

Quantity of IL-2, IL-4, IL-10, INF- γ , TNF- α and KC-Like Cytokines in Serum of Bitches With Pyometra in Different Stages of Oestrous Cycle and Pregnancy

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Contents

The occurrence of the pyometra is most common in the first half of the dioestrus when there is decreased cellular immunity associated with increased serum concentration of progesterone in females. The aim of this study was to determine the immunological profile of bitches with pyometra, studying serum levels of IL-2, IL-4, IL-10, INF- γ , KC-like and TNF- α and comparing them with those of healthy bitches in anoestrus, dioestrus and pregnant. Forty females were divided into four experimental groups: group 1 (G1): with pyometra ($n = 10$); group 2 (G2): bitches in the second week of gestation ($n = 10$); group 3 (G3): in anoestrus ($n = 10$); and group 4 (G4): in dioestrus ($n = 10$). The serum levels for IL-2, KC-like, INF- γ and TNF- α were similar for all experimental groups. The values obtained for IL-10 were found increased ($p < 0.001$) in animals in dioestrus and pyometra compared with females in anoestrus and pregnant, and the levels of IL-4 observed were significantly greater ($p < 0.001$) in bitches with pyometra when compared with others. The cytokine profile in animals with pyometra is similar to bitches in dioestrus for IL-10 and had increase in IL-4 for bitches with pyometra, which represents an anti-inflammatory these cases. This suggests the presence of an immunosuppressive state in both cases, which may explain the propensity of bitches in dioestrus to be affected by pyometra and the severity of the disease on these animals.

Introduction

Pyometra is a severe disease of female dogs characterized by chronic uterine inflammation and bacterial colonization with consequent installation of a systemic inflammatory and septic syndrome (Smith 2008). The occurrence of this disease is most common in the first half of the dioestrus when there is a decrease in cellular immunity associated with an increase in serum concentration of progesterone in females (Sugiura et al. 2004; Tsumagari et al. 2005; Smith 2006).

The progesterone in bitches presents a peculiar activity on the immune system of females, modulates the action of T lymphocytes, being able to suppress the function of these cells (Michelon et al. 2006), and directly affects the local immunity favouring the installation of uterine infection (Dabrowski et al. 2007).

The presence of a pathogenic agent to the organism promotes the activation of the innate immune system mediated by macrophages and neutrophils which recognize self-molecules of the agent and lead to intracellular signalling, activation of transcription factors and production of cytokine (Robertson and Coopersmith 2006; Dickson 2009). The cytokines are soluble proteins synthesized by various cells of the immune system that act as immune regulators and induce the production of

immunoglobulins by plasmocytes, neutralize bacterial toxins and prevent their adherence (Abbas et al. 2000; Hahn and Liewer 2007).

Recent studies in human and veterinary medicine (Denney et al. 2011; Feliciano et al. 2012) on the profiles of cytokines during gestation have demonstrated the behaviour of these units in gestational immunology. As for pyometra, there are no reports on the behaviour of cytokines in females, but it is suggested that this knowledge can prevent the occurrence of the disease in female dogs during dioestrus or improve the prognosis of females in severe cases. Considering the importance of the disease in small animal obstetrics, the aim of this study was to determine the immunological profile of bitches with pyometra, studying serum levels of interleukins 2, 4, 10 (IL-2, IL-4, IL-10), interferon- γ (INF- γ), natural killer-like cells (KC-like) and tumour necrosis factor- α (TNF- α) and comparing them with those of healthy bitches in anoestrus, dioestrus and pregnancy.

Material and Methods

Animals and experimental groups

This study was conducted following the approval of the Animal Ethics and Welfare Committee of the School of Agrarian and Veterinary Sciences of the Sao Paulo State University, Brazil (protocol No 017314/10).

A total of 40 females of various breeds from 10 to 25 kg of body weight, all adults between 4 and 8 years old, were used. The animals were divided into four experimental groups in accordance with the reproductive status observed by physical, complementary examinations (haemogram and biochemical profile) and obstetric examinations including specific reproductive tract inspection, vaginal cytology, abdominal palpation and imaging: group 1 (G1): bitches with pyometra ($n = 10$); group 2 (G2): bitches in the second week of gestation ($n = 10$); group 3 (G3): bitches in anoestrus ($n = 10$); and group 4 (G4): bitches in dioestrus ($n = 10$).

The animals belonging to group 1 were females that had presented clinical signs and ultrasonographic findings consistent with the framework of pyometra. The clinical diagnosis was confirmed by the visualization of uterine alterations during the surgical procedure performed as the treatment of choice.

To monitor and determine the oestrous period for bitches of G2, G3 and G4, vaginal cytology was carried out for four consecutive days. The material was collected using a swab, fixed with methanol and stained in glass slide by Giemsa. After this, the materials were

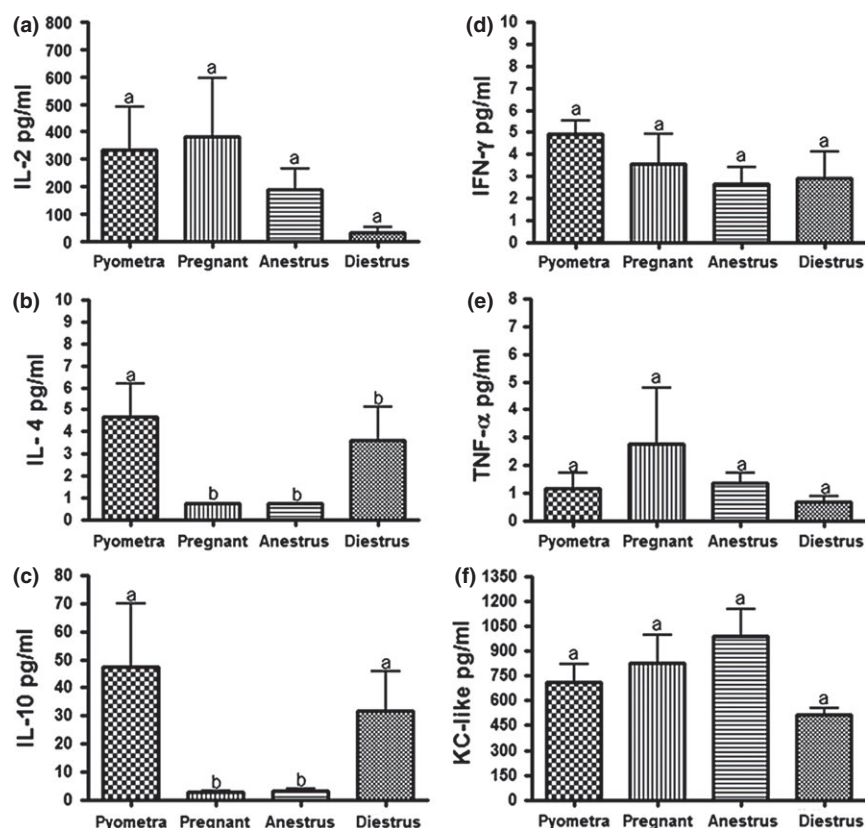


Fig. 1. Graphical representation of serum cytokine levels in bitches with pyometra, pregnant, inanoestrus and dioestrus: (a) IL-2, (b) IL-4 (c) IL-10, (d) IFN- γ , (e) TNF- α and (f) KC-like. The presence of different small letters in columns indicates significant differences between the parameters in bitches ($p < 0.05$).

analysed through direct microscopy in increments of 25 and 100 dioptres.

The oestrus signs were also monitored and associated with direct microscopy findings. After the confirmation of probable date of higher fertility, the female belonging to group 2 in oestrus and the male were put together and were separated after 3 days. While kept together, it was noticed that the female was receptive to the male mounts. The determination of pregnancy was carried out 2 weeks after mating, in analogy to that reported by Feliciano et al. (2007). These bitches were evaluated in two groups G2 and G4 according to their reproductive state.

Immunological profile

Blood samples of 5 ml were collected from each female from the jugular vein, and then, the serum was separated and frozen at -80°C for evaluation of cytokines. The immune profile was established through the determination of serum levels of cytokines: IL-2, IL-4, IL-10, IFN- γ , TNF- α and KC-like through the panel cytokines MILLIPLEX MAP (CCYTO-90K, Millipore, Billerica, 89 Massachusetts, USA), validated for dogs. The samples were standard and incubated in microspheres coupled to a specific antibody and then incubated with streptavidin-PE. Then, the reading was performed in the matrix system – MILLIPLEX (Luminex 200, Luminex Corporation, St. Charles, Mo., USA). All procedures for the analysis of samples were conducted in the laboratory GENESE (Genese LTDA, São Paulo – SP, Brasil) (Feliciano et al. 2012).

Statistical analysis

Statistical analysis was performed with the aid of R[®] software (R Foundation for Statistical Computing, Vienna, Austria). The experimental design corresponded to a completely randomized design. The normality of residuals and homogeneity of variances were tested previously, and actual or transformed data were analysed by analysis of variance, and when the difference between the treatments was significant, the means were compared by Tukey's test ($p < 0.05$).

Results

The serum levels for IL-2, KC-like, INF- γ and TNF- α were similar for all experimental groups. The values obtained for IL-10 were found increased ($p < 0.001$) in animals in dioestrus and with pyometra compared with females in anoestrus and pregnant, and the levels of IL-4 observed were significantly greater ($p < 0.001$) in bitches with pyometra when compared with the others (Fig. 1 and Table 1).

Discussion

The immune response to pyometra is associated with an inflammatory reaction of the innate immune system mediated by an increase in neutrophil leucocytes and a reduction in lymphocyte activity, which can be associated with endotoxemia or to the inhibition of lymphocyte mitogenic factors (Faldyna et al. 2001). For these reasons, the immune response in pyometra is compromised, which can change the adaptive responses and cytokine profiles.

Table 1. Values (mean $\pm$ SE) of the levels (pg/ml) of cytokines in bitches with pyometra, pregnant, dioestrus and anoestrus

Cytokines	Pyometra	Pregnant	Anoestrus	Dioestrus
IL-2	330 $\pm$ 160	380 $\pm$ 210	190 $\pm$ 76	29 $\pm$ 20
IL-4	4.7 $\pm$ 1.5	0.74 $\pm$ 0.1	0.74 $\pm$ 0.2	3.5 $\pm$ 1.5
IL-10	43.4 $\pm$ 22.6	2.6 $\pm$ 0.4	3.1 $\pm$ 0.8	31.9 $\pm$ 13.9
INF- γ	4.9 $\pm$ 0.6	3.5 $\pm$ 1.4	2.6 $\pm$ 0.8	2.9 $\pm$ 1.2
TNF- α	1.1 $\pm$ 0.6	2.8 $\pm$ 1.9	1.4 $\pm$ 0.4	0.6 $\pm$ 0.2
KC-like	714 $\pm$ 105	825 $\pm$ 172	988 $\pm$ 164	511 $\pm$ 45

It was observed that the immune profile of bitches with pyometra is altered demonstrating impairment of the immune response of these animals. The alteration in the amount of cytokines in bitches with pyometra is associated with the activation of the innate immune system in cases of uterine diseases in dogs. This immune response is characterized by an increase in leucocytes, decreased lymphocyte activity, high levels of immunoglobulins, lysozyme and the presence of immune complexes caused by endotoxemia and inhibition of lymphocyte mitogenic factors in this condition (Faldyna et al. 2001).

According to the literature (Faldyna et al. 2001; Smith 2006; Dickson 2009; Karlsson et al. 2012), the bitches in dioestrus and with pyometra present an immunosuppression that could be observed in bitches in this study. The serum concentration of IL-10 was found increased in animals with pyometra and dioestrus when compared with animals in anoestrus and pregnant. The IL-4 levels were statistically increased only in bitches with pyometra, but bitches in dioestrus also expressed high levels of this cytokine.

In humans, the IL-10 has been associated with sepsis (Urbonas et al. 2012) and patients mortality (Novotny et al. 2012). In animals, Karlsson et al. (2012) detected the presence of IL-10 only in dogs with pyometra and systemic inflammatory response syndrome (SIRS), indicating that this cytokine is an indicator of severity in these cases. In this study, it was observed increase in levels of IL-10 both in bitches with pyometra and in dioestrus, demonstrating the immunological fragility of female dogs on that stage of the oestrous cycle, resulting from hormonal alterations, and their possible predisposition to the occurrence of pyometra if the females do not become pregnant.

IL-4 has been associated with conditions of severe sepsis and as an early indicator of mortality in humans (Bozza et al. 2007). Therefore, the increased levels of IL-4 found in the female dogs may be an early indicator of pyometra and of the disease severity.

The TNF- α has been considered as an early marker of acute exposure to endotoxin in animals with experimental sepsis (Otto 2007). However, the observed results did not show the increase in this cytokine in bitches with pyometra as well as in studies by Karlsson et al. (2012).

Among the evaluated cytokines, the IL-2 and INF- γ were similar for all groups of bitches and their expression in pregnant females has been considered essential

for the development and maintenance of pregnancy (Feliciano et al. 2012), because it is related to the inflammatory process that favours the foetal implantation (Mellor and Munn 2000).

The immunological profile observed in this study by the levels of cytokines obtained demonstrated that animals with pyometra have an immune behaviour similar to that of dogs in dioestrus except for IL-4, suggesting the presence of an anti-inflammatory response in both cases. This may explain the propensity of bitches in dioestrus to be affected by pyometra and could be related to the severity of the disease. This characteristic may be related to levels of progesterone (Feliciano et al. 2012) that predispose to bacterial colonization of the uterus and induces specific endometrial receptors for the same (Fransson and Ragle 2003).

It is believed that the most important immunoregulatory effect of progesterone is related to its action on T lymphocytes, which in humans, under the allogeneic stimulus, begins to express receptors for progesterone and in high concentrations of this hormone the lymphocytes synthesize an immunomodulatory protein (PIBF) that inhibits the liberation of arachidonic acid and the activity of NK cells and also alters cytokine levels (Michelson et al. 2006). As an example of the action of PIBF on cytokines, this mediator promotes the secretion of T-helper-2 cytokines (IL-3, IL-4 and IL-10) while inhibits the T-helper-1 cytokines (INF- γ , TNF- α and IL-2) (Szekeres-Bartho et al. 2005), similar behaviour to that found in the present study.

Conclusion

The cytokine profile in animals with pyometra is similar to bitches in dioestrus for IL-10 and higher to the other groups to levels of IL-4 representing an anti-inflammatory pattern that suggests the presence of immunosuppression in both cases. This may explain the propensity of bitches in dioestrus to be affected by pyometra, and it could be related to the severity of the disease and with the progesterone action.

Studies relating these proteins with clinical signs and progression of pyometra in animals could help clarify the immune response that occurs during different stages of the disease and the IL-4 role as an evidence of disease. Evaluating other cytokines such as CRP, IL-7, IL-18 and IL-15 that have been correlated with the disease in different stages of the oestrous cycle can be interesting in the search for immune conditions that lead to pyometra establishment during dioestrus.

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Conflict of interest

The authors of this manuscript declare no conflict of interest in relation to this article.

Author contributions

Giovana Serpa Maciel and Marcus Antônio Rossi Feliciano designed the study, analysed data and drafted the paper. Ricardo Ramirez

Uscategui analysed data and drafted the paper. Vivian Tavares de Almeida and Wilter Ricardo Russiano Vicente drafted the paper. Maria Emilia Franco Oliveira analysed data.

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