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RESEARCH ARTICLE



Relationships between haptoglobin and inter-alpha trypsin inhibitor heavy chain 4 levels in local and peripheral blood and systemic inflammatory response syndrome in bitches with pyometra

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ABSTRACT

We aimed to assess the usefulness of monitoring inter-alpha trypsin inhibitor heavy chain 4 (ITI4) and haptoglobin (Hp) in peripheral and local blood in canine pyometra, and evaluation the relationships among acute phase proteins (APPs), systemic inflammatory response syndrome (SIRS) and the presence of bacteria. The material was collected from bitches with pyometra and from healthy ones. Blood was taken from the cephalic and uterine veins. APPs levels were quantified by ELISA. In the peripheral circulation, the Hp was higher in animals with open-cervix pyometra (OCP) than in the closed-cervix pyometra (CCP) and the control group. The Hp concentration was not correlated with age, with the presence of SIRS or with the type of bacteria (Gram-negative, Gram-positive or mixed flora). The ITI4 concentrations in the peripheral blood did not differ significantly in the cases of pyometra. The Hp concentration in the local circulation increased in the OCP but not in the CCP groups, although the histopathological changes in the endometrium were similar. Peripheral Hp concentrations may be a useful tool in differentiating between the types of pyometra.

KEYWORDS

bitch, pyometra, ITI4, haptoglobin, acute phase proteins

INTRODUCTION

Pyometra is one of the most common diseases in elderly non-neutered bitches. The disease is polyetiologic, and both hormonal disorders and bacterial infections (mainly *Escherichia coli*)

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are involved in its pathogenesis. These usually occur during, or immediately after, progesterone dominance (Baithalu et al., 2010). The accumulation of pus in the uterus may cause purulent vaginal discharge if the cervix is open (open-cervix pyometra, OCP), or the pus may remain inside the uterus (closed-cervix pyometra, CCP), which often results in severe intoxication and may cause uterine rupture. Pyometra may also be associated with the development of systemic inflammatory response syndrome (SIRS), which can lead to multiple organ dysfunction and death (Chakraborty and Burns, 2021). Inflammation triggers a cascade of reactions that lead to the production of acute phase proteins (APP). APPs are proteins produced primarily by the liver, and their concentration changes by at least 25% in response to proinflammatory cytokines released during pathological processes (Eckersall and Bell, 2010). APPs are classified as positive if their concentrations increase and negative if they decrease. Depending on the magnitude and dynamics of their concentration surge, APPs are also divided into major (>100-fold increase within 24–48 h), moderate (5–10-fold increase within 2–3 days), and minor (50–100% increase) (Eckersall and Bell, 2010). The following APPs are known to play an important role in animals: C-reactive protein (CRP), haptoglobin (Hp), inter-alpha trypsin inhibitor heavy chain 4 (ITIH4), serum amyloid A (SAA), α -1 acid glycoprotein, fibronectin, fibrinogen, and mannose-binding protein MBL (Eckersall and Bell, 2010; Sharif et al., 2013; Enginler et al., 2014; Jitpean et al., 2014; Nakamura et al., 2018) as positive APPs, and albumin and transferrin as negative APPs (Eckersall and Bell, 2010). In dogs, CRP and SAA are considered major APPs, while Hp, AGP, and ITIH4 are considered moderate APPs (Eckersall and Bell, 2010).

The concentration of Hp increases several-fold during inflammation (Andersen et al., 2017). This protein binds and removes iron from the bacterial microenvironment, causing bacteriostatic effects (Crawford et al., 2013; Andersen et al., 2017). Moreover, HP is responsible for capturing haemoglobin (formed during haemolysis), which leads to the formation of haptoglobin-haemoglobin complexes. It also decreases the oxidising properties of haeme, which is formed during the breakdown of haemoglobin (Andersen et al., 2017). ITIH4 is well known in human, but not in veterinary medicine (Soury et al., 1998; Hanaka et al., 2013). It is a 120 kD glycoprotein identified in humans, pigs, rats, and dogs (Soury et al., 1998; Hanaka et al., 2013). Major acute phase proteins of pigs (Pig-MAP) appears to have a structure similar to ITIH4 (Gonzalez-Ramon et al., 1995; Soury et al., 1998). More than 2/3 of the amino acid sequences showed homology with the heavy chains (H1, H2, and H3) of the inter-alpha-trypsin family inhibitor. The investigations carried out in cows showed an increase in ITIH4 concentration during experimental induction of mammary gland inflammation (mastitis) (Soler et al., 2019). Soler et al. (2016) confirmed an increase in this acute phase protein in dogs after surgery. However, the exact mechanism of the activity of this protein remains unknown. Previous studies by Bost et al. (1998) and Choi-Miura et al. (2000) have shown anti-inflammatory effects of ITIH4. Furthermore,

the possibility of binding to or inhibiting lymphocyte proliferation has been described. It has also been suggested that there is a possible positive correlation between ITIH4 and IL-6 (Hauptman et al., 1997; Soler et al., 2021).

Our hypothesis was that Hp and ITIH4 could be a prognostic biomarker of SIRS in bitches with pyometra. We aimed to compare the concentrations of the two APPs in dogs with and without pyometra, as well as in dogs with different types of pyometra (i.e., OCP and CCP). Moreover, we evaluated the accuracy of these APPs in differentiating between OCP and CCP bitches. Our study could help fill the knowledge gap because, to date, the cognition of both ITIH4 and Hp is limited. Hp test was chosen because changes in its concentration in dogs have not yet been described.

The second objective of this study was to determine the usefulness of these APPs as biomarkers and the possibility of their use in daily clinical veterinary practice for the diagnosis of purulent (also called suppurative) uterine inflammation.

MATERIALS AND METHODS

Experimental design and ethics approval

The diagnostic material used for the study originated from dogs that were taken to the clinic for ovariohysterectomy surgery by the owners. The study did not require special approval. The animals were patients, handled according to high ethical standards of the national legislation. Before all treatments, the owner's agreement was obtained. According to current regulations, committee license is not needed for routine veterinary procedures.

In three main groups, 28 female dogs were included in the study: control group ($n = 6$), OCP group ($n = 9$) including 3 SIRS+ and 6 SIRS- animals, and CCP group ($n = 13$) with 4 SIRS- and 9 SIRS+ members. The animals aged between 1 and 9 years (median: 7 years). Age was significantly different between dogs with pyometra (median: 7 years; range: 5–9 years) and the control group (range: 1–2 years). Their body weight ranged from 3 to 40 kg (median: 12 kg). Ten dogs were mixed breeds and the remaining 18 belonged to six breeds: Yorkshire Terriers (6), Beagles (4), Maltese dogs (3), German Shepherds (3), Shetland Sheepdog (1) and Border Collie (1). The breeds were evenly distributed among the groups.

Pyometra was diagnosed based on medical history, clinical signs (anorexia, polydipsia, polyuria, and ataxia), complete blood count (CBC) (Tables 1 and 2) and abdominal ultrasonography. The dogs with pyometra were further divided into groups of OCP and CCP according to the presence of purulent vaginal discharge (full field of degenerative neutrophils and bacteria) in the cytological examination. Additionally, the dogs with pyometra were classified as SIRS-positive and SIRS-negative according to the following scheme. SIRS was diagnosed if two or more of the following criteria were met: temperature under 38.1 °C or over 39.2 °C, respiratory rate: over 20/min, heart rate over 120 beats/min, white blood cell count (WBC) < 6 or >16 G/L or percentage of band neutrophils over 3% (Tang et al., 2008).



Table 1. Clinical characteristics of the study population

Clinical findings	Group			Omnibus test ^c P-value	Pair-wise comparison
	Control (n = 6)	OCP (n = 9)	CCP (n = 13)		
Body temp. [°C] ^a	38.4 (36.8–38.9)	38.7 (38.4–39.3)	39.3 (38.3–39.8)	0.004*	C vs. OCP 0.754 C vs. CCP 0.007* OCP vs. CCP 0.081
Fever (>39.2 °C) ^b	-	1 (11)	7 (54)	0.032*	
Respiratory rate (per min) ^a	14 (11–19)	16 (14–23)	24 (15–28)	0.015*	C vs. OCP – 0.679 C vs. CCP – 0.018* OCP vs. CCP – 0.229
Heart rate (per min) ^a	95 (84–100)	120 (100–130)	120 (90–140)	0.017*	C vs. OCP – 0.034* C vs. CCP – 0.023* OCP vs. CCP – 0.999
WBC [G/L] ^a	13.2 (11.4–14.9)	19.2 (14.5–28.6)	21.5 (4.5–5.7)	0.012*	C vs. OCP – 0.030* C vs. CCP – 0.014* OCP vs. CCP – 0.999
Leucocytosis (>16 G/L) ^b	-	6 (67)	9 (69)	0.999	
Leucopenia (<6 G/L) ^b	-	0	1 (8)	-	
SIRS present ^b	-	3 (33)	8 (62)	0.190	

OCP: open-cervix pyometra; CCP: closed-cervix pyometra.

^a presented as median and range.

^b presented as count and percentage.

^c Kruskal–Wallis *H* test for numerical variables and maximum likelihood G test for categorical variables.

* significant at $\alpha = 0.05$.

Table 2. Results of the bacteriological examination of the tissue samples

Bacterium ^b	Control (n = 6)	OCP (n = 9)	CCP (n = 13)
Gram-negative rods	-	5 (56%)	9 (69%)
Gram-positive cocci and mixed infections		4 (44%)	4 (31%)
<i>Escherichia coli</i>	-	5	9
<i>E. coli</i> haemolytic		4	7
<i>E. coli</i> non-haemolytic		1	2
<i>Streptococcus</i>		2	1
<i>Streptococcus</i> and <i>E. coli</i>		1	0
<i>Staphylococcus</i>		1	1
<i>Staphylococcus</i> and <i>E. coli</i>		0	2

OCP: open-cervix pyometra; CCP: closed-cervix pyometra.

Female dogs that underwent elective OVH in the diastral phase of their reproductive cycle (<4 weeks after resolution of signs of oestrus). Blood samples were taken from the cephalic vein (peripheral circulation) and the uterine vein (local circulation) into 2 ml plain tubes during surgery. The blood was centrifuged (MPW223e, 4,000 rpm, 5 min) and the serum was harvested and kept frozen at -71 °C until testing.

One swab was collected from the uterus of each dog for bacteriological examination, and tissue samples from the middle of each uterine horn was taken for histopathological examination.

Each dog underwent a general clinical examination to diagnose other abnormalities and diseases. None of them was pregnant neither was any on contraceptives.

Laboratory tests

ITI4 and haptoglobin were measured using two ELISA Sandwich tests (Acuvet Biotech, Zaragoza, Spain) that were previously developed and validated (Dąbrowski et al., 2015). The intra- and inter-assay coefficients of variation (CVs) for the ITI4 ELISA were lower than 6% and 7%, respectively. The intra- and inter-assay CVs for haptoglobin ELISA were lower than 5% and 11%, respectively.

Microbiological examination

Bacteriological studies were performed using routine bacteriological procedures to isolate aerobic and facultative anaerobic mesophilic bacteria. Briefly, the specimens were cultured in standard media, Columbia agar supplemented with 5% sheep blood (Graso Biotech, Poland), and MacConkey agar (Graso Biotech, Poland) and incubated at 37 °C under aerobic conditions for 48 h. The isolates obtained were first differentiated according to their phenotypic characteristics, such as colony morphology, cell morphology and Gram staining, as well as their ability to produce catalase and oxidase. Further identification was performed based on the biochemical properties of the isolates using the API E20 or API Staph tests (BioMérieux, France). In addition, a tube coagulase test was performed to differentiate between coagulase-positive and coagulase-negative staphylococci.

Histopathological examination

The tissue samples were fixed in 10% neutral buffered formalin. The samples were then dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin blocks, trimmed into 4 µm-thick sections, and



further processed according to the standard protocol. Finally, tissue samples were stained with the haematoxylin and eosin method (H–E). Histopathological evaluation was performed using a light microscope Olympus BX43 integrated with the Olympus cellSens imaging software (Olympus, Japan).

Statistical methods

Categorical variables were presented as counts and percentages and compared between groups using the maximum likelihood G test or Fisher’s exact test if the expected count in any cell of the contingency table was <5. Numerical variables were summarised using median, range, and interquartile range (IQR) if the number of measurements was >5. The comparison between two groups was made using the Mann–Whitney *U* test and between more than two groups using the Kruskal–Wallis *H* test followed by Dunn’s post hoc test if significant. The correlations between the numerical variables were measured using the Spearman rank correlation coefficient. The accuracy of distinguishing between the types of pyometra was assessed by estimating the area under the ROC (Receiver Operating Characteristic) curve (AUROC) and classified as low (AUROC, 50–70%), fair (AUROC, 70–80%), moderate (AUROC, 80–90%), or high (AUROC, >90%) (Pretzer, 2008). Statistical significance was set at 0.05 and was considered statistically significant. Statistical analysis was performed with TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA).

RESULTS

Demographic and clinical characteristics

The clinical characteristics of the dogs are presented in Tables 1 and 2. Fever (>39.2 °C) was present in only one dog with OCP (11%) and seven dogs with CCP (54%) (*P* = 0.032). No dogs had a fever >40.0 °C. Compared with

the control group, heart rate and WBC were significantly elevated in the OCP and CCP groups and did not differ between the pyometra groups (*P* = 0.999). Leucocytosis (WBC, >16 G/L) was present in 68% of dogs with pyometra (15/22), equally often in the OCP and CCP groups (*P* = 0.999). Leukopenia (WBC, <6 G/L) was observed in only one dog. SIRS was diagnosed in 50% (11/22) of the bitches with pyometra, and the proportions were not significantly different between the OCP and CCP groups (*P* = 0.190). Bacteria were isolated from all dogs with pyometra. A pure culture of *E. coli* was isolated from 14 dogs (64%) (haemolytic strains from 11 and non-haemolytic from three dogs), *Staphylococcus* spp. from four dogs (18%; in two cases together with haemolytic *E. coli*), *Streptococcus* spp. in four dogs (18%; in one case, together with haemolytic *E. coli*). There was no significant difference between the OCP and CCP groups regarding the percentage of Gram-negative, Gram-positive bacteria, and mixed bacterial infections (*P* = 0.662). In the control group, no bacterial growth was observed.

ITI4 and Hp concentrations. The values of ITI4 and Hp concentrations did not differ significantly between local and peripheral circulation in the entire study population (*P* = 0.990 and *P* = 0.400, respectively) or between groups separately (Tables 3 and 4).

Similarly, the ITI4 levels did not differ significantly between the control, OCP and CCP groups, between the SIRS-positive and SIRS-negative groups, or between the Gram-negative and Gram-positive bacteria and mixed bacteria groups, neither in the local nor in the peripheral blood (Tables 2 and 3). Neither in the local nor in the peripheral circulation was the concentration of ITI4 significantly correlated significantly with age (*R*_s = −0.10, *P* = 0.615 and *R*_s = −0.06, *P* = 0.764, respectively).

In the local circulation, ITI4 did not differ significantly between the control, OCP and CCP groups, between the SIRS+ and SIRS− groups, or between the Gram-negative

Table 3. The measured concentration of the acute phase proteins in the local and peripheral circulation

Acute phase proteins ^a	Group			Kruskal–Wallis <i>H</i> test <i>P</i> -value	Pair-wise comparison
	Control (<i>n</i> = 6)	OCP (<i>n</i> = 9)	CCP (<i>n</i> = 13)		
ITI4 [g L ^{−1}]					
Local	0.35 (0.24–0.74)	1.01, 0.70–1.67 (0.22–1.86)	0.54, 0.28–1.31 (0.16–1.51)	0.166	-
Peripheral	0.34 (0.28–0.77)	0.98, 0.77–1.46 (0.21–1.88)	0.53, 0.28–1.02 (0.15 – 2.03)	0.162	-
<i>P</i> -value	0.500	0.859	0.583		
Hp [g L ^{−1}]					
Local	1.54 (1.27–0.82)	5.78, 3.79–7.11 (0.07–10.11)	3.77, 1.57–5.74 (0.04–11.95)	0.062	-
Peripheral	1.51 (1.25–2.42)	5.07, 4.60–7.25 (2.81–10.95)	2.92, 1.23–4.57 (0.06–11.25)	0.007*	C vs. OCP – 0.011* C vs. CCP – 0.876 OCP vs. CCP – 0.042*
<i>P</i> -value	0.345	0.678	0.196		

OCP, open-cervix pyometra; CCP, closed-cervix pyometra.

^a presented as median, interquartile range, and range.

* significant at α = 0.05.



Table 4. Concentration of acute phase proteins in the local or peripheral circulation of animals with pyometra according to the presence of systemic inflammatory response syndrome (SIRS) or the type of bacteria isolated

Acute phase proteins ^a	Group			Group		
	SIRS-positive (n = 11)	SIRS-negative (n = 11)	P-value	Gram-negative bacteria (n = 14)	Gram-positive/mixed bacteria (n = 8)	P-value
ITIH4 [g L ⁻¹]						
Local	0.49, 0.26–1.04 (0.21–1.67)	1.31, 0.54–1.51 (0.16–1.86)	0.189	0.77, 0.25–1.37 (0.16–1.67)	1.00, 0.42–1.59 (0.28–1.86)	0.290
Peripheral	0.53, 0.20–1.02 (0.15–1.48)	0.86, 0.50–1.8 (0.21–2.03)	0.131	0.72, 0.28–1.02 (0.2–2.03)	1.00, 0.43–1.63 (0.15–1.88)	0.474
P-value	0.445	0.789		0.600	0.674	
Hp [g L ⁻¹]						
Local	5.27, 1.57–6.35 (0.07–8.88)	4.24, 3.15–7.11 (0.04–11.95)	0.742	4.00, 1.57–6.35 (0.04–11.95)	5.21, 2.98–6.75 (0.43–10.11)	0.562
Peripheral	4.10, 1.23–5.07 (0.06–7.91)	4.60, 2.81–7.25 (0.56–11.25)	0.264	4.40, 2.81–5.07 (0.56–11.25)	4.49, 1.27–6.79 (0.06–10.95)	0.811
P-value	0.286	0.789		0.594	0.999	

^a presented as median, interquartile range and range.

and Gram-positive and mixed bacteria groups. In contrast, in the peripheral circulation, the Hp concentration was significantly higher in the OCP group than in the control ($P = 0.011$) and in the CCP groups ($P = 0.042$) (Table 3). The Hp concentration was not significantly correlated with age neither in the local nor in the peripheral blood ($R_s = 0.26$, $P = 0.195$ and $R_s = 0.32$, $P = 0.099$, respectively). Hp did not differ significantly between the SIRS-positive and SIRS-negative groups or between the Gram-negative and Gram-positive bacteria and mixed infection groups, neither in the local nor in the peripheral circulation (Table 4).

Given that statistically significant difference was detected only in the peripheral Hp concentrations between the OCP, CCP and control groups, we evaluated the accuracy of peripheral Hp concentration in distinguishing between OCP and CCP. The accuracy was fair, with an AUROC of 80% (confidence interval 95%, 61–99%). However, at an optimal cut-off value of $\text{Hp} \geq 4.6 \text{ g L}^{-1}$, several dogs had Hp concentrations very close to this value.

Histopathological examination

Of the 22 cases, 20 showed suppurative endometritis, including cases showing marked acute ($n = 14$) and moderate chronic ($n = 6$) characteristics, and two cases showed mild chronic inflammatory infiltration (Fig. 1).

No histopathological changes were observed in the control group. Marked acute suppurative endometritis occurred in 44% (4/9) of the OCP cases and 77% (10/13) of the CCP cases. Endometritis was accompanied by metritis in 91% of all cases. Microscopic examination of the OCP (Fig. 1 A–D) and CCP (Fig. 1 E–H) revealed a thickened oedematous endometrial wall with congestion and focal to diffuse inflammatory cell infiltrates composed mainly of neutrophils and mononuclear cells in the endometrial mucosa. There was multifocal cystic endometrial hyperplasia of the glands (89% OCP [$n = 8$] and 92% CCP [$n = 12$]) with luminal blockage by purulent exudate, amphiphilic to

eosinophilic mucoid material. Furthermore, in 23% (5/22) of the cases, the presence of markedly dilated glands was observed a bit more frequently ($n = 3$) in the CCP group (Fig. 1F). The dilated glands were partially lined of flattened or hyperplastic columnar epithelial cells showing vacuolated cytoplasm with varying of severity. Vacuolisation of the endometrial epithelium was observed in almost all cases (91%, 20/22) (Figures 1B and 1G). In most cases, necrosis of the mucosa (82%, 18/22) and haemorrhage (77%, 17/22) were present (Figures 1C and 1G). Among the six cases (four with OCP and two with CCP), the features of chronic endometritis were characterised by moderate fibrosis and a predominant infiltration of mononuclear cells in the endometrium (Figures 1D and 1H). Furthermore, one case in each group showed less chronic infiltration accompanied by mild to moderate hyperplasia of the connective tissue and superficial and deep glandular epithelium.

DISCUSSION

Studies using mouse models have shown that ITIH4 may be a serum biomarker in the acute phase of infection. In addition, in the cited study, the concentration of this protein has been noted to differ in various microorganisms (Dąbrowski et al., 2015). In our study, we did not observe such a correlation, probably due to the lack of precise identification of some bacterial species and strains. More research on pyometra is needed in this field. However, our patient groups included too few cases to confirm the significance of these factors. The bacteriological results showed a tendency that suggests that non-haemolytic *E. coli* caused a higher increase in Hp and ITIH4 concentrations only in the CCP cases in both local and peripheral blood circulations. By comparing the concentrations of APPs in bitches infected with haemolytic and non-haemolytic *E. coli*, it can be assumed that the presence of haemolysin increases the



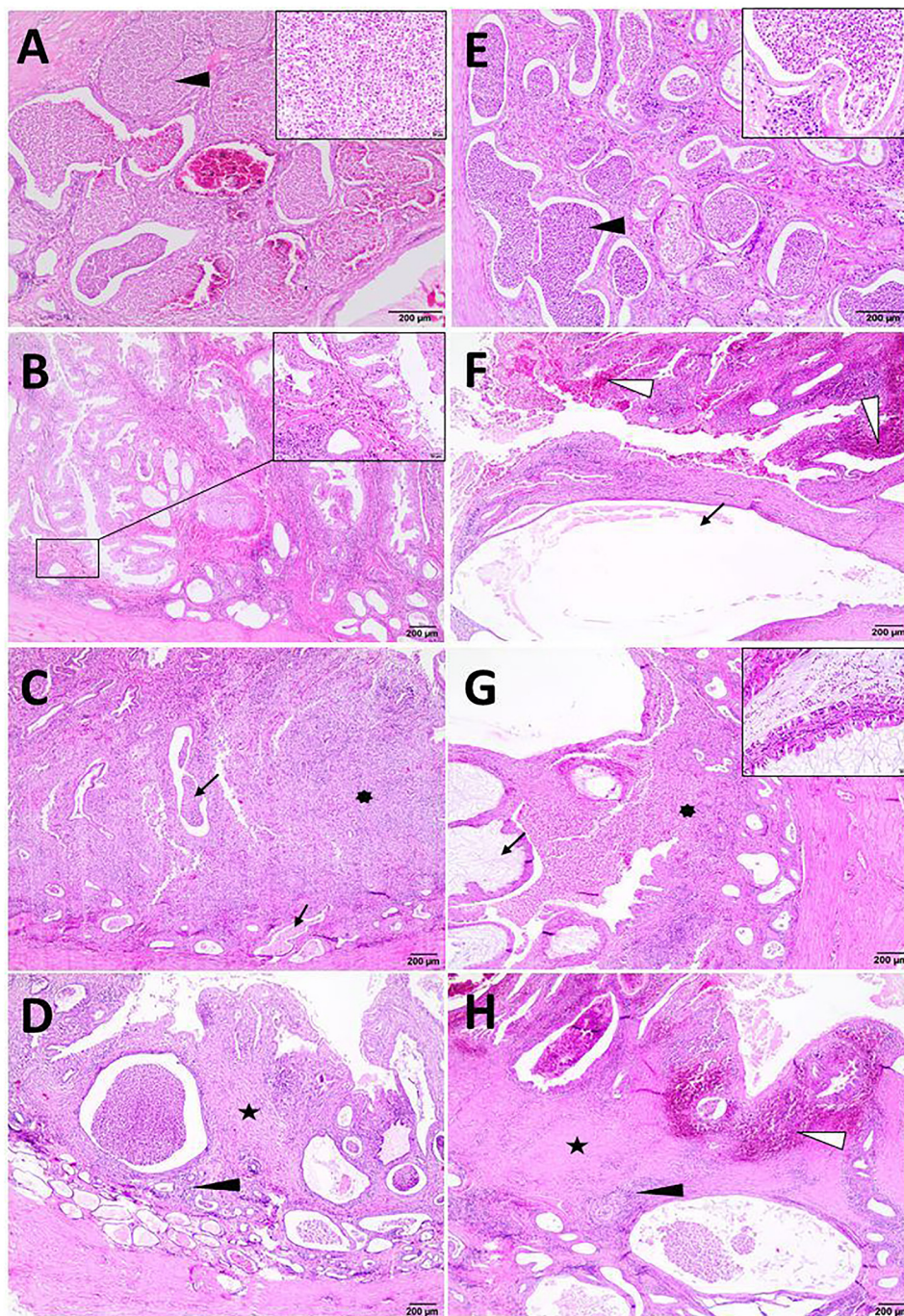


Fig. 1. Representative histopathological findings in the canine endometrium samples from the open-cervix (OCP) and the closed-cervix pyometra (CCP) groups in the left (A–D) and right (E–H) panels, respectively. Haematoxylin and eosin staining. The scale bars correspond to 200 µm. A & E: The black arrowheads indicate dilated glands densely packed with intraluminal neutrophils mixed with cellular debris. The insert pictures show the luminal content (magnification: 200×). B & F: Multifocal cystic hyperplasia of the endometrial glands. The insert picture shows hyperplastic epithelium with vacuolated cytoplasm (magnification: 100×). A large, ectatic thin-walled gland is shown by a black arrow. Areas of haemorrhage (white arrowhead) frequently occurred in the CCP group. C & G: Thick endometrial mucosa with marked, diffuse neutrophilic and lymphocytic infiltrate, areas of necrosis (star) and damaged glands with varying degrees of severity of change. The damaged glands are filled with cellular debris or eosinophilic/amphophilic mucoid material (black arrows). The insert picture shows some dilated glands lined with hyperplastic vacuolated epithelial cells and containing an amphophilic mucoid content with numerous neutrophils (magnification: 200×). D & H: The hallmarks of chronic inflammation with proliferation of fibrous connective tissue (black star) and mononuclear inflammatory cells (black arrowhead). The white arrowhead indicates a haemorrhage in the upper part of an endometrium from the CCP group

concentrations of ITIH4 and Hp in open and closed pyometra. In addition, there was a tendency for increased concentrations of ITIH4 in the CCP cases with Gram-positive bacteria in both uterine and peripheral blood. The correlation between the bacteriological results and the concentrations of APPs appears to be important from a clinical point of view, but was not found to be statistically significant. To assess the best way to check these significances, we performed a preliminary analysis on how to divide the groups of bacteria. The bacteria are divided into Gram-positive, Gram-negative, haemolytic *E. coli*, and other bacteria (Table 2). There were no statistically significant differences between the groups. The discrepancy between the results from clinical and statistical point of views results from the small sizes of examined groups. It is also necessary to perform similar studies on bitches with aseptic pyometra. Furthermore, due to discrepancies in the clinical observations and statistical analysis, research on larger groups is needed to describe the exact correlations between the type of bacteria in the uterus and its influence on APPs concentrations. It is necessary to quantify the species of bacteria and evaluate the toxins produced by individual species and strains of bacteria to describe the correlations between proteins and the type of pathogen inhabiting the uterus. All in all, it also appears necessary to assess the influence of bacteria called superantigens on the concentrations of APPs.

The levels of APPs were measured using Sandwich ELISA tests that have been previously developed and validated (Dąbrowski et al., 2013). This makes the obtained results reliable. A significantly higher concentration of acute phase protein was found in the OCP cases. The results obtained by other studies have been similar (Dąbrowski, 2009, 2015; de Carvalho et al., 2021). Therefore, Hp itself may play a role as a biomarker during OCP due to its slow increase in the serum. It is worth highlighting that bitches with OCP do not show full clinical signs for a long time or could be less systemically ill than CCP bitches (Ceron et al., 2005; Pretzer, 2008; Baithalu et al., 2010). This phenomenon suggests that the use of Hp as a biomarker of early-stage OCP may be helpful in the diagnosis of this disease.

Ceron et al. (2005) have also noted that APP responses under inflammatory conditions were higher in adult animals. In our study, the concentration of Hp was not correlated with the age of the bitch. Similar results were obtained in a human study (Milman et al., 1988) but not in pigs (Christoffersen et al., 2015). In our study, ITIH4 was not correlated with the age of the dogs.

In the study by Soler et al. (2019), the concentration of ITIH4 in peripheral blood serum has been determined after ovariohysterectomy and mastectomy. After such surgeries, a slow increase of ITIH4 concentration in the blood serum has been observed. In another study, the authors have evaluated the ITIH4 and Hp levels in bitches with pyometra, but have not investigated the link between the APPs concentration and the presence of SIRS (McGrotty et al., 2005). According to Soler et al. (2016), a delayed increase in the concentration of this protein is caused by tissue damage during surgery. We found no differences in either study group. Therefore,

according to our results, ITIH4 cannot be used as a biomarker to diagnose inflammatory reactions during pyometra in bitches. The difference between the OCP and control groups was statistically significant. This result could be due to the small number of dogs in the study groups. Meanwhile, the difference between the control and CCP groups was far from being statistically significant, contrary to the results of Soler et al. (2021). It is noteworthy that the ITIH4 concentrations in the local and peripheral blood were similar. Therefore, in this case, peripheral blood tests were sufficient to assess the degree of inflammation development during pyometra.

In the case of OCP in bitches with SIRS, there were no specific changes in the endometrium that determined the development of a generalised inflammatory reaction compared to SIRS-negative bitches. Additionally, no statistically significant changes in Hp and ITIH4 levels were observed in SIRS-positive and SIRS-negative bitches. We also observed a higher concentration of Hp in the OCP group compared to the control and CCP groups. de Carvalho et al. (2021) have reported that CCP presents a higher intensity of the inflammatory process. This contradicts our results showing that the concentrations of APPs in OCP were higher. This phenomenon probably resulted from the fact that bitches with OCP are generally less ill systemically than those with CCP (Milman et al., 1988). This is probably because usually they are presented to the veterinarian later when the inflammatory processes are more advanced. In our collected data, there was no information about the time that elapsed from the first signs of pyometra to surgery, so we could not verify this suspicion. In the future, it will be necessary to evaluate the influence of time on the degree of histopathological changes in the uterus and the concentration of APPs.

In addition, the correlation between histopathological changes in the uterus and ovaries, the presence or absence of clinical signs of SIRS, and the concentration of individual cytokines and APPs was investigated. Histopathological changes in the endometrium in bitches that underwent open and closed pyometra were similar. Changes occurring within the endometrium affect the immune response. In the case of open hidradenitis suppurativa in SIRS + bitches, there were no specific changes in the endometrium conditioning the development of a generalised inflammatory response compared to SIRS + bitches. Chu et al. (2002, 2006) as well as van Chruchten et al. (2003) have reported the apoptosis of endometrial cells in bitches.

Regarding the changes in the endometrium in closed versus to open hysterectomy, bitches with signs of SIRS are characterised by a greater degree of tissue degeneration, manifested primarily by purulent inflammation, cystic degeneration of the mucosa and vacuolisation of the epithelium. Mononuclear cell infiltration was also observed in the endometrium. In studies by Qian et al. (2020) and Resta et al. (2012), simultaneous acute and chronic inflammation characteristics have been observed, especially in the initial phase of uterine inflammation. This was associated with the infiltration of the endometrium by neutrophils and



lymphocytes, which is characteristic of acute inflammation, and the presence of plasma cells associated with chronic conditions.

In conclusion, the ITIH4 concentration did not change in the pyometra. The concentration of Hp appears to increase in OCP but not in CCP cases. Therefore, peripheral Hp concentrations may have a role in differentiating between the two types of pyometra. However, the pathophysiological background of this phenomenon requires further investigation. Neither ITIH4 nor Hp concentrations were affected by the presence of SIRS or the type of bacterial infection involved in the development of the pyometra.

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