Polish Journal of Veterinary Sciences Vol. 19, No. 2 (2016), 359–364

DOI 10.1515/pjvs-2016-0044

Original article

Evaluation of bone marrow with particular consideration of the megakaryocyte lineage and coagulation profile in the pregnant fallow deer (*Dama dama*)

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Abstract

The aim of the study was to evaluate the megakaryocyte lineage of bone marrow and coagulation parameters in fallow deer during the last month of pregnancy. The animals were managed in the barn-feeding system. Twenty female fallow deer, aged 2-3 years, divided into 2 groups were used in the study. Group 1 comprised the females in the last month of pregnancy, and the non-pregnant females were used as the control. All the animals were clinically healthy. Coagulation parameters were measured in all the deer: thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT), and plasma concentrations of fibrinogen, D-dimer, and antithrombin III. A quantitative assessment of bone marrow was carried out for the erythroblastic, myeloid, lymphoid, monocyte-macrophage, and megakaryopoietic cell lines. A detailed analysis of megakaryocyte lineage was performed after whole blood and platelet count.

There were no significant differences in the erythroblast, granulocyte, monocyte-macrophage and lymphoid systems between the animal groups. Thrombocyte count in the pregnant deer was lower than that found in the control group. Bone marrow smears revealed a slightly decreased megakaryocyte count, while the megakaryoblast and promegakaryocyte counts were unchanged. The analysis of coagulation parameters showed increased levels of fibrinogen, thrombin time, prothrombin time and activated partial thromboplastin time in the pregnant animals. The study suggested a hyperactivation of the coagulation system with a slight reduction in the megakaryocyte count in bone marrow, and a reduction in platelet count in peripheral blood at the end of pregnancy.

Key words: platelets, megakaryocyte, bone marrow, coagulation profile, fallow deer

Introduction

In fallow deer, as well as in other animals, health screening is largely based on the assessment of haematological, biochemical and coagulation parameters (Vengust et al. 2002). Coagulation disorders are most often associated with inflammatory, parasitic, viral and bacterial diseases (Mackintosh et al. 2002). Other pathological factors responsible for coagulation disorders include blood loss due to injury, and poisoning

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with aflatoxins and anticoagulants were reported by Beklova et al. (2007) and Fernandez et al. (1995). In fallow deer the influence of hormonal changes on the activity of the coagulation system during pregnancy has been observed (Poljicak-Milas et al. 2009). Platelet deficiency or functional defects can also lead to bleeding disorders reported in ruminants (Sato and Mizuno 1982, Morin et al. 1993). Haematologic parameters, enzyme activity and haemostasis are closely linked to hormonal changes during pregnancy and lactation (Vihan and Rai 1987), and during the progression of inflammatory conditions. Most changes in peripheral blood parameters arise from the modified activity of haematopoietic progenitor cells in bone marrow. Amman et al. (1996) investigated Holstein cattle and found significantly reduced counts of myeloid cells in the animals with bone marrow aplasia. Severe haematopoietic disorders, caused by a number of factors, are associated with pancytopenia and substantial deterioration in animal health. One of the most common reasons for inhibited haematopoiesis is endotoxemia accompanying inflammation within the respiratory tract caused by bacteria and viruses. Another factor strongly affecting haematopoiesis and reducing cell counts in peripheral blood is gastrointestinal bleeding associated with long-term, debilitating diarrhoea of various aetiology. Anaemia and thrombocytopenia are associated with the strong inhibition of hematopoietic processes in bone marrow and intensified coagulation disorders due to consumptive coagulopathy. Because in the available literature there is no data concerning the assessment of megakaryocytes in bone marrow of fallow deer based on the evaluation of coagulation profile, we have decided to take this research problems. To the best of our knowledge, this the first report concerning this topic.

In animals and humans the efficiency and activity of haematopoiesis determine haemostasis, which is the balance between coagulation and fibrinolysis. Haemostasis depends on the integrity of the vessels, platelet count and function, and plasma coagulation system. In mammals, platelets are small oval shreds of cytoplasm formed by the detachment of cytoplasmic fimbria from megakaryocytes. Their counts differ depending on the species and physiological status, animal age and health (Snarska et al. 2013a). In ruminants, nuclei in megakaryocytes are divided into lobes, and with age or during pregnancy the number of lobes increases, up to 32 fragments (Janicki et al. 2000, Snarska et al. 2013b). Significant differences in haematological and biochemical parameters in pregnant fallow deer caused by changes in hormone levels and metabolic rate have been reported (Slavica et al. 2000, El-Sherif and Assad 2001). Studies on coagulation parameters in these animals are relatively scarce and do not address haematopoiesis or the evaluation of bone marrow, which is why this research was undertaken. The aim of the present study was to evaluate the megakaryocytic lineage of bone marrow and coagulation parameters in barn-fed fallow deer during the last month of pregnancy, and to compare findings with those obtained the non-pregnant control animals.

Materials and Methods

Twenty female fallow deer, aged 2-3 years, managed in the barn-feeding system and divided into two groups were used in the study. The experimental group comprised 10 female fallow deer in the last month of pregnancy, and 10 non-pregnant females were used as the control. All the animals were captured from the herd using nets and sedated with 1.5 mg/kg xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), and 2.2 mg/kg zolazepam and tiletamine (Zoletil, Virbac, France). Pregnancy was diagnosed based on ultrasound scan performed with a portable I-Scan apparatus with a 5 MHz linear rectal probe. Bone marrow diseases or functional disorders were ruled out based on manual smears and the analysis of whole blood count. Smears were also analysed to rule out the presence of parasites invading blood cells. All the animals were dewormed with 0.5 ml/10 kg bw of oral Valbazen (Pfizer) three weeks before the experiment.

Bone marrow samples for smears (ca. 1 ml) were sampled by aspiration biopsy, from the third and fourth rib in the sternal region with a 63 mm biopsy needle, 13G. The sampling site was prepared according to standard surgical procedures. Bone marrow was sampled into 1 ml syringes containing no anticoagulant. Due to rapid clotting, the material was immediately smeared onto glass slides. Whole blood for haematological analyses was sampled under standard conditions from the jugular vein into test tubes prefilled with K_2EDTA.

The specimens were stained using the May Grunwald-Giemsa (MGG) method. Blood marrow was stained for 80 s (May-Grunwald protocol) and 5 min (Giemsa protocol). Manual smears were made to rule out the presence of parasites invading peripheral blood cells. Peripheral blood cells were stained for 3 min (May-Grunwald protocol) and 12 min (Giemsa protocol). Giemsa dye was diluted with phosphate buffer pH=7.2 in a ratio of 1:10. Peripheral blood count was determined with an ADVIA 2120i blood analyser. Bone marrow cells were counted with the use of the SH-96/24D haematology cell counter.

Blood for haematological analyses was sampled from the jugular vein into test tubes prefilled with 3.2% trisodium citrate. After collection and centrifuging (2000 rpm) plasma was frozen at -86°C. Coagulation parameters, including thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen level were measured us-

ing a Bio-Ksel Coag-Chrom 3003 apparatus. D-dimer and antithrombin III levels were measured using the kinetic colorimetric method at a wavelength of 405 nm. The results of coagulation assays were statistically analysed with the Newman-Keulus test and Statistica 10.0 software to determine arithmetic means, standard deviations, and the significance of differences between means at a confidence level of $p \leq 0.01$.

Results

All the deer in both groups were found to be clinically healthy. Smears of peripheral blood from all the experimental animals demonstrated no presence of parasites invading blood cells.

There were no statistically significant differences in erythroblast and granulocyte counts in bone marrow between both groups, or in the lymphocyte and monocyte-macrophage counts (Table 1). The number of thrombopoietic cells in bone marrow was higher in non-pregnant females, but the differences with the control group were insignificant (Table.1). Detailed morphological analysis of these cells revealed the smallest difference between both groups of animals in the number of megakaryoblasts and promegakaryocytes (Table 2). The megakaryocyte count in the pregnant females was slightly lower than that in the non-pregnant animals, but no statistically significant differences were found.

Platelet count (PLT) in the pregnant deer (170,000/µl) was statistically lower than in that determined in the non-pregnant animals. No statistically significant differences between both groups were found in the mean platelet volume (MPV) and plateletcrit (PCT) (Table 3).

Thrombin time (TT) was slightly longer in the pregnant deer (31.03 s). The mean prothrombin time (PT) was also slightly longer in the pregnant animals (26.45 s). Activated partial thromboplastin time (APTT) was significantly longer in the group of the pregnant females (48.39 s). D-dimer concentration was also significantly higher in the group of pregnant females (684.22 µg/l m), as was the fibrinogen level (7.76 g/l) (Table 4). A significant decrease in thrombocyte count was observed in the pregnant deer. Antithrombin III level was slightly higher in the pregnant animals as compared to that found in the control females (Table 4).

Discussion

Pregnancy in different animal species is a period in which changes in hormonal metabolism affect morphotic and biochemical parameters of blood, but also bone marrow. Sasaki and Ito (1980) reported clear differences in bone marrow cell counts, and thus in peripheral blood, during the final stage of pregnancy in mice, reflected in decreased counts of lymphocytes, monocytes and platelets. Van Merris et al. (2004) demonstrated the effect of steroid hormones and retinoid on myelopoiesis in cattle, and increased levels of all myelocytic lineages. An increased count of neutrophils and eosinophils, as well as increased erythrocyte counts in peripheral blood, were found in the erythroblastic system. Our study revealed a slight increase in erythroblast counts in the pregnant deer, but there were no differences in the lymphoid and monocyte-macrophage counts between the analysed groups. Similar findings were obtained for the lymphoid and monocyte-macrophage lineages by Van Merris et al. (2004) in a study investigating bone marrow cells in cows having progesterone levels indicative of advanced pregnancy. Megakaryocyte count in the thrombopoietic system was slightly lower, and platelet count in peripheral blood was significantly lower in the pregnant animals than in non-pregnant controls. This was probably due to a drop in the number of cytoplasm fragments cleaved from megakaryocytes, and thus decreased platelet count in peripheral blood. Due to the fact, that the number of the animals used in the present study was limited but we have observed changes in the count of megakaryocytes line cells in the group of the pregnant deer comparing to that found in the control group, in our opinion, the investigations should be repeated in the future involving larger number of the animals originating from different breeding farms. Megakaryoblast and promegakaryocyte counts were similar in both animal groups. In a study on bovine bone marrow Van Merris et al. (2004) also demonstrated a drop in megakaryocyte count during late pregnancy and parturition, with no decrease in megakaryoblast and promegakaryocyte counts.

Lin and Linzer (1999) observed a correlation between megakaryopoiesis and the activity of thrombopoietin, cytokines and various interleukins. It has been proven that megakaryocyte and platelet counts also depend on normal placental function, which in mammals is an organ with a huge impact on hormone metabolism during late pregnancy and shortly after parturition. Studies in humans (Fay et al. 1983) showed a significant decrease in platelet count in women with a normal course of pregnancy, with no effect on general health and no differences in the composition of thrombopoietic cells. Our study revealed very slight differences in thrombopoietic cell counts between pregnant and non-pregnant fallow deer, supporting the above hypothesis.

McCrae (2003) reported thrombocytopenia in 6-10% of pregnant women. In most cases, the drop in platelet count is transient and subsides without consequences after parturition. Our study also revealed a slight decrease in thrombocyte count in pregnant

Table 1. Evaluation of bone marrow in the examinated deer ($\bar{x}$ + SD).

Groups	Granulocytes system (%)	Erytroblastic system (%)	Monocytes--macrophage system (%)	Lymphoid system (%)	Megakariocytes system (%)	Undefined cells (%)
Non-pregnant females	24.82 ± 3.73	43.51 ± 3.98	3.01 ± 0.43	26.61 ± 4.15	2.02 ± 0.25	0.03 ± 0.01
Pregnant females	25.12 ± 3.73	42.21 ± 4.21	3.16 ± 0.52	28.01 ± 3.99	1.48 ± 0.23	0.02 ± 0.01

Table 2. Evaluation of the megakariocytes system of the bone marrow in the examinated deer ($\bar{x}$ + SD).

Groups	Megakarioblasts %	Promegakariocytes %	Megakariocyte %
Non-pregnant females	0.29 ± 0.087	0.19 ± 0.087	1.44 ± 0.35
Pregnant females	0.25 ± 0.12	0.15 ± 0.07	1.08 ± 0.23

Table 3. The mean values of platelets parameters in the non-pregnant and pregnant deer ($\bar{x}$ + SD).

Groups	PLT (10^3/µl)	MPV (fL)	PCT (%)
Non-pregnant females	256.4 ± 35.3	7.99 ± 0.89	0.26 ± 0.02
Pregnant females	170.3* ± 23.4	8.15 ± 1.03	0.29 ± 0.03

* – difference statistically significant at p≤0.01

Table 4. The mean values of coagulation parameters in non-pregnant and pregnant female deer ($\bar{x}$ + SD).

Groups	TT (s)	PT (s)	APTT (s)	Fibrinogen (g/l)	D-Dimer (µg/l)	AT III (%)
Non-pregnant females	23.44 129	20.42 0.78	35.72 9.49	4.42 1.04	282.15 52.46	114.1 9.1
Pregnant females	31.01 4.22	28.41 3.99	48.39* 14.93	7.76* 2.36	684.22* 156.26	81.2 6.98

* – difference statistically significant at p≤0.01

fallow deer. Apart from platelet count, two other parameters are considered, i.e. mean platelet volume (MPV) and plateletcrit (PCT), and they should be analysed together with the platelet count for the correct interpretation of changes in the thrombopoietic system. Marco and Lavin (1999) reported that MPV in animals is often overestimated due to the presence of small platelet aggregates. Similar conclusions were made by Rosef et al. (2004). No statistically significant differences in platelet count, mean platelet volume and plateletcrit between the analysed groups of animals were found in our study. Values of MPV were higher in the pregnant females. In a study by Barić et al. (2011) MPV was higher in adult deer compared to the younger ones. Poljicak-Milas et al. (2009), similar to our study, demonstrated higher MPV in pregnant fallow deer than in the non-pregnant controls. Plateletcrit is the ratio of the platelet volume to the whole blood volume. If the platelet count (PLT) is normal for a given animal species, then PCT has no significant diagnostic value. Our study revealed a slightly higher PCT in the pregnant deer compared to the non-pregnant controls. Because MPV also increased in the pregnant animals, it can be assumed that increased plateletcrit largely depends on the platelet size. Barić et al. (2011) found no increased PLC in adult fallow deer with increased MPV, which is most likely associated with the significant difference in

platelet counts between the young and adult animals. Cross et al (1994) demonstrated a correlation between platelet count, platelet size and PCT, and animal age and season of the year. In the young deer, as demonstrated by Barić et al. (2011) and Poljicak-Milas et al. (2009), values of MPV and PCT were higher than those found in the adult animals. The present findings are comparable to those obtained by Cross et al. (1994) for PLT, MCV and PCV. Platelet counts reported by Barić et al. (2011) differ from those in our study. However, Barić at al. (2011) did not assign the animals to groups considering their sex or physiological status.

Siroka et al. (2011) investigated coagulation parameters in fallow deer and observed high fibrinogen levels in the young animals similar to those found in our study for the pregnant animals. A significantly increased fibrinogen level in females during late pregnancy may indicate a lack of balance between fibrinogen synthesis and fibrinolysis associated with the rapid growth of the foetus and its impact on homeostasis.

Sutherland et al. (1985) reported 12.9 s thrombin time (TT) in fallow deer. In our study TT was significantly higher in pregnant animals. Importantly, Sutherland did not investigate pregnant animals, and animals were not grouped by sex. Thrombin time indicates the transformation of fibrinogen into fibrin and is determined by the fibrinogen level, activity of coagulation inhibitors, polymerization and stabilization of fibrin, and its degradation products. Studies carried out in cows revealed prolonged TT in animals with disseminated intravascular coagulation (DIC) associated with diseases of various aetiology (Sobiech et al. 2008).

In the present study there no significant differences in PT between the animal groups. This parameter is a measure of thrombin activation in the extrinsic pathway, and depends on factors V, VII and X, and prothrombin and fibrinogen levels. The lack of significant differences in PT between both groups of the animals indicates a lack of correlation between PT and pregnancy phase. Interestingly, increased PT in ruminants suggests an intensified degradation of coagulation factors (Heusewieser et al. 1990).

Activated partial thrombin time was significantly longer in deer. This parameter is a measure of the efficiency of the intrinsic thrombin activation pathway, and depends on a number of plasma coagulation factors, as well as prothrombin and fibrinogen levels. Prolonged APTT was reported in dogs with vitamin K deficiency (Mason et al. 2002) and hepatic dysfunction (Badylak and Van Fleet 1981). Studies carried out on cows during the perinatal period (Heusewieser et al. 1990) demonstrated a drop in APTT a few hours before parturition. In a study on non-pregnant fallow deer (Siroka et al. 2011), values of APTT were similar to those found in our study. Longer APTT in the pregnant animals suggests the development of hyperoagulation disorders indicative of physiological burden on the liver associated with late pregnancy.

Significantly increased D-dimer concentrations were observed in the pregnant deer. This parameter indicates the level of fibrinogen degradation products. Fibrin and fibrinogen degradation products have a negative effect on haemostasis by inhibiting haemostatic platelet functions, thromboplastin synthesis in plasma, and polymerization and stabilization of fibrin, which can finally result in the formation of a defective clot (Franchini 2006).

Significantly higher D-dimer concentrations in the pregnant sheep indicate intensified fibrinolysis associated with activated intrinsic and extrinsic coagulation pathways (Balikci et al. 2007).

There were no significant differences in antithrombin III activity between the deer groups. Antithrombin III is one of coagulation inhibitors and the most important physiological inhibitor of thrombin. Studies carried out in cattle (Heusewieser et al. 1990) demonstrated decreased AT III activity during late pregnancy, suggesting that hyperoagulation disorders is not associated with inactivation of AT III.

Our findings dealing with PT are similar to those obtained by Siroka et al. (2011). Mbassa and Poulsen (1991) reported significant decreases in platelet count in pregnant goats. Monagle et al. (2010) demonstrated that haemostasis during the drop in platelet count is maintained through the activation of plasma coagulation factors, which was also confirmed in our study. This suggests that changes in hormone metabolism during pregnancy strongly affect clot formation and fibrinolysis.

To summarize, in heavily pregnant female fallow deer megakaryocyte count in bone marrow is slightly reduced, which is associated with physiological thrombocytopenia and minor haemostatic disorders caused by activated fibrinolysis. The obtained results should be considered as preliminary study for further trials concerning the evaluation of reference values of coagulation profile parameters and assessment of bone marrow in the fallow deer.

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