks. The volume of blood collected each week was calculated based on the ram's weight. The total circulating blood volume in sheep is equivalent to 66 mL/kg of the body weight. Nonterminal blood collection without additional monitoring should be limited to 10% (6.6 mL/kg) of the total circulating blood volume with a single collection or with serial collections every 2 weeks (https://www.research.uky.edu/division-laboratory-animal-resources/guidelines-blood-collection-laboratory-animals). In the rams studied 8.5 - 10% of the blood volume was collected weekly, which was sufficient to cause changes in Fe metabolism and the development of anemia. All measures were taken to protect against sepsis: shaving of hair and disinfection of the phlebotomy site. Blood of the rams was collected in transfusion bags (Yiaxing Tianhe Pharmaceuticals, CO., LTD, China), according to the calculated volume.

For laboratory analyses, blood samples were taken in the morning between 6 and 8 am before collection in transfusion bags using vacuum separation tubes (catalogue number 367958, BD vacutainer, UK) and EDTA tubes (catalogue number 367873,

BD vacutainer, UK). Blood samples were transported to the laboratory in cool boxes on ice. Processing and analysis of the samples in the laboratory took place the same day. Complete blood count was determined after the blood with EDTA was mixed on a roller and wormed to room temperature. Blood serum was obtained after clotting at room temperature for 3 h and centrifugation (1500 G, 10 min), and sera were analyzed immediately. All analyses were performed in the Laboratory of Pathophysiology, Department of Veterinary Medicine, University of Novi Sad.

The first blood sampling before blood collection served as a control for the other samplings.

Laboratory analysis

Iron and hematology status were determined by the following parameters: iron concentration in blood serum (Fe), unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), transferrin saturation percentage (TS%), red blood cell count (RBC), reticulocyte count (RET), hemoglobin (HGB), mean corpuscular volume (MCV), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), white blood cells count (WBC), neutrophil to lymphocyte ratio (NLR), platelet count (PLT). Metabolic and endocrine status was assessed by measuring blood serum parameters: lactate (LACT); non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), glucose (GLU), total bilirubin (TBIL), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), triglycerides (TGC), cholesterol (CHOL), total protein (TPROT), albumin (ALB), creatinine (CREAT), urea (UREA), triiodothyronine (T3), thyroxine (T4), insulin (INS), revised quantitative insulin sensitivity check index-BHB (RQUICKI-BHB), cortisol (CORT). Hematological analyses were performed with a Mek-6550 automated hematology counter (Nihon Kohden, Japan). Biochemical analyses were performed with a Chemray analyzer automatic biochemical analyzer (Rayto, People's Republic of China) using standard kits (BioSystems, Spain). An AIA-360 automated immunoassay analyzer (TOSOH, Japan) was used for the analyses of insulin (sensitivity 0.033 μU/mL, specificity ~100%, and cross-reactivity <0.001 mol%, accuracy 98-103%), cortisol (sensitivity 0.04 mg/dL, specificity ~100%, and cross-reactivity from <0.001 to 2.8 mol%, accuracy 100-103%), T3 (sensitivity 0.2 ng/mL, specificity ~100% and cross-reactivity from <0.001 to 0.31 mol%, accuracy 99.6-100.5%) and T4 (sensitivity 0.5 μg/dL, specificity ~100% and cross-reactivity from <0.001 to 0.31 mol%, accuracy 99.6-100.5%). RQUICKI-BHB was determined by the calculation formula [19] and TS% was calculated by the online platform (https://www.omnicalculator.com/health/transferrin).

Statistical analysis

The influence of the week was determined using ANOVA analysis and the post-hock LSD test. Correlations between Fe, iron-binding capacity indicators, and other

hematological and metabolic parameters were determined using Pearson correlation analysis. Finally, a "what-if" analysis and a sensitivity analysis were performed to determine how the blood parameters studied would change in rams if the Fe value was below the minimum reference value (Fe = 9 μmol/L was taken as an example). The obtained results were presented graphically. The statistical packages SPSS (IBM, USA) and Excel (Microsoft Office, USA) were used for the analysis.

Ethic

The study was approved by Ministry of Agriculture, Forestry and Water Management - Veterinary directorate (BPG: 802204003118;HID.BR: 802204002215;VKB: RS-470-134) and University of Novi Sad (number EK-II-2022).

RESULTS

Our experimental model showed changes in Fe during the study, making it suitable for studying Fe-related changes in rams. Fe decreased from the first to the sixth week, with significantly lower concentrations in the last three weeks of the experiment. Indicators of Fe transport in blood changed, so that the value of TIBC and UIBC increased, while the value of TS% decreased ($p<0.01$). Hematological changes included a significant decrease in RBC, HGB, MCV and HCT, which was lower at week 3 or 4 to 6 compared to weeks 1 to 3 ($p<0.01$). A tendency for RET and RDW to increase was also noted ($p<0.1$). The values of MCH, MCHC, WBC, NLR and PLT did not significantly change over time (Table 1).

The metabolic response during phlebotomy was characterized by an increase in GLU concentration, CHOL, TGC and AST, while T3, T4 ($p<0.01$), CORT and RQUICKI-BHB were decreased ($p<0.05$). There was a tendency for lactate to increase and BHB to decrease ($p<0.1$). The influence of the week of trial was statistically significant, and the greatest differences were observed in the period of the last three weeks, compared to the first three weeks of the experiment. Parameters such as NEFA, TBIL, TPROT, ALB, UREA, CREAT and INS remained unchanged during the experiment (Table 2).

Correlation analysis showed that significantly changed blood parameters or blood parameters that tended to change correlated with Fe. Correlation coefficients ranged in absolute value from 0.2-0.25 for HCT, LACT and BHB ($p<0.01$); 0.3-0.55 for TS%, RBC, HGB, MCV, GLU, TGC, CHOL, AST, T3, T4, RQUICKI-BHB and CORT ($p<0.0001$), 0.71-0.72 for TIBC and UIBC ($p<0.00001$). Other parameters related to iron decrease such as TIBC, UIBC, TS%, RBC and HGB were not correlated with metabolic or other hematology parameters, except among themselves (Figure 1).

Using the linear relationship and regression allows us to investigate what would happen to the intensity of the deviation of the blood parameters when Fe = 9 μmol/L (extrapolated value for clinical Fe deficiency). The length of the bars indicates the magnitude of the impact of Fe deficiency on the blood parameters, and the position

Table 1. Blood serum iron (Fe) and hematological parameters in phlebotomized rams

Blood parameters	Week of phlebothomy						P
	1	2	3	4	5	6	
Fe µmol/L	32.1±8.2[a]	31.9±7.6[a]	32.1±7.7[a]	26.3±6.9[b]	24.2±6.3[b]	21.6±7.9[c]	<0.01
TIBC µg/dL	440±12.5[a]	460±10.9[b]	459±11.4[b]	485±10.5[c]	482±9.4[c]	495±10.9[d]	<0.01
UIBC µg/dL	271±11.4[a]	279±12.1[a]	285±10.8[b]	315±10.2[c]	322±12.1[d]	325±11.3[d]	<0.01
TS %	40.4±8.9[a]	38.9±8.9[a]	39.2±9.2[a]	30.9±8.9[b]	28.4±9.4[b]	24.3±9.5[b]	<0.01
RBC $\times 10^{12}$/L	9.92±2.4[a]	9.53±2.2[a]	9.23±2.3[a]	8.34±2.2[b]	8.11±1.9[b]	7.74±1.9[c]	<0.01
RET %	0.38±0.11[a]	0.41±0.11[a]	0.4±0.11[a]	0.44±0.1 [a]	0.45±0.1 [a]	0.45±0.11[a]	NS (<0.1)
HGB g/L	108±13.1[a]	104.2±10[a]	96.2±9.9[b]	85.2±9.2[c]	86.7±10.1[c]	79.2±9.4[d]	<0.01
HCT L/L	35.2±4.6[a]	34.5±4.8[a]	31.4±5.75[a]	29.3±4.1[b]	29.9±4.4[b]	29.2±3.16[b]	<0.01
MCV fL	35.3±1.4[a]	35.1±1.25[a]	34.7±1.35[b]	34.5±1.20[b]	33.4±1.19[c]	32.5±1.35[c]	<0.01
MCH pg	11.2±0.9[a]	11±0.89[a]	10.5±1.01[a]	10.4±1.02[a]	10.6±0.99[a]	10.5±1.03[a]	NS
MCHC g/L	308.9±3.9[a]	306.3±4.1[a]	305.1±3.9[a]	295.4±5.8[a]	315.2±6.9[a]	302.3±6.8[a]	NS
RDW %	18.2±1.5[a]	18.9±1.34[a]	18.6±1.28[a]	18.5±1.44[a]	19.6±1.37[a]	19.9±1.07[a]	NS (<0.1)
WBC $\times 10^9$/L	7.95±1.65[a]	8.15±1.48[a]	8.2±1.39[a]	8.05±1.52[a]	8.3±1.59[a]	7.99±1.71[a]	NS
NLR	0.95±0.1[a]	0.92±0.1[a]	0.93±0.1 [a]	0.99±0.1[a]	0.97±0.11	0.94±0.11[a]	NS
PLT$\times 10^9$/L	223±26[a]	209±21[a]	215±22[a]	228±27[a]	219±24[a]	231±21[a]	NS

*Abbreviations: Blood serum iron (Fe), Unsaturated iron-binding capacity (UIBC), Total iron-binding capacity (TIBC), Transferrin saturation percentage (TS%), Red blood cells count (RBC), Reticulocyte count (RET), Hemoglobin (HGB), Mean corpuscular volume (MCV), Hematocrit (HCT), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red blood cell distribution width (RDW), White blood cells (WBC), Neutrophil to lymphocyte ratio (NLR), Platelet (PLT). [a,b,c,d] - Different superscripts mean statistically significant differences between groups.

Table 2. Metabolic and endocrine blood parameters in phlebotomized rams

Blood parameters	Week of phlebothomy						P
	1	2	3	4	5	6	
LACT mmol/L	1.65±0.61[a]	1.79±0.6[a]	1.71±0.6[a]	1.7±0.59[a]	2.11±0.6[a]	2.13±0.6[a]	NS (<0.1)
NEFA mmol/L	0.25±0.12[a]	0.26±0.14[a]	0.29±0.11[a]	0.28±0.09[a]	0.29±0.12[a]	0.31±0.13[a]	NS
BHB mmol/L	0.45±0.11[a]	0.41±0.12[a]	0.40±0.09[a]	0.41±0.12[a]	0.39±0.11[a]	0.38±0.12[a]	NS (<0.1)
GLU mmol/L	3.85±0.29[a]	3.71±0.31[b]	4.02±0.32[c]	4.15±0.28[c]	4.36±0.33[d]	4.61±0.28[e]	<0.01
TBIL µmol/L	4.45±3.9[a]	4.91±3.6[a]	5.02±3.3[a]	4.52±3.7[a]	4.48±3.5[a]	5.11±3.9[a]	NS
AST IU/L	71.5±10.2[a]	78.2±11.4[a]	69.4±7.9[a]	81.3±9.9[a]	85.2±11.3[b]	84.6±10.8[b]	<0.05
GGT IU/L	38.5±5.1[a]	39.9±5.2[a]	40.1±5.3[a]	38.9±4.9[a]	40.2±5.03[a]	39.3±4.89[a]	NS
TGC mmol/L	0.15±0.03[a]	0.16±0.02[a]	0.17±0.02[a]	0.18±0.01[b]	0.19±0.02[c]	0.19±0.02[c]	<0.01
CHOL mmol/L	1.48±0.35[a]	1.56±0.32[a]	1.52±0.3[a]	1.69±0.42[a]	1.95±0.44[b]	2.25±0.48[b]	<0.01
TPROT g/L	66.5±6.4[a]	67.1±6.2[a]	67.5±6.3[a]	66.8±6.1[a]	68.1±5.9[a]	68.5±6.4[a]	NS
ALB g/L	39.2±3.1[a]	38.5±3.3[a]	38.1±3.5[a]	37.9±3.5[a]	38.5±3.6[a]	38.4±3.2[a]	NS
UREA mmol/L	6.99±2.4 [a]	6.85±2.2[a]	7.1±2.2 [a]	7.5±1.89 [a]	7.8±2.13 [a]	8.1±2.15 [a]	NS
CREAT µmol/L	91.2±23.5[a]	92.3±21.1[a]	93.1±25.2[a]	96.5±24.5[a]	100.9±20.9[a]	105.3±21.3[a]	NS
T3 nmol/L	1.85±0.09[a]	1.82±0.1[a]	1.83±0.09[a]	1.73±0.08[b]	1.70±0.09[b]	1.71±0.8[b]	<0.01
T4 nmol/L	99.8±8.1[a]	104.5±7.5[b]	105.2±7.1[b]	97.3±6.9[a]	92.4±7.2[c]	87.5±7.8[c]	<0.01
INS mU/L	4.5±1.9[a]	4.47±1.85[a]	4.48±2.21[a]	4.65±1.99[a]	5.03±2.56[a]	4.99±2.61[a]	NS
RQUICKIBHB	0.53±0.04[a]	0.53±0.02[a]	0.52±0.03[a]	0.51±0.04[b]	0.47±0.04[c]	0.48±0.04[c]	<0.05
CORT nmol/L	55.3±9.5[a]	52.5±9.2[a]	51.6±10.3[a]	48.9±9.7[a]	47.6±9.6[a]	45.8±9.2[b]	<0.05

*Abbreviations: Lactate (LACT); Non-esterified fatty acids (NEFA), Beta-hydroxybutyrate (BHB), Glucose (GLU), Total bilirubin (TBIL), Aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), Triglycerides (TGC), Cholesterol (CHOL), Total protein (TPROT), Albumin (ALB), Creatinine (CREAT), Triiodothyronine (T3), Thyroxine (T4), insulin (INS), Revised Quantitative Insulin Sensitivity Check Index-BHB (RQUICKI-BHB), cortisol (CORT). [a,b,c,d] - Different superscripts mean statistically significant differences between groups.

of the bars indicates the range of the blood parameters. The blood parameters, from the most sensitive to the least sensitive, respond to Fe deficiency as follows: a) positive deviation: UIBC, TIBC, CHOL, RET, LACT, RDW, GLU, AST, TGC, TBIL, INS, UREA, CREAT, WBC, TPROT, ALB, PLT, GGT; b) negative deviation: TS%, HGB, RBC, MCV, HCT, BHB, CORT, T4, MCH, T3, RQUICKIBHB, NEFA, MCHC, NLR (Figure 2).

	Fe	TIBC	UIBC	TS%	RBC	HGB
Fe	1.00	-0.72	-0.71	0.60	0.45	0.35
TIBC	-0.72	1.00	0.62	-0.58	0.29	0.25
UIBC	-0.71	0.62	1.00	-0.57	0.25	0.34
TS%	0.36	0.51	0.40	1.00	0.10	0.20
RBC	0.45	0.29	0.25	0.25	1.00	0.15
RET	-0.52	0.20	0.25	0.20	0.21	0.19
HGB	0.49	0.34	0.30	0.30	0.41	1.00
HCT	0.23	0.08	0.11	0.11	0.36	0.14
MCV	0.46	0.09	0.09	0.14	0.30	0.11
MCH	0.10	0.11	0.09	0.11	0.13	0.13
MCHC	0.06	0.10	0.07	0.12	0.14	0.12
RDW	-0.07	0.03	0.04	0.11	0.12	0.10
WBC	-0.06	-0.01	-0.03	0.05	-0.03	0.02
NLR	0.07	0.11	0.05	0.01	0.11	0.09
PLT	0.03	-0.02	0.01	-0.03	0.02	0.05
LACT	-0.21	0.12	0.13	0.14	0.15	0.11
NEFA	-0.07	0.04	0.06	-0.03	0.10	-0.07
BHB	0.24	0.05	0.04	0.05	0.05	0.05
GLU	-0.47	0.08	0.08	0.02	0.06	0.03
TBIL	-0.11	0.06	0.10	0.03	0.06	0.05
AST	-0.36	0.07	0.12	0.05	0.14	0.07
GGT	-0.07	0.08	0.10	0.11	0.12	0.10
TGC	-0.42	0.09	0.03	0.10	0.10	0.03
CHOL	-0.36	0.10	0.05	0.06	0.10	0.06
TPROT	0.11	0.03	0.05	0.06	0.05	0.05
ALB	0.09	0.06	-0.03	-0.02	0.04	0.03
UREA	-0.06	0.03	0.02	-0.01	-0.01	0.02
CREAT	-0.02	0.05	0.03	-0.04	0.03	-0.02
T3	0.33	0.07	0.05	0.06	0.05	0.08
T4	0.39	0.09	0.07	0.03	0.08	0.04
INS	-0.07	0.10	0.10	0.04	0.06	0.02
RQUICKIBHB	0.32	0.10	0.04	0.05	0.03	-0.04
CORT	0.34	0.06	0.04	0.01	0.02	-0.05

Figure 1. Correlation between iron status, hematological and biochemical parameters in rams.

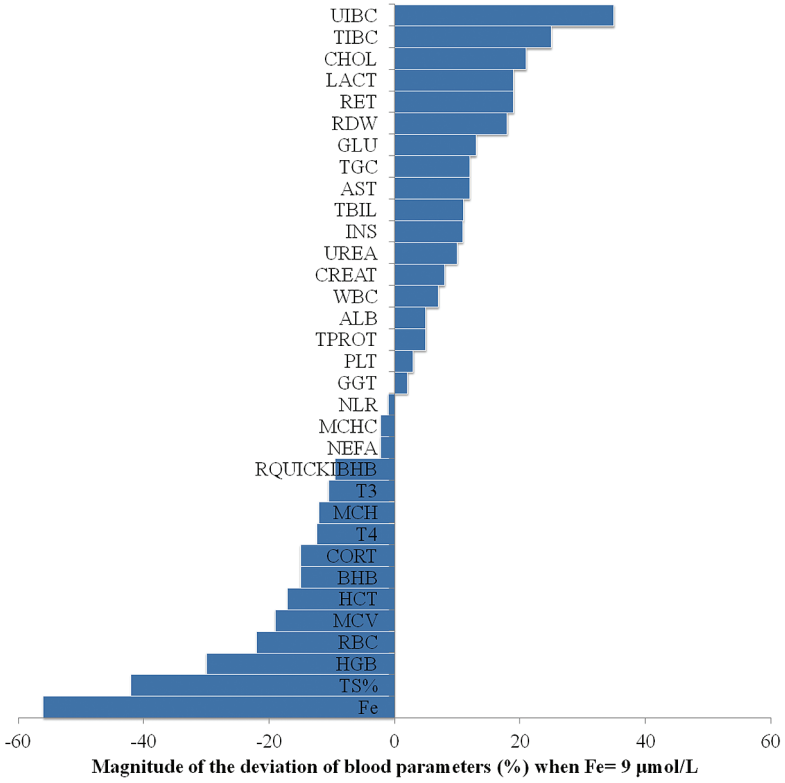

Figure 2. Percent change of blood parameters if Fe=9 µmol/L

DISCUSSION

Clinically healthy rams with blood serum Fe concentration ranging from 12.8-39.2 µmol/L and metabolic parameters within the established reference range [22-24] were included in the experiment. The reference range of Fe established in other studies is 12.5-36.5 µmol/L [20, 21]. The experiment included a complete metabolic panel that allowed us to look at the metabolism of carbohydrates, fats, proteins, and the functioning of individual organs [25].

The bleeding model used [26] resulted in the expected changes in Fe and complete blood count. In our experiment, the concentration of TPROT did not change significantly. Dehydration was not observed in any ram during the study. All included rams had HCT greater than 35%, and the HGB greater than 100 g/L, which are requirements that allow blood collection from animals (see link in Material and Method). This finding is a consequence of the participation of quality rams with optimal body weight and age, and these biological characteristics have a positive effect on the value and stability of HGB and RBC [24].

The results show that there is a decrease in Fe over time, along with a decrease in RBC, HGB, MCV, and HCT. These results are consistent with previous studies in which the following dynamic changes were noted during phlebotomy: a) decrease in HGB; b) decrease in plasma iron; c) reticulocytosis, increase in MCV and MCH; d) diminution of MCV and MCH, increase in total iron-binding proteins; and e) decrease in MCHC [27]. RBCs have the highest priority in Fe utilization after Fe supplementation in chronically phlebotomized lambs [10], confirming the connection between Fe and red blood cell parameters obtained in our work. Decreased MCV (microcytosis) occurred in lambs before the onset of hypochromasia [28].

Many hematological and metabolic parameters did not show a significant deviation during the experiment, which is the consequence of chronic exposure to phlebotomy and blood loss that allowed the animals to adapt to the condition. The number of RET did not change significantly, but a tendency to increase was noted. Sheep have many erythrocytes with low hemoglobin content and low daily turnover, so the intensity of erythropoiesis can be classified as medium, reaching the peak response after about 4-7 days [29]. The slower erythropoiesis that coincides with the frequency of blood collection may be one of the reasons for the absence of an increase in RET. In this study, we did not find significant changes in WBC, NLR, and PLT. This result differs from previous results in sheep where significant hematological changes were found. However, acute blood loss was used in the earlier models [30], whereas chronic blood loss was used in this study. LACT concentration showed a non-significant increase. An increase in LACT was seen in acute blood loss in sheep as an acute response or in very severe anemia in young animals [31]. UREA and CREAT increased during the experiment, but remained within the reference range. This result is probably a consequence of chronic blood loss because acute loss of a larger volume of blood increases the concentration of these metabolites [30, 32] due to prerenal azotemia and reduced blood volume flowing through the kidneys.

The concentration of ALB and TPROT did not change significantly, while their value showed statistically significant changes in acute hemorrhage in goats [32]. NEFA and BHB did not change significantly because they are indicators of energy balance [25], which was not disturbed in this experiment. The increase in GLU and the slight increase in INS established in this experiment stabilized NEFA and BHB [33].

In this experiment, a significant increase in GLU, CHOL, TGC, AST, RQUICKI-BHB index and a decrease in T3, T4 and CORT were found, with a tendency for an increase in LACT and a decrease in BHB. Changes in the value of these metabolites are correlated with Fe (but not with TIBC, UIBC, or TS%) and the magnitude of change in their concentration increases when Fe is extrapolated to a clinical deficit. Fe deficiency can lead to impairment of mitochondrial respiration, resulting in a change of the fatty acid oxidation process, thus potentially contributing to fatty infiltration of the liver [34]. Low serum Fe has been associated with a higher risk of nonalcoholic fatty liver disease and liver fibrosis [35]. Fe or transferrin have been found to stimulate lipolysis and induce the development of insulin resistance in isolated adipocytes, which further

increases their tendency to lipolysis [36,37]. In Fe-deficient mice, a decrease in liver β-oxidation with an increase in lipogenesis was observed [38], which is consistent with the tendency of BHB to decrease in this experiment. Rams have a greater peripheral tissue insulin resistance, increasing both INS and GLU. Some previous studies showed that mixed insulin resistance occurred in Fe-deficient animals [39]. An increase in CHOL and TGC was observed. Iron deficiency enhances hepatocyte lipogenesis, which is in relation to CHOL and TGC synthesis [40,41].

Concentrations of T3 and T4 were lower in rams with progressive blood loss by phlebotomy. These results agree with previous results, which showed that the concentration of thyroid hormones was lower in hemorrhagic shock and blood loss [42,43]. Nevertheless, numerous results in a review paper and pooled analysis confirm the association between iron deficiency, chronic anaemia, and thyroid function, while a recent case-controlled study confirmed that thyroid dysfunction affects all blood parameters [44,45]. Iron deficiency attenuates the response of cortisol to ACTH, so the decrease in CORT in our experiment is consistent with previous results [46]. Iron affects thermogenesis and thermolysis, leading to changes in metabolic adaptation via endocrine glands [16]. The relationship between body temperature and metabolic adaptations was confirmed in heat-stressed sheep [47]. Fe deficiency in rams was found to result in changes in RBC and their indices, iron-binding capacity, and TS% that correspond to the changes due to anemia and/or functional Fe deficiency [13,48].

Fe demonstrated a better linear correlation with metabolic and hematological parameters than TIBC, UIBC, HGB, and RBC. TIBC did not show a significant correlation with metabolic parameters in phlebotomized rams. The reason could be that transferrin reacts more slowly compared to Fe in the body due to its half-life and numerous factors that can affect its formation [49]. In the periparturient period of sheep, the changes in TIBC and UIBC did not follow the dynamics of Fe status [50,51], which was also noted in this study. In pregnant sheep with gastrointestinal parasitosis a decrease in TIBC was found with a decrease in Fe as part of the inflammatory response [52].

The limitation of this study is that some parameters of Fe functional status, such as ferritin, transferrin, and hepcidin, are missing, but their value changes significantly only in clinical Fe deficiency [53].

CONCLUSIONS

Frequent phlebotomy with a loss of about 10% of blood leads to a decrease in Fe. Indicators of Fe transport in the bloodstream change so that TIBC and UIBC increase, while TS% decreases. Hematological changes included a decrease in RBC, HGB, MCV, and HCT, a tendency of RET to increase, and no changes in WBC, NLR, and PLT. Chronic phlebotomy led to a specific metabolic response reflected by insulin resistance, an increase in GLU, CHOL, TGC, and AST, a decrease in T3,

T4, and CORT, with a tendency to increase in LACT, and a decrease in BHB. The mentioned blood parameters correlated with Fe and additionally showed greater changes when Fe was extrapolated to the clinical deficit level (Fe=9 μmol/L), which adds value to this study. The absence of expected significant changes in the values of certain hematological (RET, WBC, NLR) and metabolic (UREA, ALB, TPROT) parameters can be interpreted by the applied experimental model of chronic and controlled phlebotomy because these parameters change in acute and severe bleeding. The correlation of the studied parameters with Fe indicates that it is necessary to monitor the metabolic and endocrine status in addition to the usual parameters of Fe and red blood cell indicators in rams used for blood harvesting. In comparison with previous results in other species, Fe may have a direct effect on metabolic processes in rams.

Acknowledgments

This research was supported by grants No. 451-03-65/2024-03/200117 from Ministry of Science, Technological Development and Innovation.

Author Contributions

SG and MC have prepared the concept of research and manuscript. SG and MR have chosen an adequate methodology. MŽ did the software analysis and visualization. JPR and AP have done the validation. MC made a formal analysis. SG did the investigation and provided the resources. AP has done the data curation. SG and MC have written the original draft. JPR and JS have participated in the process of writing review and editing. J.S. performed supervision.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Suttle NF: Mineral nutrition of livestock. 5th edition, Cabi Publishing, 2022.
2. Tvedten H: Classification and laboratory evaluation of anaemia.
3. Schalm's veterinary haematology, 2022, 198-208.
4. Katsogiannou EG, Athanasiou LV, Christodoulopoulos G, Polizopoulou ZS: Diagnostic approach of anaemia in ruminants. J Hell Vet Med Soc 2018, 69(3):1033-1046.
5. Kumar A, Sharma E, Marley A, Samaan MA, Brookes MJ: Iron deficiency anaemia: pathophysiology, assessment, practical management. BMJ open gastroenterology 2022, 9(1):e000759

6. Egwuatu TO, Ogunsola FT, Okodugha IM, Jide B, Arewa DG, Osinupebi OA: Effect of blood agar from different animal blood on growth rates and morphology of common pathogenic bacteria. Adv Microbiol 2014, 4(16):1237.
7. Chung Y, Desiraju S, Namachivayam K, Guzman, P, He L, Mohan Kumar K: Haematological changes in neonatal mice with phlebotomy-induced anaemia. Pediatr Res 2022, 92(6):1575-1579.
8. Matzek LJ, LeMahieu AM, Madde NR, Johanns DP, Karon B, Kor DJ, Warner MA: A contemporary analysis of phlebotomy and iatrogenic anaemia development throughout hospitalization in critically ill adults. Anesth Analg 2022, 135(3):501-510.
9. FOGSI General Clinical Practice Recommendations - Management of Iron Deficiency Anaemia in Adolescent Girls citirano: De Andrade Cairo RC, Rodrigues Silva L, Carneiro Bustani N, Ferreira Marques CD: Iron deficiency anaemia in adolescents; a literature review. Nutr Hosp 2014, 29:1240-1249.
10. Faruqi A, Mukkamalla SKR: Iron binding capacity. In StatPearls [Internet]. StatPearls Publishing, 2022.
11. Guiang III SF, Georgieff MK, Lambert DJ, Schmidt RL, Widness JA: Intravenous iron supplementation effect on tissue iron and hemoproteins in chronically phlebotomized lambs. Am J Physiol Regul Integr Comp Physiol 1997, 273:R2124-R2131.
12. Chapel SH, Veng-Pedersen P, Schmidt RL, Widness JA: A pharmacodynamic analysis of erythropoietin-stimulated reticulocyte response in phlebotomized sheep. J Pharmacol Exp Ther 2000, 295:346-351.
13. Freise KJ, Widness JA, Schmidt RL, Veng-Pedersen P: Pharmacodynamic analysis of time-variant cellular disposition: reticulocyte disposition changes in phlebotomized sheep. J Pharmacokinet Pharmacodyn 2007, 34: 519-547.
14. Johns J, Heller M: Hematologic conditions of small ruminants. Vet Clin N Am - Food Anim Pract 2021, 37: 183-197.
15. Simcox JA, McClain DA: Iron and diabetes risk. Cell metabolism 2013, 17(3):329-341.
16. Marku A, Galli A, Marciani P, Dule N, Perego C, Castagna M: Iron Metabolism in Pancreatic Beta-Cell Function and Dysfunction. Cells 2021, 10:2841.
17. Szklarz M, Gontarz-Nowak K, Matuszewski W, Bandurska-Stankiewicz E: "Ferrocrinology" – Iron Is an Important Factor Involved in Gluco- and Lipocrinology. *Nutrients* 2022, 14:4693.
18. Madlool AK, Siadat SO, Ali HA, Ali RA, Brbber AM, Abbas AJ, Awad AS, Hasan Mohammed SS, Al-Rufaie MM: Association Between Serum Levels of Adropin and Insulin Resistance in Patients with Beta-Thalassemia Major. Egypt. Acad J Biolog Sci (D-Histology&Histochemistry) 2023, 15(1):127-138.
19. National Research Council (US): Committee on Nutrient Requirements of Small Ruminants. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids, 2007.
20. Petrović MŽ, Cincović M, Starič J, Djoković R, Belić B, Radinović M, Majkić M, Ilić ZŽ: The Correlation between Extracellular Heat Shock Protein 70 and Lipid Metabolism in a Ruminant Model. Metabolites 2022, 12: 19.
21. Reintke J, Brügemann K, Yin T, Wagner H, Wehrend A, Müller A, König S: Associations between minerals and meta-bolic indicators in maternal blood pre-and postpartum with ewe body condition, methane emissions, and lamb body weight development. Animal 2021, 15: 100034.

22. Silva TR, Soares PC, Dantas AF, Marques AVS, Filho EFO, Aguiar GMN, Marques ALA, Riet-Correa F: Serum and liver copper, iron, molybdenum and zinc concentration in goats and sheep in the state of Paraíba, Brazil. Pesqui Vet Bras 2018, 38:1313-1316.
23. Šimpraga M, Šmuc T, Matanović K, Radin L, Shek-Vugrovečki A, Ljubičić I, Vojta A: Reference intervals for organically raised sheep: Effects of breed, location and season on haematological and biochemical parameters. Small Rumin Res 2013, 112:1-6.
24. Shek Vugrovečki A, Vojta A, Šimpraga M: Establishing reference intervals for haematological and biochemical blood variables in Lika pramenka sheep. Vet arhiv 2017, 87:487-499.
25. Cincović MR, Belić B, Lakić I, Majkić M: Reference values of blood parameters in rams of the Bosnian Pramenka breed. Acta Agric Serb 2020, 25: 37-43.
26. Hernandez J, Benedito J L, Castillo C: Relevance of the study of metabolic profiles in sheep and goat flock. Present and future: A review. Span J Agric Res 2020, 18(3): e06R01-e06R01.
27. McGuill MW, Rowan AN: Biological Effects of Blood Loss: Implications for Sampling Volumes and Techniques. ILAR News 1989, 31(4): 5-18.
28. Conrad ME, Crosby WH: The natural history of iron deficiency induced by phlebotomy. Blood 1962, 20(2):173-185.
29. Vatn S, Framstad T: Anaemia in housed lambs: effects of oral iron on clinical pathology and performance. Acta Vet Scand. 2000, 41: 273-281.
30. Udroiu I, Sgura A: Rates of erythropoiesis in mammals and their relationship with lifespan and hematopoietic stem cells aging. Biogerontology 2019, 20: 445–456
31. Sousa RS, Sousa CS, Oliveira FLC, Firmino PR, Sousa IKF, Paula VV, Caruso NM, Ortolani EL, Minervino AHH, Barrêto-Júnior RA: Impact of Acute Blood Loss on Clinical, Haematological, Biochemical, and Oxidative Stress Variables in Sheep. Vet Sci 2022, 9: 229.
32. Widness JA, Lowe LS, Bell EF, Burmeister LF, Mock DM, Kistard JA, Bard H: Adaptive responses during anaemia and its correction in lambs. J Appl Physiol 2000, 88(4): 1397-1406.
33. Abdalla S E, Abdelatif AM: Physiological responses of goats (*Capra hircus*) to haemorrhage as influenced by splenectomy. Am.-Eurasian J Sci Res 2010, 5(2):76-87.
34. Cincović MR, Đoković R, Belić B, Lakić I, Stojanac N, Stevančević O, Staničkov N: Insulin resistance in cows during the periparturient period. Acta Agric Serb 2018, 23(46):233-245.
35. Gusdon AM, Song KX, Qu S: Nonalcoholic Fatty liver disease: Pathogenesis and therapeutics from a mitochondria-centric perspective. Oxid Med Cell Longev 2014, 1:637027.
36. Yang HH, Chen GC, Li DM, Lan L, Chen LH, Xu JY, Qin LQ: Serum iron and risk of nonalcoholic fatty liver disease and advanced hepatic fibrosis in US adults. Sci Reports 2021, 11: 10387.
37. Rumberger JM, Peters T, Burrington C, Green A: Transferrin and iron contribute to the lipolytic effect of serum in isolated adipocytes. Diabetes 2004, 53: 2535-2541.
38. Green A, Basile R, Rumberger JM: Transferrin and iron induce insulin resistance of glucose transport in adipocytes. Metabolism 2006, 55:1042-1045.
39. Sherman AR: Lipogenesis in iron-deficient adult rats. Lipids 1978, 13:473–478.
40. Soliman AT, De Sanctis V, Yassin M, Soliman N: Iron deficiency anaemia and glucose metabolism. Acta Biomed Atenei Parmensis 2017, 88: 112.
41. Davis M, Rendina E, Peterson S, Lucas E, Smith B, Clarke S: Enhanced expression of lipogenic genes may contribute to hyperglycemia and alterations in plasma lipids in response to dietary iron deficiency. Genes Nutr 2012, 7:415-425.

42. Gao H, Jin Z, Bandyopadhyay G, Wang G, Zhang D, e Rocha KC, Liu X, Huayi H, Kisseleva T, Brenner DA, Karin M, Ying W: Aberrant iron distribution via hepatocyte-stellate cell axis drives liver lipogenesis and fibrosis. Cell metabolism 2022, 34(8): 1201-1213.
43. Grill E, Strong M, Sonnad SS, Sarani B, Pascual J, Collins H, Sims CA: Altered thyroid function in severely injured patients. J Surg. Res. 2013, 179:132-137.
44. Vitek V, Shatney CH, Lang DJ, Cowley RA: Thyroid hormone responses in hemorrhagic shock: study in dogs and preliminary findings in humans. Surgery 1983, 93: 768.
45. Wopereis DM, Du Puy RS, Van Heemst D, Walsh JP, Bremner A, Bakker SJ, Bauer, D.C., Cappola AR, Ceresini G, Degryse J, Dullaart RPF, Feller M, Ferrucci L, Floriani C, Franco OH, Iacoviello M, Iervasi G, Imaizumi M, Jukema JW, Khaw KT, Luben RN, Molinaro S, Nauck M, Patel KV, Peeters RP, Psaty BM, Razvi S, Schindhelm RK, van Schoor NM, Stott DJ, Vaes B, Vanderpump MPJ, Völzke H, Westendorp RGJ, Rodondi N, Cobbaert CM, Gussekloo J, den Elzen WPJ: The relation between thyroid function and anaemia: a pooled analysis of individual participant data. J Clin Endocrinol Metab 2018, 103:3658-3667.
46. Ahmed SS, Mohammed AA: Effects of thyroid dysfunction on haematological parameters: Case controlled study. Ann Med Surg 2020, 57:52-55.
47. Saad MJ, Morais SL, Saad ST: Reduced cortisol secretion in patients with iron deficiency. Ann Nutr Metab 1991, 35(2):111-115.
48. Čukić A, Rakonjac S, Djoković R, Cincović M, Bogosavljević-Bošković S, Petrović M, Savić Ž, Andjušić L, Andjelić B: Influence of Heat Stress on Body Temperatures Measured by Infrared Thermography, Blood Metabolic Parameters and Its Correlation in Sheep. *Metabolites* 2023; 13(8):957.
49. Thomas DW, Hinchliffe RF, Briggs C, Macdougall IC, Littlewood T, Cavill I: British Committee for Standards in Haematology. Guideline for the laboratory diagnosis of functional iron deficiency. Br J Haematol 2013, 161, 639-648.
50. Short LF, Murray GF, Uptagrafft WR, Weissbach SG, Polk HC: Transferrin level derived from total iron-binding capacity: is it a reliable relationship? Am J Surg 1984, 148:621-623.
51. Cihan H, Temizel EM, Yilmaz Z, Ozarda Y: Serum iron status and its relation with haematological indexes before and after parturition in sheep. Kafkas Univ Vet Fak Derg 2016, 22: 679-683.
52. Kurpińska A, Jarosz A, Skrzypczak W: Parameters of protein and iron metabolism in dairy cows during periparturient period. Acta Sci Pol Zootechnica 2019, 18:3-10.
53. Kozat S, Yüksek N, Göz Y, Keleş İ: Serum iron, total iron-binding capacity, unbound iron-binding capacity, transferrin saturation, serum copper, and haematological parameters in pregnant Akkaraman ewes infected with gastro-intestinal parasites. Turkish J Vet Anim Sci 2006, 30:601-604.
54. Yiannikourides A, Latunde-Dada GO: A Short Review of Iron Metabolism and Pathophysiology of Iron Disorders. Medicines 2019, 6:85.

UTICAJ UČESTALE FLEBOTOMIJE NA KONCENTRACIJU GVOŽĐA U KRVI, HEMATOLOŠKE, METABOLIČKE I ENDOKRINE PARAMETRE KOD OVNOVA

Siniša GRUBAČ, Marko CINCOVIĆ, Miodrag RADINOVIĆ, Aleksandar POTKONJAK, Marina ŽEKIĆ, Jože STARIČ, Jasna PRODANOV RADULOVIĆ

Česta flebotomija, čak i kada se uzima mala količina krvi u dijagnostičke svrhe, može dovesti do razvoja nedostatka gvožđa (Fe) sa hematološkim i metaboličkim promenama. Cilj ovog istraživanja bio je da se utvrdi uticaj čestih flebotomskih gubitaka krvi na koncentraciju gvožđa, hematološke, metaboličke i endokrinološke parametre i njihov odnos. Uzorci krvi su sakupljeni od 30 ovnova tokom 6 uzastopnih nedelja. Hronična flebotomija sa gubitkom od oko 10% krvi dovodi do smanjenja vrednosti Fe. Indikatori transporta Fe u krvotoku se menjaju, pa se povećava vrednost ukupni kapacitet vezivanja gvožđa i rezervni kapacitet vezivanja gvožđa, dok se procenat saturacije transferina smanjuje. Hematološke promene su uključivale smanjenje eritrocita, hemoglobina, srednje zapremine eritrocita i hematokrita, tendenciju povećanja broja retikulocita i širine distribucije eritrocita. Hronična flebotomija je dovela do specifičnog metaboličkog odgovora, koji se ogleda u povećanju glukoze, insulinske rezistencije, holesterola, triglicerida i aspartat-aminotransferaze, smanjenim vrednostima tiroksina, trijodotironina i kortizola, sa tendencijom povećanja laktata i smanjenja beta-hidroksibutirata. Navedeni parametri krvi su korelirali sa vrednostima Fe i pokazali su dodatne veće promene kada se koncentracija Fe ekstrapolira na nivo kliničkog deficita (Fe=9 μmol/L). Ove korelacije ukazuju da je neophodno pratiti metabolički i endokrini status tokom hronične flebotomije, pored uobičajenih parametara indikatora Fe i crvenih krvnih zrnaca. U poređenju sa prethodnim rezultatima na drugim vrstama, moguće je da Fe ima direktan uticaj na metaboličke procese kod ovnova.