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Original article

Prevalence of subclinical uterine pathologies diagnosed by biopsy and cytological and bacteriological findings in cyclic bitches

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Abstract

The study was performed on 45 bitches in different cycle phases that were divided into the following groups: anoestrus (I, n=15), heat (pro-oestrus (n=7) or oestrus (n=8) (II, n=15) and metestrus (III, n=15). Moreover, all experimental dogs were grouped according to their age: younger than 5 years (Y, n=35) and older than 5 years (O, n=10). The endometrial status was evaluated using cytological, bacteriological and biopsy samples obtained after ovariohysterectomy.

The main uterine pathology diagnosed by biopsy was endometritis, since 40%–66% of bitches, independent of the experimental group, developed this condition. No significant differences were found among the cycle phase groups ($p>0.05$). By contrast, significant differences were found in the age groups; the prevalence of this pathology was higher in older bitches ($p=0.0019$). The general prevalence of cystic endometrial hyperplasia (CEH) and a normal endometrium (NE) was lower (6.7–26.7% vs 26.7–53.3%) in all groups, and no statistically significant differences were found between certain groups ($p>0.05$). The percentage of polymorphonuclear neutrophilic leukocytes (PMNs) in endometrial cytology was generally low (< 2%) and did not differ significantly among the experimental groups ($p=0.142$). In general, a low degree of correlation was found between the diagnostic results by endometrial cytology and biopsy (Kappa Coefficient=0.046). Positive bacteriological findings were found in approximately 50% of the bitches, independent of the cycle phase and health status of the endometrium. No correlation was found between the bacteriological and histopathological findings ($p=0.883$).

In conclusion, uterine cytology is not a reliable diagnostic method to detect the subclinical inflammatory and degenerative uterine pathologies in cyclic bitches.

Key words: bitches, endometrial cytology, endometritis, uterus

Introduction

Bacterial and/or endocrine conditions are considered the main causes of uterine pathologies in bitches. Persistent ovarian follicles, ovarian cysts and ovarian tumours acting together with bacterial infections have a pronounced, detrimental effect on the canine uterus, resulting in its inflammation and/or degeneration (Kida et al. 2006). Uterine lesions, whether inflammatory, hyperplastic or neoplastic, have been considered potential causes of infertility in bitches (Johnston et al. 2001, Fontaine et al. 2009, Schlafer 2012, Mir et al. 2013). Endometrial alterations might induce implantation failure or defective embryonic or foetal development in a bitch (Freshman 1991).

The most common lesion identified was fibrotic degeneration of the endometrial glands, followed by endometritis and cystic endometrial hyperplasia (CEH) (Mir et al. 2013). In other studies, CEH has been reported as the most common uterine lesion in bitches (Dow et al. 1958, Versteegen et al. 2008, Schlafer et al. 2012). Pyometra, a special form of uterine disease where pus accumulates within the uterine lumen, is also a very frequent condition (Hagman et al. 2006, Jitpean et al. 2014). However, recently, some studies have shown that endometritis is a frequent finding during the diagnosis of uterine disease. These studies showed a high percentage of endometritis, both in randomly selected, clinically healthy bitches and in those with subfertility or infertility (Christensen et al. 2012, Mir et al. 2013, Gifford et al. 2014, Garcia Mitacek et al. 2017, Pradeiro et al. 2019).

Most cases of inflammatory disease of the canine uterus are considered to be caused by vaginal infectious agents invading the uterus (Dhaliway et al. 1999, Mateus et al. 2013). The oestrous cycle phase and associated patency of the cervix may also influence whether bacteria are found in a normal uterus (Schulthesis et al. 1999). Although bacteria have been isolated from the uterus in clinically healthy bitches in all phases of the estrous cycle, the most abundant bacterial colonisation of the uterus was generally found in the pro-oestrus and estrous phases (Watts et al. 1996, Janowski et al. 2008). Some authors have detected most bacteria in the uterus during metestrus with the main species identified being *Staphylococcus pseudintermedius*, coagulase-negative *Staphylococcus*, *Streptococcus spp.*, *Micrococcus spp.*, *Bacillus spp.*, *Corynebacterium spp.* and haemolytic *Escherichia coli* (Schultheiss et al. 1999, Maksimović et al. 2012). In general, the composition of the uterine bacterial flora is similar to that of the vaginal flora and includes *Lactobacillus sp.*, *Staphylococcus intermedius*, *Streptococcus canis*, *Pasteurella multocida*, *Enterococcus sp.*, and haemolytic *E. coli* strains (Zduńczyk et al. 2006, Font-

bonne 2011). Some pathologies of the uterus, such as cystic endometrial hyperplasia (CEH) and mucometra, may lead to a secondary infection of the uterus and then subsequent pyometra, which is a life-threatening disease (Hagman 2012). *Escherichia coli* is the most common pathogen isolated from bitches with pyometra, and is believed to arise from the normal bacterial flora of the reproductive tract (Jitpean et al. 2014).

The precise diagnosis of uterine pathologies in bitches is challenging because no clinical method exists for direct examination of this organ (Günzel-Apel et al. 2001). Additionally, ultrasonography is only partially useful in diagnosing canine uterine disease (Bigliardi et al. 2004, Dawidson et Baker 2009). In contrast to livestock, biopsy has also limited importance due to sampling problems. Innovatively, endoscopic transcervical sampling by flashing has been developed for cytological and bacteriological examinations of the uterus (Watts and Wright 1995, Günzel-Apel et al. 2001). Endometrial cytology has been used to study the morphologic and morphometric features of endometrial cells from normal and pathological canine uteruses, especially for subclinical endometritis (Watts et al. 1998, Fontaine et al. 2009, Gropetti et al. 2010). In cows and mares, it is a well-established method and cut-off points for a healthy and pathological uterus, calculated as a percentage of neutrophils among endometrial epithelial cells, have been reported (Kasimanickam et al. 2004, Gilbert et al. 2005, Kasimanickam et al. 2005, Nielsen 2005, Sheldon et al. 2009, Barański et al. 2012, Overbeck et al. 2013). By contrast, in bitches, such studies have been neglected and the threshold for the percentages of neutrophils that differentiates a healthy uterus and one affected by endometritis remains undefined. Recently, Pradeiro et al. (2019) performed a cytologic investigation of the canine uterus during metestrus using endometrial smears. However, in that study, the threshold of $\geq 2\%$ polymorphonuclear neutrophilic leukocytes (PMNs) used for the diagnosis of cytological endometritis was adopted from studies performed on mares (Overbeck et al. 2013). Additionally, in an earlier study by Fontaine et al. (2009) on subclinical endometrial infection as a cause of infertility, the cytologic threshold for the canine uterine diagnosis was not established; the authors used criteria defined by Watts et al. (1998).

The main aims of this study were as follows:

- to determine the endometrial health status in different phases of the estrous cycle by comparing the uterine cytologic, bacteriologic and biopsy findings in clinically healthy bitches;
- to establish a cytologic threshold for healthy and unhealthy canine endometrium, focusing on endometritis.

Materials and Methods

Animals and study design

All the procedures were conducted according to standard veterinary clinical practices and ethical approval rules set up by the Local Ethical Commission, based on the European Commission's guidelines (Directive 210/63/EU) and with the owners' permission.

This study was carried out on forty-five privately owned, intact non-pregnant, clinically healthy bitches in different phases of the estrous cycle. The bitches were fed by their owners with a standard commercial canine diet two or three times daily, and water was available ad libitum. According to their owners, the bitches were cyclic. The animals used in this study were included in a surgical contraception program performed at the Department of Animal Reproduction with Clinic, University of Warmia and Mazury in Olsztyn, Poland.

The bitches included in this study were clinically healthy based on thorough clinical and reproductive examinations. The lack of vaginal discharges in clinical and vaginoscopic examinations was a key criterion for inclusion in the study. Ultrasonographic examination of the reproductive tract was also carried out. No change in the ultrasound image of the uterus was another important criterion for inclusion in the study. In turn, in all the tested animals, the stage of the reproductive cycle was determined using clinical and laboratory examinations, including clinical external inspection, vaginoscopy, exfoliative vaginal cytology and serum progesterone concentration measurements.

The examinations were performed according to the routine protocols at our clinic (Jurczak and Janowski 2018). The clinical and laboratory data were analysed and evaluated according to common standard diagnostic criteria for cycle phases in bitches (England 2013).

Based on the above examinations, all the bitches were divided into the following three groups: group I (n=15) – bitches in anoestrus; group II (n=15) – bitches in pro-oestrus or estrous (n=15 (7+8)); group III (n=15) – bitches in metestrus. The age (mean \pm SE) of the experimental bitches was 3.0 ± 0.25 years (range, 1–9 years). Mixed breeds comprised the most common group in each of the 3 experimental groups (n=30; 66.7%). The 15 dogs of group I, weighing from 2 kg to 32 kg, and aged from 1 year to 8 years, were of the following breeds: crossbreed (n=9; 60%), German Shepherd (n=2; 13.4%), Boxer (n=1; 6.7%), Irish Setter (n=1; 6.7%) Labrador (n=1; 6.7%) and Poodle (n=1; 6.7%). The 15 bitches of group II, weighing from 2 kg to 35 kg, and aged from 1 year to 6 years, were of the following breeds: crossbreed (n=12; 80%), Boxer (n=1; 6.7%), German Shepherd (n=1; 6.7%) and Labrador

(n=1; 6.7%). The 15 dogs of group III, weighing from 3 kg to 34 kg, and aged from 1 year to 9 years, were of the following breeds: crossbreed (n=9; 60%), Labrador (n=2; 13.4%), Cocker Spaniel (n=1; 6.7%), German Shepherd (n=1; 6.7%), Scottish Terrier (n=1; 6.7%) and Shi-Tzu (n=1; 6.7%).

The experimental bitches were also divided into 2 groups based on their age: younger than 5 years (Y, n=35) and older than 5 years (O, n=10).

All the bitches were ovariohysterectomised (OVH) to obtain cytologic, bacteriologic and biopsy uterine samples. Anaesthetic and surgical procedures were carried out according to standard procedures of good veterinary practice (Fossum 2013, Mir et al. 2013). The lack of macroscopic uterine alterations was also considered an inclusion criterion in the present study.

After ovariohysterectomy, samples for endometrial culture, endometrial cytology and biopsy to determine the status of the endometrium were collected through the incision of the uterine wall. All the samples were taken from the same region of the uterus – i.e., the transition of the uterine corpus into a randomly selected horn. The cytologic, bacteriologic and biopsy findings in the groups were collected and analysed statistically.

Diagnosis of the estrous phase

Clinical and vaginoscopic examinations

The clinical examination included inspections of the vulva and perivulvar area, as well as palpation of the mammary glands. Vulvar oedema, the colour of the mucosa, and vaginal discharge were evaluated. The vaginoscopic examinations were performed using a Richard Wolf 4200 LP endoscope (Knittlingen®, Germany). The cyclic changes of the vaginal mucosa and presence or absence, as well as characteristics of discharge, were evaluated (England 2013).

Vaginal cytology examination

Vaginal cytology samples were obtained by introducing a cotton-tipped swab into the vagina and gently rotating it against the floor and lateral walls of the vagina. The swab was gently rolled over a clean microscopic slide, and smears were air-dried and stained using Shorr's method. The stained slides were examined at $\times 100$ and $\times 400$ magnifications to count basal cells, parabasal cells, intermediate cells, superficial cells, erythrocytes, and leukocytes under an Olympus BX 50 light microscope (Olympus Optical Co®, Ltd, Japan). The presence and proportions of epithelial cells were evaluated according to standard cytological criteria for the canine reproductive cycle (England 2013).

Progesterone assay

The blood samples for progesterone concentration measurements were obtained via venipuncture (*v. saphena*) and centrifuged (1200 × g, 10 min). The obtained sera samples were analysed immediately. The progesterone levels were measured using a chemiluminescence method (Progesterone II®, Roche Diagnostics GmbH, Mannheim, Germany). The serum progesterone concentrations were compared using the following reference data described by England (2013): anoestrus – less than 0.5 ng/ml; pro-oestrus – less than 1 ng/ml. The first day of estrous was defined as the day when the progesterone level reached 1–2 ng/ml for the first time. During estrous, the serum progesterone concentration gradually increased and the ovulation time was set up when the progesterone concentration reached 5.0–8.0 ng/ml. Metestrus was considered when the concentration of progesterone increased dramatically (in the early postovulatory period >8.5 ng/ml) and reached a level of 18–20 ng/ml.

Endometrial status evaluation Endometrial bacteriology

Samples from the uterine horn were collected by introducing a sterile swab through the uterine wall incision. The swabs were immediately transported to the Microbiological Laboratory at the Faculty of Veterinary Medicine in Olsztyn and were processed immediately. The samples were seeded (Oxoid®, UK) in currently used culture media prepared on Petri dishes: TSB broth (non-selective), Columbia agar with 5% defibrinated sheep blood (non-selective), Edwards agar with 5% defibrinated sheep blood (*Streptococcus sp.*), MacConkey agar (Gram-negative), and Chapmann agar (*Staphylococcus sp.*) under sterile conditions of the laminar chamber Herasafe (Heraeus®, Germany). The colonies were then classified based on their appearance, haemolysis type and ability to produce catalase, oxidase and coagulase. Gram staining was also performed. Plates without growth were left under incubation conditions for another 24 h and re-evaluated. The number of grown colonies was determined according to the following pattern: very numerous – if, in direct culture, and overall medium intensive microbial growth has occurred; numerous – if, in direct culture, intensive microbial growth with numerous colonies occurred on the spread lines; few – if intensive growth of microorganisms in direct-culture single colonies occurred on the spread lines; single – if, in direct culture, intensive microbial growth occurred and is missing on the spread lines; sterile – if in direct culture and no microbial growth occurred on the spread lines. The final identification was carried out using a PathoDextra Strep Grouping

latex tests Kit (Oxoid®, UK) and biochemical API 20E, API 20NE, API Staph, API Strep (Bio-Merieux®, France). All the samples with heavy growth of one or two bacterial strains were considered positive (Fontaine et al. 2009).

Endometrial cytology

Samples for endometrial cytology were collected directly after bacteriological samples, introducing a cytobrush through the incision hole, rolling into the endometrium and then rotating on a microscopic slide. Finally, smears were stained using Shorr's staining. The cytological assessment was performed by counting the number of polymorphonuclear neutrophilic leukocytes (PMNs) and epithelial cells present. Three hundred cells were counted at ×100 and ×400 magnifications using 10 separate fields of view (Westermann et al. 2010). The proportion of PMNLs to epithelial cells was then calculated and expressed as a percentage. As a positive control for cytologic diagnosis, 10 samples obtained from the bitches with purulent endometritis were used. The investigator performing this examination was blind to the sample identities.

Uterine biopsy and processing of histologic samples

Uterine biopsy samples (1 cm × 1 cm) were collected from the same uterine region using a No.12 scalpel blade and forceps. The sample was then fixed in 10% buffered formalin, washed three times in PBS (3 × 24 h), and then embedded in Tissue-Tec® O.C.T.™ Compound (Sakura Europe®). The samples, 9-µm thick, were cut with a microtome, mounted on SuperFrost Plus microscope slides (Menzel-Gläser®), stained with haematoxylin and eosin and examined with a light microscope under 100× and 400× magnifications (Olympus®, Japan).

All the biopsies were assessed, and the cells and tissues were identified and categorised based on a previously used scoring system (Schlafer and Gifford 2008, Schlafer et al. 2012). The following criteria for the evaluation of histopathological changes in the endometrium were used: normal endometrium (NE) was defined as showing no changes in tissues, the absence of inflammatory cells or the presence of a maximum of three neutrophils in the superficial endometrial epithelium per ×40 field; endometritis (END) was defined as acute (A-END) or subacute (SA-END) depending on the severity of histopathological changes. A-END was defined as the presence of at least four neutrophils in the superficial endometrial epithelium per ×40 field and the presence of severe hyperaemia, vascular congestion or stromal oedema. SA-END was defined as

Table 1. Prevalence of histopathological endometrial states in the cycle phase groups of bitches.

Condition	Group I (Anoestrus) (n=15)	Group II (Pro-oestrus + Estrous) (n=15)	Group III (Metestrus) (n=15)
NE	8/15 (53.3%)	4/15 (26.7%)	6/15 (40%)
END	6/15 (40%)	10/15 (66.6%)	5/15 (33.3%)
CEH	1/15 (6.7%)	1/15 (6.7%)	4/15 (26.7%)

NE – normal endometrium, END – endometritis, CEH – cystic endometrial hyperplasia, $p > 0.05$

Table 2. Percentage of bitches with normal and abnormal endometrium by age group.

Condition	Group Y (<5 years old) (n=35)	Group O (≥ 5 years old) (n=10)
NE	18^a (51.4%)	- ^b
END	15 (42.8 %)	6 (60%)
CEH	2^a (5.8%)	4^b (40%)

$\chi^2 = 12.49$, $p = 0.0019$

NE – normal endometrium, END – endometritis, CEH – cystic endometrial hyperplasia, a, b – significant difference at $p \leq 0.05$ among the NE, END and CEH group.

fewer than four neutrophils, moderate to severe vascular congestion, hyperaemia, haemorrhage, and oedema in the stroma. Additionally, neutrophilic, lymphocytic, or lymphoplasmacytic infiltrates and hemosiderin-laden macrophages were present; cystic endometrial hyperplasia (CEH) was defined when the glands become cystic, the epithelium of the glands becomes flattened, and rare association with endometrial inflammation is observed.

Statistical analysis

In experimental groups based on the oestrus cycle (I, II, III), the percentage (%) of bitches affected by certain uterine pathologies was calculated. Additionally, the correlation between the uterine biopsy results and endometrial cytology and endometrial bacteriology was evaluated. The data are shown as means \pm SD (standard deviation) or as a percentage (%). The collected data were analysed using IBM SPSS Statistics 25.

The agreement between the endometrial cytology and biopsy results was evaluated using Cohen's kappa (κ) coefficient. The non-parametric Kruskal–Wallis test was used to compare the differences in the PMNL percentage between the groups.

Among the groups, based on age, the occurrence of the same histopathological and cytological findings was calculated and analysed statistically as above.

Statistical significance was set at $p < 0.05$, and a statistical trend at $p < 0.10$.

Results

The histopathological findings are presented in Table 1 and Table 2. END occurred in 40%–66% of bitches (Table 1). However, the differences among the experimental groups I, II and III were not statistically significant. Additionally, no significant differences were also found regarding the incidence of CEH and NE ($p > 0.05$).

By contrast, significant differences ($p < 0.01$) were found between the bitches grouped based on age (Y, O). NE was more often observed in bitches younger than 5 years (Y), while CEH occurred mainly in the group of older bitches (O) ($p = 0.0019$). Moreover, bitches aged older than 5 years had END (60%) and CEH (40%), whereas none had NE. By contrast, 40%–60% of younger bitches had NE and a much lower incidence of END and CEH (Table 2).

The percentage of PMNs observed in endometrial cytology (not illustrated) was low ($< 2\%$) and did not differ significantly among the experimental groups ($p = 0.142$). By contrast, in 10 control samples from bitches with purulent endometritis used as a methodological control, the average number of PMNs was much higher (9.8 ± 1.93). The numerically highest percentage of PMNs was detected during pro-oestrus and estrous (group II), followed by metestrus (group III) and anoestrus (group I) (2.0 ± 2.45 vs. 1.93 ± 2.25 vs. 0.8 ± 1.61 respectively; $p = 0.142$). Similarly, no significant differences were found between the percentages of PMNs in the histopathological findings ($p = 0.790$);

Table 3. Bacteriological findings – number of bitches (%) with positive (+) or negative (-) bacterial growth in all experimental groups of bitches by endometrial status.

Condition	Method of examination Bacteriology (+)	Method of examination Bacteriology (-)
NE	8/18 (44.5%)	10/18 (55.5%)
END	11/21 (52.4 %)	10/21 (47.6%)
CEH	3/6 (50%)	3/6 (50%)

$\chi^2 = 0.24$, $p = 0.883$

NE – normal endometrium, END – endometritis, CEH – cystic endometrial hyperplasia,
 + substantial, heavy growth of one or two bacteria strains,
 - no growth of bacteria.

Table 4. Bacterial strains isolated from uterus cultures in the cycle phase groups.

Strain	Group I (Anoestrus) (n=15)	Group II (Pro-oestrus + Oestrus) (n=15)	Group III (Metestrus) (n=15)	
<i>Bacillus spp.</i>	-	1 (6.7%)	3 (19.2%)	$p = 0.146$
<i>E. coli</i>	1 (6.7%)	-	-	$p = 0.360$
<i>Micrococcus spp.</i>	-	1 (6.7%)	-	$p = 0.360$
<i>Pasteurella spp.</i>	-	-	2 (13.4%)	$p = 0.123$
<i>Staphylococcus spp.</i>	2 (13.4%)	8 (53.6%)	3 (19.2%)	$p = 0.122$
<i>Streptococcus spp.</i>	2 (13.4%)	1 (6.7%)	1 (6.7%)	$p = 0.760$
Total %	33.5 %	73.7 %	58.5%	

however, the percentage was numerically highest in the END group (1.9 ± 2.51) compared with those in the CEH (1.17 ± 1.47) and NE (1.33 ± 1.94) groups. Interestingly, the percentages of PMNs in both age groups was also similar (Table 3) and not significantly different (1.60 ± 2.23 vs. 1.50 ± 2.01 ; $p = 0.868$).

A low degree of correlation was observed between the results obtained by endometrial cytology and the biopsy results (Kappa Coefficient = 0.046).

Bacteriological findings are presented in Table 3. A bacterial mixture of *Staphylococcus sp.* and *Streptococcus sp.* was the most frequently isolated bacterial population independent of the cycle stage. Twenty-three of forty-five experimental bitches (51.1 %) from all the groups showed no bacterial growth. The highest number of bitches with negative results occurred in the anoestrus group (I) (11/15, 73.3%). By contrast, in the 2 remaining groups (II and III), the percentage of bitches without bacteria was much lower (40%). The bacteriological results in certain groups were not statistically significant ($p = 0.101$).

No correlation was found between the histopathological and bacteriological findings ($p = 0.883$) (Table 4). However, positive bacteriological findings were found in 52.4% (11/21) of bitches with END, 50% (3/6)

of bitches with CEH and 44.5% (8/18) of bitches with NE (Table 1).

Discussion

This study is the first published report on the endometrial status of clinically healthy bitches in all phases of the estrous cycle diagnosed by cytology, biopsy and bacteriology simultaneously. We are aware that we combined animals in pro-oestrus and oestrus into one group due to the small number of bitches usually ovariectomised in these cycle phases. Moreover, pro-oestrus and oestrus are both related to follicular growth and are short lasting; from a practical point of view, they are considered commonly as “heat”. We believe that the animal grouping in this study allows valuable observations regarding the studied problem.

Because we used uterine cytology, biopsy and bacteriology to evaluate the endometrial status simultaneously, our experiment allows comprehensive assessment of the endometrium during the main phases of the estrous cycle. Methodologically, a similar study was reported earlier only by Pradeiro et al. (2019). However, that study was limited to metestrus bitches only. Other earlier studies on this topic performed in all cycle

stages were methodologically limited to histopathology or cytology separately (Watt et al. 1998, Christenson et al. 2012, Gifford et al. 2014). According to our knowledge, the above report by Pradeiro et al. (2019) is the only paper that applied the PMN numerical threshold in endometrial cytology in bitches until now. However, as mentioned above, the cytological threshold used by authors was adopted from studies on mares (Overbeck et al. 2013). The authors of the above study did not obtain practical and acceptable results regarding endometrium diagnosis. Interestingly, we found no statistical relationship between the cytologic results and endometritis and particular estrous cycle stage. Our findings agree with the outcomes obtained by Watt et al. (1998), who observed that the leukocyte score was low and did not change throughout the reproductive cycle. These authors also stressed that many leukocytes had undergone degeneration, which is a complication in diagnosis. Analysis of the percentage of PMNs in particular phases of the estrous cycle revealed a slightly enhanced average number of PMNs in both the pro-oestrus/estrous and metestrus groups, whereas the lowest percentage was observed in anoestrus. No correlation was found between the age of the bitches and number of neutrophils. However, numerically, we observed a slight decrease in the number of PMNs with increasing age. The above results are difficult to interpret because comparable studies in bitches are not available. Importantly, we found a low proportion of PMNs in smears obtained from canine uteri compared with that in other species such as cows and mares (Barański et al. 2012, Overbeck et al. 2013). A potential explanation of this phenomenon is unknown; however, canine cytology may have more limitations than those in other species.

An interesting and novel aspect of this study was the attempt to find a correlation between endometritis diagnosed by biopsy findings and cytologic results obtained using cytobrush, which is considered an optimal method. The cytologic outcomes may depend strongly on the sampling method in the studies in bitches in which uterine flushing was mostly used to obtain samples (Watts et al. 1998, Groppetti et al. 2010). At the same time, the present study was undertaken because such studies in dogs have not been performed. In cows and mares, endometrial cytology is a well-established diagnostic method to detect endometritis, particularly in its subclinical form (Kasimanickam et al. 2004, Gilbert et al. 2005, Kasimanickam et al. 2005, Nielsen 2005, Sheldon et al. 2009, Barański et al. 2012, Overbeck et al. 2013). Unfortunately, we found no correlation between the percentage of PMNs and endometritis diagnosed histopathologically. This finding agrees with the results of the Pradeiro et al. (2019) study in metestrus

bitches. It is noteworthy, that we generally observed a low percentage of endometrial PMNs in the bitches, independent of the cycle phase and uterine pathology diagnosed.

In our study, endometritis diagnosed by uterine biopsy was found to be the most prevalent uterine disease (46.7%, 21/45). These findings agree with a few earlier reports estimating the percentage of affected bitches at 43%–54% (Christensen et al. 2012, Gifford et al. 2014, Pradeiro et al. 2019). However, studies also showed a lower (29%) or higher (63.3%) incidence of endometritis (Mir et al. 2013, Mitacek et al. 2017). It is interesting to note that none of the experimental bitches showed clinical signs of END. This mild inflammatory reaction of the endometrium is difficult to explain. It could be only speculated that it is probably the initial phase of bacterial and/or endocrine END in progress. Unfortunately, the experimental animals have an unknown reproductive history, and it is therefore impossible to estimate the impact of this form of END on lack of fertilization and embryonal mortality. CEH was another uterine pathology diagnosed in our study. However, we detected this abnormality only in 6 of 45 bitches (13.4%). This result agrees with the finding by Pradeiro et al. (2019), who diagnosed even fewer CEH cases (4%) in metestrus bitches. By contrast, other studies reported a much higher prevalence of CEH, up to 30% of the population studied (Christensen et al. 2012, Gifford et al. 2014). It has been assumed that the incidence of CEH strongly depends on the age of the examined bitches (Moxon et al. 2016). This phenomenon could also explain our results. Our experimental canine population was relatively young – 77.8% (35/45) of bitches were younger than 5 years. Comparing the incidence of particular uterine pathologies diagnosed by biopsy is difficult because the obtained results strongly depend on the age and size of the population studied (Moxon et al. 2016). The number of bitches affected by uterine pathologies is still increasing with the growing age of animals (Christenson et al. 2012, Moxon et al. 2016). We observed the same trend in our study since all bitches aged older than 5 years showed endometritis or CEH. Additionally, the reproductive history of bitches, especially the number of cycles with a long dominance of steroid hormones and eventually hormonal treatment, acting together with invading bacteria are causes of uterine diseases (Fontbonne 2011).

Although many studies have reported on this issue, the role of bacteria in the etiopathology of uterine disease, except for pyometra, remains unclear. In our study, in approximately 50% of diagnosed uterine pathologies, we observed a bacteriological background. However, a high proportion of bitches with a normal

endometrium showed significant bacterial growth. The interpretation of the bacteriologic results in the bitches is not easy (Kustritz 2006, Mir et al. 2013). Based on our results, it seems that, in approximately half of uterine disturbances only, bacteria are present and may potentially play a role in the development of disease. However, it is also possible that our bacteriological results may be underestimated because some pathogens were not detected by our methodology. Our microbial findings are similar to those reported earlier by other authors (Watts et al. 1996, Zduńczyk et al. 2006, Janowski et al. 2008, Groppetti et al. 2012, Maksimović et al. 2012). We also observed a typical bacterial density fluctuation during cycle stages, with a peak during pro-oestrus and estrous, continued in dioestrus, and followed by a decrease in bacteria during anoestrus. This phenomenon is likely caused by bacteria invading through the open cervix in pro-oestrus and estrous and their persistence in the uterine lumen due to the immunosuppressive action of progesterone in metestrus and their further elimination during anoestrus (Dhaliwal et al. 2001).

In conclusion, the results obtained in this study are difficult to explain, and our working hypothesis to set up cytologic thresholds to categorise the states of canine endometrium has not been confirmed. Interestingly, in bitches, the cytologic findings do not precisely reflect endometritis and other uterine pathologies diagnosed by biopsy, which is considered a reference method. The explanation of this phenomenon may be that biopsy allows the assessment of all layers of the uterine wall, whereas cytology evaluates only the superficial layers of the endometrium (Madoz et al. 2014). Until now, only a few reports have been published on uterine cytology in bitches (Fontaine et al. 2009, Pradeiro et al. 2019), but they did not evaluate this diagnostic method positively. Fontaine et al. (2009) recommended its use with transcervical sampling by flushing in all unexplained cases of infertility; however, in that study, the authors did not use a biopsy method as a reference diagnostic method. In their opinion, such a comparison would allow for a more precise interpretation of cytologic examination. However, our findings and those of Pradeiro et al. (2019) did not confirm their suggestion. In bitches, endometrial cytology is likely even more unfavourable than in farm animals. In these species, cytobrush cytology, despite a few limitations, is recognised as a valuable diagnostic method in uterine diseases, including subclinical endometritis. Importantly, in this study, the cytobrush method was used, a method that is considered an optimal sampling method for endometrial cytology in farm animals (Kasimanickam et al. 2005, Barlund et al. 2008).

Uterine cytology has not been confirmed as a reliable

diagnostic method for inflammatory and degenerative diseases of canine uteri. We could not set up a cytological threshold to diagnose canine endometritis. Interestingly, using biopsy, we observed a high percentage (50%) of bitches affected by subclinical endometritis, despite their good clinical and macroscopic uterine health.

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