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Acute phase proteins, hematological and serum biochemical profiles of female dogs in diestrus, mucometra and pyometra

[*Proteínas de fase aguda, perfis hematológicos e bioquímicos séricos de cadelas em diestro, mucometra e piometra*]

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ABSTRACT

Blood samples from 200 female dogs divided into 6 groups (diestrus, mucometra, pyometra) were evaluated, with the pyometra group categorized according to the ASA classification (American Society of Anesthesiologists), that is, from ASA II to V, totaling six groups. Aiming to analyze the acute phase proteins (APP), hematological and serum biochemical profiles of the female dogs in the study, establishing the differential diagnosis and prognosis according to the group. The SDS-PAGE method was used for protein fractionation, complete blood count using an automated hematological analyzer and histogram, biochemical tests performed using a semi-automatic spectrophotometer and measurement of serum concentrations of sodium and ionic calcium using the selective ion method. The results showed that female dogs with pyometra categorized in the ASA II to V classification revealed biochemical alterations between the study groups, as well as the acute phase proteins, presented variations according to the systemic involvement, degree of inflammatory response and ASA classification of the female dogs with pyometra, in which ceruloplasmin ($p=0.07$) and transferrin ($p=0.07$) did not show statistical significance, but showed an increase in their concentration according to the inflammatory evolution, albumin ($p<0.0001$), IgGCP ($p <0.0001$), haptoglobin ($p<0.0001$), alpha acid glycoprotein ($p<0.0001$), IgGCL ($p<0.0001$) and the one identified by its atomic weight 23,000 Da ($p=0.0031$), enabling the assessment of the acute phase response and aiding in the early detection of the systemic inflammatory response and better therapeutic guidance for the patient.

Keywords: female dog, inflammation, pyometra, acute phase proteins, uterus

RESUMO

Foram avaliadas amostras de sangue de 200 cadelas, divididas em seis grupos (diestro, mucometra, piometra), sendo o grupo piometra categorizado de acordo com a classificação ASA (American Society of Anesthesiologists), ou seja, de ASA II a V, totalizando seis grupos. O objetivo deste estudo foi analisar as proteínas de fase aguda (APP), os perfis hematológicos e bioquímicos séricos das cadelas do estudo, estabelecendo-se o diagnóstico diferencial e o prognóstico de acordo com o grupo. O método SDS-PAGE foi utilizado para fracionamento de proteínas. Foi feito hemograma completo com analisador hematológico automático e histograma, exames bioquímicos com espectrofotômetro semiautomático e medição das concentrações séricas de sódio e cálcio iônico pelo método de íons seletivos. Os resultados mostraram que cadelas com piometra categorizadas na classificação ASA II a V revelaram alterações

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bioquímicas entre os grupos de estudo, assim como as proteínas de fase aguda apresentaram variações de acordo com o envolvimento sistêmico, o grau de resposta inflamatória e a classificação ASA das cadelas com piometra, nas quais a ceruloplasmina (P=0,07) e a transferrina (P=0,07) não apresentaram significância estatística, mas apresentaram aumento em sua concentração conforme a evolução inflamatória, albumina (P<0,0001), IgGCP (P<0,0001), haptoglobina (P<0,0001), glicoproteína alfa ácida (P<0,0001), IgGCL (P<0,0001) e aquela identificada pelo seu peso atômico 23.000 Da (P=0,0031), o que possibilitou a avaliação da resposta de fase aguda e auxiliou na detecção precoce da resposta inflamatória sistêmica e na melhor orientação terapêutica para o paciente.

Palavras-chave: cadela, inflamação, piometra, proteínas de fase aguda, útero

INTRODUCTION

Pyometra, also called complex cystic endometrial hyperplasia, is an endometrial disease that most frequently affects adult non-sterilized female dogs, characterized by the uterine inflammatory process with accumulation of purulent secretion in the uterine lumen, generally in the luteal phase, resulting in systemic, hematologic, and variable imaging changes (Coggan *et al.*, 2008; Oliveira *et al.*, 2016; Riquelme and Ruiz, 2017). The etiology of pyometra can be established by the action of hormones, that is, the exacerbated endometrial response to chronic exposure to progesterone and estrogen, whether endogenous or exogenous, making the uterus vulnerable to bacterial infection (Chen *et al*., 2007; Hagman, 2016).

Female dogs with pyometra that present endotoxemia and sepsis frequently present dysfunctions in various systems and organs, especially in the maintenance of homeostasis (Tanja *et al*., 2006), which is why authors comment on possible changes in the concentrations of Acute Phase Proteins (APF) in the serum of female dogs with the disease, which may vary according to the severity of the clinical condition (Frasson *et al.*, 2004; Ceron *et al.*, 2005; Eckersall and Bell, 2010; Hagman, 2011; Kuleš *et al.*, 2020; Malin and Pitaszewicz, 2022). APFs are a group of serum proteins related to innate nonspecific immunity, maintenance of homeostasis and microbial growth, furthermore, their serum concentrations tend to be altered in the face of infectious and inflammatory processes, surgical procedures, trauma and some cases due to stress (Murata *et al.*, 2004; Petersen *et al*., 2004; Ceron *et al*., 2005; Dąbrowski *et al.*, 2013; Ruggerone *et al.*, 2021; Malin and Pitaszewicz, 2022).

During an acute inflammatory response, APFs may have their serum concentration altered by up to 25% in response to the pro-inflammatory cytokines that act in the focus of the lesion, stimulating the synthesis and release of APF mainly by the liver, and later by extra-hepatic tissues, such as adipose tissue, breast and lungs (Murata *et al.*, 2004; Ceron *et al.*, 2005; Gruys *et al.*, 2005). In addition, the development of pyometra is related to the release of chemokines, cytokines, extravasation of inflammatory cells, antibacterial action, complement system and innate immune responses (Hagman *et al*., 2009).

In view of the growing interest in evaluating acute phase responses in animals, innovative practices and advantages related to APF monitoring began to be described as advances with clinical and experimental purposes in many animal species, especially canines. In veterinary medicine, APP clinical applications have been documented in diagnosis (Hagman, 2011), prognosis/treatment (Kogika *et al*., 2003), staging (Eckersall, 2000; Mitchell *et al.*, 2009; Lucas *et al.*, 2010; Yuki *et al*., 2010) and evaluation of the chronic inflammatory process (Petersen *et al*., 2004; McGrotty *et al*., 2005; Eckersall and Bell, 2010).

In human medical practice, APFs such as CRP (C-Reactive Protein) and procalcitonin are commonly used as biomarkers for the diagnosis and prognosis of various pathologies (Hansson *et al.*, 1993; Werra *et al.*, 1997). In veterinary medicine, the use of APFs as biomarkers is arousing interest in several studies (Jitpean *et al.*, 2014), as in the evaluation of various proteins through inflammatory response in infected and inflamed uterine tissue during pyometra (Hagman, 2012), as markers in the differentiation between pyometra and cystic endometrial hyperplasia (Frasson *et al.*, 2004) and between different stages of the estrous cycle.

The diagnosis of pyometra occurs through ultrasound examination, which may be associated with microscopic and microbiological analysis of the contents of the uterine horn, however, these tests are not used as a criterion for prognosis or evaluation of the response to treatment in critically ill patients, showing the importance of assessing APF since they are considered as prognostic markers, assisting in decision-making regarding treatment (Ceron *et al*., 2005). APF analysis is considered a reliable test when measuring the systemic response of an inflammatory stimulus and is potentially more sensitive to inflammatory variations than other tests used in clinical practice, such as leukocyte counts (Dąbrowski *et al*., 2013), since the leukogram may not show changes in canine patients with pyometra (Kogika *et al*., 2003).

Pyometra has been associated with increased plasma levels of APF, as well as other bacterial infections (Ceron *et al.*, 2005; Fransson *et al.*, 2007), reflecting the important role of these proteins in modulating the inflammatory immune response, praising the relevance of the identification of APFs in understanding the staging, prognosis, and progression of diseases (Petersen *et al.*, 2004; Gruys *et al*., 2005; Nikunen *et al.*, 2007; Planellas *et al.*, 2009; Cetinkaya *et al.*, 2011; Dąbrowski *et al*., 2013). Some authors state through their research that APFs are useful biomarkers for predictive postoperative complications in female dogs with pyometra. They also report an increase in haptoglobin proportional to the inflammatory response compared to healthy female dogs (Yamamoto *et al*., 1993; Dabrowski *et al.*, 2009; Yoon *et al.*, 2021). Most proteins found as abundant proteins significantly differ between pyometra and control groups such as haptoglobin (Dabrowski *et al.*, 2013; Jitpean *et al.*, 2014; Yoon *et al.*, 2021), C-reactive protein, alpha acid glycoprotein -1 (Hagman, 2011; Dabrowski *et al*., 2013), ceruloplasmin, transthyretin, paraoxonase-1 (PON-1), H1 heavy chain inter-alpha-trypsin inhibitor, alpha-2-HS-glycoprotein and transferrin, findings which confirm the activation of the acute phase response in canine pyometra (Kuleš *et al*., 2020).

The present scientific study aims to compare hematological, biochemical and serum protein laboratory findings of female dogs in diestrus, mucometra or pyometra, the latter being organized according to the ASA classification.

The analysis of the severity of the inflammatory process related to the complex physiology of mucometra and mainly pyometra, materializes significant alterations in the concentration of acute phase proteins, knowing that the values in the plasmatic concentration of some of the proteins allow providing information on tissue damage and monitoring recovery from inflammation.

MATERIAL AND METHODS

The study began in March 2014 after approval by the Animal Ethics and Welfare Committee (CEUA) with protocol n° 7.797/16 of the *Faculdade de Ciências Agrárias e Veterinárias* - Campus of Jaboticabal (FCAV-UNESP).

The experiment included 200 female dogs, aged between 1 and 20 years and different breeds (94 mixed race, 20 Poodles, 14 Rottweilers, 13 Labradors, 12 Pitbulls, 9 Boxers, 6 German Shepherds, 6 Cocker Spaniels, 4 Pinschers, 3 Teckels, 3 Brazilian Queues, 2 Yorkshires, Weimaraner, Neapolitan Mastin, Lhasa Apso, Pointer, Chow Chow, 1 Schnauzer, Golden Retriever, Great Dane and Beagle), being distributed in six groups, organized in Table 1.

The mean weight of the animals in the diestrus group was 12.72±7.12; mucometer 17.88±11.67; and pyometra 21.23±15.33.

Female dogs were divided into groups according to the classification by Benson *et al*., 1997 (Table 1).

The inclusion criteria for the animals screened for the experiment were hematological laboratory evaluations (hemogram, biochemical – described in Table 3 and 4), US with characteristics of pyometra, mucometra and healthy animals.

The excluded animals were those with concomitant illnesses, such as mammary neoplasms, skin wounds, continuous treatments for other illnesses. The exclusion of these female dogs with these characteristics aimed to prevent interference in the responses of Acute Phase Proteins, as well as possible changes in the

results of hematological and biochemical analytes.

For the proper distribution of female dogs in their respective groups, detailed anamnesis, physical examination, hematological and biochemical analyses, and abdominal ultrasound were performed. The sonographic findings that led to the diagnosis of pyometra were an increase in uterine diameter, filled with hypoechoic or anechoic contents and cysts on the endometrial surface.

Table 1. Distribution of female canines into six groups according to uterine involvement and ASA classification (American Association of Anesthesiologists)

Group	Description	N° of animals
G1	Diestrus, healthy females without alterations in clinical routine laboratory tests, being submitted to elective ovariohysterectomy.	39
G2	Mucometra.	43
G3	Pyometra, ASA II classification - mild systemic disease, geriatric patients, and localized infections.	4
G4	Pyometra, ASA III classification - moderate systemic disease, moderate dehydration, hypovolemia, anorexia, cachexia, and anemia.	50
G5	Pyometra, ASA IV classification - severe systemic disease, shock, uremia, toxemia, severe dehydration, hypovolemia, severe anemia, decompensated kidney disease.	48
G6	Pyometra, ASA V classification - encompassing dying females with no expectation of survival with or without a surgical procedure within 24 hours of diagnosis, multiple organ failure and shock.	16

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Blood collection from female dogs was performed with local antisepsis with iodized alcohol and jugular vein puncture with a vacuum collection system in silicone plastic tubes with EDTA anticoagulants and without anticoagulant type 16x100mm vacutainer tube, volume of 10 mL for pre-anesthetic and pre-surgical evaluation. The hematological analysis used an automatic hematological analyzer to evaluate the hemogram and histogram parameters, consisting of the analysis of the red series with data on erythrocyte counts, hemoglobin dosage, hematocrit, medical corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, analysis white series with total and differential leukocyte count. The tubes without anticoagulant were centrifuged at 1.400g for 20 minutes, and 1.5mL of serum were aliquoted for biochemical profiling and storage in eppendorf tubes, frozen at -20°C until APF analysis was performed. The biochemical profile consisting of cholesterol, triglycerides, creatinine, urea, alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (AP), sodium, ionic and total calcium (Ca), chloride, magnesium and phosphorus were performed using commercial reagents *Labtest Diagnóstica*® on a *Labquest/Labtest Diagnóstica*® semi-automatic spectrophotometer, while the serum concentrations of sodium and ionic calcium were analyzed using the ion-selective method equipment 9180 electrolyte analyzer.

Total serum protein concentration was determined by the biuret method, using a set of commercial reagents (*Labtest Diagnóstica*). Sample readings were performed in a semi-automatic spectrophotometer (*Labquest, Labtest*), with specific wavelengths for the test.

For protein fractionation, the SDS-PAGE technique proposed by Laemmli (1970) was used. After performing the electrophoresis, the gels were stained for 10 minutes in a colloidal solution of 0.25% Coomassie Brilhante blue R solution and subsequently placed in a 7% (v/v) acetic acid solution to remove excess dye and 25% (v/v) methanol until protein fractions are clear.

The molecular weight (MW) and the concentration of protein fractions were determined using computer-assisted densitometry (CS-9301PC, Shimadzu Corporation). The densitometric evaluation of the protein bands was performed using the standard wide-range marker solution (MW 6.500 to 200.000 Da, Sigma-Aldrich S8445). Reference curves were created and used to generate computer graphics that would allow analysis of MW and protein concentrations of interest, which were calculated based on the total protein previously measured by the biuret method. Of the 10 fractions analyzed, seven were nominally identified, among them: ceruloplasmin, transferrin, albumin, heavy chain IgG (IgGCH), haptoglobin, alpha-1 acid glycoprotein, light chain IgG (IgGCL).

The variables age, weight, biochemical profile, serum proteinogram were submitted to analysis of variance (ANOVA-procedure GLM) and Tukey's test for comparison between pairs of means, at a 5% significance level. Statistical analyzes were performed using a computerized statistical program (SAS-version 9.1).

RESULTS AND DISCUSSION

Patients diagnosed with pyometra belonging to groups G3, G4, G5 and G6 showed several clinical signs, such as anorexia (27%), hyporexia (25%), vaginal discharge (23%), apathy (22%), polydipsia (19%), emesis (15%), weight loss (11%), dyspnea (11%), hyperthermia (9%), prostration (9%), polyuria (7%), pain abdominal (6%), hematuria (4%), oliguria (3%), hypoplasia (3%), diarrhea (3%) and dysuria (2%).

Pyometra can result in varying degrees of systemic disease, since the colonization capacity of bacteria, toxin production, and activation of inflammatory mediators directly influence the clinical manifestation of the animal, and clinical signs are of paramount importance in establishing the severity of clinical condition and prognosis (Noakes *et al.*, 2001; Pretzer, 2008; Hagman, 2022).

The exclusion of was based on the animals that felt concomitant illnesses (Tothova *et al*., 2016), as described in trauma (Conner *et al*., 1988), polytrauma (Ruggerone *et al*., 2021), intestinal parasites (Akdogan *et al*., 1999), leishmaniasis (Martinez-Subiela *et al*., 2002), hemoparasites (Lobetti *et al*., 2000), neoplasms (Battisti *et al*., 2013), pancreatitis (Yoon *et al*., 2021), in physiological states such as age (Fayos *et al*., 2005), pregnant female dogs, embryonic implantation, placental growth and hormonal alterations were elucidated in several studies (Eckersall *et al*., 1993; Vanucchi *et al*., 2002; Kuribayashi *et al*., 2003; Ulutas *et al*., 2009). Similarly, animals with alterations in the results of hematological and biochemical analytes (Barsanti, 2006; Verstgen *et al*., 2008; Küplülü *et al*., 2009).

The data for the races described in this experiment differ from those reported by Hagman (2022), probably due to the incidence of certain races in different areas, but they are similar to those reported by Ahn *et al*. (2021).

Female dogs with open cervix pyometra frequently present vaginal discharge ranging from mucopurulent to serosanguinolent and abdominal pain, in addition to clinical signs such as prostration, anorexia, polyuria, polydipsia, emesis (Johnston *et. al*., 2001), dehydration and hyperthermia (Verstegen *et al.*, 2008) while tachycardia and tachypnea commonly occur as a result of the systemic effects of infection (Fransson *et al*., 2007).

Regarding the use of progestogens, female dogs in the diestrus phase (G1) showed a predominance of the absence of the use of these drugs (81%) compared to the use (19%). Female dogs with mucometra (G2) showed a predominance of females with a negative history of progestogens use (63%) compared to females who underwent progestogens administration (37%). In relation to females with pyometra (G3, G4, G5 and G6), females that did not use progestogens (76%) were also predominant in the number of females that used these drugs (24%), it should be noted that female dogs with pyometra were not evaluated in isolation for the use of progestogens and the ASA groups.

As described in Table 2, there is a higher mean age in months in female dogs in the pyometra group, especially in those in ASA III, IV and V. It is believed that older female dogs with uterus that have undergone several estrous cycles are more predisposed to the development of pyometra (Chen *et al*., 2007), due to the repeated stimulation of progesterone in the luteal phase (Melo *et al.*, 2020).

Table 2. Means± Standard deviation (SD), minimum and maximum values, and age range in months of female dogs in the diestrus phase and affected by mucometra and pyometra (ASA II, III, IV, and V)

			Pyometra (n=118)			
Age (months)	Diestrus (n=39)	Mucometra (n=43)	ASA II (n=4)	ASA III (n=50)	House IV (n=48)	House V (n=16)
Means ± SD	41.27 ±22.60	96.61±43.41	41.0±22.0	88.67±32.22	98.68±40.20	109.84±41.52
(min-max)	(8-110)	(23-201)	(20-72)	(24-156)	(12-244)	(60-204)
Amplitude	102	178	52	132	232	144

Table 3. Mean, minimum and maximum blood count and leukogram values in the diestrus, mucometra and pyometra groups (ASAII, III, IV, and V)

		He (µL)	Hb (g/dL)	Ht (%)	Le (µL)	Bas (Vr)	Eos (Vr)	NBas (Vr)	NSg (Vr)	Lymph (Vr)	Mon (Vr)	Plat (µL)
Diestrus	Means	5508±	12.1±	38.1±	9425.9±	2.8±	575.9±	201.7±	6307.8±	2003±	365.7±	261037.3±
	± SD	1432	3.2	10.6	4492.7	0.1	5.9	2.7	11.0	8.3	1.9	152933.9
	Min	2620	5.6	18.6	3500	0	942	0	3581	471	188	68000
	Max	8790	20.3	65.2	23200	942.5	2167	1131	7540	4524	9425	886000
Mucometra	Means	5793±	13.94±	43.8±	15043±	15.0±	1037±	605.3±	10805±	2381±	500±	349448±
	± SD	1992	3.2	9.9	13305	0.4	4.4	2.3	9.7	8.4	1.2	174703
	Min	3.5	8.1	26.5	4000	0	0	0	5423.6	451.2	300.8	124000
	Max	8720	21.5	67.6	59900	300.8	2557	25573	14140	6017	1053	900000
Pyometra	**ASA II**											
	Means	4235±	9.92±	30,22 ± 14	33950±	0	594.1±	3564±	25207±	2970±	1612±	212750±
	± SD	212	4		19747.4	0	1.5	6.4	5.1	2.7	2.2	102265
	Min	1560	4.1	11.7	7000	0	339.5	2716	22746	2037	679.0	118000
	Max	6670	15.4	46.8	50700	0	1358	6790	26820	4074	2376	358000
	ASA III											
	Means	4998±	11.6±	35.1±	23750±	9.5±	964.2±	1645±	17195±	2959±	983.2±	279163±
	± SD	1496	3.3	10.5	23749	0.1	4.3	8.0	10.1	6.8	3.0	153517
	Min	1090	2.6	7.9	12.3	0	0	0	9480	711	237	29000
	Max	8110	18.5	57.2	80000	237.0	2137	7110	21330	7584	4740	754000
	ASA IV											
	Means	5598±	12.78±	39.68±	26892.2±	83.3±	968.1±	1718±	19279±	3834±	1175±	272459±
	± SD	1327	3.5	10.7	23831.9	1.5	3.5	8	10.8	9.5	4.1	149909
	Min	3030	6.3	19.6	24.3	0	0	0	35	3	1	83000
	Max	8250	21.5	67.9	150000	10	16	40	89	57	27	593000
	ASAV											
	Average	4943±	11.28±	34.3±	32178.5±	0	10554±	3539±	23329±	3102±	894.5±	280714,1±
	± SD	1323	3.0	9.9	23145		3.6	15.3	12.9	4.1	1.0	144180.6
	Min	2020	4.7	12.5	8100.00	0	0	0	3192.9	185.9	321.7	108000
	Max	6460	15.8	47.0	76800	0	4183	4183	27968	5786	1608	523000

Vr= relative value (mm^3) d; He= Red Blood Cells; Hb=Hemoglobin; Ht=Hematocrit; Le=Total Leukocytes; Bas=Basophils; Eos=Eosinophils; NBas=Neutrophils Rods; NSg=Segmented Neutrophils; Lymph=Lymphocytes; Mon=Monocytes; Plat=Platelets.; Min= Minimum; Max=Maximus.

Mild anemia was detected in female pyometra with ASA II, III and V classification, an expected event in patients with pyometra, either due to the loss of red blood cells due to diapedesis into the uterine lumen or due to toxic depression of erythropoiesis, it should also be noted that the anemic condition is normally reestablished when pyometra is properly treated (Martinuzzi *et al.*, 2016). Leukocytosis due to left-sided neutrophilia was found in female dogs with pyometra classification ASA II, III, IV, and V, and this leukocytosis with or without toxic changes in neutrophils is considered to be common and predominant findings in females with pyometra, with leukocytosis tending to be more elevated in female dogs with closed cervix pyometra (Pretzer, 2008). Leukocytosis is refractory to neutrophilia resulting from the inflammatory response and to the shift to the left that occurs as a result of the exacerbated synthesis and release of immature neutrophils into the bloodstream (Torres *et al.*, 2019). Hematologic changes in blood counts and leukograms in patients with pyometra are expected to reach normal levels after surgical treatment (Bartoskova *et al*., 2007).

Table 4. Means, Standard Deviation and maximum and minimum values of the biochemical profile of female dogs on the diestrus, mucometra and pyometra (ASA II, III, IV and V)

	Diestrus	Mucometra	Pyometra				P value
			ASA II	ASA III	ASA IV	ASA V	
Cholesterol (mg/dL)	B	B	AB	AB	AB	A	0,0013
Means ± DP	195.13±14.78	187.20±41.19	200.85±68.70	230.04±68.70	228.18±92.74	268.29±92.81	
Min.	105.16	119.00	120.91	95.99	92.74	133.50	
Máx	197.50	264.20	260.49	470	556.65	497.45	
Triglycerides (mg/dL)	A	AB	AB	B	AB	B	0,01
Means ± DP	70.17±5.13	83.87±55.40	60.24±30.5	104.29±65.58	97.29±39.64	118.46±93.19	
Min.	38.91	27.06	20,17	26.32	37.43	37.59	
Máx	71.00	305.05	93.42	386.60	197.27	428,58	
Creatinine	A	A	AB	AB	B	AB	0,0137
(mg/dL)	0.99±0.05	1.04±0.24	0.83±0.07	1.37±1.27	1.77±1.81	1.62±1.17	
Means ± DP	0.64	0.54	0.76	0.42	0.49	0.74	
Min.	1.00	1.55	0.93	6.61	7.08	5.42	
Máx							
Urea(mg/dL)	A	A	A	A	A	A	0,0606
Means ± DP	40.30±1.88	31.0±14.60	30.90±13.26	45.95±43.95	65.07±95.64	65.82±75.53	
Min.	40.00	10.76	17.66	7.56	9.34	14.39	
Máx	51.75	91.58	49.06	204.43	484.22	313.55	
ALT (U/L)	A	B	AB	B	B	B	<0,0001
Means ± DP	48.46±3.33	35.81±23.64	22.26±14.42	27.15±20.19	129.97±118.1	21.60±12.22	
Min.	28.19	10.48	5.23	5.23	16.58	5.23	
Máx	49	131	36.67	89.05	501.60	41.90	
AP (U/L)	AB	B	AB	AB	A	A	<0,0007
Means ± DP	85.37±2.32	56.50±60.48	80.84±28.22	104.87±92.46	129.97±118.1	140.70±102.0	
Min.	85.00	8.29	41.46	24.88	16.58	41.46	
Máx	99.50	340.00	107.80	472.60	501.60	368.95	
AST(U/L)	A	B	BC	CD	CD	BD	<0,0001
Means ± DP	48.54±2.81	21.73±6.76	19.64±11.61	35.45C±25.47	34.25±20.83	28,98±18.34	
Min.	31.43	10.48	10.48	10.48	2.95	10.48	
Máx	49.00	36.67	36.67	125.70	94.28	73.33	
Na (mmol/L)	A	AB	AB	B	B	AB	0,0009
Means ± DP	151.07±0.48	149.45±4.37	146.25±5.18	146.29±8.38	146.30±6.16	148.73±5.56	
Min.	151.00	139.00	139.00	118.00	127.00	134.00	
Máx	154.00	161.00	150.00	163.00	160.00	157.00	
Ionic Ca (mEq/dL)	A	B	B	B	B	B	<0,0001
Means ± DP	5.48±0.71	0.94±0.212	0.90±0.24	0.98±0.28	1.03±0.21	0.96	
Min.	1.16	0.64	0.64	0.38	0.54	±0.290.32	
Máx	5.60	1.34	1.24	1.47	1.36	1.33	
Total Ca (mg/dL)	AB	A	AB	B	A	AB	0,0019
Means ± DP	9.91±0.06	9.28±1.27	9.01±0.75	10.25±1.79	9.01±1.70	9.69±1.81	
Min.	9.90	6.34	8.30	7.14	5.23	3.78	
Máx	10.32	12.57	10.00	14.53	12.44	11.91	
Chloride (mEq/dl)	B	A	AB	AB	B	AB	0,0027
Means ± DP	110.00±5.06	119.31±7.27	114.60±3.99	112.92±12.92	112.49±13.23	117.24±15.66	
Min.	105.00	107.00	11.50	85.60	69.40	84.50	
Máx	115.00	138.10	120.10	140.65	144.20	152.15	
Magnesium (mg/dL)	A	A	A	A	A	A	0,5848
Means ± DP	2.08±0.12	1.65±0.32	1.40±0.40	5.71±23.73	1.72±0.55	1.83±0.62	
Min.	1.32	1.10	1.11	0.65	0.95	0.59	
Máx	2.10	2.46	2.00	152.00	3.04	2.91	
Phosphorus (mg/dl)	A	B	ABC	BC	BC	C	<0,0001
Means ± DP	3.85±0.04	5.79±1.75	5.61±0.58	7.40±3.61	6.44±2.84	8.21±3.85	
Min.	3.85	2.90	5.23	3.80	2.81	3.45	
Máx	4.15	11.21	6.47	23.65	20.30	19.26	

Different letters on the same line differ from Tukey's yesye ($p<0.05$).
ALT= Alanine Amino Transferase; AP= Alkaline Phosphatase; AST= Aspartate Amino Transferase; Na= Sodium; Ca= Calcium. Min= Minimum; Max=Maximus.

There was a statistical difference between the groups studied for cholesterol (p=0.0013), especially the patients with a pyometra classification ASA V compared to the other groups, and the increase in cholesterol levels may be related to the increase in AF, also with a statistically significant difference (p=0.0007) occurred in the mentioned group, as a consequence of a possible intrahepatic cholestasis related to the systemic inflammatory response and primary hepatocyte injury (Barsanti, 2006). The increase in FA can also be justified by the possible septicemia and tissue hypoxia as an evolution of the clinical condition of the patient with pyometra (Nelson and Couto, 1998). Serum triglyceride levels (p=0.01) were statistically different mainly in relation to the ASA III and V groups compared to the other groups, related to possible lipolysis and anorexia in female dogs with pyometra (Voorwald, 2014). Regarding creatinine, there was a statistical difference between groups (p=0.137), especially in relation to female dogs with pyometra classification ASA III, IV and V compared to the other groups, a fact that may be associated with protein catabolism, as well as with dehydration, reduced renal perfusion and toxemia (Fransson and Ragle, 2003; Fransson *et al.*, 2007; Küplülü *et al*., 2009), a fact that may also explain the increase in urea, although without statistical difference, in the pyometra ASA V and VI groups. Enzymatic activity ALT values remained within the reference values, however a reduction in the serum activity of this enzyme was observed in female dogs with pyometra (ASA II, III, IV and V), a fact that may reflect the inhibition of the hepatic synthesis of this enzyme by the action of endotoxins produced by the inflammatory process (Schepper *et al.*, 1987; Verstegen *et al.*, 2008; Hagman *et al.*, 2009; Küplülü *et al*., 2009). Serum AST levels were shown to be high in female dogs with pyometra classification ASA III and IV compared to the other groups, due to the possible increase in the permeability of the hepatocyte membrane due to pyometra (Meyer *et al*., 1992; Küplülü *et al.*, 2009).

Serum sodium values remained within normal limits in all groups, as also observed by Hagman *et al.* (2009). Both the ionic Ca ($p < 0.0001$) and total Ca ($p = 0.0019$) values showed significant statistical differences between groups, however, with a slight reduction from the diestrus group to the ASA IV pyometra group, in addition, with respect to the ionic Ca, there was a significant reduction in concentrations serum levels of this in the groups of female dogs with inflammatory disease, a fact also reported by Hagman *et al.* (2009) which indicate that patients with septic shock tend to have a reduction in serum calcium levels. Serum chloride concentrations showed a statistical difference (p=0.0027), with a mild increase in the mucometra group and in the ASA V pyometra group, and hyperchloremia may be due to dehydration (Voorwald, 2014). There were no significant differences in serum magnesium concentration (p=0.5848) with oscillatory values between the groups studied, with a significant increase in the pyometra ASA III group, which may be due to renal impairment or alteration in urinary excretion in females with pyometra (Kaneko, 1997). Regarding the serum phosphorus level, there was a statistically significant difference ($p<0.0001$) between the groups studied, especially those with more significant systemic involvement, such as the mucometra and pyometra group (ASA II, III, IV and V), so that phosphorus retention in the circulation blood is one of the factors for the progression of kidney disease, both acute and chronic, including decreased phosphorus excretion leading canine patients to death (Figueiredo *et al*., 2017). Thus, evaluating the results of the present study, the altered calcium and phosphorus ratio is observed, in addition to the increases in creatinine, and even though the urea was not statistically significant, its concentration was high, perhaps justified by renal impairment in female dogs affected by the evolution of pyometra.

Alterations in the profile of serum proteins by the electrophoresis method were observed in female dogs with pyometra as in the study described by Yoon *et al.* (2021).

Of the 10 fractions analyzed, seven were nominally identified (described in Table 5), among them: ceruloplasmin, transferrin, albumin, IgG heavy chain (IgGCP), haptoglobin, alpha-1 acid glycoprotein, IgG light chain (IgGCL).

In serum concentrations of total PTN, it was observed statistical difference (p=0.0005) between the groups studied, a fact that highlights the use of acute phase proteins to assess the

severity of inflammatory processes in female dogs with pyometra, where there may be an alteration in the serum protein level due to the action of endotoxins (Faria Junior, 2004).

In serum concentrations of ceruloplasmin and transferrin, was not observed statistical difference between the groups studied (p=0.07 and p=0.66 respectively), but ceruloplasmin showed an increase in its concentration in relation to the increase in the inflammatory degree and the severity of the clinical condition of the groups studied, as also observed by Serin and Ulutas (2010), who found an increase in this protein in female dogs after ovariohysterectomy as treatment for pyometra, as well as Ulutas *et al.* (2009) showing an increase in female dogs in the final third of pregnancy. Ceruplasmin acts as an inflammatory agent, as it can minimize the formation of the superoxide anionic radical, generated by polymorphonuclear leukocytes, during the inflammatory process, which may cause greater tissue damage (Broadley and Hoover, 1989).

Table 5. Means, Standard Deviation and maximum and minimum values of the serum total protein concentration (total PTN) and of the protein fractions obtained from female dogs on the diestrus, mucometra and pyometra (ASA II, III, IV and V)

		Total PTN (g/dL)	Ceruloplasmin (mg/L)	Transferrin (mg/L)	Albumin (mg/L)	IgGCH (mg/L)	Haptoglobin (mg/L)	α-1 acid glycoprotein (mg/L)	IgGCL (mg/L)	23000 Da (mg/L)
	Diestrus	A	A	A	A	C	A	AB	A	A
	Means ± DP	6.85	28,37	173.25	4259.92	896.34	93.3	57.12	471.83	658.61
		±1.06	±22.68	±107.75	± 599.56	±377.68	±87.83	±37.16	±379.33	±182.09
	Minimum	5.80	0.95	0.21	2860.62	399.21	12.33	4.31	77.54	356.06
	Maximus	7.90	114.6	562.52	5262.51	2076.92	463.67	187.21	1725.58	974.96
	Mucometra	B	A	A	B	BC	A	A	A	ABC
	Means ± DP	5.98	38.83	1146.0	3226.15	1061.65	130.09	45.78	506.24	582.70
		±1.06	±26.50	±61.11	±638.99	±543.45	±172.4	±22.47	± 315.40	±175.18
	Minimum	2.62	3.85	31.73	854.98	32.30	8.36	4.94	133.18	164.69
	Maximus	8.86	148.92	290.06	4613.10	2490.36	908.86	98.32	1710.33	934.57
	ASA II	AB	A	A	ABC	ABC	A	ABC	AB	AB
	Means ± DP	7.36	17.43	163.39	3673.38	1093.28	195.06	74.15	1014.26	798.01
		±1.57	±18.27	±122.5	±409.07	±389.98	±271.8	±34.2	±747.68	±144.56
	Minimum	5.05	1.52	24,23	3167.76	552.21	21.14	28.93	202.60	636.95
	Maximus	8.49	43.75	289.08	4133.82	1458,15	600.44	111.72	1960.93	938.48
	ASA III	A	A	A	BC	BC	A	B	B	ABC
	Means ± DP	7.21	36.60	146.33	3039.59	1713.78	356.32	77.96	962.66	583.43
		±1.76	±20.64	±77.76	±857.02	±883.01	±449.65	±55.74	±647.79	±217.25
	Minimum	4.24	10.70	18.59	1419.98	175.81	5.36	11.31	150.02	237.21
	Maximus	15.00	104.26	386.0	5969,00	4663.00	313 9.23	302.79	3464.85	981.12
	ASA IV	A	A	A	CD	CD	A	B	B	BC
	Means ± DP	6.91	41.43	139.39	2722.42	1653.07	371.64	88.13	1019.07	525.14
		±1.39	±26.97	±83.17	±771.64	±991.57	±218.96	±55.42	±814.25	±245.25
	Minimum	4.12	2.71	14.35	804.54	396.30	39.39	12.12	73.68	147.02
	Maximus	10.52	152.40	451.0	4659.76	5198.24	822.49	306.09	4196.32	1349.98
	ASA V	AB	A	A	D	D	A	C	B	C
	Means ± DP	6.97	45.39	152.34	2399.78	1626.26	519.29	130.23	1181.10	467.58
		±1.46	±37.39	±62.23	±474.93	±778.73	±330.88	±73.49	±778.91	±160.33
	Minimum	3.67	1.06	43.96	1585.99	392.02	29.73	5.12	62,97	299.91
	Maximus	9.33	135.26	251.42	3268.36	3161.57	1057.10	313.86	2457.78	797.64
	P value	0.0005	0.07	0.66	<0.0001	0.0391	<0.0001	<0.0001	<0.0001	0.0031

Pyometra

Total PTN= total protein; Da= Dalton; IgGCH= Heavy Chain Immunoglobulin G; IgGCL= Light Chain Immunoglobulin G.

Transferrin despite the fluctuations between the groups studied, serum values decreased according to the aggravation of the disease, so that the function of transferrin is to bind to Fe free, reducing it and providing an unfavorable environment for the survival of bacterial agents,

as verified in the groups studied (Jain *et al*., 2011). The same observed by Fam (2012), stating the decrease of this fraction due to the increase in the hepatic synthesis of anti-inflammatory proteins, decreasing the synthesis of less active protein fractions during inflammation (Eckersall, 2008).

Serum albumin concentrations showed a statistical difference between the groups ($p<0.0001$) mainly between the control groups and the mucometra and pyometra groups (ASA II, III, IV, and V), its concentration showed decrease in inflammation or infection, by deficiency in production or an increase in vascular permeability, as well as the presence of proteinuria (Céron *et al.*, 2005), so that hypoalbuminemia may be the result of liver damage resulting from pyometra or sepsis (Ralphs *et al*., 2003; Trhall, 2007; Yoon *et al.*, 2021; Janković *et al*., 2022).

The serum concentrations of IgGCH and IgGCL showed a statistical difference between the groups studied ($p<0.0001$ and $p<0.0001$ respectively), with an increase in serum concentrations according to the progression of the inflammatory process and the consequent severity of the disease, so that the increase in this protein may be justified by the production of immunoglobulins to be synthesized by plasmocytes, cells derived from the activation of B lymphocytes, after stimulation by cytokines produced at the site of inflammation from activated T-helper lymphocytes (Tizzard, 2002).

The serum concentrations haptoglobin showed a significant difference between the groups studied ($p<0.0001$), behaving as a positive acute phase protein, because the serum levels of this protein may be high as a result of a chronic inflammatory process (Yamamoto *et al.,* 1993; Dabrowski *et al.,* 2007), as reported by Dabrowski (2013), Hagman (2012) and Yoon *et al.* (2021) suggesting an inflammatory monitoring marker in pyometra.

Elevated concentrations of haptoglobin were also observed in female dogs with benign and malignant mammary neoplasms (Battisti *et al*., 2013), hyperadrenocorticism (Caldin *et al.*, 2009), nasal neoplasia (Sheahan *et al.*, 2010), parvovirus (Kocaturk *et al.*, 2010). Yang *et al.* (2003) state that this increase may be related to the fact that haptoglobin prevents tissue oxidative damage by binding to free hemoglobin, since haptoglobin has a high oxidative capacity. In this study, we obtained 4.1 times increase in haptoglobin in G2 when compared to G1 and can be considered as a moderate protein for pyometra. In this sense, other authors report that the haptoglobin protein is considered a slow-reacting protein, increasing its levels gradually (Dabrowski *et al.,* 2007, 2009, 2013; Eckersall and Bell, 2010; Serin and Ulutas, 2010.

Alpha-1 acid glycoprotein positive acute phase with increased concentration in female dogs with inflammatory and infectious conditions, showed statistical difference in the studied groups ($p<0.0001$). Hagman (2011) observed in his studies with pyometra in female dogs an increase in the average concentration of this protein four times higher compared to healthy female dogs. Previously, the same was reported by Hayashi *et al.* (2001) in female dogs submitted to ovariohysterectomy. In systemic tissue lesions, the change in the concentration of AGA is expected due to the protective effect it plays with its elevation in the process (Hochepied *et al.*, 2003), as an immunomodulatory agent and as a plasma transport protein (Céron *et al.*, 2005).

CONCLUSIONS

Agarose gel electrophoresis allowed detecting changes in serum concentrations of ceruloplasmin, transferrin, albumin, heavy chain IgG (IgGCP), haptoglobin, alpha-1 acid glycoprotein, light chain IgG (IgGCL) fractions in the determined animals.

The alterations revealed are due to the inflammatory progression and consequently the severity of the disease, mainly in the pyometra group in female dogs with the ASA classification II, III, IV and V, justifying the alterations in the serum concentrations of the biochemical tests between the study groups, being the most relevant in cholesterol, triglycerides, creatinine, ALT, ALP, AST, Na, Ca ionium and total, chloride and phosphorus.

The alterations in the laboratory evaluations in the six groups demonstrated are fully related to the worst prognosis, but there was no postoperative clinical and laboratory evaluation,

not obtaining data to determine how long the alterations would remain.
Acute phase proteins show changes according to systemic involvement, degree of inflammation and ASA classification, especially albumin, haptoglobin, alpha-1 acid glycoprotein.

The data obtained from acute phase proteins associated with alterations evidenced in hematological and serum biochemical analysis, reveal acute phase proteins as potential biomarkers for monitoring the clinical picture and prognosis with greater precision.

The findings of both protein and analyte concentrations do not replace diagnostic tests, as they are values that suggest the clinical condition of the patient and do not define the disease, requiring different diagnostic means for each suspicion.

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