The effects of *in vitro* exposure to progesterone and estradiol-17β on the activity of canine neutrophils

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ABSTRACT: To date, only limited information about the influence of ovarian hormones on canine immune system cells has been available. The study investigated the *in vitro* influence of progesterone and estradiol-17β on the activity of canine neutrophils. Treatment of cells by both hormones led to a significant decrease in phagocytosis-induced oxidative burst as detected using luminometry after stimulation with opsonised zymosan. The increase in oxidative burst, not connected with phagocytosis, was recorded after stimulation with a soluble stimulator. Using flow cytometry, the tendency of both hormones to decrease the production of reactive oxygen species associated with phagocytosis of *Escherichia coli* was also evident, although not significant. Suppression of canine neutrophil activity is not connected with pathogen recognition capabilities, since the expression of Toll-like receptor 4 was unaffected. This study reveals that both hormones have a suppressive effect on the activity of canine neutrophils and thus might contribute to the aetiology of pyometra.

Keywords: TLR4; flow cytometry; oxidative burst; phagocytosis; pyometra; dog

Although it is well known that ovarian hormones, besides their direct influence on the reproductive system, affect the whole organism of females in many ways, there is still a lack of data available on how the different parts of the immune, reproductive and in particular endocrine systems interact. As early as 1982, the function of neutrophils obtained from steers treated with ovarian hormones was proven to be affected by progesterone which enhanced their random migration under agarose, but significantly depressed phagocytosis (Roth et al. 1982). With respect to human neutrophils, it is known that these respond to both oestrogen and progesterone. Oestrogens are generally recognised for their antiinflammatory effects, mainly due to their effects on the production of nitric oxide and inhibition of chemotaxis (Miyagi et al. 1992; Garcia-Duran et al. 1999; Stefano et al. 1999). Conversely, progesterone is acknowledged for its positive effect on chemotaxis and its more proinflammatory role in general (Miyagi et al. 1992; Bouman et al. 2005).

The oestrous cycle in bitches is unique in terms of the length of the follicular and mainly luteal phases. In this respect, it differs from the cycle of not only humans, but also other domestic animals (Jochle and Andersen 1977; Johnston et al. 2001). This long-lasting effect of ovarian hormones is considered to predispose this species to its most common reproductive tract disorder – pyometra (Noakes et al. 2001; Smith 2006) whose etiopathogenesis has not yet been fully elucidated, despite decades of research (Kida et al. 2010). Pyometra is a serious and potentially lethal disease with a poor prognosis and, in the bitch, is not only a disease of the reproductive tract. It is very often associated with systemic inflammatory response syndrome or with septic shock (Hardie 1995; Fransson and Ragle 2003).

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There is evidence that the etiopathology of canine pyometra, unlike other kinds of infectious inflammatory diseases, has not only a bacterial cause but also a hormonal background (Fransson and Ragle 2003). As for the bacterial cause, the most common bacterial agent is *E. coli*, which causes pyometra in 56–96 % of cases (Nomura 1984; Dhaliwal et al. 1999). Even though in the past hormonal imbalance or disorders of uterine oestrogen and progesterone receptor expression had been suggested as important causes, none of the aforementioned has been fully proved (Noakes et al. 2001; Fransson and Ragle 2003). Yet, the underlying changes in sex hormone levels during heat and dioestrus and subsequent physiological responses of the canine uterus are crucial for the onset and progression of pyometra (Johnston et al. 2001, Noakes et al. 2001; Fransson and Ragle, 2003). The impaired immunological status of bitches with pyometra has already been described (Faldyna et al. 2001a; Bartoskova et al. 2007) but whether and to what extent sex hormones contribute to the immunological aspect (i.e. potentially altered susceptibility) of the development of this disease in bitches has remained open to question.

Sugiura et al. (2004) demonstrated the influence of ovarian hormones on antigen-specific immune responses, particularly secondary responses. In their research, oestrogen appeared to upregulate, while progesterone downregulated the activity of lymphocytes *in vivo* and *in vitro*, effects which were also dependent on the stage of the oestrous cycle.

The predominant immunological response in dogs exposed to bacterial infection is represented by a massive increase in the number of neutrophils. This is by no means surprising, as dogs belong to the species with a physiologically high neutrophil: lymphocyte ratio (Jain 1993). However, even nowadays little is known regarding their activity under the influence of ovarian hormones. Therefore, the aim of our study was to investigate whether the ovarian sex steroids estradiol-17β and progesterone also affect the functions of canine neutrophils, which are an important part of the first line of defence against the progression of *E. coli* growth in the canine uterus.

MATERIAL AND METHODS

Animals and blood sampling. Six healthy adult bitches of various breeds (three Beagles, two mongrels, and one Jack Russell Terrier) ranging from two to eight years of age were used. Bitches were referred to the Department of Reproduction, Clinic of Dog and Cat Diseases, University of Veterinary and Pharmaceutical Sciences Brno for elective ovariectomy or ovariohysterectomy. Peripheral blood was collected from the *vena cephalica antebrachii* under pyrogen-free conditions. The blood was collected under informed consent of owners as part of a pre-surgical general examination. All dogs were clinically healthy, in the stage of anoestrus, which was confirmed by serum progesterone measurement (concentrations lower than 0.2 ng/ml). Their general health was also confirmed by the results of haematological tests and basic metabolic panel examination, none of which showed any alterations from the physiological range.

Detection of respiratory burst by chemiluminescence. White blood cells (WBCs) were isolated by hypotonic lysis of erythrocytes as follows: 5 ml of heparinised blood was mixed with 40 ml of apyrogenic distilled water for 20 s and then the osmotic pressure was normalized with 5 ml of ten-times concentrated Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, Life Technologies, USA). The WBCs were washed twice with DPBS and finally resuspended in Hank's balanced salt solution (HBSS, Cambrex, USA) at a concentration of 10^7 per ml. These samples contained 60–70% of neutrophils as detected from blood smears stained with May-Grünwald and Giemsa-Romanowski.

One million WBC from each bitch were mixed with ovarian hormones – progesterone and estradiol-17β (both from Sigma-Aldrich, USA). Both hormones were prepared in the same concentrations (progesterone: 0.01, 0.1 and 1μM; estradiol-17β: 0.02, 0.2 and 2nM) and manner as described by Suigura et al. (2004). WBCs were preincubated at 37 °C in 5% CO_2 atmosphere for 4 h. In control samples, the ovarian hormones were replaced with dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) which was used to dilute the hormones.

The release of reactive oxygen species was measured using 8-amino-5-chloro-7-phenylpyridopyridazine (L-012, Wako Pure Chemical Industries, Ltd., Japan) – enhanced luminometry. Samples were run in triplicates with a 20μM concentration of L-012 using a multi-detection microplate reader Synergy 2 (BioTek Instruments, USA). WBCs were stimulated with serum-opsonised zymosan particles (InvivoGen, USA) or phorbol 12-myristate 13-acetate as a soluble activator of protein-kinase C

(PMA, final concentration 1μM, Sigma-Aldrich, USA) or were left non-stimulated.

Detection of phagocytosis and respiratory burst by flow cytometry. For flow cytometry analyses of phagocytosis and respiratory burst, *E. coli* was used as a stimulus as described previously (Ondrackova et al. 2012). The *E. coli* (O149:K88 serotype) was transformed by electroporation with pDsRed-Express Vector plasmid (Clontech Laboratories, Inc., USA). A 48 h culture was prepared from a frozen stock by cultivation at 37 °C. The *E. coli* was resuspended in HBSS immediately before use. The concentration of *E. coli* was evaluated using spectrophotometry.

Peripheral blood diluted 1 : 1 with HBSS was mixed with ovarian hormones and pre-incubated for 4 h as described in section 2.2. In control samples, the ovarian hormones were replaced with DMSO alone. The incubation of the samples was performed in 96-well plates. All samples were run in quadruplicates. All incubations were performed at 37 °C in a 5% CO_2 atmosphere.

Five μM of oxygen-sensitive fluorescent dye DCF (5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester, Invitrogen, Life Technologies, USA), and 1×10^7 *E. coli* were added to each well and the samples were subsequently incubated for 30 min. Finally, haemolysis of red blood cells was performed with ice-cold haemolytic solution (154.4mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, all Sigma Aldrich, USA). The cells were washed with ice-cold PBS, mixed with propidium iodide (Sigma-Aldrich, USA) and immediately measured.

The measurements were performed using a FACSCalibur flow cytometer (Becton-Dickinson, USA). At least 60 000 events were acquired. The post-acquisition analysis was performed using Summit software (Dako, Denmark). Viable neutrophils were gated according to their light scatter properties and propidium iodide-staining. Viable neutrophils were further characterised by green and yellow fluorescence. Green fluorescence reflected the fluorescence of the DCF probe and was represented by neutrophils exhibiting a respiratory burst while yellow fluorescence reflected the fluorescence of DS-RED represented by neutrophils associated with *E. coli*. The percentage of viable neutrophils which had ingested *E. coli* and then engaged in a respiratory burst was evaluated.

Toll-like receptor 4 expression. Since lipopolysaccharide is one of the most relevant components of the *E. coli* bacterial wall, the expression of Toll-like receptor 4 (TLR4) was measured using flow cytometry. Peripheral blood diluted 1 : 1 with HBSS was mixed with ovarian hormones and incubated for 4 h as described in section 2.2. In control samples, the ovarian hormones were replaced with DMSO alone. Then, haemolysis of red blood cells was performed with ice-cold haemolytic solution and the cells were washed with ice-cold PBS.

Cells were stained using an indirect technique as described previously (Faldyna et al. 2001b). Twenty μl of heat-inactivated filtered goat serum and 0.5 μl of anti-TLR4 antibody (clone HTA125, eBioscience, Inc., USA; usability for canine TLR4 detection was proven by Burgener and Jungi 2008) were added to the cell suspension, mixed and incubated for 20 min at 4 °C. The sample was then washed with cell washing solution (CWS, PBS containing 1.84 g/l EDTA, 1 g/l sodium azide and 4 ml/l gelatine, all Sigma–Aldrich, USA) and 50 μl of secondary R-PE-conjugated goat anti-mouse IgG2a antibody (Southern Biotech, USA) diluted 1 : 500 was added for next 20 min. Finally, the sample was washed with CWS, propidium iodide was added and the measurement was performed using a BD LSRFortessa cell analyser (Becton-Dickinson, USA). At least 60 000 events were acquired.

The post-acquisition analysis was performed using Summit software (Dako, Denmark). Viable neutrophils were gated according to their light scatter properties and propidium iodide-staining. The median of fluorescence intensity of TLR4-expressing neutrophils was established.

Statistical analysis. The data were analysed with the use of the non-parametric paired Wilcoxon test. P values < 0.05 were considered significant. All calculations were performed with GraphPad Prism software, version 3.03 (GraphPad Software, Inc., USA).

RESULTS

The aim of our study was to investigate whether the ovarian sex steroids oestradiol-17β and progesterone affect the functions of canine neutrophils.

Detection of respiratory burst by chemiluminescence

Using luminometry, we found a clear tendency of oestrogen and progesterone, in some cases statis-

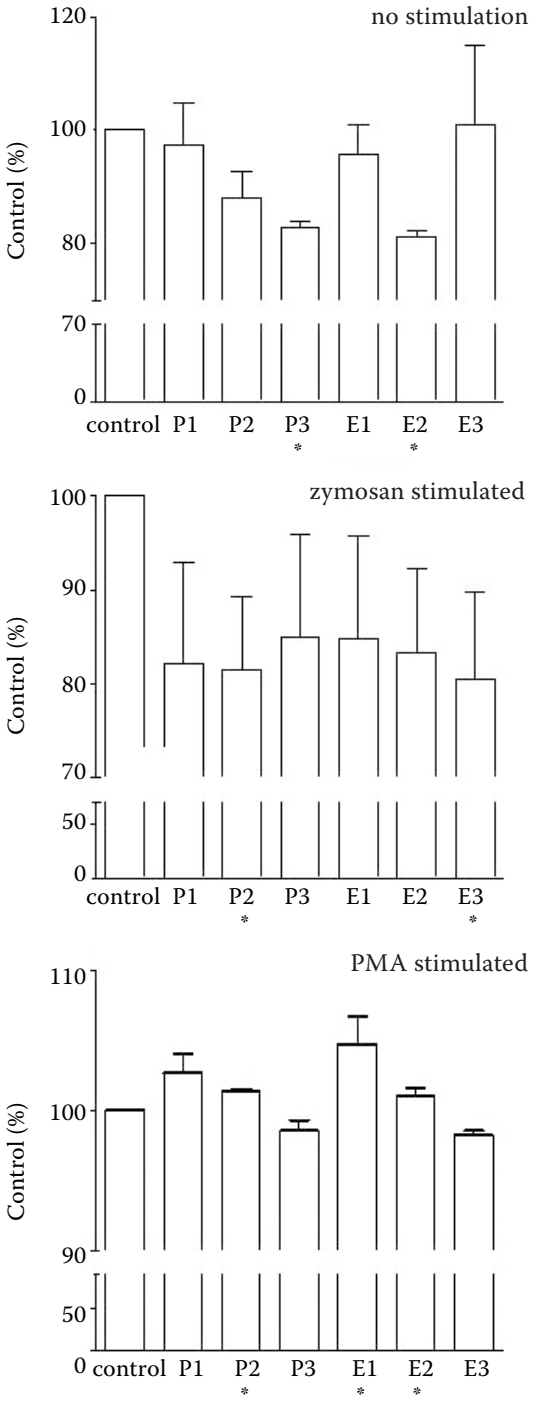

Figure 1. The effect of progesterone (P) and oestradiol-17β (E) on the respiratory burst of canine neutrophils with no stimulation and after stimulation with opsonised zymosan or PMA. Concentrations of progesterone used were as follows: P1: 0.01μM, P2: 0.1μM, and P3: 1μM. Concentrations of oestradiol-17β used were as follows: E1: 0.02nM, E2: 0.2nM, and E3: 2nM. Results are shown as mean ± SD of percentage of control, i.e. activity of neutrophils without any hormonal treatment

*$P < 0.05$ vs. control

tically significant, to suppress the oxidative burst induced by phagocytosis after stimulation by opsonised zymosan. We observed these tendencies, to a lesser extent, even in non-stimulated neutrophils (Figure 1).

In order to test whether or not the impaired ability of neutrophils to phagocytise was behind the decreased oxidative burst, we performed the luminometry assay after stimulation with a soluble nonspecific artificial stimulant of protein kinase C – PMA (Figure 1). In this case no decrease was observed. On the contrary, the intensity of the oxidative burst was increased with the some concentrations of both hormones.

Detection of phagocytosis and respiratory burst using flow cytometry

Our aforementioned results were in accordance with the results obtained using flow cytometry. In control samples, i.e. in samples without any hormonal treatment, 83.8 ± 2.9% of neutrophils phagocytosed *E. coli* with subsequent induction of respiratory burst. After treatment with progesterone or oestradiol-17β, the trend towards a decrease

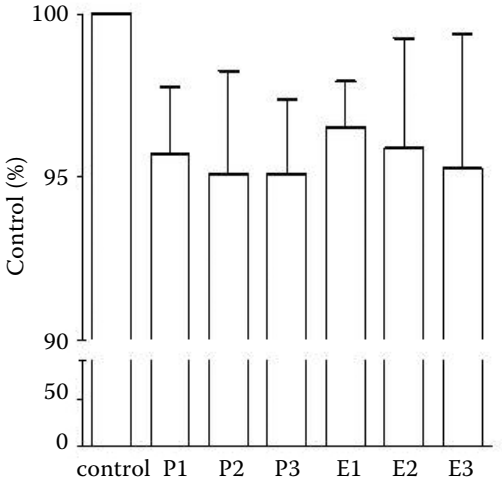

Figure 2. The effect of progesterone (P) and oestradiol-17β (E) on the percentage of canine neutrophils capable of *E. coli* phagocytosis and subsequent respiratory burst as determined by flow cytometry. Concentrations of progesterone used were as follows: P1: 0.01μM, P2: 0.1μM, and P3: 1μM. Concentrations of estradiol-17β used were as follows: E1: 0.02nM, E2: 0.2nM, and E3: 2nM. Results are shown as mean ± SD of percentage of control, i.e. activity of neutrophils without any hormonal treatment

in oxidative burst was also evident although not statistically significant (Figure 2).

Toll-like receptor 4 expression

Subsequently, we wanted to discover whether suppression of phagocytosis-induced oxidative burst was caused by impaired ability to recognise lipopolysaccharide (LPS) by TLR4 because LPS is the main component of the outer membrane of *E. coli*, the most common bacterial agent associated with canine pyometra (Fransson et al. 1997; Dhaliwal et al. 1999) (Figure 3). Median fluorescence intensity of TLR-expressing neutrophils appeared not to be influenced by oestrogen and progesterone.

DISCUSSION

The sex steroid-induced suppression of oxidative burst recorded in the present study confirms some of the previous investigations in this field (Bekesi et al. 2000, 2007; Abrahams et al. 2003). For example, Bekesi et al. (2000) found that both oestradiol and progesterone suppress the release of superoxide anion from neutrophils. Furthermore, in the rodent model, neutrophil respiratory burst activity was higher in ovariectomised rats subjected to trauma or haemorrhagic shock than in hormonally intact animals (Doucet et al. 2010).

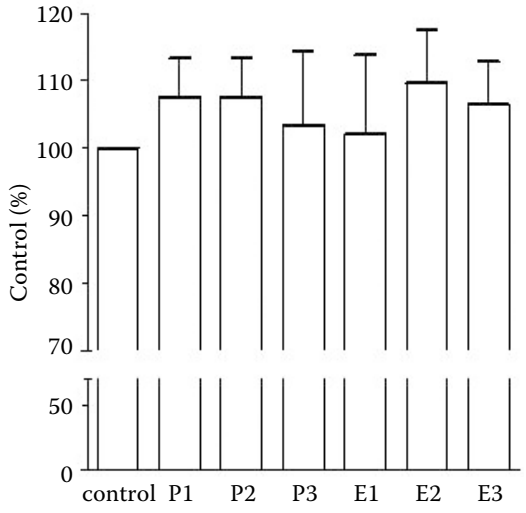

Figure 3. The effect of progesterone (P) and estradiol-17β (E) on median fluorescence intensity of TLR4 expression on canine neutrophils as detected by flow cytometry. Concentrations of progesterone used were as follows: P1: 0.01μM, P2: 0.1μM, and P3: 1μM. Concentrations of oestradiol-17β used were as follows: E1: 0.02nM, E2: 0.2nM, and E3: 2 nM. Results are shown as mean ± SD of percentage of control, i.e. activity of neutrophils without any hormonal treatment

The ability to produce reactive oxygen species under the influence of sex hormones has been studied several times with varying results (da Silva et al. 1998; Cassidy 2003; Molloy et al. 2003; Bouman et al. 2005; Bekesi et al. 2000). The reported PMA-induced increase in the intensity of the oxidative burst is in accordance with the findings of Molloy et al. (2003). They also noted an increase in reactive oxygen species production after stimulation of human female neutrophils with PMA, from which they concluded that sex steroid hormones enhance the oxidative burst in neutrophils. However, other researchers documented an inhibitory influence of sex hormones on neutrophil oxygen radical production (Abrahams et al. 2003; Bekesi et al. 2007). Moreover, the vast majority of other studies have also reported an inhibitory role of oestrogens on oxygen radical production in various cell lines (reviewed in Straub 2007).

Taking into consideration the limited number of studies performed so far, which all vary with respect to methods, concentrations of hormones, and times of exposure, it is very difficult to compare our results with previous findings. Moreover, to the best of our knowledge, this is the first study to investigate the influence of ovarian hormones on canine neutrophil activity. Nevertheless, it has been proven that both oestrogen and progesterone do affect the functions of immune system cells, including neutrophils (reviewed in Bouman et al. 2005; Straub 2007).

The unchanged intensity in TLR4 expression is in accordance with the findings of Giannoni et al. (2011) who reported that neither oestradiol nor progesterone modulated the expression of TLR4 on human cord blood mononuclear cells. Furthermore, other studies have documented that TLR4 expression on innate immune system cells after *in vitro* exposure to oestrogens was not altered (Vegato et al. 2004; Pioli et al. 2007; Rettew et al. 2009) whereas *in vivo* oestrogens augment TLR4 expression on murine macrophages (Rettew et al. 2009).

There are many factors that might induce changes in the impact of ovarian steroids, including species sensitivity, gender, stage of the oestrous cycle, individual variability, concentration of endogenous hormones or the dose of administered hormones, duration of exposure, health status, and interac-

tion with other hormones and substances. For instance, dogs and ferrets are extremely susceptible to oestrogen-induced lethal bone marrow depression compared to other species (Johnson 1989; Hart 1990; Farris and Benjamin 1993; Sontas et al. 2009). Even though the mechanism has not been fully elucidated, the progression of this condition was reported already in 1948 by Crafts who described a greatly increased initial release of neutrophils into the bloodstream, followed by congestion, oedema and destruction of the bone marrow.

The interaction of sex steroid hormones and the innate immune system is complex and the research performed so far in this field seems to bring more paradoxes and questions than answers. For example, oestrogen exerted a suppressive effect on the migration of neutrophils and monocytes in most tissues *in vivo*, whereas in the uterus, the effect was completely the opposite (Straub 2007). Other studies have reported that NK cell activity is inhibited or stimulated by oestrogen in a dose-dependent manner (Straub 2007). Recent findings (Chotimanukul and Sirivaidyapong 2011) also describe close yet complicated relationships between reproductive functions and innate immunity in the model of canine pyometra, where TLR4 expression in various layers of endometrium changed significantly throughout the oestrous cycle. It is noteworthy that TLR4 expression was at the lowest level in the surface epithelium during oestrus which prevented unnecessary post-mating inflammation, whereas, in bitches with pyometra the expression was markedly increased.

Giannoni et al. (2011) revealed that sex steroid hormones are one of the possible underlying causes that lead to high susceptibility to sepsis in human new-borns. New-borns are naturally exposed to high levels of sex steroid hormones and in this study they were proven to have a strong inhibitory effect on the innate immune response.

Regarding neutrophils, oestrogen and progesterone were reported to mediate delayed apoptosis in both human genders (Molloy et al. 2003) and female sex hormones have been recognised for their protective role in sepsis, trauma and haemorrhage, and organ ischaemia reperfusion injury (Wichmann et al. 1996; Schroder et al. 1998; Angele et al. 1999; Kraemer 2000; Mizushima et al. 2000; Knoferl et al. 2002; Molloy et al. 2003). On the other hand, over-delayed neutrophil apoptosis is connected with systemic inflammatory response syndrome, subsequent tissue damage and multiple organ failure (Jiminez et al. 1997; Molloy et al. 2003). Systemic inflammatory response syndrome is also a common complication of pyometra in dogs (Fransson and Ragle 2003).

In the present study we found that progesterone and estradiol-17β cause a decrease in the activity of neutrophils via suppression of phagocytosis-induced oxidative burst. However, this suppression was not caused by the decreased expression of the molecule responsible for recognition of lipopolysaccharide: TLR4 on the neutrophil surface. Thanks to the prolonged exposure of the canine organism to increased levels of both hormones during heat the described suppression of neutrophil function caused by ovarian steroids might be one of the underlying conditions contributing to the high susceptibility of bitches to pyometra.

Although the results did not show a clear dose-dependent decrease in neutrophil activity, the biological relevance of the obtained data should not be dismissed. The most consistent decrease was recorded when the concentrations (0.1μM progesterone and 0.2nM estradiol-17β) of both hormones corresponded to the naturally elevated ovarian hormone levels in canine serum during the heat.

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