Acute-phase Protein Profile of Healthy Bitches in Diestrus: the Influence of Steroid Hormones

Camila Infantosi Vannucchi, Cristina de Fátima Lúcio, Fernanda Machado Regazzi, Daniel de Souza Ramos Angrimani, Maira Morales Brito & Renata de Azevedo Abreu

ABSTRACT

Background: Acute-phase proteins are known as significant indicators of inflammation for several animal species, allowing more accurate follow-up of diseases, particularly when other clinical signs are not apparent. Nevertheless, the difficulty of data interpretation due to the absence of reference values for canine species represents an imperative obstacle for systematic clinical application. Moreover, no influence of hormonal status has yet been established for dogs. The aim of this study was thus to propose reference values of fibrinogen, haptoglobin, ceruloplasmin, seromucoid and glycoprotein for diestrous healthy females and therefore verify the influence of the hormone status.

Materials, Methods & Results: Data consisted of weekly blood sampling from 20 Great Dane diestrous bitches during 9 weeks. Each animal’s health condition was verified through physical assessment and the absence of acute inflammatory or infectious processes was guaranteed by a total white cell count. Sampling started with diestrus onset and continued until the beginning of anestrus, evaluated by vaginal cytology. From blood samples, the concentrations of fibrinogen, haptoglobin, ceruloplasmin, seromucoid, glycoprotein, progesterone and estrogen were determined accordingly. No statistical variations (P < 0.05) on plasmatic fibrinogen, serum haptoglobin and ceruloplasmin concentrations were observed between weeks of diestrus nor a correlation between the hormonal profile. Significant increase in seromucoid concentration was verified between the 1st and 6th week of diestrus, while significant variation (P < 0.05) of glycoprotein was measured between the 1st and 3rd weeks of diestrus, as well as between weeks 2 and 9. The reference values proposed were: 182.22 (±46.40) mg/dL for fibrinogen, 80.22 (±9.80) mg/dL of HbCN binding capacity for haptoglobin, 8.19 (±1.68) IU/L for ceruloplasmin, 79.88 (±15.78) mg/dL for seromucoid and 11.89 (±0.92) % for glycoprotein.

Discussion: As no correlation was verified among fibrinogen, haptoglobin and ceruloplasmin and hormone concentrations in diestrous bitches, such laboratorial determination can thus be utilized for patients regardless of progesterone concentration. However, hormone status resulted in seromucoid increase during progesterone decline in diestrus. During the first half of the diestrous period (1st to 5th weeks), there is an intense corpus luteum steroidogenesis which will require seromucoid plasmatic increase for progesterone transportation. There was higher glycoprotein level at early diestrus (3rd week), during which there is high progesterone concentration. Therefore, the possible explanation for the increase in glycoprotein concentration is the progesterone profile, nonetheless the lack in statistical correlation between these variables. In conclusion, the hormonal profile has no influence on fibrinogen, haptoglobin and ceruloplasmin concentrations. On the other hand, the levels of seromucoid and glycoprotein vary according to the diestrous phase (or progesterone concentration), suggesting a differential acute-phase protein profile in relation to the hormonal profile. However, additional studies are required to clearly establish the influence of reproductive hormones on inflammatory or non-inflammatory acute-phase protein synthesis in the canine liver and reproductive tissues. In addition, research on proestrous and estrous bitches may clarify the modulation system by which relates to estrogen and the acute-phase protein profile.

Keywords: acute-phase proteins, canine, progesterone, estrogen, diestrus.
INTRODUCTION

Acute-phase proteins (APP) are broad indicators of an inflammatory process, extremely useful when other clinical signs are not evident [20]. However, variation on their concentration can occur during pathological or physiological processes [8,16]. Therefore, the complete usefulness of these tests for clinical diagnosis is only achieved unless the physiological parameter is previously known. To date, the only APPs with reported physiological variation in dogs are haptoglobin, C-reactive protein and ceruloplasmin.

Variation of acute-phase protein is associated with the type and extent of stimuli, including pregnancy [31]. Sex hormones may play a role in regulating inflammatory responses, especially in the female gender. For example, estrogen has a proinflammatory effect, while progesterone replacement therapy decreases the levels of circulating inflammatory markers in women [21]. Progestins attenuate the proinflammatory effects of estrogens and may actually be responsible for changes in acute phase proteins [32]. Previous work reported that hormone replacement decreases the plasma concentration of fibrinogen, thereby suggesting a possible hormone-induced anti-inflammatory effect [27].

Acute-phase protein analysis is not widely employed in small animal medicine, although relevance has been pointed out. In fact, no reference values as a systematic clinical procedure have yet been established for canine species. The aim of this study was thus to propose reference values of certain APPs for healthy dogs during a specific period of the estrous cycle (diestrus) and, therefore, verify the influence of reproductive hormones on APP synthesis.

MATERIALS AND METHODS

Animals

Twenty Great Dane bitches aging 2-7 years old, clinically healthy and with no infectious or inflammatory process records were used. All the dogs were fed with the same commercial dog food and water was provided ad libitum. Sanitary management was uniform, all animals being helminth-free, periodically immunized and serologically tested for heartworm disease and brucellosis. Each animal’s health condition was verified through physical assessment and the absence of acute inflammatory or infectious processes was guaranteed by a total white cell count.

Blood sampling

Weekly blood samples were drawn from each bitch during 9 weeks starting with diestrus onset and continued until the beginning of anestrus, evaluated by vaginal cytology [24]. Therefore, a total of 180 samples were used for each acute-phase protein assay.

Animals were kept fasting for 12 h and blood samples were then taken by venipuncture (left or right cephalic vein). Sterile tubes containing gel and no anticoagulant were used for haptoglobin, ceruloplasmin, seromucoid and glycoprotein. Tubes containing EDTA as anticoagulant were used for fibrinogen and white blood cell count. Blood samples without EDTA were immediately centrifuged for 10 min at 1500 g. Blood samples with EDTA were processed at a maximum of 24 h, whereas serum aliquots were kept at -70°C until processing.

Acute-phase proteins assays

Plasmatic fibrinogen was measured according to the method based on fibrinogen denaturation caused by heat [18]. Fibrinogen concentration was assayed by refractometry and expressed as mg/dL.

Ceruloplasmin was determined through oxidase activity measurement, in which ceruloplasmin reacts with o-dianisidine dihydrochloride in acid medium in the presence of oxygen [25]. Color change from amber to violet was measured by spectrophotometry at 540 nm. Ceruloplasmin enzymatic activity was referred as IU/L.

Haptoglobin was assessed based on cyanmethemoglobin resistance to acid denaturation when bound to haptoglobin [9]. Results in mg/dL of HbCN binding capacity were obtained by spectrophotometry at 380 nm and 400 nm.

Seromucoid or mucoprotein was measured by means of a previously described method [5]. With the exception of seromucoid, proteins are precipitated by perchloric acid and then filtered away. Mucoprotein in solution then reacts with phosphotungstic acid and the turbidity was measured by spectrophotometry at 400 nm. Seromucoid values were expressed in mg/dL, using a factor of 964.33 obtained from a calibration curve based on turbidity produced by known levels of bovine serum albumin.

Glycoprotein was analyzed by a binding assay [7]. Serum glycoprotein binding to concanavalin A, with turbidity increase, was measured by spectrophotometer coupled to a computerized kinetic analysis system at 37°C. Results were expressed in percentage relative to a standard of known glycoprotein high level concentration.
Hormonal Assay

Serum progesterone and estrogen concentrations were measured by radioimmunoassay using a commercial kit (Progesterone Coat-a-Count and Estrogen Coat-a-Count - DPC®). The sensitivity of the progesterone assay at 96% binding was 0.026 ng/mL and the low and high intra-assay coefficients of variation were 12.82 and 2.50%, respectively. The low and high interassay coefficients of variation were 0.11 and 1.51%, respectively. For the estrogen assay, the sensitivity at 92% binding was 7.81 pg/mL and the low and high intra-assay coefficients of variation were 10.01 and 6.83%, respectively. The low and high interassay coefficients of variation were 2.96 and 4.27%, respectively.

Statistical Analysis

Statistical analysis was performed by means of the computer program SAS (Statistical Analysis System). All values of acute-phase proteins and hormonal concentrations were expressed as mean ± SD (standard deviation). The Tukey test for coupled samples (different weeks) was utilized. The statistical significance level of 5% (P < 0.05) was used to compare average values and to analyze variance.

RESULTS

No statistical variations (P < 0.05) on plasmatic fibrinogen, serum haptoglobin and ceruloplasmin concentrations were observed between weeks of diestrus (Table 1) nor a statistical correlation between the hormonal profile (Table 2). The reference values obtained for fibrinogen ranged from 145 to 220 mg/dL at 6th and 4th weeks of diestrus, respectively (182.22 ± 46.40 mg/dL). Minimum and maximum values of haptoglobin were 71.84 and 88.79 mg/dL, respectively, at 9th and 1st week of diestrus (80.22 ± 9.80 mg/dL of HbCN binding capacity for haptoglobin). Average values of ceruloplasmin ranged between 7.12 and 9.68 IU/L at 5th and 7th weeks of diestrus (8.19 ± 1.68 IU/L) [Table 1].

Regarding seromucoid concentration, significant variation (P < 0.05) of mean values was detected mainly between the first two and the following weeks (Table 1), although no statistical correlation was verified between seromucoid and hormone profile (Table 2). Significant increase in seromucoid concentration was verified between the 1st and 6th week of diestrus. Seromucoid mean values ranged from 66.7 to 90.4 mg/dL at 1st and 5th weeks of diestrus, respectively (79.88 ± 15.78 mg/dL).

Significant variation (P < 0.05) of glycoprotein was measured between the 1st and 3rd weeks of diestrus, as well as between weeks 2 and 9 (Table 1). No statistical correlation between glycoprotein and hormone profile was verified (Table 2). Mean values ranged between 11.28 and 12.72%, respectively, at 1st and 9th weeks of diestrus (11.89±0.92 %) [Table 1].

Regarding the progesterone profile, decreased values from the 2nd week onward was verified, hence resulting in a statistical difference between the initial weeks of diestrus and the final weeks (Table 1). In relation to the estrogen status during diestrus, no such differences were assessed (Table 1). It is important to explain that the sensitivity of the estrogen test utilized was low, therefore, the results obtained in all weeks, except for the 1st and 5th, were below 7.81 pg/mL. However, for statistical proposes those results were considered as equal as 7.81 pg/mL.

Table 1. Mean and standard deviation (X ± SD) of fibrinogen (mg/dL), haptoglobin (mg/dL), ceruloplasmin (IU/L), seromucoid (mg/dL), glycoprotein (%), progesterone (ng/dL) and estrogen (pg/dL) concentrations in Great Dane female dogs (n = 20) during 9 weeks of diestrus.

<table>
<thead>
<tr>
<th>Week</th>
<th>Fibrinogen</th>
<th>Haptoglobin</th>
<th>Ceruloplasmin</th>
<th>Seromucoid</th>
<th>Glycoprotein</th>
<th>Progesterone</th>
<th>Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>155 ± 60.48</td>
<td>88.79 ± 23.03</td>
<td>9.11 ± 4.21</td>
<td>66.70 ± 14.47</td>
<td>11.28 ± 1.63</td>
<td>25.07 ± 8.35</td>
<td>8.38 ± 1.58</td>
</tr>
<tr>
<td>2</td>
<td>155 ± 58.95</td>
<td>78.32 ± 23.00</td>
<td>7.91 ± 4.71</td>
<td>70.59 ± 19.96</td>
<td>11.40 ± 1.75</td>
<td>28.25 ± 9.26</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>210 ± 165.53</td>
<td>80.26 ± 26.82</td>
<td>8.28 ± 4.48</td>
<td>77.05 ± 20.70</td>
<td>12.29 ± 1.61</td>
<td>24.06 ± 9.26</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>220 ± 80.47</td>
<td>82.17 ± 35.43</td>
<td>7.22 ± 3.36</td>
<td>85.20 ± 23.11</td>
<td>11.85 ± 1.58</td>
<td>19.66 ± 6.09</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>185 ± 90.97</td>
<td>78.81 ± 34.14</td>
<td>7.12 ± 4.47</td>
<td>90.40 ± 27.47</td>
<td>11.82 ± 1.23</td>
<td>11.01 ± 4.63</td>
<td>8.11 ± 0.48</td>
</tr>
<tr>
<td>6</td>
<td>145 ± 144.57</td>
<td>84.44 ± 32.97</td>
<td>8.31 ± 4.77</td>
<td>89.23 ± 23.20</td>
<td>12.27 ± 1.55</td>
<td>6.44 ± 5.28</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>7</td>
<td>170 ± 72.63</td>
<td>81.49 ± 36.17</td>
<td>9.68 ± 5.74</td>
<td>80.90 ± 15.58</td>
<td>11.83 ± 1.50</td>
<td>4.52 ± 5.32</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>8</td>
<td>165 ± 90.00</td>
<td>75.82 ± 30.75</td>
<td>8.43 ± 4.59</td>
<td>80.28 ± 23.07</td>
<td>11.54 ± 1.69</td>
<td>2.30 ± 3.15</td>
<td>8.00 ± 0.0</td>
</tr>
<tr>
<td>9</td>
<td>185 ± 65.38</td>
<td>71.84 ± 32.20</td>
<td>7.68 ± 4.16</td>
<td>78.54 ± 32.90</td>
<td>12.72 ± 1.84</td>
<td>1.35 ± 1.96</td>
<td>8.00 ± 0.0</td>
</tr>
</tbody>
</table>

*a* Significant difference between the 1st and the 4th, 5th and 6th weeks (P < 0.05). b*b* Significant difference between the 2nd and the 5th and 6th weeks (P < 0.05). a* Same subscript letter between rows differ significantly (P < 0.05).
Table 2. Correlation coefficient (r) and probability value (P) of acute-phase proteins (fibrinogen, haptoglobin, ceruloplasmin, seromucoid and glycoprotein) and hormones (progesterone and estrogen) in Great Dane female dogs (n = 20) during 9 weeks of diestrus.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Progesterone</th>
<th>Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>r = 0.15; P = 0.69</td>
<td>r = -0.28; P = 0.46</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>r = 0.47; P = 0.20</td>
<td>r = 0.62; P = 0.07</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>r = -0.09; P = 0.81</td>
<td>r = 0.27; P = 0.48</td>
</tr>
<tr>
<td>Seromucoid</td>
<td>r = -0.56; P = 0.12</td>
<td>r = -0.49; P = 0.19</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>r = -0.46; P = 0.21</td>
<td>r = -0.50; P = 0.16</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The laboratorial diagnosis of subclinical acute diseases, follow-up of therapy and tumor evolution, establishment of specific prognosis and pregnancy diagnosis in bitches are among the various indications to undertake APP tests [31]. Nevertheless, the difficulty of data interpretation due to the absence of reference values for canine species represents an imperative obstacle for systematic clinical application. No research employing considerable number of samples from dogs under controlled sanitary management had been previously undertaken. Therefore, with the intention of facilitating clinical assessment of these parameters, physiological values for various acute-phase proteins were proposed in the present work, with special regard to hormonal influence.

In this work, the mean values of fibrinogen ranged from 145 to 220 mg/dL, which is slightly inferior to values proposed earlier for non-pregnant bitches in diestrus (< 230 mg/dL) [2]. Additionally, no statistical correlation was verified between fibrinogen and hormone concentrations in diestrous bitches in the present experiment. Conversely, in women, there is a relationship between fibrinogen and progesterone levels in early pregnancy. That is, progesterone mediates the relationship between low levels of coagulation factors and spontaneous abortion in women and seems to be the primary marker of this pregnancy disturbance [22]. On the other hand, no changes in fibrinogen level during short-term hormonal replacement therapy (17β-estradiol plus medroxyprogesterone acetate) occurs in women [33]. Fibrinogen determination can thus be utilized for dog patients with inflammatory process, coagulation disturbance and also to confirm pregnancy in bitches regardless of progesterone concentration.

Exogenous estradiol, and to a lesser degree progesterone, have been shown to stimulate haptoglobin secretion by isolated bovine liver parenchymal cells* in vitro* [14]. Additionally, increases in estrogen concentration or changes in progesterone/estrogen ratio during estrus or follicular waves throughout the cow estrous cycle induce haptoglobin synthesis in the bovine liver in *vivo* [19]. However, the present research was not able to demonstrate the influence of the hormonal status on hepatic synthesis of haptoglobin in diestrous bitches. This study failed to demonstrate any relationship between reproductive hormones and haptoglobin synthesis, especially in relation to progesterone profile. However, no such knowledge was reported in relation to proestrous or estrous bitches, a fact that definitely deserves further studies. For this reason, it is possible to infer that haptoglobin values can be employed as clinical analysis in spite of progesterone concentration. Thereby, haptoglobin assay could be requested when there is a suspicion of several diseases or pregnancy, regardless of the progesterone concentrations. The mean reference value of haptoglobin obtained in this study (80.22 ± 9.8 mg/dL) was similar to that verified by others for healthy dogs [4,12,28]. It is important to emphasize that during intravascular hemolysis reduced values are expected [4,17].

Ceruplasmin assay can be performed in cases of gestation diagnosis, inflammation, bacterial, viral and parasitic infections (increased values are expected). Reduced concentrations are found during birth, malnutrition, nephrosis and hepatic insufficiency caused by copper intoxication [8,17,31]. In this research, no variation among weeks of diestrus was observed, nor a statistical correlation between hormone profile. In respect to means values, we obtained results of 8.19 ± 1.68 IU/L, similar to previous reports for healthy dogs [28].

Seromucoid is considered the plasma protein responsible for the progesterone, cortisol and drug transportation [26]. This APP has the ability to bind steroid hormone such as progesterone in a physiological way, as progesterone interacts with the carbohydrate residues of seromucoid [1]. In the present experiment, although without statistical correlation between seromucoid and hormone profile, increased seromucoid was verified at mid-diestrus (4th, 5th and 6th weeks). Therefore, we can suggest an indirect relationship between progesterone and seromucoid levels. During the first half of the diestrous period (1st to 5th weeks), there is an intense corpus luteum steroidogenesis which will require seromucoid plasmatic increase for progesterone transportation. In fact, we observed decreased values of seromucoid from the 6th week onward as canine corpus luteum starts to
suffer luteolysis. The results on seromucoid (79.88 ± 15.78 mg/dL) are similar to mean values obtained by others for healthy dogs [3,10]. Dello et al. [6] and Ogilvie et al. [23], however, suggested a lower reference value for healthy dogs: 0.47 ± 0.09 g/dL and 310.5 ± 134.2 µg/mL, respectively. Different values are probably due to distinct assay techniques, but also relatively to the breed of the dog, as small dogs have lower seromucoid concentrations than larger dogs [30]. Nevertheless, these researches do not specify bitches estrous phase, which could have interfered with seromucoid reference values. Effectively, seromucoid determination can be requested to confirm suspicion of acute inflammation or neoplasia, including prognosis and tumor response during chemotherapy [8,23]. However, it is important to state that seromucoid determination should be requested concomitant with estrous cycle phase identification, in order to perform a realistic interpretation of the exam.

The acute-phase protein group consists mainly of glycoproteins, which have their synthesis stimulated during infectious and/or inflammatory episodes [3,8,11]. In addition, glycoprotein assay is useful in cases of degenerative diseases and pregnancy diagnosis [15,31]. Therefore, glycoprotein assay can indicate the overall profile of inflammatory process, being thus useful as a triage method [8]. However, steroid hormones are known to stimulate hepatic metabolism and protein synthesis in order to support hormonal biotransformation. In fact, in the present research, there was a higher glycoprotein level at early diestrus (3rd week), during which there is high progesterone concentration. Therefore, the possible explanation for the increase in glycoprotein concentration is the progesterone profile, nonetheless the lack in statistical correlation between these variables. The glycoprotein results of 11.89 ± 0.92% were similar to the reference value of 13% proposed elsewhere for healthy dogs [3], thereby suggesting as a reference value.

Progesterone and its derivatives have anti-inflammatory and immunosuppressive properties that are relevant for maintenance of pregnancy [29]. In addition, the progesterin component of hormonal replacement therapy lowers the estrogen-induced increase in APP concentrations [27]. Therefore, hormones may cause a changed steady-state metabolism, that is, increased synthesis or decreased clearance of acute-phase proteins [27]. For these reason, APP profile in bitches under progesterone influence has to be carefully interpreted. Hormonal relationship may alter protein results, leading to inferior results or false-negative data in case of inflammatory process simultaneously to progesterone influence (diestrus). Thus, this research has practical implications for the interpretation of laboratory exams of known physiological conditions, in special reference to endocrinological profile.

CONCLUSIONS

In conclusion, the hormonal profile has no influence on fibrinogen, haptoglobin and ceruloplasmin concentrations. On the other hand, the levels of seromucoid and glycoprotein vary according to the diestrous phase (or progesterone concentration), suggesting a differential APP profile in relation to the hormonal profile. However, additional studies are required to clearly establish the influence of reproductive hormones on inflammatory or non-inflammatory APP synthesis in the canine liver and reproductive tissues. In addition, research on proestrous and estrous bitches may clarify the modulation system influencing (diestrus). Thus, this research has practical implications for the interpretation of laboratory exams of known physiological conditions, in special reference to endocrinological profile.

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