LYMPHOCYTE SUBPOPULATIONS IN THE BITCHES WITH PYOMETRA TREATED WITH AGLEPRISTONE

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Abstract

The purpose of the study was to compare flow cytometric and haematologic variables in dogs with spontaneous endometritis pyometra complex (EPC) treated with aglepristone to healthy controls. Peripheral blood mononuclear cells: CD3, CD4, CD8, and B lymphocytes (CD21) were analysed by flow cytometry and white blood cell count. Significant differences were observed (P ≤ 0.01) between control (C) and study (S) group in the total number of leukocytes, monocytes and granulocyte populations (without lymphocytes) on beginning, after 7 d, and return to reference value after 14 d of the treatment. The percentage of the T-cell (CD3+) at the beginning was 47.22 ±9.64% of total lymphocytes, in contrast to B lymphocytes (CD21+) that represented the smallest percentage of 14.24 ±7.74% (P ≤ 0.01). The percentage of the lymphocytes CD4+ was 27.42 ±5.53% and CD8+ was 25.18 ±4.36%.

The percentage of CD3+ lymphocytes was increasing throughout the experiment in group C and gradually decreased in group S from 14th to 28th d of dioestrus. No differences in the number of CD3+, CD4+, CD8+, and CD21+ lymphocytes between group C and S on the 14th and 28th d of dioestrus (P ≥ 0.05) were observed. The number of CD8+ cells in group S decreased gradually from day 14 to 28 but no statistical differences were noted. Treatment of pyometra with aglepristone decreased the number of leukocytes, monocytes, and granulocytes to referential value but statistically significant influence on the level of subpopulations of T and B lymphocytes was not observed. The results enabled to estimate for the first time the number of lymphocyte subpopulations in dioestrus in both healthy bitches as well as in those suffering from pyometra.

Key words: bitches, pyometra, lymphocytes, aglepristone.

Pyometra is a common infectious disease of the uterus in bitches (1, 3, 9, 21, 22). The onset of the disease characteristically occurs in the first half of the dioestrus stage in the oestrous cycle, during which the blood level of progesterone peaks and oestradiol-17β is the lowest (1, 3). Many dogs affected by pyometra present corpora lutea together with follicles within the ovaries (1, 3). Pyometra is often observed after treatment with progesterone, used to block oestrus (1, 13, 22). Ovarian hormones affect the systemic immune system, since oestrogen appears to directly stimulate the activity of immune cells including T cells (5) and B cells (4). In contrast, progesterone appears to inhibit the proliferation and activation of lymphocytes (12, 15, 17). Although a significant increase in the number and a left shift of neutrophils has been observed in the peripheral blood in pyometra, many bitches show lymphopenia (6). This evidence suggests that status of systemic immunity affected by the ovarian hormones may play a critical role in preventing and inducing bacterial growth in the uterus (12, 20). Anti-progestagen aglepristone is a synthetic steroid, which binds to progesterone receptors with threefold greater affinity than progesterone and prevents its biological effects. It was suggested that the increase in nonspecific immunity after aglepristone treatment (20) is one of the factors that may accelerate the recovery, although clinical investigations in this field were not performed. Blocking the progesterone receptors (PR) by aglepristone can affect the progesterone's activity on immunological cell function and its direct influence on these cells remains unknown.

The aim of this study was to estimate whether PR blocking may have influence on some immune cells. It seems that expected results could be useful in the clinical treatment of pyometra and may help in more efficient disease prevention.

Material and Methods

Animals, treatment, and sample collection.
The study was carried out on 12 bitches (six clinically healthy – control group (C), aged 2.91 ±1.5 years, and six with pyometra – studied group (S), aged 6.95 ±2.5 years) of different breeds and mean weight of 39.2 ±4.72 kg. All bitches were in dioestrus (confirmed by cytology and progesterone level). Blood samples were taken on days 14, 21, and 28 of dioestrus. Smears for
microbiological analysis were taken from the vulva from all bitches. The aglepristone (Alizine, Virbac, France) was applied subcutaneously at a dose of 10 mg/kg on 1, 2, and 7 d.

**Haematological analysis.** The total number of leucocytes, neutrophils, lymphocytes, and monocytes was determined by an Abacus Junior Vet Automated Haematology Analyser (Diatron, Austria). The leukogram was obtained by microscopy and its results werecounterechecked by flow cytometry.

**Flow cytometry.** Cytometric analysis was carried out in a FACScalibur flow cytometer (Becton-Dickinson, Germany). Data acquisition was performed using CellQuest software. The proportions of selected lymphocyte subpopulations (CD3+, CD4+, CD8+, CD21+) were determined.

**Blood samples.** Peripheral blood from the supraradial vein was collected by venipuncture into tubes containing 5 mM EDTA-2K. A 50-µl aliquot of blood was used for each staining with monoclonal antibodies.

**Monoclonal antibodies and fluorochromes.** The labelled monoclonal antibodies used in this study were purchased from Serotec (UK) and their characteristics are shown in Table 1.

**Preparation of samples.** Cells were labelled by adding 10 µl of monoclonal antibodies and incubating for 20 min at room temperature. Peripheral blood leukocytes were labelled in whole blood, followed by lysis of erythrocytes in 1 ml of FACS lysing solution (Becton-Dickinson, Germany). Erythrocyte cell debris was eliminated by washing in PBS. Finally, blood cells were suspended in PBS with 0.5% formaldehyde.

**Hormonal profile.** Progesterone (P4) concentrations were measured using commercial test (immunoenzymatic test for quantitative determination of progesterone, Pointe Scientific, Poland) after previous extraction by ethyl acetate. The optical density was checked by Pointe 2000 analyser. All tests were done twice in each of the series. Efficiency of extraction of P4 ranged from 92% to 99% and sensitivity of determinations as well as intra serial mistake amounted for progesterone 0.05 ng/mL (0.8 nmol/L) and 8.0%, respectively.

**Microbiological analysis.** Bacteriological examination of the vulvar leakage of bitches with pyometra revealed *Escherichia coli* in five (83%) bitches, *Staphylococcus intermedius* in one (16%) bitch, and *Pasteurella multocida* in one (16%) bitch. Bacteriological examination of smears from bitches’ vagina from group C showed *Pasteurella multocida* in two (33.3%) bitches, *Escherichia coli* in one (16%) bitch, and no bacteria in three bitches.

### Table 1

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Molecule</th>
<th>Isotype</th>
<th>Cell type expressing molecule</th>
<th>Labelling</th>
</tr>
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<tbody>
<tr>
<td>CA17.2A12*</td>
<td>CD3</td>
<td>mouse IgG1</td>
<td>T lymphocytes</td>
<td>FITC</td>
</tr>
<tr>
<td>YKIX302.9*</td>
<td>CD4</td>
<td>rat IgG2</td>
<td>helper/inducer T lymphocytes</td>
<td>RPE</td>
</tr>
<tr>
<td>YCATE55.9*</td>
<td>CD8</td>
<td>rat IgG1</td>
<td>cytotoxic/suppressor T lymphocytes</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>CA2.1D6</td>
<td>CD21</td>
<td>mouse IgG1</td>
<td>B lymphocytes</td>
<td>RPE</td>
</tr>
</tbody>
</table>

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**Statistical analysis.** Results are presented as arithmetic means and standard deviations. The significance of differences was calculated using Student's *t*-test and the Mann–Whitney–Wilcoxon test.

**Results**

**Haematology.** In group S, the total leucocyte count was found to be 17.58 ±3.72×10⁹/L, among them lymphocytes accounted for 3.06 ±1.01×10⁹/L, monocytes - 2.88 ±2.01×10⁹/L, and granulocytes - 16.01 ±4.31×10⁹/L. Significant differences (P<0.01) between group C and S were observed in all points (without lymphocytes) (Fig. 1). In group S, application of aglepristone over 7 d caused significant differences in the total number of lymphocyte, monocyte, and granulocyte populations (P<0.01). After 2 weeks, significant differences (P<0.001) between group C and S in all points (without lymphocytes) were observed (Fig. 1). The total count of monocytes during the observations showed significant differences between group C and S (P<0.01). There were no statistical differences in the number of the total lymphocyte count between group C and S (Fig. 1).

**Progesterone.** The level of P4 in group C started from 23.15 ±2.35 ng/mL, increased to 27.63 ±2.73 ng/mL after 7 d, and remained at the same level (27.43 ±1.67 ng/mL) in the 2nd week. In group S, at the same time, the level of P4 was 24.97 ±1.17 ng/mL, 29.78 ±1.3 ng/mL, and 28.73 ±0.55 ng/mL, respectively. There were no statistical differences in P4 level between group C and S.

**Microbiological analysis.** Bacteriological examination of the vulvar leakage of bitches with pyometra revealed *Escherichia coli* in five (83%) bitches, *Staphylococcus intermidius* in one (16%) bitch, and *Pasteurella multocida* in one (16%) bitch. Bacteriological examination of smears from bitches’ vagina from group C showed *Pasteurella multocida* in two (33.3%) bitches, *Escherichia coli* in one (16%) bitch, and no bacteria in three bitches.
Fig. 1. The number of leukocytes (WBC), neutrophils (NEUT), lymphocytes (LIMF), and monocytes (MON) in groups C (n=6) and S (n=6) on days 14, 21, and 28 of dioestrus. Statistical differences between group C and group S in all points (without lymphocytes) (P from ≤0.01 to ≤0.05).

Fig. 2. The number of T lymphocytes (CD3+, CD4+, CD8+) and B lymphocytes (CD21+) in groups C (n=6) and S (n=6) on days 14, 21, and 28 of dioestrus.
**Lymphocytes.** Cytometric analysis with selected monoclonal antibodies revealed the proportions of lymphocyte subpopulations in healthy bitch. The percentage of the T-cells with CD3 surface markers at the beginning of the experiment was 47.22 ±9.64% of lymphocytes (2.43 ±1.12×10⁹/L) (Fig.2). There were no differences in the number of these cells between group C and S on the 14th d of dioestrus (1.94 ±0.1×10⁹/L, P≥0.05). The number of CD3+ cells was increasing throughout the experiment in group C and gradually decreased in group S from day 14 to day 28 of dioestrus (1.46 ±0.39) (Fig. 2). In two cases the percentage of the T-cells with CD3 surface markers on days 2 and 28 of dioestrus were between 91%-94%. However, a wide range of standard deviation were the reason of no statistical differences between group C and S in the number of CD3+ cells on days 14 and 28. In contrast, the percentage of subpopulation with CD21 surface markers (B lymphocytes) was the smallest (14.24 ±7.74%, 0.5 ±0.19×10⁹/L) of the total lymphocyte count (Fig. 2). There were no statistical differences in the number of CD21+ cells between group C (0.5 ±0.19×10⁹/L) and S (0.28 ±0.13×10⁹/L) on day 14 of dioestrus and in other days of observation. The percentage of the total T-cell with CD4 surface markers at the beginning of the experiment in group C was 27.42 ±5.53% of lymphocytes (1.06 ±0.37×10⁹/L) and with CD8 markers was 25.18 ±4.36% of lymphocytes (0.9 ±0.33×10⁹/L) (Fig. 2). There were no statistical differences in the number of CD4+ cells between group C (1.06 ±0.37×10⁹/L) and S (0.99 ±0.28×10⁹/L) on day 14 of dioestrus and in other days of observation. The number of CD8+ cells in group S decreased gradually from day 14 (1.19 ±0.39×10⁹/L) to day 28 (0.79 ±0.25×10⁹/L) but there were no statistical differences (Fig. 2).

**Discussion**

The incidence of pyometra in dogs increases in the first half period of dioestrus, a period in which the blood concentrations of progesterone are the highest and those of oestrogen are the lowest (2, 3, 6, 23). In contrast, the incidence of pyometra is relatively low in the oestrous stage, during which the blood concentration of oestradiol-17β is the highest and that of progesterone is the lowest (13, 22). The balance of oestrogen and progesterone in the blood affects antigen specific immune responses, particularly secondary responses (22).

In healthy bitches, intra cell neutrophil metabolism activity in NBT, and lymphocyte proliferation on mitogen phytohaemagglutynin (PHA) significantly increases in prooestrus and oestrus compared with anoestrus and significantly decreases in dioestrus (12).

It has been observed that increasing level of progesterone and decreased level of oestrogen induces the degradation of immune resistance, which increases the incidence of pyometra in the early period of dioestrus (19). Inhibition of mitogen - driven lymphocyte proliferation was the characteristic feature of the immunological profile in bitches affected by pyometra, and this corresponded with the impairment of the general state of health (6). Sera from bitches affected by pyometra also had higher levels of immunoglobulins, lysozyme, and circulating immune complexes (6).

The activation and recruitment of monocytes and neutrophils may not relate however, to the activation of antigen-specific immunity, but may be induced by the direct contact with bacterial wall via Toll-like receptors (TLRs) or chemokines produced by epithelial cells (19). Therefore, the antigen-specific immunity altered by ovarian hormones seems to play an important role in preventing infectious disease in the uterus (20).

Raise in leukocyte count in pyometra is typical for this disease and was observed by many authors (6, 15). In this research, the level of leukocytes was not very high, probably because of early diagnosis (day 14 of dioestrus) of the disease. Significant raise in neutrophil and monocyte numbers may be explained by the activation of non-specific immunity mechanisms, which was confirmed by other authors (6, 12, 15). Lack of statistical differences in lymphocyte numbers between both groups was caused by high values of standard deviation. However, lower average leukocyte level in group S is clearly evident and suggests inhibition of the specific immunity as the result of high progesterone concentration in this phase of oestrus cycle of a bitch.

The increased incidence of pyometra in the early phase of dioestrus results partially from the suppression of antigen-specific Th1 cell responses and cellular immunity by progesterone (18).

Mifepristone (RU 486) and aglepristone (RU-534) are antiprogestins that bind with progesterone receptors with greater affinity than progesterone. Mifepristone is one of better known drug from this group used in humans. Aglepristone is the only approved agent for veterinary use. Many intensive clinical trials have been dedicated to this drug over the past few years, while basic tests are still severely lacking. Aglepristone blocks progesterone receptors, leading to inhibition of the activity of this hormone in tissues, accelerating the development of pyometra. (12).

Inhibition of the progesterone receptor results in a significant improvement in white blood cell profile in bitches after 2 week treatment, which usually returns to reference values (24). No relation between the changes in progesterone concentration and demonstrated levels of total leukocytes, neutrophils or monocytes were observed. Such observation might be indicative of less significant inhibiting action of progesterone on the first defense line of the immune system comparing to selective specific immunity mechanisms.

Even in dioestrus, once bacteria significantly increase in number and infiltrate into uterus tissue, monocytes in the uterus are activated and a significant number of neutrophils are recruited into the uterus and peripheral blood from the bone marrow, in which the production of the cells significantly increases (6, 8). The predominant pathogen in pyometra is Escherichia coli isolated in 60%-85% of pyometra cases and this was
confirmed in our study. Shiga toxin 1 from *Escherichia coli* blocks activation and proliferation of bovine lymphocyte subpopulations *in vitro* (17). This fact may be of importance in estimation of lymphocyte subpopulation in the bitches with pyometra, but such *in vivo* or *in vitro* studies in bitches were not conducted. The research regarding bacteria in healthy bitches in dioestrus confirms that the presence of bacteria in clinically healthy individuals and the possibility of their growth strictly depend on local and systemic immunity status (25).

In the lymphocyte subpopulation, lymphocytes T CD3+ dominate, comprising almost 50% of the subpopulation, and in some cases their number exceeds even 90%. The predominant number of CD3+ cells was observed in both groups but in group C their value gradually increased in contrast to group S where a gradual decline was noted (Fig. 2). However, significantly high standard deviations make it impossible to assume statistically significant differences between the groups. The use of aglepristone did not cause a statistically significant difference in CD3+ cell level in medically treated bitches. The lack of similar studies in bitches makes the interpretation of obtained results even more difficult. In women, T lymphocytes were observed in the endometrium with use of a specific monoclonal CD3 antibody. After 14 d of DMPA (depot-medroxyprogesterone acetate) treatment of women, the predominant number of CD3+ cells was maintained but did not reach a significant level (16). We did not confirm a similar pattern in bitches but the differences may be explained by the fact that clearly distinct research methods were used.

Relatively low number of B lymphocytes (CD21+) in our observations is in contrast with others lymphocyte subpopulations that may suggest low immunogenicity of pathogens found in this disease.

According to one of few studies on lymphocyte subpopulation in dogs with congestive heart failure, CD4+ cells comprise 23.91% and CD8+ cells 48.75% of investigated subpopulation and average CD4/CD8 ratio was 2.19 (7). The percentage of CD4+ cells observed in our research was similar (27.42%) whereas percentage of CD8+ cells was twofold lower (25.18%), what gave the CD4/CD8 ratio around 1.

It is quite difficult to compare the results of particular studies/authors as various antibodies were used and different diseases were diagnosed in dogs of unknown hormonal status that additionally complicates the issue.

During evaluation of the results it should be considered that aglepristone reaches its therapeutic concentration after 24-48 h, whereas the process of changes in lymphocyte population may take less time: from several to 24 h. Another factor influencing the results was the time of the observed clinical symptoms of pyometra. Currently, there is no diagnostic method that would affirm unanimously diagnosis of the first stage pyometra. The obtained results suggest that to evaluate potential aglepristone activity, or its lack, additional observations in shorter intervals of time (e.g. every few hours) may be needed. Next phase of our research has been designed in a way that will allow such observations. The results of this study allowed estimating for the first time the number of lymphocyte subpopulations in dioestrous of healthy bitches, as well as those with pyometra. They also characterised the influence of inhibiting progesterone function on selected immune system cells.

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