

Haematological and chosen biochemical parameter assessment of the antioxidant system in red deer (*Cervus elaphus*) blood in early and late pregnancy

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Abstract

Introduction: The aim of this study was to investigate changes in haematological parameters and the antioxidant system in the early and late pregnancy of red deer (*Cervus elaphus*). **Material and Methods:** Blood samples were collected from 30 red deer females 50 days after impregnation and 40 days before calving. Complete blood counts and stained blood smears were assessed. Paraoxonase 1 (PON1), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) activities, glutathione disulphide (GSSG), total glutathione, total bilirubin, total protein, albumin, uric acid, malondialdehyde (MDA), beta-hydroxybutyrate (BHB), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, cholesterol levels and total antioxidant status (TAS) were measured. **Results:** The haematological characteristics of blood collected in the third trimester showed higher haemoglobin levels, haematocrit, mean corpuscular volume ($P < 0.05$) and a lower mean cell haemoglobin concentration ($P < 0.05$) in comparison to samples collected on the 50th day after mating. Activity of CAT and MDA, LDL, and triglyceride levels were lower while GR, GSSG, total glutathione, total protein and BHB levels were increased in blood samples drawn in the third trimester compared with the first trimester blood samples. There were no changes in SOD, PON1 activities, TAS, bilirubin, albumin, HDL, cholesterol or uric acid concentrations. **Conclusion:** Red deer's compensatory mechanisms facilitate its optimal adaptation to seasonal changes evidenced by mild pronounced haematological disturbances and an effective antioxidant system during pregnancy.

Keywords: red deer, haematology, antioxidant system.

Introduction

Pregnancy leads to dynamic changes in the ruminant metabolism (7, 11), which might be amplified in red deer (*Cervus elaphus*) when they are exposed to environmental factors in winter. There are very few studies on deer haematology (4, 23) and even fewer on blood parameter variations due to the season (10); there is also no information about the potential impact of season on blood, antioxidant status and lipid alterations in pregnancy. Red deer haematological values may vary according to physiological factors, sex, and the methods of restraint used to obtain the blood samples.

Deer are prone to stress, which may result in red blood cell (RBC) values significantly increasing in excited animals in comparison with resting ones, possibly due to spleen contraction (28). Significant differences in deer blood morphology were found in samples obtained using different animal restraint methods. The physical means used may increase the RBC parameters and segmented neutrophil, lymphocyte, monocyte and total leukocyte counts, while conversely, anaesthesia could lead to a higher eosinophil count (21). Blood parameters may vary between stags and hinds and significantly higher leukocyte counts were measured in unsedated male red deer and chital deer (*Axis axis*) than

in female deer of these species (4, 12). Seasonal changes also have an influence on deer haematology; it was observed that the RBC count was higher during summer and lower during winter, while the white blood cell (WBC) count as mean corpuscular volume (MCV) was found to be higher in winter (10).

Environmental adjustments may also be associated with dynamic changes of redox status and reactive oxygen species (ROS) generation. Metabolic changes in the body involve the production of ROS, the ROS balance being controlled by the enzymatic and non-enzymatic antioxidant systems. The superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and paraoxonase 1 (PON1) enzymes diminish the excess of ROS, and are supported by the non-enzymatic components glutathione, bilirubin and uric acid in stabilisation of the redox state (8). Also, plasma proteins such as albumins prevent the production of free radicals by binding free metals (27). Working in concert in various biochemical reactions, these systems and enzymes all play important roles in establishing an optimal balanced redox state in the organism. Ruminant pregnancy imposes greater metabolic demands and causes extensive changes in protein synthesis in the uterus and as foetal development needs. It may lead to oxidative stress when ROS generation exceeds ROS neutralisation because of imbalanced antioxidant systems (7, 11). Free radical damage done to the placental tissues by protein and lipid peroxidation can complicate the pregnancy (16). The red deer breeding season includes a shortening photoperiod, the gestation and parturition falling within the 7 months from October to April, when seasonal adjustments also take place in deer ecology (1, 2). The first months of foetal development fall in autumn and early winter, and place little nutritional demand, which rises over time, having its peak in the last trimester. The major conceptus mass (more than 70%) is acquired in the latter half of gestation and takes the calf weight to 20% of the total red deer hind's mass at the parturition. Experiments showed a substantial increase in daily dry matter intake in the third trimester, reflecting the increasing demands of the foetus. Restricted diets were associated with significant effects on body condition score and changes in mammary development in comparison to hinds allowed *ad libitum* forage intake at this stage of gestation (2). Deer employ physiological strategies, including seasonal changes in their metabolism, as a response to environmental perturbations and limited access to feed in winter (2, 5). In summer, which is viewed as a period of nutritional abundance, their somatic reserves (mostly consisting of fat) increase, to be used in winter to prevent them from becoming undernourished. Furthermore, in mule deer (*Odocoileus hemionus*), body fat was used preferentially over protein during winter, with lean body mass catabolised to support survival only when fat reserves were depleted (22). Limited nutrition and increased lipid

metabolism in winter, higher nutritional demands and metabolic activity due to pregnancy might be factors leading to an imbalance in antioxidant systems and oxidative stress.

The aim of this study was to investigate for the first time the haematological changes, blood redox and lipid status in red deer (*Cervus elaphus*) in early gestation and a month before calving.

Material and Methods

Animals. The examined group of animals comprised 30 pregnant 3- to 4-year-old female red deer (*Cervus elaphus*), which were kept on an experimental farm providing natural living conditions with open access to grass pastures, greenchop, water and mineral salts. Blood samples were collected twice: on the 50th day after being mated (in October) and 40 days before planned parturition (in April). The animals were captured using cage traps according to standard techniques, without any pharmacological restraining.

Blood sampling. Blood samples were taken from the jugular vein and collected into the ethylenediaminetetraacetic acid (EDTA)-K2 tubes (Promed Collection Test tube; FL MEDICAL s.r.l. Torreglia, Italy) and the clotting test tubes (Promed Collection Test tube; FL MEDICAL). Blood samples with EDTA-K2 were used for haematological analysis and then centrifuged at $1000 \times g$ for 10 min at 4°C. Serum and plasma were separated and stored at -80°C until further analysis. To obtain the red blood cell lysates, the following procedure was used: blood was centrifuged at $1000 \times g$ for 10 min at 4°C and red blood cells were taken and lysed in four times their volume of ice-cold high-performance liquid chromatography-grade water and then centrifuged at $1000 \times g$ for 15 min at 4°C, and finally the supernatant was collected and stored at -80°C. Blood smears were stained using May-Grünwald-Giemsa staining.

Haematological and biochemical blood analysis. Peripheral blood count was determined with an Abacus Junior Vet haematology analyser (Diatron, Budapest, Hungary). Serum (extracellular) and hemolysate (intracellular) superoxide dismutase (SOD) activities were evaluated using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) and an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). The samples were diluted with commercial buffer to a final 1:5 dilution for serum and 1:100 dilution for red blood cell lysates. The results were expressed in U/mL for the serum and U/g of haemoglobin for the erythrocyte lysate.

Glutathione reductase (GR) was measured in the serum using the Glutathione Reductase Assay Kit (Randox Laboratories, Crumlin, UK) and a Point 180 bichromatic semi-automated chemistry analyser (Pointe Scientific, Canton, MI, USA). The results were presented in U/L.

The catalase (CAT) activity in serum was determined by a commercial assay kit (Cayman Chemical) and the Epoch Microplate Spectrophotometer (BioTek Instruments), and the final results were expressed as U/mL.

Paraoxonase activity was assessed using a slightly modified method (19) on a UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). A 20 μ L volume of serum was added to 800 μ L of 100 mM Tris-HCl buffer, pH 8.0, containing 2 mM paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma-Aldrich, St. Louis, MO, USA), 1 mM NaCl and 2 mM CaCl₂. The production of p-nitrophenol at 25°C was measured at a 412 nm wavelength. The molar extinction coefficient used to calculate the rate of hydrolysis was 18.290 M⁻¹cm⁻¹. One unit of paraoxonase activity produced 1 nmol of p-nitrophenol per min. A blank sample was run simultaneously to correct for spontaneous substrate breakdown. Reactivity with phenyl acetate was analysed in a reaction mixture (3 mL) containing 1 mM substrate and 2 mM CaCl₂ in 100 mM Tris-HCl buffer, pH 8.0. After adding 10 μ L of serum to the mixture, the increase in absorbance at 270 nm for 1 min at 37°C was measured. One U was established to hydrolyse 1 μ mol of phenyl acetate/min, and a molar extinction coefficient to calculate the rate of hydrolysis of 1,310 M⁻¹cm⁻¹ was used. The results were expressed in U/mL. Activity of PON1 was expressed as units per millimole of high-density lipoprotein (HDL) (PON1/HDL ratio), which represents HDL-standardised paraoxonase activity (9, 29).

Plasma samples were assayed for total antioxidant status (TAS) on the Pointe 180 analyser (Pointe Scientific), using a Total Antioxidant Status Kit (Randox Laboratories). The results were expressed as mmol/L of Trolox equivalents.

The total glutathione and glutathione disulphide (GSSG) concentrations were assayed in haemolysates using the Glutathione Assay Kit (Cayman Chemical). The absorbance was measured by the Epoch Microplate Spectrophotometer (BioTek Instruments). The results were expressed in μ mol/L.

Serum total protein (TP), albumin, total bilirubin, total cholesterol, triglycerides, HDL, low-density lipoprotein (LDL), uric acid and beta-hydroxybutyrate (BHB) concentrations were evaluated using Pointe Scientific kits (Pointe Scientific) and Miura One fully-automated clinical chemistry analyser (I.S.E., Rome, Italy).

The level of lipid peroxidation was assessed in plasma using a modified method (3, 26), which detects the concentration of thiobarbituric acid reactive substance recognised as malondialdehyde levels (MDA). Briefly, 250 μ L of plasma was mixed with 250 μ L of 0.9% sodium chloride and 2.5 mL of 20% trichloroacetic acid in 0.6 M hydrochloric acid. After 10 min, 1.5 mL of 0.67% thiobarbituric acid was added to all the samples. The mixture was boiled for 20 min

and cooled to room temperature, then 4 mL of 1-butanol was added. The prepared samples were centrifuged at 5000 \times g for 10 min. The supernatant absorbance was measured at 535 nm using the Epoch Microplate Spectrophotometer (BioTek Instruments). The MDA values were expressed in μ mol/L of serum. The intra-assay 6.8% (n = 10) and inter-assay 7.6% (n = 10) coefficients of variation were established.

Statistical analysis. The D'Agostino-Pearson omnibus normality test was used for testing the data for normality. Differences between investigated groups were assessed by non-parametric analysis using the Kruskal-Wallis test with a *post-hoc* Dunn's multiple comparison test. The results were presented as mean \pm SE. A P value <0.05 was considered statistically significant. GraphPad Prism software, version 5.00 (GraphPad Software, San Diego, CA, USA) was used for the statistical evaluation of the results.

Table 1. Red deer blood complete blood count and serum biochemistry panel on the 50th day after being mated (October) and 40 days before planned parturition (April)

Parameter	First collection (October) (n = 30)	Second collection (April) (n = 29)
RBC (10 ¹² /L)	10.52 \pm 1.80	11.53 \pm 1.92
HGB (g/L)	16.19 \pm 2.11 ^a	17.77 \pm 2.63 ^a
HCT (L/L)	0.43 \pm 0.05 ^a	0.54 \pm 0.08 ^a
MCV (fL)	41.72 \pm 4.98 ^a	47.00 \pm 4.10 ^a
MCH (pg)	15.56 \pm 1.45	15.50 \pm 1.22
MCHC (g/dL)	37.52 \pm 1.30 ^a	33.25 \pm 0.80 ^a
WBC (10 ⁹ /L)	6.20 \pm 1.80	5.35 \pm 1.74
LYM (10 ⁹ /L)	3.50 \pm 1.04	3.11 \pm 1.17
MID (10 ⁹ /L)	0.41 \pm 0.28	0.42 \pm 0.20
GRA (10 ⁹ /L)	2.30 \pm 1.07	1.82 \pm 1.12
PLT (10 ⁹ /L)	211.07 \pm 69.69	212.57 \pm 85.40
MPV (fL)	6.01 \pm 0.52	5.90 \pm 0.36
Total protein (g/L)	66.00 \pm 4.44 ^a	73.07 \pm 8.96 ^a
Albumin (g/L)	37.17 \pm 3.72	39.47 \pm 8.49
Cholesterol (mmol/L)	1.82 \pm 0.34	1.83 \pm 0.31
HDL (mmol/L)	1.25 \pm 0.23	1.20 \pm 0.22
LDL (mmol/L)	0.62 \pm 0.19 ^a	0.47 \pm 0.20 ^a
Triglyceride (mmol/L)	0.20 \pm 0.11 ^a	0.15 \pm 0.08 ^a

RBC – red blood cells; HGB – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume; MCH – mean cell haemoglobin; MCHC – mean cell haemoglobin concentration; WBC – white blood cell count; LYM – lymphocyte; MID – monocyte; GRA – granulocyte; PLT – platelet count; MPV – mean platelet volume; HDL – high-density lipoprotein; LDL – low-density lipoprotein
Values are mean \pm SD, ^a indicates significant differences (P < 0.05)

Results

The blood collected in April had higher levels of haemoglobin (HGB) and haematocrit (HCT), a greater

mean corpuscular volume (MCV) ($P < 0.05$) and a lower mean cell haemoglobin concentration (MCHC) ($P < 0.05$) in comparison to samples collected on the 50th day after mating. No parasites or cell morphological changes were found in stained blood smears.

A total protein concentration increase and lower serum triglyceride and LDL levels were found in red deer blood samples collected in April in comparison to those drawn in October, whereas albumin, cholesterol and HDL levels showed no significant changes (Table 1).

In the enzymatic antioxidant system, lower CAT and higher GR activities were detected in April, but no changes were noted in SOD, PON1 or standardised PON1 activities in comparison to the activities of those enzymes in October. Also, there were no changes in the total bilirubin or uric acid concentration as nonenzymatic parameters, nor in the TAS concentration. Plasma malondialdehyde (MDA) was in lower and serum beta-hydroxybutyrate (BHB) in higher concentration in spring than in autumn (Table 2).

Table 2. Blood biomarker concentrations in red deer blood on the 50th day after being mated (October) and 40 days before planned parturition (April)

Parameter	First collection (October) (n = 30)	Second collection (April) (n = 29)
Serum		
SOD (U/mL)	21.96 ± 6.70	20.01 ± 5.62
CAT (U/mL)	50.33 ± 19.23 ^a	27.53 ± 15.15 ^a
GR (U/L)	160.58 ± 82.74 ^a	224.29 ± 71.94 ^a
PON1 (paraoxonase) (U/mL)	239.60 ± 62.19	242.63 ± 61.96
PON1 (arylesterase) (U/mL)	171.57 ± 36.25	186.53 ± 52.90
PON1/HDL ratio (U/mmol)	207.27 ± 65.30	200.13 ± 61.16
TAS (mmol/L)	0.71 ± 0.25	0.79 ± 0.14
total bilirubin (µmol/L)	5.99 ± 3.25	6.70 ± 3.11
uric acid (mmol/L)	0.02 ± 0.01	0.02 ± 0.03
Haemolysate		
SOD (U/mL)	256.34 ± 66.44	292.27 ± 107.24
SOD (U/g Hb)	1608.82 ± 462.90	1676.88 ± 611.50
total glutathione (µmol/L)	71.16 ± 30.67 ^a	94.91 ± 39.89 ^a
GSSG (µmol/L)	59.31 ± 23.64 ^a	73.96 ± 30.37 ^a
Plasma/serum		
MDA in plasma	1.38 ± 0.35 ^a	1.19 ± 0.31 ^a
BHB in serum (mmol/L)	0.26 ± 0.07 ^a	0.43 ± 0.12 ^a

SOD – superoxide dismutase; CAT – catalase; GR – glutathione reductase; PON1 – PON1 paraoxonase 1; HDL – high-density lipoprotein; TAS – total antioxidant status; GSSG – glutathione disulphide; MDA – malondialdehyde; BHB – beta-hydroxybutyrate. Values are mean ±SD, ^a indicates significant differences ($P < 0.05$)

Discussion

Seasonal changes and environmental perturbations may have an impact on the deer metabolism, especially in the demanding period of pregnancy, which could be reflected in their blood parameters and redox state. Deer compensate for these demands on their metabolism by employing physiological strategies and adjustments in their ecology. The elevation of the HGB and HCT parameters in the third trimester might indicate a haemoconcentration due to dehydration, which can accompany undernutrition (25) during winter. The TP increase and the increasing tendency in albumin levels measured in April seems to confirm this; however, the dehydration is mild since the TP values from both October and April fall within the ranges observed in brown brocket (*Mazama gouazoubira*) and Chinese water deer (*Hydropotes inermis*) (10, 23). Similar changes were observed in female white-tailed deer (*Odocoileus* spp.) with additional anaemia occurring in the early spring (14, 15). Our data showed no depletion in RBCs and a decrease of MCHC in April, but with values within normal limits (4). Furthermore, with the fall in MCHC in spring in comparison to autumn, there was no anaemia due to hypochromia seen in the evaluated blood smears, indicating more effective adjustments of the red deer to the season. This is also substantiated by the low extent of the increase of MCV in April without any macrocytosis or anisocytosis seen in the blood slides. In contrast, less well adjusted captive brown brocket deer showed macrocytosis of red blood cells accompanied by elevation in leukocytes after winter as a consequence of higher erythroleukopoietic activity, where young cells led to an increased MCV without raising the RBC count (10).

Deer are prone to stress conditions and their blood parameters are easily altered by stressors. Our animals' morphology values measured in the first and third trimesters were not elevated and were within the normal limits, similar to those found in red deer, brown brocket and Chinese water deer (4, 10, 23). The red and white blood cells counts were closer to the lower values of the normal ranges (4, 10), suggesting a minimum impact of stress. Even when blood sampling was performed on restrained animals, the red blood parameters were comparable with those of red deer restrained using anaesthetics, which is presented as a less stressful manner of handling (21).

Parturition in ruminants might be associated with an imbalance in redox equilibrium, especially when red deer are exposed to harsh winter conditions. They reduce their appetite in order not to expend energy on the unproductive search for limited food and they increase the efficiency of nutrient exploitation and digestion from forage to attenuate the forthcoming potential negative energy balance (1). The BHB level rise shown in the third trimester could indicate such a moderate energy balance disturbance; however, there

were no SOD or PON1 activity changes, suggesting an optimal redox state. Similar observations were made in pregnant Holstein-Friesian and Polish Red dairy cows with no negative energy balance shortly after calving. Individuals of these breeds with increased BHB levels had only a decrease in PON1 activity without any changes in SOD activity; in contrast, Norwegian breed cows with subclinical ketosis (higher serum BHB values of 1.331 mmol/L postpartum) had a significant elevation in SOD activity and a decline in that of PON1 (17, 18). The PON1 and SOD values having been overall approximately 50 U/mL and 10 U/mL higher in red deer than in bovines may also show a higher capacity of the deer enzymatic antioxidant system; however, it also might be associated with a smaller impact of milk production in deer. Also, there were no significant differences in ruminant serum in non-enzymatic constituents such as uric acid, albumins or bilirubin, known for its free radical-diminishing abilities, suggesting optimal scavenging capacity (27).

As a component of the ungulate strategy for winter season adaptation, body fat reserves are used in addition to food, which could also affect ROS formation, leading to oxidative stress and lipid peroxidation (1, 8). In our study we found lower values of plasma MDA in the third trimester, which could be an indirect result of CAT activity and the elevation of total glutathione and GSSG pools, suggesting an effective antioxidant system compensation (20). Any stress-mediated response to high lipid oxidative damage such as unbalanced MDA formation can maximise plasma antioxidant activity, whereas in our experiment TAS, which effectively indicates dynamic equilibrium in the redox state in the plasma compartment (11), showed no difference in early and late pregnancy.

The significant depletion of triglycerides in red deer in April, also observed in brown brocket deer serum in comparison to levels in autumn but with statistical insignificance (11), might be due to limited access to natural feed in winter and the deer being undernourished. Moreover, it could be associated with parturition metabolic changes (2) similar to those in dairy cows, which have the lowest triglyceride rates shortly after parturition (18). Very low-density lipoprotein (VLDL) is proposed as the main triglyceride carrier in ruminants (6). In the plasma of deer and cattle, VLDLs are present at very low levels (13). One of the reasons might be its very short half-life, ranging from only 2 to 11 min. Its normal liver secretion rate is slower in ruminants than in non-ruminants, and in late ruminant pregnancy, VLDL levels could be even more diminished because of the increased demands of mammary gland for fatty acids, effecting the triglyceride serum levels. Lower VLDL synthesis might also reduce LDL serum levels (24). Depletion overall of lipoproteins produced by the liver and consequently falls in triglyceride values could

additionally be effects of the reduction in the mass of the red deer liver by approximately $\frac{1}{4}$ in winter as one of its seasonal adjustments, to reduce the energy needs of alimentary visceral organs (1).

Deer adaptation to seasonal changes, winter time undernutrition and the gestation period involve significant metabolic and physiological changes which are associated with oxidative stress. Our data suggest that red deer have an effective compensatory response, leading to the haematological disturbances being less pronounced and the antioxidant system balanced, having an optimal adaptation mechanism in pregnancy as an adjustment to seasonal changes.

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