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HYDROGEN PEROXIDE PRODUCTION OF NEUTROPHILS DURING STAPHYLOCOCCAL INFECTION IN OBESE DOGS

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Summary

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Experimental *Staphylococcus* infection was induced in obese and non-obese mongrel dogs, at the age of 4–7 years in order to study its effects on reactive oxygen species (H_2O_2) production of neutrophils, which are one of cell components of innate immunity. Prior to infection, dogs of experimental group I (obese dogs), were submitted to overfeeding with a high-fat diet for a period of 3 months in order to induce experimental obesity. Both the experimental group II (non-obese dogs) and the control group were fed a standard maintenance diet. *Staphylococcus* infection was induced in dogs of groups I and II by subcutaneous application of bacterial suspension of *Staphylococcus intermedius.* Neutrophil counts and their phagocytic activity (measured by H_2O_2 production) were estimated at the following time intervals: initial levels (before infection – hour 0), hours 3, 24, 48 and days 7 and 14 after infection. In non-obese dogs, leukocyte counts increased by the 24th and 48th hours and were higher up to 7th day; neutrophil counts were higher from the 3rd hour up to 7th day; H_2O_2 production of neutrophils augmented and was significantly higher on hour 24 (as compared to controls) and on day 14 (as compared to initial levels). In obese individuals leukocyte counts were also higher on hours 24, 48 and on day 7; neutrophil counts were elevated from the 3rd hour up to the 14th day; but H_2O_2 production decreased significantly by hours 3 and 24.

Key words: dog, infection, neutrophil ROS production, obesity, *Staphylococcus intermedius*

INTRODUCTION

Staphylococcus intermedius is one of the normal commensals of animals, including dogs, and also a causative microorganism of various infections in animals (Hajek, 1976; Phillips & Kloos, 1981; Raus & Love, 1983; Talan *et al*., 1989) including staphylococcal pyoderma, which is the most common dermatological problem in dogs, both healthy and atopic (Simou *et al*., 2005).

Phagocytosis, as a response of organism to infections, is known to be an essential component of innate immunity. Neutrophils have proved to possess the highest phagocytic activity of all leukocytes. The production of H_2O_2 (a component of reactive oxygen species-ROS) is one of the main microbicide factors of neutrophils (Sawyer *et al*., 1989).

Recently, obesity has become a problem, which could negatively influence the pathogenesis of infection, not only in humans but also in domestic dogs bred as pets. Obesity in humans has been reported

to decrease granulocyte activity, that may lead to decreased immunological defense (Debczynski *et al*., 1996). Some researchers have reported obesity as linked to higher monocyte and granulocyte phagocytosis and oxidative burst activity (Nieman *et al.*, 1999). In dogs, obesity may adversely affect immune response to infection, and infection is reported to occur more frequently in obese individuals (Gottschlich *et al*., 1993). The alterations in immune function in obese individuals, as well as during bacterial infections, have been described by different studies as highly controversial. The aim of our study was to evaluate the alterations in H_2O_2 production of neutrophils, during experimental local *Staphylococcus intermedius* infection in obese dogs and dogs in normal body condition.

MATERIALS AND METHODS

Experimental animals

We used twenty-three healthy male, mongrel dogs, 4-7 years of age. The animals were kept in individual cages (situated indoor, at constant room temperature) and went for walks twice a day – half an hour in the morning and another walk in the evening. So, the conditions were similar to the those of pets. The adaptation period continued one month. At its beginning, the dogs were treated against parasites with Prazimec-D tablets containing abamectin 2 mg and praziquantel 50 mg (Biovet, Peshtera, Bulgaria) at a dose of 1 tablet/10 kg. Also, they were treated against ectoparasites with antiparasite shampoo – Bolfo-Shampoo containing 0.11 g propoxur/100 mL (Bayer AG, Germany).

Dogs were divided into three groups: control group, consisting of nine animals and two experimental groups. The first experimental group (group I, obese dogs) consisted of six animals. They were submitted to overfeeding with a high-fat diet: standard maintenance diet ("Jumbo Dog", Gallisman S. A., Bulgaria; containing protein – 17%, fats – 8%, fibre – 4%, vitamin D_3 – 3000 IU/kg, vitamin E – 200 mg/kg, vitamin A – 11000 IU/kg, Zn – 35 mg/kg, Na – 0.4%, Mg – 50 mg/kg, Ca – 0.95–1.3%, Cl – 0.95%, Cu – 9 mg/kg, with humidity – 9%) plus lard supplement – 10g/kg body weight, for a period of three months, in order to induce experimental obesity. In group I, the initial body weight before the overfeeding was 12.86 ± 3.50 kg and by the end of the 3rd month it increased to 16.54±4.10 ($p<0.001$). The second experimental group (group II, non-obese dogs) weighed 13.65±3.32 kg and this weight did not change significantly within the period of experiment. It consisted of eight animals, which were fed the aforementioned standard maintenance diet. Experimental staphylococcal infection was induced in animals of group I and group II. Control animals received the same standard maintenance diet as the experimental non-obse ones and were submitted to no other treatment.

Experimental infection

Local *Staphylococcus intermedius* infection was induced by subcutaneous application, in the lumbar region, of 5 mL bacterial suspension (1×10^9 CFE/mL). The suspension was prepared from bacterial culture after 24-hour growth. A terrain strain, isolated from a dog with a clinical infection, was used. Microbiological identification was done by BD BBL Crystal Gram Positive ID System. The strain was determined as catalase-positive, oxidase-negative, producing plasmocoagulase and desoxyribonuclease.

Sample collection

Blood samples of 2 mL were collected in sterile glass tubes by punction of *vena cephalica* at the following time intervals – right before infection (hour 0), and on post infection hours 3, 24, 48 and days 7 and 14. As anticoagulant, heparin (Biochemie, Vienna, Austria), at 10 IU per sample was used.

Nitroblue tetrazolium reduction test

Microscopic slide histochemical nitroblue tetrazolium chloride reduction test was performed to evaluate ROS (H_2O_2) production of neutrophils (H_2O_2 converts the colorless nitroblue tetrazolium – NBT, to a deep blue coloured compound while in active cells, insoluble NBT formazan is visible as fine purple deposits). The number of ROS (H_2O_2) producing neutrophils was calculated as percentage of total neutrophil count (100) on a blood smear (safranin staining) (Baehner & Nathan, 1968).

Total leukocyte counts and neutrophil counts

Leukocytes ($\times 10^9$/L) were counted using the Bürker chamber. The neutrophil counts ($\times 10^9$/L) were determined on blood smears (May-Grünwald-Giemsa staining).

Statistical analysis

Results were submitted to standard F- and t-tests (StatMost, version 2.5, DataMost Corporation). The data were presented as means±SD. Differences were considered statistically significant at the $P<0.05$ level.

RESULTS

Total leukocyte counts, neutrophil counts and H_2O_2 production of neutrophils in dogs from the control group did not change significantly within the period of experiment (Table 1).

In group I, total leukocyte counts increased by the 48th hour and 7th day, compared to hour 0 ($P<0.05$). In group II, as compared to hour 0, leukocyte counts were increased from the 24th hour to the 7th day ($P<0.01$).

Total leukocyte counts showed statistically significant differences between groups as followed: by hour 24 they were higher in groups I and II, compared to control group ($P<0.05$); by hour 48 – higher in group I vs controls ($P<0.05$).

At each time interval, neutrophil counts, showed statistically significant differences between groups as followed: by hour 3 they were higher in group II, compared to both group I and the control group ($P<0.05$); by hours 24 and 48 and on the 7th day in group I and group II and on the 14th day in group I neutrophils were also significantly higher vs. controls. Compared to initial levels after infection, neutrophils were higher in group I by hours 24 and 48 ($P<0.001$) and by days 7 ($P<0.01$) and 14 ($P<0.05$); in group II – by hours 14 and 48 ($P<0.001$) and by day 7 ($P<0.01$).

During infection, H_2O_2 production in group I, evaluated in the abovementioned dynamics was significantly lower on hour 3 ($P<0.05$) and 24 ($P<0.01$) vs hour 0. H_2O_2 production rose significantly on day 7, compared to hour 0 ($P<0.05$).

In group II, H_2O_2 production increased significantly on the 24th hour and the14th day, compared to hour 0 ($P<0.05$).

The comparison of H_2O_2 production between the three groups revealed the following statistically significant differences: on hour 3, it was higher in group II and the control group, compared to group I ($P<0.01$); on hour 24, it was lower in group I vs controls ($P<0.05$) and higher in

Table 1. Total leukocyte counts ($\times 10^9$/L), neutrophil counts ($\times 10^9$/L) and % of ROS (H_2O_2) producing neutrophils in dogs: control group (n=9, non-obese and non-infected animals), experimental group I (n=6, obese dogs submitted to infection*) and experimental group II (n=8, non-obese dogs submitted to infection*). Results are expressed as mean ± SD

Time#	Groups	Leukocytes ($x10^9$/L)	Neutrophils ($x10^9$/L)	% ROS (H_2O_2) producing neutrophils
hour 0	Control group	8.02±1.55	0.55±0.04	25.44±9.59
	Obese dogs (I)	8.70±3.31	0.59±0.06	21.5±5.45
	Non-obese dogs (II)	7.23±1.44	0.60±0.04	25.80±14.18
hour 3	Control group	8.43±1.29	0.54±0.03	33.71±9.62
	Obese dogs (I)	9.6±4.94	0.47±0.16	9.80±6.02 a^2,c^1,b^2
	Non-obese dogs (II)	7.75±2.53	0.65±0.11 a^1,b^1	34.40±11.04
hour 24	Control group	8.49±1.94	0.53±0.10	29.11±15.96
	Obese dogs (I)	13.82±4.76 a^1	0.79±0.08 a^3,c^3	8.20±5.76 a^1,c^2,b^2
	Non-obese dogs (II)	11.36±3.06 a^1,d^2	0.84±0.04 a^3,d^3	50.00±22.03 a^1,d^1
hour 48	Control group	8.16±2.22	0.53±0.06	36.20±15.48
	Obese dogs (I)	14.75±5.61 a^1,c^1	0.79±0.06 a^3,c^3	24.60±17.74
	Non-obese dogs (II)	11.08±3.26 d^2	0.81±0.07 a^3,d^3	43.40±11.13
day 7	Control group	8.53±2.89	0.57±0.04	43.20±24.71
	Obese dogs (I)	13.07±2.80 a^1,c^1	0.74±0.09 a^3,c^2	45.25±14.22 c^1
	Non-obese dogs (II)	11.25±2.37 d^2	0.69±0.05 a^3,d^2	47.00±20.12
day 14	Control group	7.58±2.98	0.55±0.05	34.00±13.77
	Obese dogs (I)	10.02±2.83	0.68±0.06 a^3,b^1,c^1	41.20±19.61
	Non-obese dogs (II)	8.66±1.40	0.60±0.06	56.50±19.99 a^1,d^1

* experimental infection produced by *Staphylococcus intermedius*; # time intervals after experimental infection. Significant differences between and within groups indicated as follows: a^1 $P<0.05$, a^2 $P<0.01$, a^3 $P<0.001$ vs. controls; b^1 $P<0.05$, b^2 $P<0.01$ between group I and group II; c^1 $P<0.05$, c^2 $P<0.01$, c^3 $P<0.001$ within group I vs. hour 0; d^1 $P<0.05$, d^2 $P<0.01$, d^3 $P<0.001$ within group II vs. hour 0.

group II vs both group I ($P<0.01$) and controls ($P<0.05$). H_2O_2 production in group II was also higher, compared to the control group on the 14th day ($P<0.05$).

DISCUSSION

The experimental *Staphylococcus intermedius* infection causes significant changes in the non-specific defense mechanisms in dogs. A large number of cell types including mononuclear phagocytes, neutrophils, vascular endothelial cells and platelets are affected as primary targets (Day, 1994; Moulding *et al*., 1999). However, phagocytes are considered to be the most critical cells in the host response to staphylococci and in the recruitment of components of the immune and inflammatory responses. The ability of canine neu-

trophils to phagocytize particles of staphylococci has been studied by some authors (Kroese *et al*., 1981; Shearer, 1997).

In the conditions of our experiment in non-obese dogs, the experimental local *Staphylococcus intermedius* infection caused an increase in leukocyte and neutrophil counts, related to a higher ROS (H_2O_2) production of neutrophils. This reveals an activation of some cell components of innate immunity.

Staphylococcus antigen is capable to stimulate neutrophil oxidative burst. This is probably due to the fact that staphylococci produce a number of components interfering with the process of opsonization, which is an important prerequisite for the performance of phagocytosis. Furthermore, pyoderma in dogs causes an evaluation in serum levels of *Staphylococcus*- specific IgG antibodies that represent specific opsonins (Shearer, 1997). In our experiment this may be the reason for the observed higher values of NBT-test on 7^{th} day after infection in obese dogs and on 14^{th} day in non-obese dogs.

In obese dogs, the infection caused a similar, but longer-lasting increase in total leukocyte (from the 24^{th} hour to the 7^{th} day) and neutrophil counts (from the 24^{th} hour to the 14^{th} day), which was connected with a significantly lower H_2O_2 production by post infection hours 3 and 24. So, in obese dogs, the H_2O_2 producing capacity of neutrophils seemed to be impaired. It is to be suggested that obese dogs could probably be more susceptible to *Staphylococcus intermedius* infection. In addition, previous investigations of ours have proved this infection to be more severe in obese dogs (Andonova *et al.,* 2006).

Several lines of evidence have supported links between the adipose tissue and the cells of immune system. This interaction is illustrated in obesity, where excess adiposity and impaired immune function have been described in both humans and rodents (Marti *et al.,* 2001). Data concerning dogs are very limited, and are based only on clinical and epidemiological information. Often, controversial data exists comparing immunity in obese and non-obese subjects (Gottschlich *et al.*, 1993; Nieman *et al.*, 1999). Leptin and other adipokines, investigated recently mainly in rodents and humans, might play a key role in the nutritional status and activity of neutrophils and all immune cells (Shirshev & Orlova, 2005). Therefore, we may say that the clarification of the mechanisms of impaired neutrophil functions needs further investigations.

CONCLUSION

The experimental *Staphylococcus intermedius* infection in obese dogs caused a longer-lasting increase in total leukocyte and neutrophil counts, which was connected with a suppression of H_2O_2 production of neutrophils.

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