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IMPLICATIONS OF BLOOD COAGULATION AND FIBRINOLYTIC DISORDERS IN SEVERE ENDOMETRITIS-PYOMETRA COMPLEX IN BITCHES

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Abstract

Twenty bitches with acute endometritis-pyometra complex (EPC) and 20 clinically healthy bitches were examined. The following coagulation parameters were determined in haemostatic evaluations: prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), fibrinogen concentrations (FBG), D-dimer concentrations (D-D), antithrombin activity (AT), and blood platelet counts (PLT). Morphological and biochemical blood parameters were also analysed. Examinations of animals affected by EPC revealed blood coagulation and fibrinolytic disorders, and the noted results (PT 13.7 ±1.06 s, aPTT 23.4 ±1.04 s, TT 15.6 ±0.68 s, FBG 2.2 g/L, D-D 785.4 ±103.05 μg/L, AT 111.1 ±13.51%, PLT 169.30 ±126.31 10^3/μL) point to a high risk of disseminated intravascular coagulation. The findings indicate that the coagulation parameters of bitches affected by EPC should be analysed before treatment as the noted disorder can significantly complicate therapy and ovariohysterectomy, and endanger the patients' life.

Key words: bitches, endometritis, pyometra, blood disorders.

In bitches, the endometritis-pyometra complex (EPC) is the most life-threatening condition that affects reproductive organs and their function. The disease has complex aetiopathogenesis, which has not been completely elucidated yet. EPC is the most common uterine disorder that affects middle-aged (>6 years) and older bitches. In recent years, the disease has been increasingly often reported in younger bitches that completed 1-2 reproductive cycles, in particular in animals subjected to hormonal treatment. Hormonal and bacterial factors play a crucial role in the development of EPC (1). In a normal reproductive cycle, the oestrogen to progesterone ratio changes in a controlled manner, whereas pathological secretion of the above hormones is noted in EPC. Prolonged oestrogen secretion leads to endometrial hyperplasia. Progesterone enhances the level of secretions from endometrial glands, decreases uterine contractility, closes the cervix, and lowers uterine resistance (3). The above factors contribute to the development of bacterial infections. In a closed form of EPC, which turns out to be significantly more serious from the clinical point of view, the accumulation of pus inside the uterus may lead to endotoxaemia. Clinical symptoms of EPC are observed when the detoxifying capacity of the liver is impaired and endotoxins are not removed from the bloodstream. The quicker is the diagnosis and treatment of the disease, the better is the prognosis. The therapeutic method should always be selected in view of the patient's clinical condition. In addition to blood morphology tests, analyses of blood coagulation and fibrinolytic parameters also support the diagnosis of EPC. Coagulation and fibrinolytic disorders are caused by various factors, including bacterial, viral, and parasitic infections, and they can complicate the therapeutic process in animals (14).

In haemostatic analyses of bitches with EPC, Plavec *et al.* (11) and Sobiech *et al.* (16) focused on disorders observed after surgical treatment. Progressing endotoxaemia in animals affected by EPC reveals very serious consequences and leads to the release of large quantities of inflammatory mediators, which affect haemostatic parameters as well as platelet and leukocyte activation. Bacterial endotoxins impair vascular endothelial function and deepen the prothrombin-antithrombin imbalance. As a result, the clinical condition of bitches with EPC may significantly deteriorate and, in critical situations, could lead to disseminated intravascular coagulation (DIC). Such cases require a cautious prognosis, and a surgical treatment involves a high risk of death.

The objective of the study was to evaluate clotting and fibrinolytic activity based on analyses of selected coagulation parameters in bitches diagnosed with acute EPC.

Material and Methods

A total of 40 bitches were examined. The first group (I) comprised of 20 animals with clinical symptoms of the endometritis-pyometra complex (including polyuria, polydipsia, fever, vomiting, apathy). In this group, ultrasonographic examinations of the abdominal cavity have revealed an enlargement of the horn and body of the uterus, which were filled with hypoechogenic fluid, a further indication of EPC. The second group (II) of 20 clinically healthy bitches served as a control. The animals of both groups represented various breeds and were between 8 and 11 years old. The animal use protocol was approved by the local Ethics Commission.

The coagulation, fibrinolytic, morphological, and biochemical parameters of blood collected from the animals were analysed. Blood samples were collected from the cephalic antebrachial vein before pharmacological treatment and surgical removal of the uterus to prevent the above treatments from influencing the haemostatic system. Coagulation and fibrinolytic profiles were determined in platelet-depleted plasma samples from 2,000 g blood specimens, which were collected into test tubes with 3.2% sodium citrate and centrifuged for 15 min. The following parameters were determined immediately after plasma separation: prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), fibrinogen concentrations (FBG), D-dimer concentrations (D-D), and antithrombin activity (AT). Coagulation tests were carried out with the use of the Coag-Chrom 3003 device (Bio-Ksel Ltd.) and reagents supplied by the manufacturer.

Morphological parameters were determined in blood samples collected into test tubes with K_2EDTA in the ADVIA 2021i haematology analyzer (Siemens) using laser-based flow cytometry. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP), and the concentrations of total protein (TP) and albumins (ALB) were determined by kinetic and colorimetric methods, respectively, using the ACCESS 200 chemistry analyser (Cormay).

The results were verified statistically by the Newman-Keulus test with the use of Statistica 6.0 software to determine arithmetic means, standard deviations, and the significance of differences between means at a confidence level of P=0.01 and P=0.05.

Results

The mean values of coagulation, morphological, and biochemical parameters are presented in Tables 1, 2, and 3, respectively. Significant differences were noted between the coagulation profiles of bitches with EPC and clinically healthy animals. A comparison of the results noted in both groups revealed significantly higher PT, PTT, and TT values and an increase in FBG and D-D concentrations in affected dogs. AT values were also significantly higher in animals of group I than of group II (Table 1).

Morphological and biochemical blood analyses showed significant differences in selected parameters between the two groups. With regard to PLT values, lower thrombocyte counts were reported in the blood of bitches with EPC than in healthy animals (Table 1). In diseased dogs, average leukocyte counts reached 20.16 $10^3/\mu L$, and they differed significantly ($P \leq 0.01$) from the results reported in animals of group II (Table 2).

Significant changes in enzymatic activity were observed in serum biochemical profiles. In affected bitches, ALT, AST, and AP activity increased in comparison with healthy individuals. TP and ALB concentrations were lower in dogs with EPC, reaching 60.22 g/L and 32.48 g/L, respectively (Table 3).

Table 1
Mean values of coagulation parameters in bitches with EPC (group I) and healthy animals (group II).

Group	PT (s)	aPTT (s)	TT (s)	FBG (g/L)	D-D (μg/L)	AT (%)	PLT ($10^3/\mu L$)
I	13.7**	23.4**	15.6**	2.2**	785.4*	111.1	169.30*
SD	1.06	1.04	0.68	0.28	103.05	13.51	126.31
II	9.5	13.1	10.5	1.3	256.3	138.6	220.20
SD	0.55	0.73	0.60	0.35	25.28	11.24	114.60

* $P \leq 0.05$; ** $P \leq 0.01$.
PT - prothrombin time, aPTT - activated partial thromboplastin time, TT - thrombin time, FBG - fibrinogen, D-D-D-dimer, AT - antithrombin, PLT - platelets.

Table 2
Mean values of morphological blood parameters in bitches with EPC (group I) and healthy animals (group II).

Group	WBC (10^3/μL)	NEUT (10^3/μL)	LYMPH (10^3/μL)	MONO (10^3/μL)	EOS (10^3/μL)	BASO (10^3/μL)
I	20.16**	15.37	3.29	1.03	0.21	0.06
SD	2.13	2.36	1.21	0.28	0.19	0.03
II	15.77	11.73	2.47	0.79	0.21	0.05
SD	5.63	4.89	1.35	0.34	0.26	0.02
Group	RBC (10^6/μl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
I	5.28*	12.27*	34.28*	64.72	23.20	35.86
SD	0.70	2.09	5.39	2.87	2.56	3.68
II	6.15	14.46	41.13	66.26	23.62	35.70
SD	1.10	2.58	8.84	3.61	2.60	3.87

* P≤0.05; ** P≤0.01.
WBC - white blood cells, NEUT - neutrophils, LYMPH - lymphocytes, MONO - monocytes, EOS - eosinocytes, RBC - red blood cells, HGB - haemoglobin, HCT - haematocrit, MCV - mean corpuscular volume, MCH - mean corpuscular haemoglobin, MCHC - mean corpuscular haemoglobin concentration.

Table 3
Mean values of biochemical blood parameters in bitches with EPC (group I) and healthy animals (group II).

Group	ALT U/L	AST U/L	AP U/L	ALB g/L	TP g/L
I	40.70**	56.80*	122.40	32.48**	60.22*
SD	8.62	13.17	32.98	1.56	4.08
II	25.20	27.20	87.65	47.13	63.09
SD	8.26	5.78	34.01	3.66	2.73

* P≤0.05; ** P≤0.01.
ALT - alanine aminotransferase, AST - aspartate aminotransferase, AP - alkaline phosphatase, ALB - albumins, TP - total proteins.

Discussion

The results of the present experiment revealed blood clotting and fibrinolytic disorders in bitches with clinical symptoms of EPC. A detailed analysis of obtained findings indicates that the discussed disease led to adverse changes not only in the mechanism of primary haemostasis, which is responsible for the formation of the platelet plug, but also in secondary haemostasis, which relies on the coagulation cascade. The latter mechanism involves enzymes (proteins), cell surface phospholipids, cofactor proteins, calcium ions, and coagulation inhibitors. Any disturbances in their function lead to changes in terms of the values of PT, aPTT, and TT, which are indicators of haemostatic disorders (5). In this study, PT, aPTT, and TT were extended in bitches with EPC in comparison with healthy animals (Table 1), which points out the initiation of haemostatic processes and changes in the extrinsic (tissue factor) pathway, intrinsic (contact activation) pathway, and the common pathway with the ultimate conversion of fibrinogen into fibrin. Similar results were reported by Sobiech *et al.* (16) in the analysis of coagulation profiles before ovariohysterectomy, which produced the following values: PT – 14.95 ±3.11 s, aPTT – 30.35 ±5.21 s, and TT – 13.98 ±2.29 s. In the work of Plavec *et al.* (11), the extension of PT, aPTT, and TT was not as significant as in our study, and only the values of aPTT exceeded the reference limit. In bitches of group I, the noted changes were mainly caused by endotoxins produced by Gram-negative bacteria in the uterus. Microbiological evaluations were not performed, but leukocytosis, high concentrations of fibrinogen (acute phase protein), acute clinical symptoms, and duration of the disease are strongly indicative of endotoxaemia. *Escherichia coli* is the predominant pathogen in cases of EPC, but the presence of other bacteria has also been noted, among them *Staphylococcus* sp., *Streptococcus, Pseudomonas, Klebsiella, Proteus, Haemophilus, Pasteurella,* and *Serratia* (1, 7, 13). *Escherichia coli* produces an endotoxin, which, after bacterial decomposition, is released into the uterine lumen and absorbed into the bloodstream. Clinical symptoms such as vomiting,

hypothermia, nervous system disorders, and septic shock are observed when endotoxin concentrations in the blood serum exceed 0.05 ng/mL. Due to releasing the endotoxin, Gram-negative bacteria initiate the extrinsic pathway, activate factor XII, trigger the intrinsic pathway, activate prothrombin and factor X, and initiate secondary fibrinolysis with the involvement of various mediators of immunological and inflammatory responses, such as interleukin 1 (IL-1), IL-6, IL-8, tumour necrosis factor (TNF), leukotrienes, thromboxanes, prostaglandins, platelet-activating factor (PAF), and reactive oxygen species (4, 6). Proinflammatory and prothrombotic interleukins play an vital role in the process. The results of recent research on the blood coagulation system emphasise the importance of IL-6, which increases the concentrations of factor VIII, von Willebrand factor (vWF) and enhances tissue factor (TF) expression. IL-6 lowers AT levels, activates the vascular endothelium, and it is believed to be a vital marker of endothelial activation next to the tissue plasminogen activator (t-PA), thrombomodulin (TM), and vWF. IL-1 inhibits TM expression, induces TF synthesis, stimulates the endothelial release of plasminogen activator inhibitor (PAF-1), and has an overall prothrombotic effect (9, 12).

Bacterial endotoxins directly affect the vascular endothelium, monocytes, and blood platelets, stimulate platelet aggregation, enhance the effect that TF and platelet factor 3 (PT-3) have on the activation of factor X, which, in turn, leads to the conversion of fibrinogen into fibrin. In this experiment, the PLT counts of bitches with pyometra were lower in comparison with healthy animals (group II) and the reference values for this species. Plavec *et al.* (11) reported similar results at the first stage of their study where thrombocyte counts were determined at around 184.4 10^9/L. A drop in PLT counts results from platelet damage and aggregation. As noted by Plavec *et al.* (11), PAF released from endothelial cells plays an essential role in infections caused by Gram-negative bacteria. PAF increases vascular permeability and stimulates the adhesion and aggregation of thrombocytes and leukocytes, which also affects their counts. In a study on humans, elevated PAF levels are noted in patients with DIC caused by sepsis (8).

Evaluations of increased FBG concentrations in control bitches should account for the fact that fibrinogen, an essential component of the coagulation system (plasma factor I), is also an acute phase protein, and elevated FBG levels are observed in various types of inflammations. Therefore, FBG concentrations in the plasma of affected animals are the product of two processes. Firstly, the activation of the coagulation cascade coverts fibrinogen into fibrin, and FBG concentrations decrease. The effectiveness of the above processes is marked by TT, which reached a high 16.7 s in one of the examined bitches. Secondly, the synthesis of FBG can be enhanced by several factors, including IL-6. In the cited study by Plavec *et al.* (11), fibrinogen levels in the first test were high (up to 4.48 ±0.72 g/L), and a decrease was reported at successive stages of the experiment. It should be noted that an increase in FBG levels is also mediated by prostaglandins, and according to Hagman *et al.* (6), prostaglandin concentrations are higher in bitches with EPC. The products of fibrin and fibrinogen degradation (FDP), including D-D, the smallest fragments also contribute to an increase in FBG concentrations.

In our experiment, elevated D-D levels were observed in bitches with EPC in comparison with healthy animals. In studies presented by Plavec *et al.* (11) and Sobiech *et al.* (16), the highest D-D concentrations were reported in blood samples collected 24 h after ovariohysterectomy, suggesting that the above increase could have resulted directly from surgical treatment (2). In human subjects, D-D levels in excess of 500 μg/L point to the activation of fibrinolysis. In the examined animals, D-D concentrations reached nearly 800 μg/L, indicating a high rate of plasminogen conversion into plasmin, a proteolytic enzyme that breaks down fibrin, circulating fibrinogen, factors XII, II, and V, as well as thrombin, which ultimately leads to the release of FDP. Elevated D-D levels are among the key indicators of DIC (17).

A drop in AT activity levels in bitches with EPC probably resulted from its excessive mobilisation and "depletion". The above indicates that diseased animals are unable to compensate the increased demand for antithrombin in a clotting disorder. Antithrombin activity also decreases in response to high levels of IL-6, which accompany various inflammations, including EPC. A drop in the activity of the discussed glycoprotein, in particular in DIC, also results from increased vascular permeability and migration of AT to sites outside the vascular lumen, thus impairing its function.

The aim of the complex and mutually dependent procoagulation and anticoagulation processes in healthy and diseased animals is to maintain a balance between the two reactions. A disruption of that balance can lead to life-threatening conditions of blood hypercoagulability or hypocoagulability. An analysis of coagulation parameters of bitches affected by pyometra suggests that the studied disease leads to the development of haematological disorders characteristic of disseminated intravascular coagulation (DIC). Prolonged PT, aPTT, and TT, decreased AT activity, and increased D-D concentrations are also specific symptoms of DIC (10, 15). A drop in FBG concentrations was the only DIC symptom that was not observed in animals with EPC. Nevertheless, a study of human subjects revealed a decline in FBG levels in less than half of the patients diagnosed with DIC (15). In an absence of acute clinical symptoms of DIC, the noted disorders can be indicative of a chronic form of DIC, which often involves only changes in blood indicators. The above statement was confirmed by haemorrhage during ovariohysterectomy, which obliged to the administration of whole blood or fresh frozen plasma to the patients.

The clinical condition of the studied animals was additionally examined by morphological and biochemical blood tests. A significant increase in WBC counts was observed in diseased bitches, and similar

results were also reported by Plavec *et al.* (11). The above findings confirm that the immune system participates in the development of clotting disorders in animals with EPC. Leukocytes exert a prothrombotic effect not only by synthesising and releasing various substances, but also by damaging vessel walls. Monocytes and macrophages are characterised by the strongest activity. Neutrophils play a less important role in the physiopathology of haemostasis. Only when activated, neutrophil granules secrete highly aggressive proteases: elastase, and cathepsin G. These enzymes damage plasma coagulation factors V, VII, VIII, IX, XII, and XIII, plasminogen, coagulation and fibrinolysis inhibitors, and they degrade platelet membrane glycoproteins (15).

In this experiment, biochemical analyses were performed to determine the activity of liver enzymes, ALT, AST, and AP, and the concentrations of total protein and albumins. The results reported in group I are indicative of liver damage and impaired protein production. Similar findings were reported by Plavec *et al.* (11), but at the early stages of the disease. These observations are significant since the majority of plasma coagulation factors, including AT, are produced by the liver, and impaired liver function affects their secretion. In this case, liver damage could be caused by circulating endotoxins, which, in healthy animals, are neutralised in the liver.

The results of haemostatic analyses should be considered while evaluating the effectiveness of chemotherapeutic agents, which modulates the coagulation system, and blood-based products in bitches with EPC. In treatments that target underlying cause of DIC, the pathological process is inhibited without the use of antithrombotic drugs. In human medicine, the most important stage in DIC treatment in gynecological patients involves the removal of uterine contents or hysterectomy, and antithrombotic agents (in particular heparin) are very rarely administered. Blood-based products, such as platelet concentrate, fresh frozen plasma, or even whole blood, should be readily available during surgical treatment of bitches with advanced pathological changes in coagulation and fibrinolytic systems. Treatment of EPC is complicated, and therefore, it requires continuous monitoring of the patient's haemostatic condition.

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