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Research paper

Leucocyte phagocytosis during the luteal phase in bitches

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ABSTRACT

Pyometra is a disease that affects a large proportion of intact bitches, and typically is seen during the latter half of dioestrus. Several factors contribute to the development of pyometra, including genetic factors, an infectious component (most often *Escherichia coli*), and hormonal factors. Hormones may act directly on the endometrium, and also affect the immune system. In dogs, the phagocytic ability has been shown to decrease with age, and ovarian hormones have also been shown to affect immune resistance. The aim of the present study was to examine whether phagocytosis by canine leucocytes varies significantly during the luteal phase. Eight bitches were followed by repeated blood sampling. Samples were taken at the calculated optimal day for mating (Day 1), and thereafter on days 8, 15 and 22 (early luteal phase) and 29, 43, 57 and 71 (late luteal phase). Blood was collected from the cephalic vein into EDTA tubes for leucocyte counts and heparinised tubes for testing of phagocytosis and oxidative burst using commercial kits and flow cytometry. The cell activity of the phagocyting leucocytes, expressed as mean fluorescence activity, MFI, was significantly lower during late luteal phase than during early luteal phase. The proportion of leucocytes that was induced to phagocyte did not differ significantly. The percentage of cells stimulated by *E. coli* to oxidative burst was significantly lower during late luteal phase. Their activity did not differ between the two periods. The number of cells stimulated to oxidative burst by a low stimulus was too low to evaluate, and leucocytes stimulated with the high stimulus did not vary in oxidative burst between the two periods. The changes in phagocytic activity and in the number of leucocytes that showed oxidative burst were not associated with any change in the proportion of different leucocytes. The decreased phagocytic capacity possibly contributes to the higher incidence of diseases such as pyometra during the latter part of the luteal phase.

1. Introduction

Uterine diseases are common in intact bitches, and pyometra affects nearly 25% of all bitches by the age of 10 years (Egenvall et al., 2001). Several factors contribute to the development of pyometra, including genetic, infectious and hormonal factors. The importance of genetics is demonstrated by the strong breed predilection for pyometra (Egenvall et al., 2001). Risk factors such as parity and oestrogen administration have been demonstrated (Niskanen and Thrusfield, 1998), but risk and protective factors may vary between breeds (Hagman et al., 2011). There is an infectious component in pyometra,

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and *Escherichia coli* is the bacterium most commonly isolated (Fransson et al., 1997; Hagman and Greko, 2005). Subclinical and clinical urinary tract infection is associated with pyometra (Hagman and Kuhn, 2002; Sandholm et al., 1975), and the gastrointestinal tract may also serve as a reservoir (Tsumagari et al., 2005). Pyometra typically occurs during dioestrus, and most cases are seen from 4 weeks to 4 months after oestrus (Dow, 1957; Smith, 2006). Bacteria may enter the uterus during oestrus (Watts et al., 1998), and the influence of progesterone on the endometrium following oestrogen stimulation is thought to predispose to pyometra (Dow, 1959). The simultaneous presence of corpora lutea and follicles has been described in bitches with pyometra (Strom Holst et al., 2001), presumably reflecting a hormonal imbalance. It has also been suggested that the down regulation of oestrogen receptors in the endometrium under the influence of progesterone is defective in bitches with cystic endometrial hyperplasia (De Cock et al., 1997), predisposing to pyometra.

Hormonal changes during the oestrous phase may also increase the risk of pyometra by immunological mechanisms, via changes in the innate immune system. The innate immune system is the first line of host defence against invading microorganisms, and includes, among other mechanisms, phagocytosis of pathogens and cells (Kobayashi and DeLeo, 2009). Neutrophils are the predominant phagocytic cells in peripheral blood and are the most important cellular component of innate immunity. The phagocytic process can be separated into several steps: chemotaxis, attachment of the particles to the surface of the phagocytic cell, phagocytosis (ingestion), and intracellular killing by oxygen-dependent (oxidative burst) and oxygen-independent mechanisms (Hostetter, 2012).

In septic dogs, phagocytic activity has been shown to increase whereas oxidative burst decreases (Webb et al., 2007). The innate immune response, including phagocytic ability, also changes during different physiological states. It has been shown to decline with age in beagle dogs (Hall et al., 2010). Innate immunity has been described to vary with reproductive state in humans. In pregnant women, among the most substantial immunologic deviations are an increase in circulating granulocytes and a decrease in lymphocytes, with additional activation of monocytes and granulocytes (Luppi et al., 2002). In bovines, progesterone reduces oxidative burst in vitro when added to leucocytes (Chaveiro and Moreira da Silva, 2010). Ovarian hormones have been shown also to affect immune resistance in dogs (Sugiura et al., 2004). The proliferative response of peripheral blood mononuclear cells (PBMNCs) to *E. coli* increased in proestrus/oestrus and decreased by day 10 of dioestrus (Sugiura et al., 2004). The proliferative response and expression of gamma interferon of cell cultures of PBMNCs collected in anoestrus was also enhanced upon addition of estradiol -17β, and suppressed by progesterone (Sugiura et al., 2004). A differential localisation and expression of toll-like receptor 4, also part of the innate immune system, has been described in the canine endometrium throughout the oestrous cycle and in pyometra (Chotimanukul and Sirivaidyapong, 2011).

The aim of the present study was to examine whether phagocytosis by leucocytes in peripheral blood varies significantly during the luteal phase in bitches.

2. Materials and methods

2.1. Animals and sampling procedure

Eight bitches were included in the study: five beagles, one German shepherd, one rottweiler and one cross bred bitch. Their mean age was 3.4 years (SD 1.2). The study period was from September 2009 to May 2010. Two mL whole blood samples were collected from the cephalic vein in EDTA Vacutainer tubes (K_3EDTA, Vacuette, Hettich Labinstrument AB, Sollentuna, Sweden) and in heparinised Vacutainer tubes (Lithium heparin, Vacuette, Hettich Labinstrument AB, Sollentuna, Sweden), which were properly filled and kept at room temperature until analysis. All samples were analysed within 2 h of collection. The study was approved by the Uppsala Ethical Committee of Animal Experimentation (C23/9) and the Swedish Board of Agriculture (31-1365/09).

2.2. Analysis of oestrous cycle stage

The bitches were followed with vaginal cytology and blood samples for progesterone assays during oestrus. Cytological staging was done according to Schutte (1967a,b). The day of ovulation was defined as the day when progesterone levels reached 15–24 nmol/L.

2.3. Experimental design

Based on progesterone levels, day 0 of the study was set at as the day optimal for mating, 2–5 days after ovulation, with progesterone levels higher than 30 nmol/L and cytological oestrus. Thereafter, blood samples were taken on days (±2) 1, 8, 15 and 22 (early luteal phase) and days 29, 43, 57 and 71 (late luteal phase). Progesterone values during the luteal phase are shown in Fig. 1.

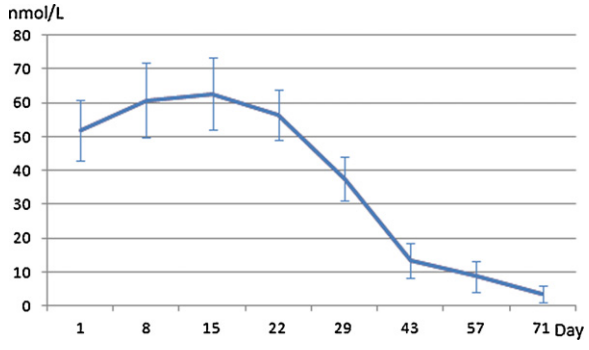

Fig. 1. Serum concentrations of progesterone (nmol/L) during the luteal phase. Mean values and 95% confidence intervals. Early luteal phase: Days 1–22; late luteal phase: Days 29–71.

2.4. Analysis of leucocyte number and function

The total number and differential count of leucocytes in EDTA-blood were analysed using the ADVIA 2120 haematology system (Siemens Healthcare Diagnostics, Erlangen, Germany) with canine settings. Advia 2120 does not detect basophils in canine blood (Lilliehook and Tvedten, 2011), thus data for basophils are not described further in the study.

2.4.1. Phagocytic activity

Bacterial phagocytic function was determined quantitatively using a commercial test kit (Phagotest™, Glycotope Biotechnology GmbH, Heidelberg, Germany). Briefly, 100 μL of heparinised whole blood was incubated at 37 °C for 10 min with 20 μL *E. coli* bacteria that were opsonised with immunoglobulin and complement and fluorescein (FITC) labelled. Control samples were incubated in an ice bath. Phagocytosis was stopped by placing the sample on ice and adding a solution that quenches the FITC fluorescence of surface bound bacteria, leaving the fluorescence of internalised particles unaltered. After two washing steps, erythrocytes were lysed by adding a lysing solution and incubating for 20 min at room temperature, followed by an additional washing. A DNA staining solution was added prior to flow cytometric analysis, to exclude aggregation artefacts of bacteria or cells.

2.4.2. Oxidative burst

Oxidative burst was measured using a commercial test kit (Phagoburst™, Glycotope Biotechnology GmbH, Heidelberg, Germany). In this test, unlabelled opsonised *E. coli* bacteria are used as the particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetat (PMA) as the high stimulus and the chemotactic peptide N-formyl-MetLeuPhe (fMLP) as the low physiological stimulus. A sample without a stimulus serves as the negative background control. Briefly, 100 μL of heparinised whole blood was incubated for 10 min at 37 °C with 20 μL of *E. coli*, PMA, fMLP, or wash solution (negative control). The reactive oxygen metabolites produced were monitored by addition and oxidation of 20 μL dihydrorhodamine (DHR) 123, followed by incubation at 37 °C for 10 min. The reaction was stopped by adding a lysing solution and incubation at room temperature for 20 min. This also removes erythrocytes, and results in a partial fixation of leucocytes. After a washing step, a DNA staining solution was added to exclude aggregation artefacts of bacteria or cells.

2.5. Flow cytometry

Samples were kept on ice, and analysed within 30 min of preparation using a LSR flow cytometer (Becton Dickinson, San José, CA, USA). Single laser analysis was carried out using an argon ion-laser, operating at 488 nm wavelength. Fluorescence was measured using a $\log_{10}$ scale. For each sample, at least 10,000 events were acquired within an acquisition gate in the red fluorescence channel (FL-3), to exclude erythrocytes and debris. All leucocytes were included. The acquired data were analysed with CELLQuest software (Becton Dickinson). Analysis regions based on fluorescence were established with control samples. The regions were maintained and used to determine the percentage and mean fluorescence intensity (MFI) of stimulated leucocytes exhibiting phagocytic activity or oxidative burst. The MFI of the control samples was subtracted from the MFIs of the samples.

2.6. Statistical analysis

For statistical analysis, a two-factor orthogonal analysis of variance using Minitab statistical software was used to evaluate the effect of bitch and day, or of period (early or late luteal phase), on the absolute and relative numbers of leucocytes, and for the percentage of stimulated cells and their MFIs for the Phagotest™, and of percentage of stimulated cells using *E. coli* or PMA, and the MFIs of the stimulated cells, for the Phagoburst™ test. There were three missing values for the Phagoburst™ test and five missing values for the Phagotest™. The missing data were imputed using the formula of Cochran and Cox (Cochran and Cox, 1952), and the ANOVA was then adjusted by reducing the residual degrees of freedom. In Fig. 2, there are missing values; the imputed values are not shown.

3. Results

3.1. Differential count of leucocytes

The total number of leucocytes varied significantly with bitch ($P < 0.001$), as did both neutrophils ($P = 0.001$), lymphocytes, eosinophils, and monocytes ($P < 0.001$). The absolute number as well as the proportion of eosinophils differed significantly ($P < 0.001$) between the two periods. Neither the absolute numbers nor the proportions of the other leucocyte populations varied significantly between the two periods (Table 1).

3.2. Phagocytic activity

The percentage of cells that phagocytised was not affected by bitch, day or period, or by the interaction between bitch and period. The activity of the phagocytising cells, described as MFI, decreased during the study, and was significantly lower during late compared to early luteal

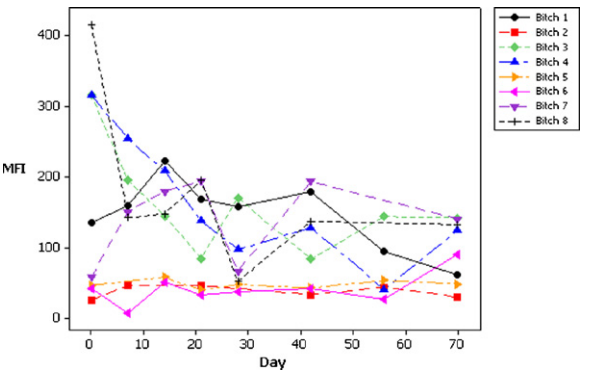

Fig. 2. Mean fluorescence intensity (MFI) of leucocytes phagocyting opsonised *E. coli* bacteria (using Phagotest™) in the individual bitches.

Table 1
Total number of leucocytes, neutrophils, eosinophils and lymphocytes ($\times 10^9$ L^{-1}), and percentage of the different leucocyte populations in canine serum during the luteal phase. Values given are median and interquartile range (IQR).

	Early luteal phase				Late luteal phase			
	Day 1	Day 8	Day 15	Day 22	Day 29	Day 43	Day 57	Day 71
Leucocytes	9.2 (4.7)	9.4 (2.1)	8.0 (7.5)	8.0 (2.0)	8.8 (3.2)	9.6 (4.0)	10.0 (5.6)	9.5 (5.0)
Neutrophils	5.5 (3.3)	5.5 (1.7)	4.9 (5.6)	4.9 (0.9)	5.4 (3.3)	6.0 (3.9)	6.5 (3.8)	6.3 (4.6)
%	61.6 (8.2)	58.3 (10.4)	61.0 (21.1)	58.0 (4.2)	57.6 (8.5)	60.7 (13.4)	61.4 (11.2)	61.9 (13.2)
Eosinophils	0.4 (0.4)	0.3 (0.4)	0.5 (0.6)	0.5 (0.3)	0.4 (0.2)	0.5 (0.5)	0.6 (1.0)	0.6 (0.6)
%	3.5 (5.1)	3.6 (5.4)	4.3 (5.8)	5.7 (4.5)	4.9 (4.2)	5.2 (5.5)	6.0 (8.4)	6.3 (7.0)
Lymphocytes	2.8 (1.0)	3.0 (1.2)	2.3 (1.2)	2.6 (1.5)	2.8 (0.6)	2.8 (0.8)	2.6 (0.7)	2.6 (0.8)
%	30.6 (6.8)	31.0 (8.6)	28.8 (19.7)	31.1 (3.0)	31.3 (8.5)	31.2 (7.1)	27.1 (8.2)	26.6 (9.0)
Monocytes	0.5 (0.4)	0.4 (0.2)	0.3 (0.5)	0.5 (0.2)	0.4 (0.6)	0.4 (0.5)	0.4 (0.4)	0.3 (0.2)
%	4.8 (2.1)	4.3 (2.1)	4.0 (2.3)	5.5 (2.9)	4.7 (3.6)	4.5 (2.4)	4.1 (1.9)	3.2 (3.0)

Table 2
Phagocytic activity of leucocytes, measured as the percentage of cells stimulated by opsonised *E. coli* bacteria, and their mean fluorescence intensity (MFI), shown as the median values and interquartile range (IQR), and oxidative burst, described as the median percentage of cells that are stimulated by the particulate stimulus (*E. coli*) or the high stimulus phorbol 12-myristate 13-acetat (PMA) and their mean fluorescence intensity (MFI).

	Early luteal phase				Late luteal phase			
	Day 1	Day 8	Day 15	Day 22	Day 29	Day 43	Day 57	Day 71
Phagocytic capacity								
% stimulated cells (IQR)	66.3 (15.0)	58.0 (23.3)	67.2 (28.6)	63.6 (12.4)	60.9 (49.6)	65.3 (7.4)	60.6 (65.7)	65.9 (9.8)
MFI in stimulated cells (IQR)	96.5 (273.2)	146.5 (168.2)	146.3 (149.6)	111.2 (146.5)	59.4 (102.8)	106.0 (126.6)	42.6 (77.6)	106.9 (86.5)
Oxidative burst								
% stimulated cells, *E. coli* (IQR)	49.8 (18.5)	47.3 (10.0)	53.2 (14.8)	43.4 (5.9)	40.7 (35.6)	49.3 (17.5)	38.2 (14.0)	49.6 (12.5)
MFI *E. coli* (IQR)	40.5 (59.8)	63.7 (50.3)	6.9 (54.7)	26.9 (34.7)	29.1 (29.9)	23.8 (18.7)	31.1 (53.3)	37.6 (58.7)
% stimulated cells, PMA (IQR)	61.4 (23.1)	58.2 (23.3)	61.1 (23.2)	44.5 (11.8)	50.8 (31.7)	52.4 (3.8)	50.1 (19.9)	53.6 (21.5)
MFI, PMA (IQR)	28.7 (55.9)	54.2 (36.8)	10.0 (77.3)	41.4 (42.3)	31.5 (41.6)	33.9 (39.8)	36.0 (36.5)	40.3 (41.1)

phase ($P = 0.003$, Table 2 and Fig. 2). It also varied significantly with bitch ($P < 0.001$), but not with the interaction between bitch and period.

3.3. Oxidative burst

The percentage of cells stimulated by *E. coli* differed significantly between bitches ($P = 0.009$). It was significantly lower in late compared to early luteal phase ($P = 0.049$) but was not significantly affected by the interaction between bitch and period. The activity of leucocytes stimulated by *E. coli* was not significantly affected by bitch, period or their interaction. The percentage of leucocytes stimulated by PMA, and their MFI, was significantly affected by bitch, but not by day, period or the interaction between bitch and period. Approximately half of the leucocyte population was stimulated by *E. coli* and a slightly higher amount with PMA (Table 2). The proportion of cells stimulated by the low physiological stimulus fMLP was very low, <1%, and these data are not included in the table or in the statistical calculations.

4. Discussion

The main finding of the present study was a reduced phagocytic ability of leucocytes, and a smaller percentage of leucocytes triggered by *E. coli* to oxidative burst, during late luteal phase. One possible explanation is that in late luteal phase, there exists a subpopulation of cells which have the capacity to phagocytise bacteria, but not to kill them by oxidative burst. Although eosinophils and monocytes have phagocytic and oxidative burst activities, the concentrations of eosinophils and monocytes were low in the present samples, and the results thus most likely reflect phagocytic and oxidative burst activities of neutrophils. As the number of neutrophils did not change significantly between early and late luteal phase and the number of eosinophils increased, the results point to a reduced capacity of the cells, and not to a decline in the number of phagocytising cells. These results can also be compared to those of a previous study, in which the total number of neutrophils was not different during the luteal phase compared to other stages of the oestrous cycle (Willson et al., 2012).

Studies on the effect of sex hormones on neutrophils in other species give various and sometimes contradictory results. In ovariectomized mares treated with progesterone or oestradiol benzoate, progesterone treatment resulted in significantly more uterine luminal neutrophils 24 h after induction of endometritis (Watson et al., 1987). Neutrophils from progesterone-treated mares had a lower bactericidal activity than from oestradiol-treated or control mares (Watson et al., 1987), while in a study on

cycling mares, no effect on neutrophil function could be related to cyclus stage (Roberto Da Costa et al., 2003). In ovariectomized cows, sex steroid hormones did not influence oxidative burst activity of neutrophils in vitro (Winters et al., 2003). However, in an in vitro study using polymorphonuclear leucocytes from high yielding dairy cows, progesterone decreased the oxidative burst activity (Chaveiro and Moreira da Silva, 2010). The activity of polymorphonuclear leucocytes has also been shown to increase during the follicular phase (Chaveiro and Moreira da Silva, 2009). In humans, the chemotaxis of polymorphonuclear leucocytes was enhanced by progesterone (Miyagi et al., 1992).

When evaluating oxidative burst, the low physiological stimulus (fMLP) did not give rise to measurable amounts of leucocyte activity. This stimulus thus seems too weak for studying physiological variations in the dog. The high stimulus, PMA, stimulated a large proportion of the leucocytes. No difference was seen between periods regarding the proportion of leucocytes that was triggered to burst, in contrast to the results using the particulate stimulus, opsonised *E. coli*. In this limited material, opsonised *E. coli* thus seemed to be the best stimulus for evaluation of physiological variations in oxidative burst. This is interesting considering that *E. coli* is a common bacterium isolated from cases of pyometra (Fransson et al., 1997; Hagman and Greko, 2005). When studying tumour-bearing dogs, a difference in the proportion of neutrophils exhibiting oxidative burst activity between dogs with tumours and control dogs was seen with PMA as stimulus, but not with *E. coli* (LeBlanc et al., 2010).

Several parts of the innate immune system were not investigated in the present study. The first phase of the phagocytic process, chemotaxis, was not evaluated. Neutrophil extracellular traps, NETs, that may be released by neutrophils upon activation and kill bacteria extracellularly (Brinkmann et al., 2004) were also not evaluated. In dairy cows, the ability for NET expression has been shown to vary during the different physiological states (Revelo and Waldron, 2010). Another important component of the innate immune system that would have been interesting to study is the toll-like receptors, e.g. toll-like receptor 4, TLR 4, as the expression of TLR 4 has been shown to vary during the oestrous cycle in the canine endometrium and in endometrial leucocytes (Chotimanukul and Sirivaidyapong, 2011).

The commercial tests in the present study have previously been used for bovine samples (Kampen et al., 2004). They have also been used for studies of phagocytic activity and oxidative burst in tumour-bearing dogs (LeBlanc et al., 2010). High variation of phagocytosis and oxidative burst of neutrophils from healthy dogs has previously been described using other methods (Eickhoff et al., 2004). In the present study there was a large variation, both within and between dogs, especially for oxidative burst, but also for the test of phagocytosis. This may be natural variation between dogs, or a reflection of the difficulties of keeping laboratory conditions constant. Both phagocytosis and oxidative burst are temperature dependent, and it is therefore important that temperature is observed and that the laboratory work is standardised. On the other hand, storage of blood for 24 h at room temperature did not have any negative effect on phagocytosis or ROS-production in dogs (Eickhoff et al., 2004). In the present study, significant differences in leucocyte phagocytosis during the luteal phase could be detected even though the study population was limited to eight dogs. There appeared to be slightly different patterns between dogs regarding phagocytic activity (Fig. 2), but there was no statistically significant interaction between dog and period. However, due to the large inter-individual variations, a large sample would be desirable for analysis of physiological changes of phagocytosis and oxidative burst.

It has previously been speculated that the morphological and physiological changes of the endometrium during the luteal phase predispose the bitch to pyometra (Dow, 1959). The bacteria, most often *E. coli*, may attach to the endometrium more easily. It has been shown that the same receptor that increases the pathogenic potential of uropathogenic *E. coli* also facilitates bacterial attachment to the endometrium (Krekeler et al., 2012). The bacteria may reach the uterus through the cervix during oestrus, or via haematological spread from the urinary or gastrointestinal tract. A reduced phagocytising capacity of the neutrophils during late luteal phase may facilitate haematological spread, but it may also facilitate development of the infection locally. In either case, an increased risk of disease such as pyometra will be the result.

5. Conclusion

The phagocytic capacity of canine leucocytes, and the percentage of cells in which oxidative burst is triggered by *E. coli*, decrease during the late compared to the early luteal phase. This is not associated with a reduction in the number of phagocyting cells. The decrease in the innate immune response during late luteal phase may contribute to the higher incidence of diseases such as pyometra during this period.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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