

Immunohistochemical studies of the uterine wall in bitches with different states of the reproductive system

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Abstract. Dysfunctions of the reproductive system in bitches are a severe problem. Studies were conducted to understand the main differences in the receptor activity of immunocompetent cells in the different conditions of the reproductive system to determine the functional activity of lymphocyte and progesterone surface receptors. Three groups of breeding bitches (a total of 15 animals) were developed to analyze the immunohistochemistry characteristics of the uterus. The immunohistochemical method was used to type cells using monoclonal antibodies. Uterine tissue samples from all groups showed variable amounts of cytotoxic lymphocytes (those with CD8 receptors). Nonetheless, isolated cells were discovered strewn across the stroma in groups 2 and 3. In the anaesthetic group, cytotoxic lymphocytes were scattered in the functional layer of the mucosa in the part closer to the uterine lumen, and lymphocytes were located singly in the stroma around the excretory parts of the glands and among the epitheliocytes. The study of the activity and topography of progesterone receptors in the uterine structures of the studied animals showed that the glandular epithelium of all the groups had extremely low progesterone cell reactivity. All of the examined samples showed a clear positive response (progesterone receptors) in the endothelium of the endometrial capillaries. The established peculiarity of progesterone receptor distribution in different structures of the uterus depending on the functional state of the reproductive system confirms the significant role of progesterone in the genesis of dysfunctions and is likely to be a sensitizing factor.

1 Introduction

In recent years, small animal veterinary medicine has undergone significant development and changes in the world. The growing importance of breeding and obtaining healthy offspring leads to an increase in reproductive disorders in dogs [1]. The immune system determines the survival of an individual, distinguishing itself from another. The body uses several protective mechanisms at the same time to ensure the absence of the disease [2].

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The physiological changes that occur in the body of bitches during the sexual cycle are directly triggered by neuroendocrine control of the immune systems. In this case, the main organ that is exposed to all three systems is the uterus itself. The potential for fertilization during the sexual cycle and later on in pregnancy will depend on its functionally sufficient state [3]. Based on the fundamental principle of positive and negative feedback, physiologically active chemicals produced by the immune and endocrine systems regulate uterine function [4]. A pyometra and, thus, an ovariohysterectomy are frequently the outcome of reproductive system dysfunctions that arise from disruptions of the delicate balance between regulatory systems, particularly at the level of the organ itself [5].

Despite the prevalence and recognized importance of the dog pyometra, we still don't fully understand its epidemiology, etiology, and pathophysiology [6]. Interestingly, most dogs with pyometra are colonized by *E. coli* from phylogroup B2 [7], the same phylogroup that is often isolated from the uterine contents of affected animals [8]. In contrast, healthy dogs are more likely to be colonised by other phylogroups, including B1 [9]. Many studies in bitches have shown that excessive amounts of progesterone or excessive uterine sensitivity to progesterone can lead to pyometra [10].

In addition, pyometra usually occurs during the period of diestrus, since high levels of progesterone during this stage contribute to endometrial proliferation and gland activity, while suppressing myometrial contraction and leukocyte suppression in the uterus [11]. Little is yet known about the state of local (uterine), particularly cellular, immunity as a crucial component of the body's defense in the scientific literature now in publication. It is the understanding of the mechanisms of interaction of immunoregulatory cell populations at the local level that will provide an opportunity to prevent the development of dysfunctions of the reproductive system.

The aim of the research was to establish the functional activity of surface receptors of immunocompetent cells and progesterone in uterine tissues in different states of the reproductive system.

2 Material and methods

Three groups of breeding bitches (a total of 15 animals) were developed for the purpose of studying the immunohistochemistry parameters of the uterus: 1 group – bitches in the anestrus stage of the sexual cycle ($n = 5$); 2 group – bitches in the state of the sexual cycle ($n = 5$); Group 3 – bitches with a pyometra ($n = 5$). All animals underwent ovariohysterectomy in compliance with all requirements for surgical intervention under ketamine-sedative neuroleptanalgesia.

The uterine tissue was placed in a glass dish with a tightly closed lid and preserved in a 10% formalin solution. In immunohistochemical studies, deparaffinisation, rehydration and high-temperature demasking of antigens were performed in a PT module (DAKO, USA) using HIER buffer (pH=9.0), after which the IHC reaction was performed in an AutostainerLink 48 (DAKO, USA) according to the protocol of the manufacturer of each antibody.

The immunohistochemical reaction was visualised using a DAKO EnVision+ System with diaminobenzidine (DAKO, USA) without amplification. Sections were additionally stained with Mayer's haematoxylin and embedded in Canadian balsam.

Evaluation of the results and photofixation of all examination stains and immunohistochemical reactions were performed in the standardized field of view of a Leica DM2000 LED microscope (Germany), at a magnification of $\times 40$ (eyepiece $\times 10$, objective $\times 4$), $\times 100$ (eyepiece $\times 10$, objective $\times 10$), $\times 200$ (eyepiece $\times 10$, objective $\times 20$), $\times 400$ (eyepiece $\times 10$, objective $\times 40$).

Table 1. List of monoclonal antibodies that were used to detect the receptor activity of immunocompetent cells in the uterus.

No	Antibody name	Producer	Clone	Expiration date
1	CD68	"DAKO", Denmark	2B11+PD7/26	2024-07-31
2	CD138	"DAKO", Denmark	CD138/144B	2024-05-31
3	CD8	"DAKO", Denmark	CD8/144B	2024-02-29
4	CD4	"DAKO", Denmark	4B12	2024-10-31
5	CD20	"DAKO", Denmark	L26	2024-04-30
6	Progesterone receptor	"DAKO", Denmark	PgR 636	2023-12-31

Statistical processing of the obtained results was carried out in the software "STATISTICA® for Windows 6.0" (StatSoft Inc., USA, license No. AXXR712D833214FAN5).

3 Results

According to the results of our own research, it was established that in the histological preparations of the endometrium in all studied groups, infiltration by round cell elements was found, which were located singly, scattered in the stroma periglandularly, and also in the form of infiltrates. Moreover, infiltration of immunocompetent cells was observed both in the stroma and around the vessels, close to the glands, or even intraepithelially (Figure 1).

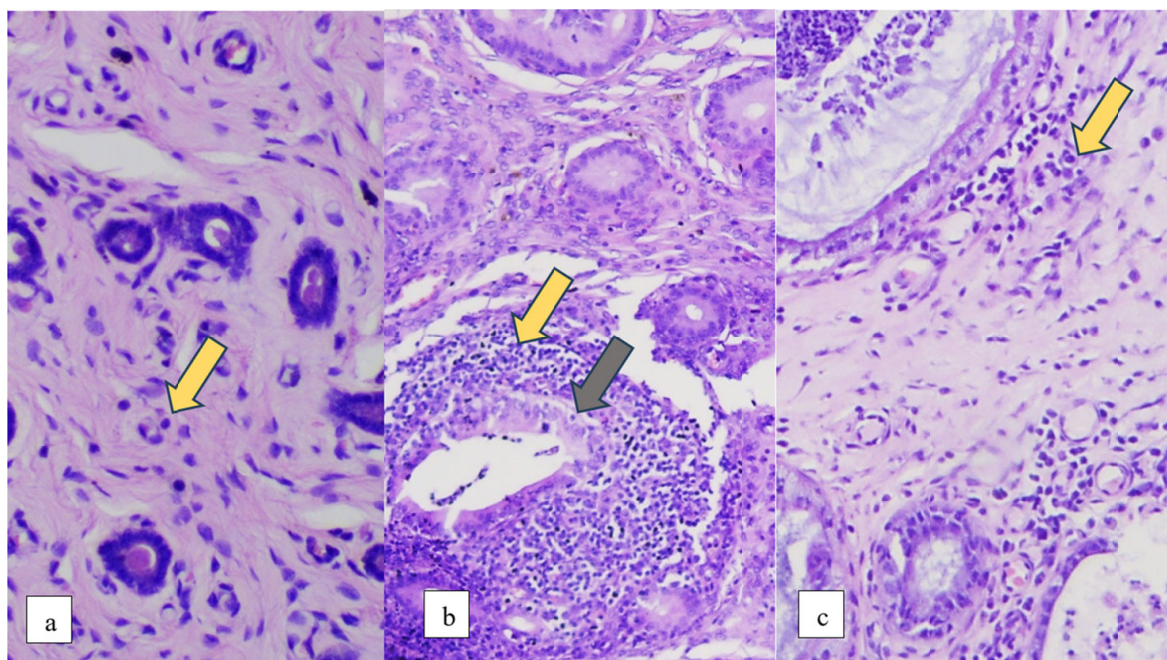


Fig. 1. Scattered cells, infiltration in the stroma (a, yellow arrow) of the mucous membrane of the uterine horn in animals in the anestrus period, periglandular cuff-like (b, yellow arrow) and intraepithelial (b, gray arrow) infiltration in the second group of animals, stromal infiltration (c, yellow arrow) in samples from animals with pyometra. Collection: x200. Staining with hematoxylin-eosin.

Infiltrating cells are immunocompetent cells that, depending on the type, perform a certain function to provide local immune protection. Using the immunohistochemical method, cell typing was carried out using monoclonal antibodies.

It is known that clusters of differentiation (CD) are used to differentiate lymphocyte subtypes, where CD45 is a general lymphocyte receptor, CD4 is characteristic of T-helpers, CD8 is for cytotoxic lymphocytes, CD20 is a marker of all B cells, of which mature B cells have CD138 and also a marker of histiocytic, macrophage cells – CD 68. The interpretation of the results of immunohistochemical reactions is based on the appearance of specific staining (depends on the type of chromogen, we used DAB – brown chromogen) in different parts of the cells. It should also be noted that nuclear, cytoplasmic and membrane staining are distinguished.

Lymphocytes were detected in all samples of the studied groups (Figure 2), which was confirmed by an immunohistochemical reaction with CD45, most of them were detected in cases of the 3rd group as part of the inflammatory infiltrate of the stroma (Figure 2 c, yellow arrow) and periglandularly (Figure 2 c, gray arrow), less in the 1st group, where lymphocytes were scattered singly in the stroma (Figure 2 a, yellow arrow), had no tropism either to vessels or to glands. Regarding the 2nd group, it should be noted that the presence of a significant number of lymphocytes (CD45) located close to the glands, or even intraepithelially was established (Figure 2 b, yellow arrow).

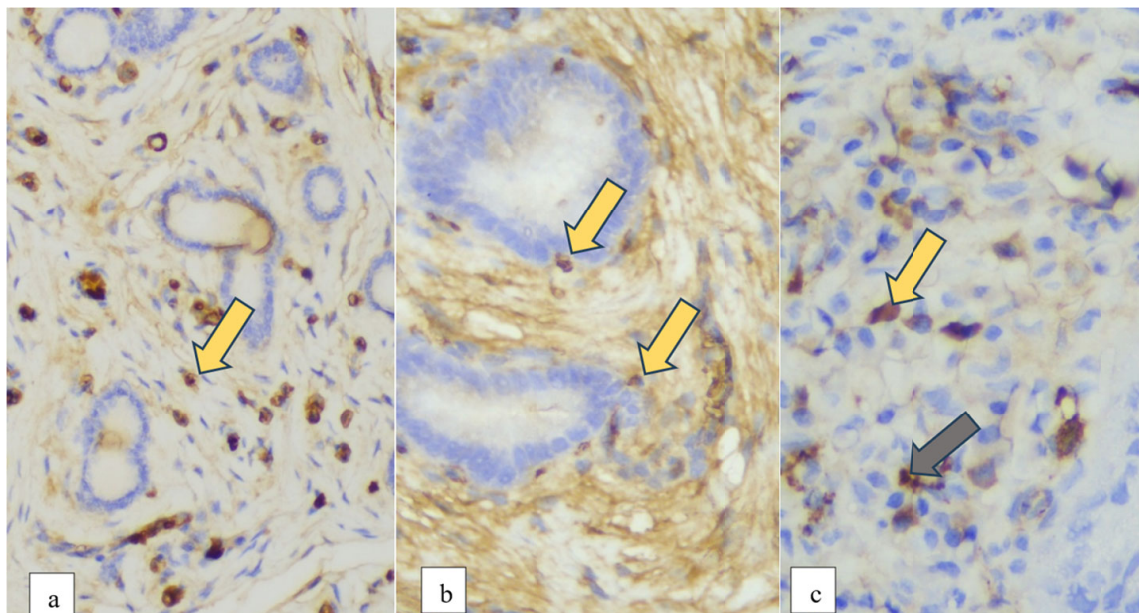


Fig. 2. Lymphocytes scattered singly in the stroma (a, yellow arrow) of the mucous membrane of the uterine horn in animals in the anestrus period and periglandularly (b, yellow arrow) during the sexual cycle, lymphocytes in the inflammatory infiltrate of the stroma (c, yellow arrow) and periglandularly (c, gray arrow) in the uterine mucosa of animals with pyometra. Collection: x200. Immunohistochemical reaction with CD45.

Because of such distinct heterogeneity of the morphotopographic features of the location of CD45 lymphocytes in the endometrium of animal uteri, we decided to specify the type of lymphocytes in each case. T-lymphocytes were verified from two populations – T-helpers (have CD4 receptors, which became the basis for the selection of appropriate monoclonal antibodies) and T-cytotoxic lymphocytes (have CD8 receptors).

Lymphocytes with characteristic brown cytoplasmic staining with membrane accentuation (positive reaction with CD4 monoclonal antibody) were verified as T-helpers. T-helpers were located both in the stroma, scattered single cells (without connection with vessels and glandular epithelium), which was characteristic of samples from the 1st group (Figure 3 a, yellow arrow), and at the border of glands and stroma in samples of the 2nd group (Figure 3 b, yellow arrow) with a significant location of CD4 positive lymphocytes intraepithelially.

Among the preparations of the uterus of the 3rd group (Figure 3 c, yellow arrow), CD4 positive cells were present in the stroma, but most of them – as a significant component of the inflammatory mixed cell infiltrate.

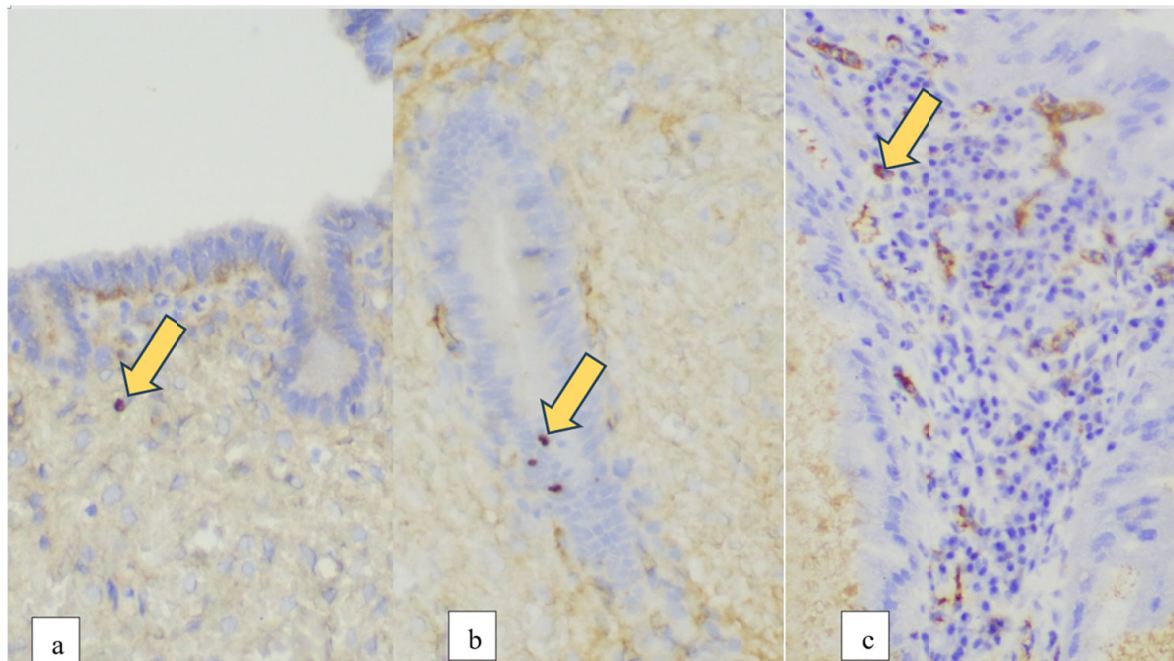


Fig. 3. Localization and density of T-lymphocytes in the stroma (a, yellow arrow) of themucous membrane of the uterine horn in animals in the anestrus period, periglandular and intraepithelial (b, yellow arrow) infiltration in uterine samples during the sexual cycle and stromal (c, yellow arrow) in the inflammatory infiltrate of the mucous uterus of animals with pyometra. Immunohistochemical reaction with CD4. Collection: x200.

The presence of the CD8+ receptor on the surface of the lymphocyte membrane is characteristic of T-suppressor and cytotoxic T-lymphocytes. The staining of the cell nucleus as a result of an immunohistochemical reaction with the appropriate monoclonal antibody is positive.

The presence of cytotoxic lymphocytes (that is, those with CD8 receptors) in varying amounts was detected in uterine tissue samples of all groups (Figure 4). At the same time, single cells scattered in the stroma were found in the 2nd (Figure 4 a) and 3rd (Figure 4 b) groups. In the group during the anestrus period (Figure 4 c), cytotoxic lymphocytes were scattered in the functional layer of the mucous membrane in the part closer to the lumen of the uterus, lymphocytes are located singly, in the stroma around the excretory parts of the glands and among the epitheliocytes.

In addition to determining the presence of T-lymphocytes in the tissue of the uterus, our research also established the presence of B-lymphocytes. The general B-cell marker is CD20, it is found in all B-lymphocytes before their differentiation into plasma cells. Not only the number of B-lymphocytes and their topography were verified (using monoclonal antibodies to CD20, the cytoplasmic and membrane reaction was positive), but also the same parameters were clarified regarding the mature forms of B-lymphocytes, namely plasma cells using an immunohistochemical reaction with antibodies to CD138.

In general, a significant presence of B-lymphocytes (Figure 5) was detected both in the samples of the 3rd group of bitches with pyometra (Figure 5c), and in the mucosa of the 1st group of animals (Figure 5a). Mature B-lymphocytes have a positive reaction with CD138 monoclonal antibodies, so we objectively investigated the presence of such positive cells with specific staining in the studied samples.

CD68 is considered a marker of histiocytic-macrophage cells, and it is characteristic not only for functionally active cells, but also for cells in the phase of apoptosis. The study of the population of these cells is important for characterizing the state of tissue immunity and the course of the immune response to inflammatory stimuli, which is manifested by an increase in the macrophage response to the lysosomal marker CD68. Dotted granular and diffuse staining of the cytoplasm is considered positive.

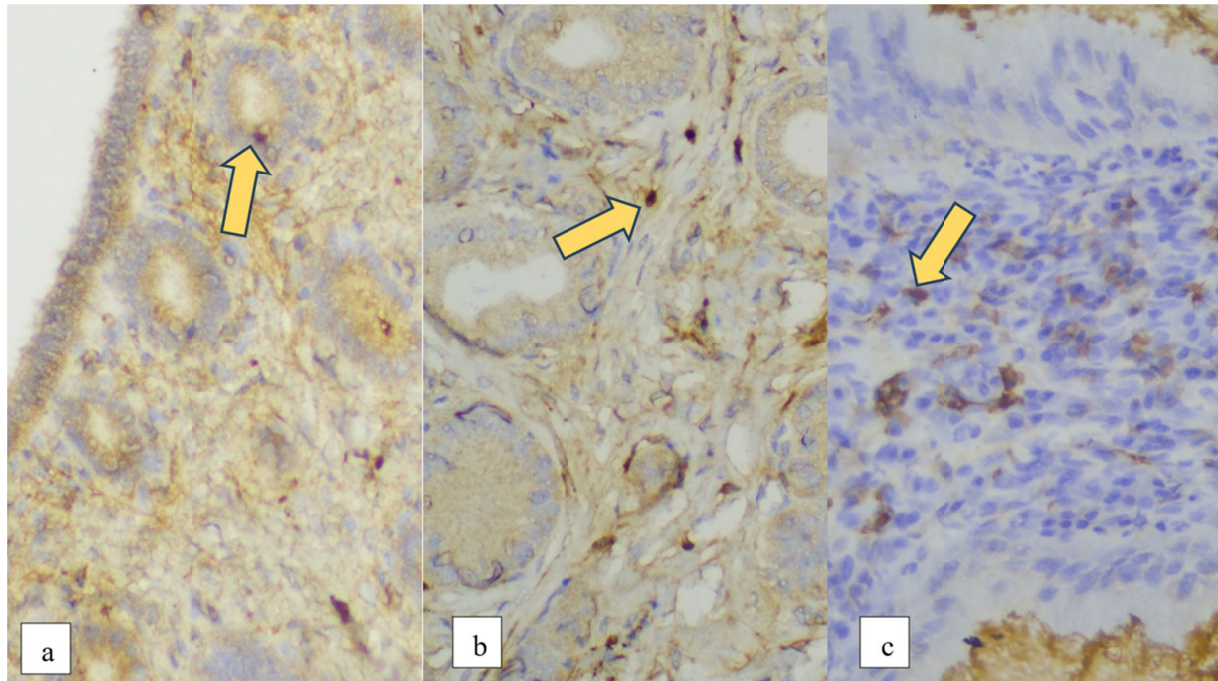


Fig. 4. The location of T-cytotoxic lymphocytes between the epitheliocytes of the endometrial glands in animals in the anestrus period (a, yellow arrow) of the mucous membrane of the uterine horns, stromal infiltration in the second group (b, yellow arrow) and in samples of animals with pyometra (c, yellow arrow). Immunohistochemical reaction with CD8. Collection: x200.

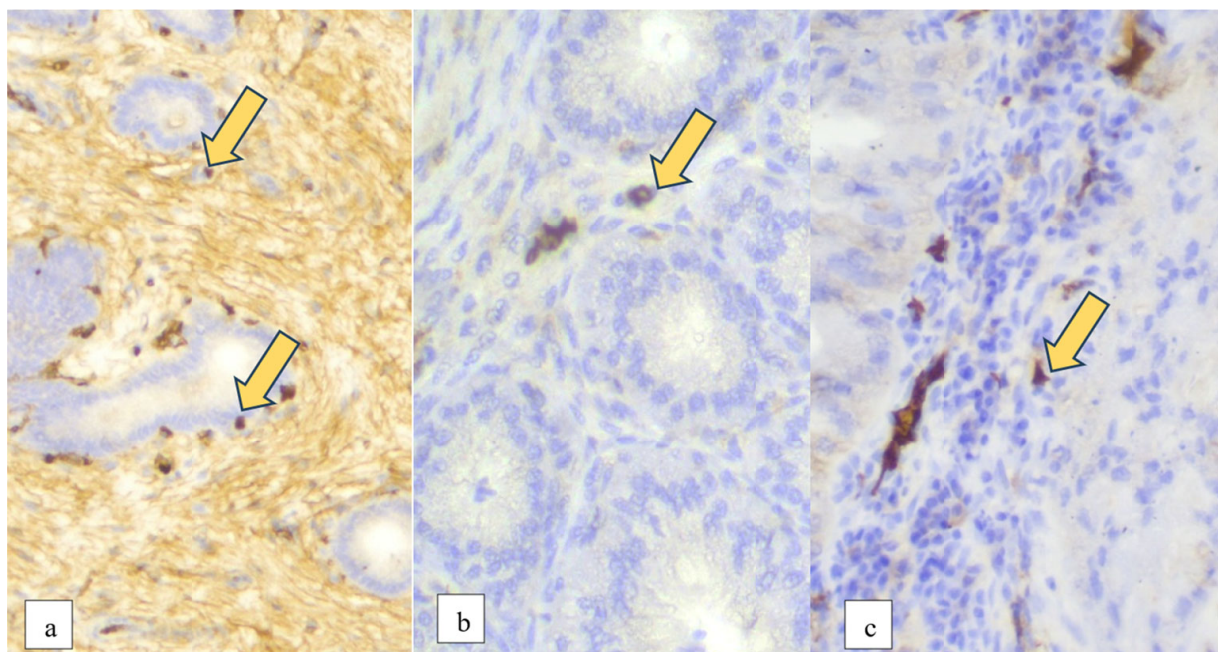


Fig. 5. B-lymphocytes around the endometrial glands of the 1st group of animals (a), scattered cells in the stroma of uterine samples of animals during the sexual cycle (b) and in uterine samples of animals with pyometra (c). Immunohistochemical reaction with CD20. Collection: x200.

It should be noted that membrane-type staining with a variable cytoplasmic component (brown small granules located in the cytoplasm of the cell) is considered positive for CD138 monoclonal antibodies. Predictably, mature B-lymphocytes were detected in the infiltrate in uteri with pyometra (Figure 6 c). Single lymphocytes with CD138 immunophenotyping were detected in samples of the uterus of bitches during the anestrus period (Figure 6 a). The detection of mature B-lymphocytes (CD138) in the samples of bitches uterus during the sexual cycle (second group) showed that they occur as single positive cells, which is illustrated in Figure 6 b.

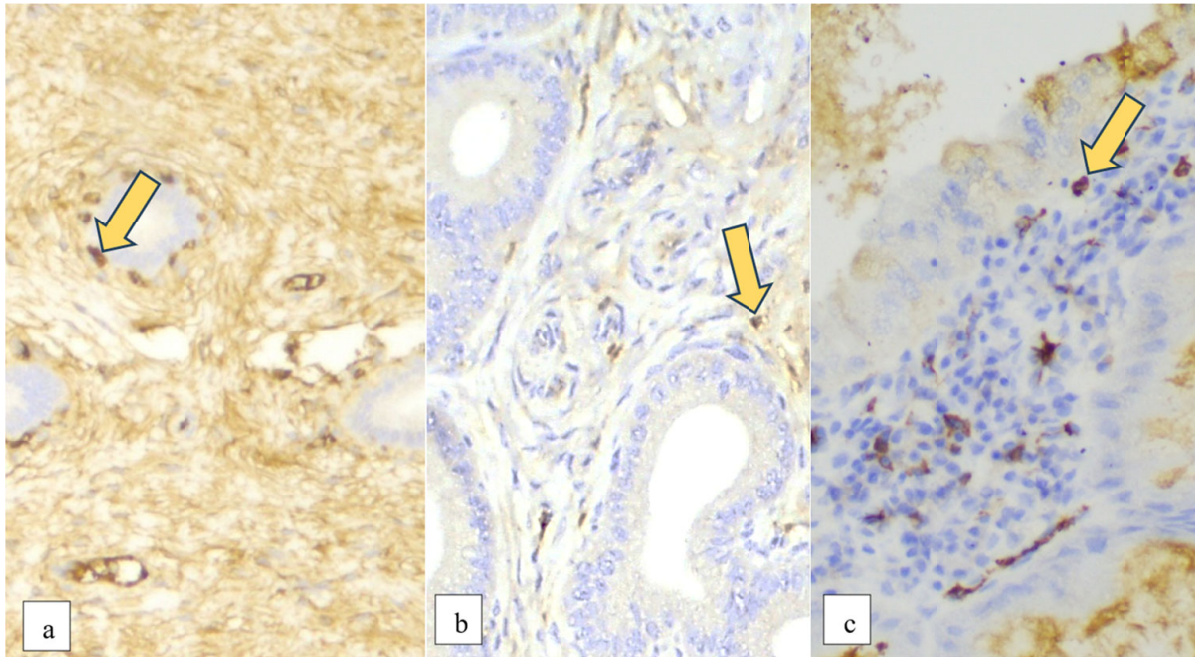


Fig. 6. Mature B-lymphocytes in the stroma of the endometrium in animals in the anestrus period (a) and during the sexual cycle (b), impurities of mature B-lymphocytes in the stroma of the uterine mucosa of bitches with pyometra (c). Immunohistochemical reaction with CD138. Collection: x200

A significant accumulation of CD68-positive cells of the macrophage line was established in the stroma, in the infiltrate, and in the lumen of the glands among the mucus and exudate in the group of animals with pyometra (Figure 7 c). Analysis of samples of the uterus of bitches in the anestrus period revealed an accumulation of CD68-positive cells on the surface of the uterine mucosa, both single and in small groups (Figure 7 a). In the group of animals during the sexual cycle (the second group), the indicated cells were found in small numbers in the samples, and were topographically localized in the stroma. (Figure 7 b).

Separately, it should be noted in the cytoplasm of macrophages found in the stroma of the uterine mucosa of all groups, no golden-brown pigment was detected, which would indicate the presence of hemosiderin, and the cells could be called hemosiderophages. The presence of such a pigment in macrophages indicates hemorrhages in the past, because after the breakdown of erythrocytes, resorption of this pigment occurs. Therefore, we did not find indirect signs of previous hemorrhages or hematomas in any of the studied samples of all groups of animals.

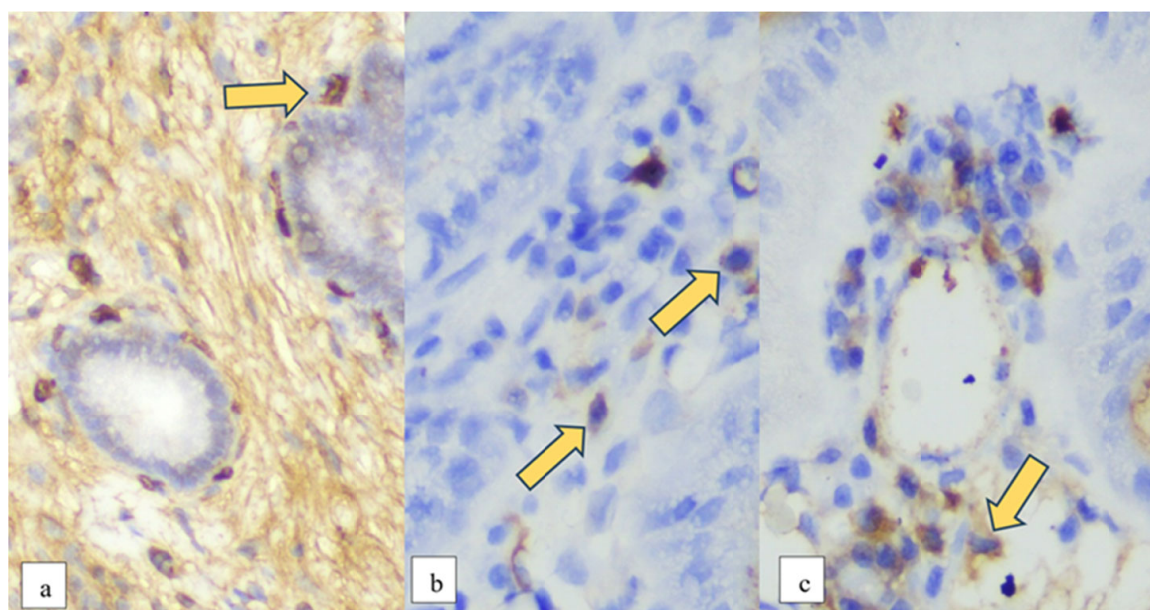


Fig. 7. Single and in groups of CD68-positive cells under the epithelial plate (a, yellow arrow) of the mucous membrane of the uterine horn in animals in the anestrus period, in the stroma of the endometrium (b, yellow arrow) of animal samples of group 2, stromal (c, yellow arrow) in animal samples with pyometra. Immunohistochemical reaction with CD68. Collection: x200

Quantitative indicators regarding the verification and accounting of lymphocyte subtypes are summarized in Table 1. It was established that for pyometra, the average number of CD4 lymphocytes was $13,40 \pm 4,20$ cells, which is the highest indicator compared to other groups. The population of CD8 lymphocytes, on the contrary, was the smallest for pyometra ($0,75 \pm 0,2$ cells). A sufficiently large number of these cells were found in the anestrus period.

Table 2. Analysis of the quantitative composition of subtypes (CD) of lymphocytes depending on the functional state of the reproductive system.

Group	The average number of lymphocyte subtypes detected (in 10 fields of view)				
	CD4	CD8	CD138	CD20	CD68
1st group; anestrus period (n=5)	$4,60 \pm 1,10$	$5,20 \pm 0,30$	$3,40 \pm 0,60$	$1,30 \pm 0,60$	$0,60 \pm 0,03$
2nd group; state of estrus (n=5)	$7,20 \pm 1,40$	$0,80 \pm 0,03$	$0,20 \pm 0,01$	$0,40 \pm 0,02$	$0,10 \pm 0,01$
3rd group; state of pyometra (n=5)	$13,40 \pm 2,20$	$0,75 \pm 0,20$	$9,80 \pm 2,20$	$4,50 \pm 1,40$	$10,30 \pm 1,60$

Note: the result of the reliability test is $p=0.043$, $t=2.07$.

Immunocompetent cells with the CD138, CD20 and CD68 phenotype were also found in pyometra in greater numbers than during the sexual cycle and in the anestrus period. During the sexual cycle, the population of cells with CD138, CD20 and CD68 phenotypes was the smallest, compared to the group after pyometra and during the anestrus period.

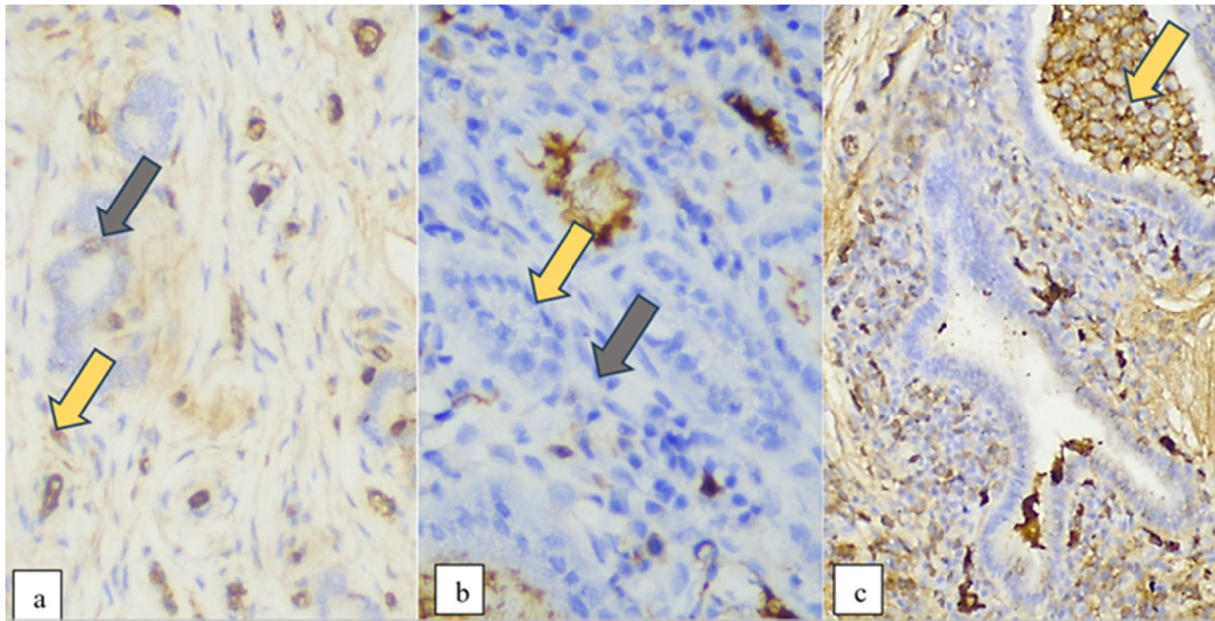


Fig. 8. Positive reaction to progesterone receptors of the stroma (a, gray arrow) of single cells of the endometrium and cells of the stroma (a, yellow arrow) of the mucous horns of the uterus in animals in the anestrus period, a negative reaction to progesterone receptors in the glandular epithelium (b, gray arrow) and of stromal cells (b, yellow arrow) in animals in a state of estrus, positive reaction of a part of endometrial stromal cells in animals with pyometra (c, yellow arrow). Immunohistochemical reaction with Pr. Collection: x200.

The study of the activity and topography of progesterone receptors in the structures of the uterus of the studied animals showed that the glandular epithelium of all the studied groups has extremely low progesterone reactivity of cells. The stroma in the histological samples of both the first group (Figure 8 a) and, to a lesser extent, the samples of the second group (Figure 8 b) had a distinct positive reaction (as an internal control of the quality of the reaction – positive staining of myometrial fibers). It is worth noting the distinct positive reaction (presence of progesterone receptors) in the endothelium of the endometrial capillaries in all the studied samples.

Comparing the tissue samples of the studied groups of animals, a low reactivity of the glandular epithelium of the mucous horns of the uterus (single positive cells) and a moderate positive reaction in part of the stroma cells in the first (Figure 8 a) and second (Figure 8 b) groups were established. In animals with pyometra (third group), the epithelium of the endometrial glands was microfocally weakly positive, and the stroma contained a significant proportion of progesterone-positive cells (Figure 8 c).

4 Discussion

There are few scientific studies describing the phenotypes of immunocompetent cells in the uterine wall of bitches under different conditions of the reproductive system in the available literature. There are difficulties with the interpretation of the obtained results in connection with the peculiarity of the physiology of the sexual cycle in bitches. Our research presents the results of the quantitative composition for subtypes of (CD) lymphocytes depending on the functional state of the reproductive system, namely, during pyometra, sexual cycle and anestrus period, as well as the activity and topography of progesterone receptors in the tissues of the uterus. De Bosschere et al. [12] emphasizes the importance of research in the histological diagnosis of uterine diseases in bitches. There is a certain list of studies indicating the role of the uterus microflora in the development of inflammatory processes in bitches [6, 13].

The microbiota plays a fundamental role in the induction, training and functioning of the host's immune system. Meanwhile, the immune system evolved significantly as a means for maintaini

ng the host's symbiotic relationship with these highly diverse, evolving microbes [14]. There is a lack of information on the state of local immunity, especially the innate one, during the physiological course of the sexual cycle and during the anestrus period. There is also a lack of information on the state of immune competent cells during the pathogenesis of inflammatory processes in the uterus. The immune cells found in the reproductive tract must play paradoxical roles as they support immunity against pathogens and also establish immune tolerance to sperm and embryos [15].

Leukocytes in the bitches' reproductive tract are distributed in an aggregated or dispersed form in the epithelial layer, lamina propria, and stroma [15]. Although distributed differently in each organ of the bitches' reproductive tract, the predominant immune cells are T cells, macrophages/dendritic cells, NK cells, neutrophils, and mast cells [15]. B cells are rare in the bitches' reproductive tract [15]. The data proves that high levels of estradiol and progesterone present during days 14-28 of the menstrual cycle reduce CD 8 activity in the uterus [16]. Our studies confirmed that during the sexual cycle, compared to the anestrus period, the activity of CD 8 receptors decreased by 6.5 times, namely from 5.20 ± 0.30 to 0.80 ± 0.03 lymphocytes in 10 fields of vision. A decrease in the activity of the subpopulation of lymphocytes CD 8, was established for pyometra as well, at the level of 0.75 ± 0.20 lymphocytes in 10 fields of vision.

Slightly different results were obtained in the study of subtypes of lymphocytes in the uterus in pyometra [17]. The authors claim that the CD 8 population in pyometra increased in contrast to the period of the normal sexual cycle. Although, according to histological assessment, the total number of cells in the uterus with pyometra was clearly higher compared to the healthy one.

A decrease in the cytotoxic activity of T-lymphocytes (CD 8) was established in bitches during the secretory stage, but at the same time, their number does not decrease [18]. The likely decrease in the number of T-cytotoxic lymphocytes (CD 8) and other subtypes during the sexual cycle is physiologically justified and necessary for the successful fixation of the embryo. Much higher expression of CD4 than cytotoxic CD8 has been also established by other authors [19]. The increase in the subtype of CD4 lymphocytes during the sexual cycle to 7.20 ± 1.40 compared to the anestrus period is probably a hormonally dependent process. According to the literature, this is mostly due to an increase in the level of progesterone [20].

Studies of the distribution of progesterone receptors in the uterus showed their high density in all types of cells in pyometra. Similar data were obtained by other researchers. The authors suggest that these receptors can be triggered by bacterial infections [21, 22].

5 Conclusions

Conducted immunohistochemical studies of the receptor activity of immunocompetent cells showed their heterogeneity depending on the functional state of the reproductive system. The physiological significance of the activity of CD8 lymphocytes in the anestrus period and CD4 in estrus and pyometra requires more detailed research. The established feature of the distribution of progesterone receptors in various structures of the uterus and depending on the functional state of the reproductive system confirms the significant role of progesterone in the genesis of the development of dysfunctions and is probably a sensitizing factor.

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