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Global hemostasis in healthy bitches during pregnancy and at different estrous cycle stages: Evaluation of routine hemostatic tests and thromboelastometry

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ABSTRACT

This study assessed the global hemostasis (including prothrombin time [PT], activated partial thromboplastin time [aPTT], antithrombin activity [ATA], fibrinogen and d-Dimer concentrations, platelet count, plateletcrit and thromboelastometry) in healthy pregnant bitches, comparing the results with those of healthy bitches at different estrous cycle stages, and assessed whether hemostatic changes during pregnancy are associated with serum progesterone concentration or the presence of fetuses *in utero*. The results show that pregnant bitches have higher fibrinogen concentration, platelet count and platelatecrit, and that fibrin and global clot formations occur faster than in non-pregnant bitches at different estrous cycle stages. Additionally, clot strength was higher in pregnant bitches than in non-pregnant ones. There were no differences in PT, ATA, and D-dimer concentration between all study groups. The aPTT was significantly shorter in bitches at the fourth and last pregnancy weeks, compared to the anestrus group, and shorter in both the fourth and last pregnancy weeks groups, compared to diestrus group. These results all support a hypercoagulable state in healthy pregnant bitches, unassociated with progesterone concentration.

1. Introduction

The bitch (*Canis familiaris*) is monoestric. Its 7-month long estrous cycle includes four stages; proestrus (lasting nine days on average), estrus (nine days), diestrus (also termed metestrus; 60 days) and anestrus (120 days) [1–4]. The estrous cycle stages are clinically determined by combining vaginoscopy and vaginal cytology, as well as exogenous signs (e.g. vulvar swelling, discharge), while ovarian changes can be characterized ultrasonographically, and confirmed by measuring serum progesterone (P4), and luteinizing hormone (LH) concentrations [3,4].

Hemostasis is maintained by adynamic equilibrium between procoagulants, anticoagulants and the fibrinolytic system [5]. In healthy women, the normal pregnancy is associated with hemostatic changes, with procoagulant dominance [6]. These changes are hypothesized to be part of complex physiological adaptation mechanisms, ensuring prompt, effective control of bleeding from placental sites when the placenta separates at parturition, while allowing maternal and fetal circulations to expand at the uteroplacental interface during pregnancy [7]. Placental separation presents a profound acute local hemostatic challenge. This process occurs rapidly, and a maternal blood flow of up to 700 mL/min to the placental sites in pregnant women has to be restricted by the combined effects of myometrial extravascular compression and thrombotic occlusion of the sheared maternal vessels [7]. Hemostatic alterations in pregnant women, including hypercoagulability, manifested by activation of both coagulation and fibrinolysis, might lead to both bleeding and thromboembolic complications, such as deep vein thrombosis (DVT) and obstetric disseminated

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intravascular coagulation (DIC) [8–10]. Such hemostatic changes include increased concentrations of most clotting factors, while the concentrations of natural anticoagulants and the fibrinolytic activity are decreased [11,12]. These changes are hypothesized to occur in order to decrease the likelihood of pregnancy- and delivery-related hemorrhage [9,12]. Additionally, the platelet count is decreased during pregnancy, possibly due to augmented destruction and hemodilution [13].

In concordance, certain hemostatic alterations were also described in pregnant bitches, including hyperfibrinogenemia, increased fibrinogen degradation products (FDPs) concentration and decreased antithrombin activity (ATA) [14–18]. In contrast to pregnant women, in pregnant bitches, thrombocytosis has been recorded rather than thrombocytopenia [17].

In contrast to pregnant women, in pregnant bitches, the hemostatic changes are considered to be unassociated with the hormonal status, but rather, are thought to be associated with a systemic inflammation, induced by the presence of fetuses *in utero* [16–18]. In pregnant queens, hemostatic changes are unrelated to the presence of fetuses *in utero* or to serum progesterone concentration [19]. To the best of our knowledge, comprehensive data of the hemostatic status of pregnant bitches are currently unavailable.

Thromboelastometry (TEM) and thromboelastogrphy (TEG) provide global assessment of hemostasis, with a continuous recording of whole blood viscoelastic changes, from the initial clot formation up to its fibrinolysis [20]. Routine citrated-plasma-based hemostatic assays are sometimes limited in their capacity to predict hemorrhage or thrombosis, while TEM tracings may better reflect the cell-based model of hemostasis [21], thereby providing better prediction of the kinetics of hemostasis (i.e., clot formation, stabilization and degradation) [20,22–25]. Several reagents are available for TEM analyses, to differentiate between various potential hemostatic abnormalities and drug effects. Several diagnostic algorithms have been proposed and were clinically validated [26]. Contact activation of hemostasis in TEM (INTEM) allows assessing the intrinsic and common coagulation pathways, fibrinogen concentration, platelet count and function and fibrinolysis, while tissue factor (TF) activation of hemostasis in the TEM (EXTEM) assesses the extrinsic and common pathways, fibrinogen concentration, platelet count and function and fibrinolysis. TEM has been validated in dogs and other animals [20,22–25,27–29].

The aims of this study were: 1) perform a comprehensive assessment of hemostasis in healthy pregnant bitches; 2) compare these results with those of healthy bitches at different estrous cycle stages; and 3) assess whether hemostatic changes during pregnancy are associated with serum P4 concentrations or the presence of fetuses *in utero*.

2. Materials and methods

2.1. Selection of dogs and blood collection

This study was approved by the Hebrew University Veterinary Teaching Hospital (HUVTH) Committee of Animal Handling and Experimentation. The study included 37 healthy, intact adult bitches, with their owners signed consent, deemed healthy based on their history and physical examination. In 10 of these, blood samples were obtained at three different time-points; during estrus and at the fourth and last weeks of pregnancy. Ten other healthy intact bitches were tested during anestrus, and 10 additional ones during diestrus, while 7 additional ones were sampled during proestrus/estrus. Dogs receiving any medication and those with a history of any pre-existing disease or hemostatic abnormality were excluded.

The estrous cycle status stage was determined by a single, board-certified veterinary theriogenologist (S.T.), based on the history, physical examination, vaginoscopy, vaginal cytology, abdominal and ovarian ultrasonography and serum P4 concentration.

Blood samples were collected via jugular venipuncture into 3.2% tri-sodium citrate and potassium-EDTA tubes, and plain tubes containing no anticoagulant, with gel-separators.

2.2. Laboratory tests

All laboratory tests were performed at the HUVTH Diagnostic Laboratory. Citrated plasma was harvested by centrifugation within 20 min from collection, and analyzed immediately. Whole blood in plain tubes was allowed to clot, centrifuged within 30 min from collection, and sera were harvested and analyzed within 90 min from collection. Whole blood in EDTA was analyzed within 20 min from collection.

Citrated plasma samples were analyzed using coagulometrican analyzers (ACL 200 or ACL-9000, Instrumentation Laboratory, Milano, Italy) for measurement of prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration (Claus method) and antithrombin activity (ATA) [chromogenic substrate; after incubating the dog's plasma with factor Xa reagent (reagents HemosIL 0020008910 and HemosIL 0020008920, respectively; Instrumentation Laboratory, Milano, Italy), in presence of heparin excess]. D-dimer concentration was measured using a latex particle-enhanced immunoturbidimetric assay (Tina-quant D-dimer Gen 2; Roche, Mannheim, Germany) using an autoanalyzer (Cobas-Integra 400 Plus, Roche, Mannheim, Germany, at 37 °C). Thromboelastometry (INTEM and EXTEM) was performed using the ROTEM® *delta* analyzer (ROTEM, Munich, Germany).

Whole blood samples collected in potassium-EDTA were used for complete blood count (CBC; Advia 120, Siemens Medical Solutions Diagnostics, Erfurt, Germany). Blood smears were prepared within 30 min from collection, and stained with modified Wright's staining solution (Hematek slide strainer; Siemens, NY, USA), and were evaluated microscopically by a single experienced board-certified internal medicine clinician (I.A), blinded to the estrous cycle or pregnancy status of the bitches. Evaluation included blood cell morphology and a manual platelet count. When the difference between the manual and automated platelet count was >20%, the automated platelet count was excluded from the statistical analysis.

Serum P4 concentration was measured by electrochemolumiscence (Elecsys 2010; Roche, Mannheim, Germany).

For all tests (CBC, coagulation tests and TEM), excluding P4 concentration, reference intervals were generated by the HUVTH Diagnostic Laboratory using reference populations of at least 40 healthy dogs.

2.3. Statistical analysis

The distribution pattern of continuous variables was assessed using the Kolmogorov-Smirnoff test. Continuous variables were compared among more than two independent groups using the Kruskal-Wallis non-parametric test, followed by pair-wise-non-parametric Mann-Whitney *U* test comparisons, with Bonferroni's correction of the significance level. Pearson's and Spearman's correlation tests, depending on the data distribution pattern, were used to investigate the association between two continuous variables. Fisher's exact test was used to examine the association between two categorical variables. In the 10 bitches in which three consecutive measurements were made (i.e., during estrus and at the fourth and last pregnancy weeks), for the continuous variables, due to the limited group size, the Friedman non-parametric test was applied for testing the difference between the three