The morphological changes in bitches endometrium affected by Cystic Endometrial Hyperplasia - Pyometra complex – the value of routine histopathological examination

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Research article

Keywords: uterine disease, bitch, endometrium, reproductive pathology

DOI: https://doi.org/10.21203/rs.3.rs-26656/v2

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Abstract

Background: The aim of the analysis was to designate the morphological symptoms that appear during the Cystic Endometrial Hyperplasia - Pyometra complex (CEH-P), diagnosed by microscopic examination. The investigation were conducted on the uteri of 120 bitches in age between 1 – 16. The microscopic examinations were based on histological stainings. The aim of examinations was to find the differences in morphology of endometrium in the specimens of the uteri wall (H&E stain). All of the uteri were divided into three pathological groups (GΙ – GΙΙΙ), created on the basis of macroscopic examinations of analyzed diseases. Uteri without macroscopic pathological changes were classified as a control group (C).

Results: Histopathological analysis that were conducted have confirmed preliminary macroscopic evaluation for control group with unchanged uteri, group GΙΙ with cystic endometrial hyperplasia of uteri (CEH), and group GΙΙΙ with uteri with pyometra. The confirmation of compatibility of both macroscopic and microscopic evaluation of the uteri were observed in groups GΙΙ and GΙΙΙ. In the uteri of the group GΙ a severe congestion of endometrium have been observed – it is typical for the inflammation – which was not confirmed during histopathological examinations. Those examinations revealed only endometrial haemorrhage.

Conclusions: The results are showing that during classification of research material microscopic examinations are required. The diagnose based on the macroscopic changes in typical for CEH-P symptoms might be incorrect, if it is not supported by detailed research. More than that, in all uteri with the closed pyometra the CEH was also observed. It suggest that pyometra may occur as a consequence of cystic endometrial hyperplasia and bacterial infections ended with inflammation. The results obtained can be used to create a basis for pathologic classification of endometrial hyperplasia and indicate typical morphological changes in endometrium during development of CEH-P complex in bitches.

1. Background

Over the last few decades, the growing trend of problems associated with canine reproduction, which has been a major obstacle in the practice of breeding these animals, can be observed. Despite the significant advancement in the technology of assisted reproduction, as well as the new diagnostic methods of reproductive system diseases in female animals, their use in routine veterinary practice is comparatively low. A Syndrome in the literature known as cystic endometrial hyperplasia - pyometra complex (CEH-P), identified within the reproductive system of female dogs is the most serious and most frequently diagnosed pathological condition of the uterus in this species. It occupies a leading position among uteropathies found in the elderly females. During the last years very young individual cases have been frequently reported [1–3]. In spite of the numerous studies conducted on determining the causes of the development of the most common uterine pathologies included in CEH-P, the etiopathogenesis of this disease remains unclear. In addition, the prevalence of the diagnosis of this type of uteropathies is significantly higher in bitches compared to females of other species. This points to the existence of
specific mechanisms leading to disturbances in the functioning of the endometrium characteristic for female domestic dogs [4, 5].

According to the current knowledge, the origination of CEH-P in bitches is the result of the simultaneous influence of hormonal factors and infectious agents. Abnormal ovarian activity is, therefore, important for the development of degenerative processes of the endometrium. When the activity of the ovaries is well preserved and normal, the ovarian hormones are able to regulate the functioning of the uterus - through specific receptors (estrogen - ER, progesterone - PR) [6]. Physiologically, these receptors are located in the endometrium in a certain balance. It is possible that an abnormal expression and or distribution of the named receptors may constitute a ground for the observed pathological changes [7–10]. On the other hand, it is worth noting that the proper functioning of the reproductive system of females depends - besides hormones produced by the ovary – on a number of growth factors (GFs) with a broad spectrum of biological effects [11]. Considering their wide range functions in many regulatory processes, the participation of these factors in the pathogenesis of CEH-P seems highly likely.

The pathogenesis of endometrial hyperplasia in relation to conditions such as endometritis, cystic endometrial hyperplasia glands (CEH), pyometra as well as the possibility of their progression to a life-threatening stage, has become the research topic of numerous scientific papers. Changes occurring in the structure of the mucosa during the menstrual cycle of bitches can be considered as a disease when they have a detrimental effect on reproduction, or in a relatively direct way, threaten the patient's life. Both endometritis, CEH and CEH-P complex are all pathological conditions affecting the uterus, which are substantially distinguishable from each other microscopically. The aim of the study was to emphasize the importance of carrying out histopathological tests while differentiating uterine diseases as well as to reliably classify them in terms of the observed macro- and microscopic changes. Thus, the obtained results constitute a basis for reliable diagnosis that is clearly conclusive of the degree of the actual conditions.

2. Results

2.1 Control group

Assessing microscopic preparations made from slices of normal uterine horns, no abnormalities in the morphology of its individual layers were found (Figure 1B, C, D). The obtained microscopic image was typical of the expected stage of anestrus in the reproductive cycle of bitches on the basis of which the qualification of the uteri in the control group was made. Thus, approving the initial macroscopic evaluation, thereby showing no apparent degenerative changes on the surface of the endometrium.

2.2 Experimental group I

Preliminary macroscopic evaluation of the surface of the uterine mucosa within that group indicated the possibility of the occurrence of pathological changes in the endometrium. The endometrium was clearly reddened and swollen along the uterine horns, which prompted the suspicion of advanced endometritis.
However, in the histopathological examination there were no changes observed to indicate an ongoing inflammatory process. Microscopically, the uteri classified into this group showed a moderate to high degree of thickening of the endometrium, occurring uniformly within the tested section of the horn. In addition, acute, mostly multifocal endometrial haemorrhage of mild to moderate severity was observed. In rare cases, there were reports of earlier bleeding that underwent a process of resorption, as well as small congestion of serous membrane. The appearance of endometrial glands was characteristic of the anestrus phase (Figure 2B, C, D).

### 2.3 Experimental group II

In the macroscopic evaluation, the CEH covered uterus showed thickened endometrium on its longitudinal section, which was as a result of the presence of numerous cysts on its surface. The degree of histological changes within the endometrial glands in cystic hyperplasia was mild to severe. The microscopic evaluation showed a mild to moderate swelling of the endometrium. Glands of different sizes, occurring focally and multifocally, characterized by a great diversity in size and chaotically distributed in endometrium. Histologically, a significant flattening and progressive atrophy of glandular epithelium was observed. In some cases, a slight to moderate degree of adenomyosis was identified. There was also an acute, mostly multifocal, mild to moderately severe haemorrhage of the endometrium (Figure 3B, C, D). In most of the analyzed preparations, there was no infiltration of inflammatory cells, but in rare cases, a moderate lymphoplasmacytic, purulent inflammation of the uterine lining was observed.

### 2.4 Experimental group III

The uteri obtained from bitches with diagnosed closed (n=22) or open (n=8) type of pyometra were significantly enlarged compared to normal uteri. The increase in volume of the uterine horns was caused by surging purulent secretions in their lumen. Depending on the severity and duration of the inflammatory process and the degree of degenerative changes, discharge took on the color from pale yellow through green to brown. Histologically, in most of the analyzed cases it was also diagnosed multifocal cystic endometrial hyperplasia, with varying degrees of severity. During microscopic examination, neutrophil migration into the lumen of endometrial glands was observed. However, in the endometrial stroma, there were lymphocytes and plasma cells found, which demonstrates the chronic inflammatory process. In all uteri, it was observed a considerable degree of inflammation of the endometrium of a purulent, partly lymphoplasmacytic/lymphohistiocytic character, usually diffuse or multifocal, which also comprised of a layer of muscle (Figure 4B, C, D). Additionally, though in rare cases, there was a moderate to significant degree of, mostly, multifocal fibrosis of the endometrium, adenomyosis and endometrial haemorrhage.

### 3. Discussion

During the estrous cycle, the endometrium of the bitch undergoes distinct morphological and biochemical changes [9, 12–15]. The important role in regulating these changes is mainly attributed to steroidal hormones [10, 15]. Both the endometrium and myometrium of the uterus undergo changes during the different phases of the menstrual cycle, pregnancy and menopause [14, 16]. Changes to the
morphological structure, which occur under the influence of steroidal hormones are in turn controlled by local factors of an autocrine - and paracrine signalling [17, 18]. It was also agreed that a certain regulatory function is played by the metalloproteinases, so-called MMPs, as well the processes of apoptosis occurring in the cells of the uterus [15, 19]. In bitches, contrary to other animal species, such as: the horse [20], rabbit [21], rat [22], mouse [23], and also man [23, 24], determining the relationship between the patterns of cell proliferation of the endometrium and the level of hormones synthesized by the ovaries is difficult.

The problem of the co-occurrence of ovarian and uterine pathology was noted by Katkiewicz and Boryczko [6]. Lesions involving the uterus were classified according to the severity of CEH-P. In the macroscopic and microscopic assessment of the uterus of a bitch with a significant advancement of the disease, the changes typical of classical pyometra were observed. The author's personal observations fully confirm the findings of the authors mentioned above. In both cases, we observed a significant enlargement of the organ, congestion and thickening of the endometrium as well as residual purulent exudates in the lumen of the uterus. Microscopic examination of the sections of the uterine horns showed structural changes typical of CEH, including proliferation, together with the presence of cysts on the surface of the endometrium (Figure 4B, C). In addition, the proliferation of endometrial epithelial lining was observed as well as the transformation of the endometrial glandular epithelium (secretory phase).

The results presented in this study as well as the authors cited for all cases of the analyzed uteri, a considerable degree of endometritis, mainly purulent, was observed. Inflammatory infiltration primarily of mononuclear cells, demonstrating a chronic, inflammatory process was identified within the endometrial stroma. However, on the mucosal surface and in the lumen of the uterine glands a plurality of neutrophils was observed (Figure 4 C). In addition, though rare, there were cases of moderate to significant degrees of, mostly, multifocal fibrosis and bleeding of the endometrium, which was not observed in the studies of the authors cited.

A picture typical of CEH-P was also observed by Groppetti et al. [25]. Apart from cystic and inflammatory processes, instances of CEH-P were accompanied by swelling of the endometrial stroma, infiltration of inflammatory cells and mucosal bleeding, which has also been described in this research.

The research findings by Younis, et al. [26] also confirm the existing changes in the macroscopic and microscopic image observed in this study. In the group of uteri affected by CEH-P, the authors chose three characteristic histological changes typical of the disease. The first one included the purulent uterine content, which, in some cases, occupied the deeper layers of the uterus. Another one was referred to the changes associated with pathological enlargement and proliferation of endometrial glands (CEH). The last, third group of changes included - progesterone dependent - proliferation of glandular epithelium, showing vacuolization of the cytoplasm and a single pyknotic nucleus. To a lesser or greater extent, the observed histopathological changes were also recorded by Bigliardi et al. [1].

De Bosschere et al. [9] points to the importance of research in the pathological diagnosis of the diseases of the uterus in female dogs. According to the author, the very frequent mistakes encountered while
making definitive diagnoses of specific diseases of the reproductive system is that the doctors only clinically assess the state of the organ, while it is not accompanied by detailed research. In women, histopathological examination is the golden rule in the diagnosis of diseases of the uterus [27, 28]. In such cases, an abnormal bleeding or a suspected infertility are the indicators for advanced diagnostics. It is a routine test in case of a chronic inflammation of the lining of the uterus, and the identification of plasma cells in the endometrial stroma is the basis for a diagnosis [27].

Moreover, in the assessment of endometrial hyperplasia in women, a clear division is used according to the system recommended by the WHO as well as the International Society of Gynecological Pathologists [28]. Taking into consideration the severity of changes in the glands of endometrial hyperplasia, we can distinguish simple hyperplasia (SH) from complex hyperplasia (CH). While the intensity of atypical changes in the nuclei of the lining of uterus enables the division into simple atypical hyperplasia and atypical complex hyperplasia. No unified system of differentiation of the early histological changes covering the mucous membrane of the uterus in the bitches leaves - in contrast to the classification used in humans - many uncertainties in the specification and differentiation of particular disorders.

Referring to the system of the distribution of diseases of the uterus used in human medicine, an attempt to create an analogous way of classifying the observed changes in the endometrium accompanying CEH bitches in the present study would amount to the category of complex hyperplasia (CH).

The routine clinical examination commonly used in veterinary practice is an insufficient method for early diagnosis of pathological conditions of the uterus [26, 29]. Biopsy of the uterus is a recommended test, which is considered to be one of the most accurate procedures for providing information about the actual condition of the organ [30]. Christiansen et al. [31] using a transcervical biopsy, demonstrated both its high sensitivity and accuracy in identifying CEH, *edometritis* as well as uterine fibrosis. The results of this study were later confirmed following a full analysis of sections of the uterus of the same bitches. An essential element of the research that undermines the credibility of the method used, was the detection of pyometra during the complete autopsy of the sections of the uteri of the bitches, which was not demonstrated by the histologic evaluation of the tissue specimen obtained using the transcervical method. The authors’ explanation is that a mismatch in the stated results can have a twofold base. These observations may be explained by the fact of the insufficient sensitivity of the method used in the identification of pyometra or else the application of the procedure itself as being the cause of disease development [31]. Moreover, these results were obtained only in bitches in *diestrus* phase, pointing to the unique sensitivity of the uterus during this period of the menstrual cycle.

The importance of the diagnosis of infertility using surgical biopsy through laparotomy is also emphasized by Mir et al. [30]. According to the authors, the risk of complications is minimal if the procedure is carried out in accordance with the guidelines of the protocol and a post-operative treatment is scheduled with the use of aglepristone or prostaglandin in bitches in the *metestrus* phase. However, transcervical uterine biopsy in order to evaluate the condition of the organ, is not a problem in most species, although, in dogs it is relatively difficult and involves the risk of organ damage and the
development of infection [13, 25, 30–32]. Therefore, this method is not often practiced in the diagnosis of uterus diseases and etiology of the reduced fertility.

The usefulness of histopathological examinations in the differentiation of uterine diseases has been, especially, emphasized in presented researches. During the initial evaluation of the research material, the uteri of the bitches from the experimental group I (GI) have been characterized as falling within the range of advanced inflammation. Such a finding is possible thanks to the macroscopic evaluation of the endometrium of bitches in the anestrus phase. A clearly reddened and swollen endometrium, in some cases, with a small amount of serous-bloody discharge, suggested the classic picture of endometritis (Figure 2A). Meanwhile, microscopic research has not confirmed any inflammation of the lining of the uterus, and, what is more, there was no presence of any cells that provide for ongoing inflammatory process within this organ, but only multifocal endometrial haemorrhage with mild to moderate severity (Figure 2B, C). The results obtained by our studies confirm the need to perform detailed analyzes in the differentiation of conditions of the uterus in bitches due to the lack of consistency of macroscopic assessment with histological analysis. The presented results, however, require the continuation of studies and broader biological analysis, due to lack of data clarifying the described condition.

New information relating to the search for key factors in the development and progression of the disease could be provided by the experimental models comprising both the induced spread of the infection by inoculation of pathogens as well as its development as a result of mechanical irritation of the lining of the uterus. A significant part of bacterial and hormonal factors as well as a specific range of sexual cycle characteristic for the development of CEH-P can be confirmed by Arora's et al. experiment [33]. The cited authors suggest that intrauterine inoculation of *E. coli* during the simulated diestrus phase in bitches after ovariectomy is associated with a high risk of CEH-P. During poststerilization examinations, the uteri were enlarged, and containing a yellow to reddish purulent discharge in the lumen of their horns. Microscopic analysis of the studied organs highlighted a picture typical of complex CEH-P, also listed under our work as well as in the studies conducted by the aforementioned authors [6, 9, 25, 26, 28]. Interestingly, intravaginal introduction of *E. coli* during physiologic or induced estrus phase did not lead to the development of CEH-P [33].

In these cases, despite the repeated intravaginal inoculation of *E. coli*, the uteri did not show any macroscopic lesions and histologically there was neither the inflammatory process nor the presence of endometrial cysts [33]. The conclusion is that during *estrus*, a normal - healthy uterus has strong enough defense mechanisms to ensure the elimination of a large number of pathogens [33, 34]. Recently it has been suggested that other specific factors may also be involved in the pathogenesis of CEH-P, which facilitate the initiation, development and maintenance of bacterial infection in the uterus during the diestrus phase [33].

Using the same experimental model, the microbial suspension of *E. coli* was inserted into the horns of the bitches in their metestrus phase [35]. Such a location of pathogens led to disease progression similar in terms of histology and immunohistochemistry to naturally occurring syndrome of CEH-P. According to the
authors, the cystic endometrial hyperplasia of the glands is not a prerequisite for the development of advanced uteropathy. The disease was induced in the physiologic metestrus phase in bitches in which CEH had been ruled out.

The reactivity of endometrium to different stimuli has been demonstrated in several experiments. Undoubtedly, the endometrium is very sensitive, during the reproductive cycle (diestrus), to both mechanical stimulation as well as to exogenous substances. The influence of mechanical stimulation on the endometrial lining was observed during the biopsy, resulting in segmental mucosal hyperplasia [36]. Proliferative changes induced by experimental manipulations in the uterine horns have also been described by Nomura et al. assembly [37]. The series of test carried out by them has been the basis for further experiments conducted by other researchers around the world. Various materials and substances such as silk thread, olive oil, broth, uterine transplants or previously mentioned bacteria E. coli were deposited inside the uterine horns [33, 35, 36].

Minor mechanical irritation of the uterus caused by the introduction of olive oil, silk thread or a delicate scarification of endometrium, induce potent mitotic cell surface of epithelial and stromal fibroblasts creating complex and irregular passages of endometrium [37, 38]. Mechanically induced CEH, in histological cases, reflects the spontaneous development of the disease described by De Bosschere et al. [9] however, with regard to the expression of the receptors of sex hormones, it corresponds more to the stages of creating the bearing or the development of pyometra [38].

As indicated by the results of their data and published literature, the clinical condition of the patient does not constitute grounds for exclusion of uterine diseases. De Bosschere et al. [9] reported that the majority of bitches, which were classified as clinically healthy, were found to be having uterine disorders in varying degrees of progression. This problem concerned 20 out of 26 patients identified as having hyperplastic changes of the endometrium from mild (8) to heavy CEH (9) - both in the diestrus and closed anoestrus. Additionally, in some cases, stromal oedema, congestion and local endometrial bleeding was diagnosed. These results are consistent with our own observations, where the majority of cases of bleeding of the endometrium and hyperplasia of glands, occurred in clinically healthy bitches, who had undergone ovariohysterectomy for the purpose of reducing the risk of later development of uterine diseases and depression of fertility.

According to citied authors females affected by diseases like CEH- mucometra do not show any visible clinical symptoms, while diseases such as CEH - pyometra are obvious disease conditions. The diagnosis given solely on the basis of clinical symptoms, which in the case of pyometra may be very diverse, can have serious errors. Pyometra accompanied by other symptoms such as: insomnia, depression, loss of appetite, polyuria, thirst and vomiting are also characteristic of many other disease conditions [9, 39–41]. Basing the diagnosis solely on simple clinical examination and general symptoms, pyometra can easily be confused with kidney failure, liver disease, diabetes or adrenal insufficiency [9].

The available literature describing the problems of the pathogenesis of uterine diseases in bitches is dominated by the view that the proliferation of glandular cystic endometrial hyperplasia and pyometra
are distinct disease conditions with a common. Thus, pyometra can coexist with cystic endometrial hyperplasia of the glands, although this is often not necessary the case. Both conditions may be separate uteropathies and are often diagnosed independently [6, 9, 29].

Independent occurrence of CEH and pyometra is also confirmed by the fact that most cases of both diseases have been described in bitches in their diestrus rather than anestrus phase. This indicates a significantly more frequent development of this pathology in the diestrus phase, confirming the hypothesis that the cystic endometrial hyperplasia of the glands is not always preceded by pyometra. The average age of bitches with CEH and pyometra is very close, and that is why pyometra does not always appear in bitches older than those with the CEH [9].

An analysis of the age structure of bitches with particular uterine diseases in our study, confirms the development of pyometra occurring more often in older females, aged about - 9.3 years, while CEH – affects bitches over 6 years of age. Although both diseases are distinct conditions included in the complex pathology of the uterine diseases of bitches, the importance of CEH in the development of pyometra is unquestioned [9, 29, 42].

Relating our own results to the works cited, it should be noted that among the analyzed cases of pyometra, there were also advanced, multifocal cystic hyperplasia of endometrial glands in all of them (Figure 4B, C). The histological picture refers to class VI of the classification of uterine diseases described by de Bosschere et al. [9] as pyometra (hyperplastic) with severe inflammatory response, numerous cysts, an overstated relation of endometrium to myometrium as well as moderate proliferation of fibroblasts.

Given the dynamic changes occurring in the uterus during implantation and development of the embryo and fetus, it is not surprising, that we observed a high sensitivity of the structures of the endometrium to the influence of various factors, both endogenous and exogenous. During the luteal phase of each reproductive cycle, regardless of the outcome of fertilization, the endometrium is subject to rapid processes of hyperplasia and hypertrophy. There has been a significant increase in both the diameter and the overall weight of the uterus [36]. In female dogs and cats, endometrial cysts, which are not accompanied by inflammation of the mucous membrane, are derived, in most cases, from the glandular epithelium of the endometrium. In mares, on the other hand, often formations of lymphoid origin are much often diagnosed [13, 32].

Although cystic endometrial hyperplasia glands (CEH) is encountered in both bitches and queens, the disease more often affects the bitches. This is associated with a longer period of impact of progesterone on the endometrium during the diestrus [13, 38, 43]. In the literature, many studies describing abnormal endometrial hyperplasia, in relation to diseases like CEH, mark also a likelihood of its progress to life-threatening stages [8, 33, 36, 38, 39, 42–45]. Cysts developing in the endometrium classified as CEH differ significantly in terms of number, size, distribution and histomorphology [36]. Groppetti et al. [25], in their microscopic evaluation of CEH, observed a clear hyperplasticity and thickening of the tissue with endometrial stromal oedema and a significant extension and branching of glands. The diameter of the cysts identified by them amounted to a few millimeters, and the interior was filled with clear secretions of
mucous character. Both the superficial and glandular epithelium of the endometrium formed high and irregular stratified columnar cells.

Compatibility of the described results was also achieved in the research presented by us; microscopic analysis of histologic preparations of uteri affected by CEH, revealed a wide range of observed changes in the degree of mild to advanced. The histologic evaluation was dominated by swollen glands of endometrium, with the occurrence of both focal and multifocal. In addition, we observed a significant flattening and progressive atrophy of the glandular epithelium as well as the often acute, multifocal bleeding of the endometrium, which has not been included in the analyses of previously cited authors (Figure 3B, C). Using the classification of uterine diseases used by De Bosschere et al. [9], the presented conversion, which do not include inflammatory response, are eligible for the third group - described as a severe form of CEH with numerous large cysts and increased relation of endometrium to myometrium.

4. Conclusions

Specific description of cases of proliferative lesions of endometrial glands with different forms of the disease is not easy and often misleading. In addition, the physiology of the reproductive cycle, during which the endometrium of bitches is a subject to dynamic reconstruction, does not make the task easier. The research presented recognizes significant changes in the microscopic structure of the endometrium observed in diseases of the uterus, forming part of a complex disease known as CEH-P. Moreover, they indicate the importance of research in the pathological diagnosis of uterine diseases in bitches, showing a frequent lack of consistency in the macroscopic assessment and histological analysis. In order to continue the research it is necessary to carry out further histological analysis of the uterus, extended to similar attempts obtained from healthy females in the diestrus phase. Since these pathologies are often identified in this phase of the reproductive cycle, the achieved results will facilitate the assessment, classification and differentiation of benign hyperplastic endometria as a physiological process with pathological degenerative changes.

5. Methods

Material for the study consisted of uteri of 120 female dogs, aged 1 to 16 years, obtained during routine ovariohysterectomies. In order to standardize the research material, the analysis included only the wombs of females in the anestrus phase. The phase of the sexual cycle was determined on the basis of cytology smears taken from the vagina. This division does not apply to animals showing clinical symptoms characteristic for diseases of the reproductive system. The animals from which the material was collected had not previously received hormone therapy or gave birth. More details about their status are presented in table 1.

Bitches diagnosed with pyometra presented different stages of typical symptoms such as purulent or purulent–hemorrhagic discharge, lethargy, vomiting, polydipsia, polyuria, dehydration, anorexia, abdominal enlargement, temperature disturbances and changes in the colour of mucous membrane.
Hematological and blood biochemistry changes also were observed. In WBC count a differential left shift has been observed as well as leukocytosis with neutrophilia. Moreover normocytic or normochromic anemia and thrombocytopenia has been noted. In biochemistry analysis of the blood C—reactive protein, alkaline phosphatase, bilirubin, cholesterol, creatinine, blood urea nitrogen and electrolytes were increased.

No significant changes in blood morphological and biochemical parameters were recorded for bitches classified for prophylactic ovariohysterectomy.

The animals from which tissue was used in our research were private property. These animals had a routine ovariohysterectomy performed in private veterinary practices. The scientists of our team obtained tissues, treated as medical waste, with the consent of the owner of the Veterinary Clinic.

| Table 1. Details of the bitches used in the research |
|-----------------------------------------------------|
|                                                      |
| **Control group** | **Group I Endometrial Haemorrhage** | **Group II CT** | **Group III Pyometra** |
|-----------------------------------------------------|
| Number of bitches | n= 30 | n= 30 | n= 30 | n= 30 |
| Age (years)                                               |
| max            | 8     | 14    | 13    | 16    |
| min            | 1     | 1     | 3     | 2     |
| medium         | 2.2   | 4     | 6.4   | 9.3   |
| Body weight (kg)                                        |
| max            | 54    | 42    | 40    | 53    |
| min            | 2.3   | 5     | 6     | 3     |
| medium         | 17.1  | 18.7  | 20.2  | 19.5  |
| Breed          |
| crossbreed     | 22    | 15    | 12    | 14    |
| in breed type  | 8     | 15    | 18    | 16    |

Macroscopic evaluation and preliminary classification of research material in regard to listed pathological changes

Following ovariohysterectomy, both uteri and ovaries were transported to the laboratory, where they were subjected to macroscopic evaluation and preliminary classification of the individual experimental groups. The division was made on the basis of visual evaluation of the endometrium after a longitudinal incision of the uterine horns. The control group (C, n = 30), however, consisted of normal organs, which in their macroscopic evaluation presented no degenerative changes (Figure 1A). The uteri with pathological changes in the mucous membranes were divided into three groups. The experimental group I (GI, n = 30) consisted of uteri which were initially evaluated as being in the state of inflammation. They were strongly congested and the mucous membrane of the uterus was swollen with serosanguinous discharge frequently occurring in the lumen of the horns (Figure 2A). Group II (GII, n = 30) consisted of uterus
affected by cystic endometrial hyperplasia (CEH), and in such cases numerous multifocal cysts on the surface of the endometrium, filled with serous exudates, were found (Figure 3A). The last experimental group consisted of uteri with accumulation of purulent secretions, residual in the lumen of their horns (GIII, n = 30). These uteri were significantly enlarged, mainly presenting pyometra of a closed type (Figure 4A).

5.1 Histological staining and visualization of microscopic preparations

Obtained fragments of the sections of the uterine horns, of approximately 2-3 cm, were fixed in buffered 10% formalin, and then dehydrated in series of increasing concentrations of ethanol and then placed in xylene with the aim of exposing the tissues and removing alcohol from them. Tissues prepared in this way were then embedded inside a block of paraffin. The paraffin blocks were cut into slices using a rotary microtome. Paraffin was removed using xylene, and then the preparations were hydrated in a series of decreasing concentrations of alcohol and they were, finally, placed in water. Staining of the preparations was performed on the basis of the staining method of Böck [46] using hematoxylin and eosin. After staining and dehydration of the preparations in a series of increasing concentration of alcohol, they were secured by gluing them to cover slippers using a lotion (Leica CV MOUNT). The preparations were evaluated under a light microscope (Olympus WX41). The pictures were made using the Image Analysis Software “analySIS FIVE”. The obtained results were confirmed by a certified pathologist.

6. Abbreviations

**CEH**: cystic endometrial hyperplasia

**CEH-P**: cystic endometrial hyperplasia - pyometra complex

**CH**: complex hyperplasia

**ER**: estrogen receptor

**GFs**: growth factors

**H&E**: hematoxylin and eosin

**MMPs**: metalloproteinases

**PR**: progesterone receptor

**SH**: simple hyperplasia

**WBC**: white blood cells

**WHO**: World Health Organization
7. Declarations

7.1 Ethics approval and consent to participate

The authors declare that during the work, no animal studies were conducted. The study is based only on tissues obtained from bitches, which were spayed. Tissues are treated as medical waste. Animal owners have agreed to use the tissues for our analyses. In case of work on tissues of animal origin, the consent of the Ethics Committee is not required.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

Not applicable.

7.4 Competing interests

The authors declare that they have no competing interests.

7.5 Funding

The research project was financed by the Polish National Science Centre under decision number DEC2013/09/N/NZ5/01835. The research was co-financed within the framework of the Ministry of Science and Higher Education programme as "Regional Initiative Excellence" 2019-2022, Project No. 005/RID/2018/19.

The founders had no role in designing the research, conducting experiments, collecting and analysing data, deciding to publish or prepare a manuscript.

7.6 Authors' contributions

M.W-W.-Writing manuscript draft, collected uteri, performed macroscopic classification of uterine diseases, collected fragments of uterine horns, interpretation of histological results and reclassification in relation to the results of microscopic analysis.

M.R.-Performed the histological examinations, contributed to the macroscopic and microscopic classification.

B.B.-Performed the histological examinations, analysis and interpretation of obtained data.

B.M.J.-Performed cytological examinations, contributed to the collected uterine fragments, collected and processed of microscopic results.

M.K.-Writing part of manuscript draft, editing.
J.M.J.-Contributions to conception of data, drafting manuscript, critical revision, final approval of the revision to be published.

All authors read and approved the final manuscript.

7.7 Acknowledgements

We would like to thank professor Dorota Bukowska (Department of Diagnostics and Clinical Sciences, Institute of Veterinary Medicine, Nicolaus Copernicus University in Torun, Poland), for help with research material collecting and scientific advising.

7.8 Authors’ information (optional)

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**Figures**

![Figure 1](image_url)
A-D Physiological uterus of bitch during anoestrus. Physiological uterus of two years old bitch. A – macroscopic image, longitudinal incision of the uterine horns. No visible degenerative changes on the surface of the endometrium. B, C, D – H&E stained histological images of transverse uterine horn fragment. An microscopic image of a typical physiological condition, without degenerative changes in the structure of each layer of the uterus. B- 20x; C-100x, D-400x.

Figure 2

A-D Endometrial haemorrhage Uterus from the annual bitch during anoestrus. A – macroscopic image, longitudinal incision of the uterine horns. Macroscopically visible redness and thickening of the endometrium. B, C, D – H&E stained histological images of transverse uterine horn fragment. Microscopically confirmed acute multifocal haemorrhage of endometrium with visible extravascular erythrocytes, a moderate degree of severity. B- 20x; C- 100x, D-400x.
Figure 3

A-D Cystic endometrial hyperplasia (CEH) Uterus from the 8 year old bitch. A – macroscopic image, longitudinal incision of the uterine horns. Macroscopically observed cysts on the surface of the endometrium. Thickened and reddened mucosa. A small amount of serous-bloody discharge was observed inside the horns. B, C, D – H&E stained histological images of transverse uterine horn fragment. Microscopically visible multifocal cystic hyperplasia of glands with a clear flattening of the glandular epithelial cells (C-D) and mild oedema of the endometrium. There are also acute, multifocal endometrial haemorrhage of slight degree. B- 20x; C- 100x, D-400x.
Figure 4

A-D Pyometra Uterus from the 8 year old bitch. The uterus increased considerably, horns entirely filled with surging purulent secretions. A – macroscopic image, longitudinal incision of the uterine horns. Macroscopically endometrium damaged, presenting significant degenerative changes. A number of differently sized cysts on the surface of the endometrium were observed, most of them already ruptured. B, C, D – H&E stained histological images of transverse uterine horn fragment. Chronic, purulent endometritis, with places of lymphoplasmacytic changes has been observed. The inflammation of a severe degree occurred in the muscular layer. Multifocal cystic endometrial hyperplasia occurred as well as adenomyosis and mild multifocal endometrial fibrosis. Endometrial glands interior filled mainly by neutrophils. B- 20x; C- 100x, D-400x.