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Research paper

# Preactivation of neutrophils and systemic oxidative stress in dogs with hyperleptinemia

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ABSTRACT

High occurrence of obesity currently constitutes the main nutritional disease of the canine species. There is evidence that leptin increases during obesity in dogs. Hyperleptinemia is associated with increased neutrophil oxidative metabolism in obese humans and contributes to oxidative stress. However, in obese dogs, the probable relationship between this condition and the activation of the oxidative metabolism of neutrophils has yet to be established. Thus, we investigated the hypothesis that neutrophil activation and systemic oxidative stress occur in dogs with hyperleptinemia. A control group of 24 healthy dogs with a body condition score (BCS) of 4–5, an overweight group of 25 dogs with a BCS of 6–7, and 27 obese dogs with a BCS of 8–9, were composed. Two subgroups were formed composed of dogs with and without hyperleptinemia, grouped according to the 95% confidence interval obtained for plasma leptin values of the control group. Changes in obesity markers (body condition score, adiponectin and plasma leptin) and plasma oxidative stress (lipid peroxidation, total antioxidant and oxidant capacities and oxidative stress index) were measured in all the dogs selected. Neutrophil oxidative metabolism was evaluated in flow cytometry by superoxide production with the probe hydroethidine and by hydrogen peroxide production with the probe 2′,7′-dichlorofluorescein diacetate, with or without phorbol myristate acetate (PMA) stimulation. Apoptosis and neutrophil viability were quantified in a capillary flow cytometer using Annexin V—PE, with or without camptothecin apoptosis inducing effect. Obese dogs presented higher systemic oxidative stress, hyperleptinemia and preactivated neutrophils with accelerated apoptosis. Dogs with hyperleptinemia and obese dogs presented higher neutrophil superoxide production under PMA stimulation and the presence of systemic oxidative stress compared with control. To our knowledge, this is probably the first evidence that preactivation of the oxidative metabolism of circulating neutrophils occurs in dogs with hyperleptinemia, a condition that can induce systemic oxidative stress in the canine species.

## 1. Introduction

Obesity is the most common nutritional disease in dogs and its incidence, similar to human obesity, has grown rapidly, becoming one of the most important canine health problems (Zoran, 2010). Overweight and obesity are associated with several diseases, affecting the quality of life and reducing the average life expectancy of animals (German, 2006).

Leptin is secreted mainly by adipocytes (Ishioka et al., 2002), it has a pro-inflammatory effect and correlates positively with adipose tissue (Abella et al., 2017). Obese dogs show a significant increase in serum leptin concentrations and a decrease in adiponectin, which has anti-inflammatory action (Park et al., 2014). One of the adverse effects of hyperleptinemia includes oxidative stress mediated by the activation of NADPH oxidase (Blanca et al., 2016). In humans, leptin receptors (OB-R) are present in neutrophils (Zarkesh-Esfahani et al., 2004). The OB-R is responsible for the activation of the Janus kinases/ STAT pathway and stimulation of human PMNs by leptin leads to an enhanced accumulation of ROS (Caldefie-Chezet et al., 2001). Leptin Indirectly Activates Human Neutrophils via Induction of TNF-α secretion by monocytes (Zarkesh-Esfahani et al. 2004). Unlike adiponectin, which inhibits the generation of neutrophil superoxide(Magalang et al., 2006) and is associated with a low risk of cardiovascular diseases (Han et al., 2007).

The production of superoxide and its derivatives exert an important bactericidal function, however excessive production of these oxidants can result in oxidative stress (Babior, 2000), inducing increased lipid peroxidation and apoptosis (Kato et al., 2008). Oxidative stress plays an important role in the pathophysiology of various diseases (Kao et al.,

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2010; Russo and Bracarense, 2016) including chronic renal failure (Bosco el al., 2017) and leishmaniasis (Almeida et al., 2013).

Even in humans, very little is known about the impact of obesity on neutrophil function. The main hypothesis is that the innate immunity is activated (Nijhuis et al., 2014). The neutrophils of obese individuals are activated in the absence and presence of stimuli (*N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and zymozan) that can induce oxidative stress (Brotfain et al., 2015). However, in dogs, no differences were observed in antioxidant activity, lipid peroxidation and neutrophil production of reactive oxygen species (ROS) in animals with obesity induced over a short period of time (Van De Velde et al., 2012). The alteration of neutrophil viability in obesity remains poorly understood, but it is known that an increase in an apoptotic marker (M30) occurs in the peripheral blood cells of obese humans (Trellakis et al., 2012).

The evidence that leptin regulate neutrophil function and oxidative stress in human obesity has yet to be established in canine obesity. This study was conducted to confirm the hypothesis that neutrophil activation and systemic oxidative stress occur in obese dogs with hyperleptinemia.

## 2. Materials and methods

### 2.1. Animal selection

The experiment was conducted in accordance with the ethical principles of the use of animals, following approval by the Ethics Committee for Animal Experimentation of São Paulo State University (UNESP), under protocol no. FOA- 2014/01343.

Seventy-six adult male and female, castrated and uncastrated dogs of different breeds were selected (Table 1). According to the body condition score (BCS) of Laflamme (1997), the control group consisted of 24 dogs with a BCS of 4–5 and considered clinically healthy after a complete clinical examination and laboratory evaluation: complete blood count, CBC; plasma concentration of albumin; total bilirubin; alkaline phosphatase, ALP; gamma-glutamyltransferase, GGT; uric acid; alanine aminotransferase, ALT; aspartate aminotransferase, AST; urea, creatinine; calcium; glucose; cholesterol; and triglycerides. Another 25 dogs were categorized as overweight with a BCS of 6–7, and 27 as obese with a BCS of 8-9. Dogs from all groups underwent clinical and laboratory tests (Table 2). Two more subgroups were formed composed of dogs with and without hyperleptinemia, grouped according to the 95% confidence interval obtained for plasma leptin (PL) values of the control group. Hyperleptinemia was considered as dogs presenting values greater than 13.09 ng/mL.

The causes associated with canine obesity reported by German (2006) were investigated, and all dogs suspected of hyperadrenocorticism and hypothyroidism were excluded from the study. In addition, dogs that were recently treated with drugs that may induce obesity (megestrol acetate, cortisone, phenobarbital and primidone), antioxidants or that affect metabolism neutrophils (corticosteroids, vaccines, anti-inflammatories and antibiotics) or diagnosed with canine visceral leishmaniosis by indirect ELISA (Lima et al., 2003), were not included in the study.

**Table 1**
Characterization of dogs according to body condition score (BCS): Control (BCS 4–5), overweight (BCS 6–7) and obese (BCS 8–9).

| Characteristics | Control (n = 24) | Overweight (n = 25) | Obese (n = 27) |
|---|---|---|---|
| **Sex (%)** | | | |
| Female | 72 | 68 | 74.07 |
| Male | 28 | 32 | 25.93 |
| **Reproductive status (%)** | | | |
| Castrated | 45.83 | 48 | 48.15 |
| Uncastrated | 54.17 | 52 | 51.85 |
| **Age group (years) (%)** | | | |
| 1 - 6 years | 72 | 65 | 59.26 |
| > 6 years | 28 | 55 | 40.74 |
| **Breed (%)** | | | |
| Mixed-breed | 50 | 40 | 37.04 |
| Poodle | 8.33 | 8 | 14.81 |
| Labrador | 0 | 12 | 18.52 |
| Pitt bull | 12.5 | 0 | 0 |
| Fox Paulistinha | 4.17 | 0 | 11.11 |
| Other | 25 | 40 | 18.52 |

### 2.2. Sample collection and laboratory analysis

The dogs were fasted for 8–12 h prior blood collection, then 10 mL of blood were collected by jugular puncture, 1 mL was placed in EDTA tube for CBC and 9 mL was collected in heparinized tubes for neutrophil isolation and to obtain plasma for biochemical analyses, measurement of oxidative stress markers and quantification of plasma adipokines.

Plasma biochemical analyzes were performed in an automated spectrophotometer (BS 200, Shenzhen Mindray Bio-Medical Electronics Co., Nanshan, China) previously calibrated with a calibrator and control serum levels I and II and using commercial reagents (Biosystems, Barcelona, Spain). The levels of the following analytes were determined: urea (UV urease/glutamate dehydrogenase enzymatic method); glucose (glucose oxidase/peroxidase method); creatinine (alkaline picrate kinetic method); albumin (bromocresol green method); cholesterol (oxidase/peroxidase enzymatic method); triglycerides (glycerol phosphate oxidase/peroxidase enzymatic method); alkaline phosphatase (diethanolamine method), GGT (enzymatic UV urease/glutamate dehydrogenase method); total bilirubin (diazotized sulfanilic method); uric acid (uricase/peroxidase enzymatic method); total calcium (cresolphthalein complexone colorimetry); AST and ALT (IFCC kinetic method). All the biochemical reactions were performed at 37 °C, following the manufacturer's protocol.

CBC was performed using a veterinary automated cell counter (BC-2800 Vet, Shenzhen Mindray Bio-Medical Electronics). The differential leukocyte count was performed on blood smears stained with commercial hematological dye (Instant-Prov, Newprov, Pinhais, PR).

### 2.3. Determination of plasma oxidative stress

The total antioxidant capacity (TAC) was determined by the method described by Erel (2004) and the results expressed in μmol of Trolox/L equivalent. The total oxidant capacity (TOC) was quantified by the method described by Erel (2005) and the results expressed in μmol of equivalent hydrogen peroxide/L. The oxidative stress index was calculated as follows: OSi (%) = 100x [TOC/TAC] (Aycicek et al., 2005).

Lipid peroxidation was determined by the quantification of thiobarbituric acid reactive species (TBARS), according to Hunter et al. (1985), with the aid of an automatic ELISA reader (Robonik, Elisa Plate Analyzer, India) at absorbance 540 nm.

### 2.4. Determination of plasma adipokines

The concentrations of adiponectin (Cloud-clone Corp, Houston, TX, USA) and leptin (Millipore Corp., St. Charles, Missouri, USA) were determined using commercial reagents by species-specific ELISA with the aid of an automated 96-well microplate (Robonik, Elisa Plate Analyzer, India) at absorbance 450 nm.

### 2.5. Isolation of neutrophils

Neutrophils were isolated from heparinized whole blood as described by Bosco et al. (2017). Briefly, 4 mL of heparinized blood were used to isolate neutrophils in a double separation gradient containing 3 mL of Histopaque-1119® and 3 mL of Histopaque-1070® (Sigma-Aldrich Co., St. Louis, USA). The isolated cells were then washed twice with aqueous ammonium chloride solution (0.14 M) and once with

**Table 2**
Hematological and biochemical parameters (mean and standard deviation) of dogs according to body condition score (BCS): Control (BCS 4–5), overweight (BCS 6–7) and obese (BCS 8–9).

| *Laboratory exams* | Control (n = 24) | Overweight (n = 25) | Obese (n = 27) | p-value |
|---|---|---|---|---|
| Cell blood count | | | | |
| PCV (%) | 49.53 ± 4.5[a] | 48.91 ± 6.57 [a] | 49.56 ± 5.86 [a] | 0.9770[#] |
| Red blood cells ($10^{12}$/L) | 6.76 ± 0.6 [a] | 6.95 ± 0.82 [a] | 6.81 ± 0.73 [a] | 0.1409[# #] |
| Hemoglobin (g/dL) | 16.96 ± 1.5 [a] | 17.76 ± 2.15 [a] | 17.53 ± 2.06 [a] | 0.1343[#] |
| MCV (fL) | 73.25 ± 1.9 [a] | 72.73 ± 2.62 [a] | 72.51 ± 2.88 [a] | 0.3840[#] |
| MCHC (%) | 34.17 ± 1.0 [a] | 34.48 ± 2.47 [a] | 34.95 ± 2.16 [a] | 0.2376[#] |
| Total leukocytes ($10^9$/L) | 10.68 ± 3.9 [a] | 10.35 ± 3.29 [a] | 11.79 ± 3.66 [a] | 0.4546[#] |
| Biochemical | | | | |
| Uric acid (mmo/L) | 0.03 ± 0.04 [a] | 0.05 ± 0.04 [b] | 0.05 ± 0.07 [b] | 0.0163 [#] |
| ALT (IU/L) | 35.73 ± 11.7 [a] | 48.33 ± 37.01 [ab] | 49.79 ± 22.75[b] | 0.0492[# #] |
| AST (IU/L) | 28.03 ± 9.1 [a] | 27.39 ± 10.30 [a] | 28.58 ± 8.80 [a] | 0.6559[# #] |
| Cholesterol (mmol/L) | 5.37 ± 2.01 [a] | 4.39 ± 1.27 [a] | 5.61 ± 1.50 [a] | 0.0651[#] |
| Creatinine (μmol/L) | 5.01 ± 0.79 [a] | 4.99 ± 0.81 [a] | 5.16 ± 0.82 [a] | 0.3142[# #] |
| Glucose (mmol/L) | 90.26 ± 14.2 [a] | 89.99 ± 14.60 [a] | 92.92 ± 14.80 [a] | 0.7587[# #] |
| Urea (mmol/L) | 6.16 ± 1.25 [a] | 5.17 ± 1.53 [a] | 5.49 ± 1.83 [a] | 0.1085[#] |
| ALP (IU/L) | 39.87 ± 27.8 [a] | 50.94 ± 49.95 [ab] | 59.62 ± 37.12 [b] | 0.0349[# #] |
| Triglycerides (mmol/L) | 0.64 ± 0.24 [a] | 1.12 ± 0.77 [ab] | 1.45 ± 1.27 [b] | 0.0008[# #] |
| Calcium (mmol/L) | 2.38 ± 0.23 [a] | 2.32 ± 0.33 [a] | 2.50 ± 0.30 [a] | 0.0939 [#] |
| Albumin (g/L) | 31.12 ± 3.8 [a] | 29.85 ± 5.41 [a] | 32.88 ± 4.62 [a] | 0.0859 [#] |
| Total Bilirubin (μmol/L) | 11.46 ± 1.71 [a] | 11.80 ± 4.46 [a] | 12,65 ± 633 [a] | 0.6928 [#] |
| GGT (IU/L) | 3.55 ± 1.5 [a] | 4.10 ± 2.40 [a] | 3,76 ± 226 [a] | 0.8736[# #] |

Different letters indicate a significant statistical difference ($p < 0.05$) for the ANOVA test with the Tukey post-test (#) or Kruskal-Wallis with the Dunn post-test (##).

Hanks' balanced salt solution (Sigma-Aldrich Co., St. Louis, USA). After hemocytometer counting, the cells were diluted in RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, USA) at a concentration of $10^6$/mL. Viability was estimated by the trypan blue exclusion method and purity was determined by cytology. Only the isolates that obtained viability and purity above 95% and 93%, respectively, were included in the study.

### 2.6. Evaluation of neutrophil oxidative metabolism and apoptosis

Neutrophil oxidative metabolism and apoptosis were evaluated according to previously described methodology (Bosco et al., 2017). Briefly, using isolated neutrophils, oxidative metabolism was evaluated by determining superoxide production with a 10 μM hydroethidine probe (HE) (Invitrogen, Eugene, OR, USA), while hydrogen peroxide production was measured using a 5 μM 2′,7′-dichlorofluorescein diacetate probe (DCFH) (Sigma-Aldrich Co., St. Louis, USA), both with and without stimulation by 0.55 μM PMA (Sigma-Aldrich Co., St. Louis, USA) for 10 min at 37 °C. The mean red (superoxide) or green (hydrogen peroxide) fluorescences were quantified by flow cytometry (BD C5 Accuri flow cytometer, Ann Arbor, MI, USA), with the acquisition of 10,000 events within the population of cells presenting neutrophil characteristics.

Viability and total apoptosis (early + late apoptosis) were determined using Annexin V-PE (Guava nexin Kit, Guava Technologies, USA) in flow cytometry, with the acquisition of 10,000 events, following the manufacturer's recommendations. Briefly, 100 μL of neutrophils ($10^6$/mL) was incubated in the presence or absence of the induce 6 mM camptothecin (CAM) (Sigma-Aldrich Co., St. Louis, USA). Following incubation for 4 h at 37 °C with shaking for 1 min (600 rpm) every 15 min in a microprocessed thermocycler (Thermomixer, Eppendorf, Mod. Comfort, Hamburg, Germany), 100 μL of Annexin V-PE was added to the cell suspension and then incubated for 20 min at room temperature in the dark.

### 2.7. Statistical analysis

All the variables were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test). To determine the significance of the differences between the groups, the ANOVA test and the Tukey post-test were performed for parametric variables, and Kruskal-Wallis with the Dunn post-test were performed for non-parametric variables. To determine the degree of correlation, Spearman's coefficient was used. Dogs with and without hyperleptinemia were grouped according to the 95% confidence interval obtained for the control group. Differences of $p < 0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism (GraphPad software, version 5.01, 2007).

## 3. Results

Normal CBC and biochemical profile values were observed in the control group, while obese dogs showed higher plasma concentrations of triglycerides, uric acid, ALP and ALT activities (Table 2). These changes did not occur in overweight dogs, except for increased uric acid levels, which were equally significant (Table 2).

### 3.1. Systemic oxidative stress in obese dogs

Lipid peroxidation concentration (7.05 ± 4.90 vs. 11.96 ± 6.40 μmol/L, p = 0.0142), TOC (102.10 ± 41.78 vs. 178.00 ± 68.24 μmol/L, p = 0.0017) and OSi (13.59 ± 8.16 vs. 25.47 ± 14.89%, p = 0.0036) were higher in obese dogs compared with control dogs (Fig. 1), already the overweight group there was no significant difference in Lipid peroxidation concentration (7.05 ± 4.90 vs. 11.08 ± 5.76), TOC (102.10 ± 41.78 vs. 1383 ± 47,264), OSi (13.59 ± 8.16 vs. 18.73 ± 10.89).The TAC no significant was also observed in obese dogs (0.92 ± 0.19 vs. 0.85 ± 0.17 mmol/L, p = 0.1090) and overweight (0.92 ± 0.19 vs. 0.820 ± 0.16) (Fig. 1).

### 3.2. Disequilibrium in the production of leptin and adiponectin in canine obesity

Obese dogs had a higher concentration of PL than control dogs (27.45 ± 27.72 vs. 9.79 ± 6.62 ng/mL, p = 0.0144), while overweight dogs showed no significant difference (15.43 ± 11.88 vs. 9.80 ± 6.60 ng/mL, p > 0.05) (Fig. 2). There was a positive correlation between PL and BCS (r = 0.4597, p = 0.0004).

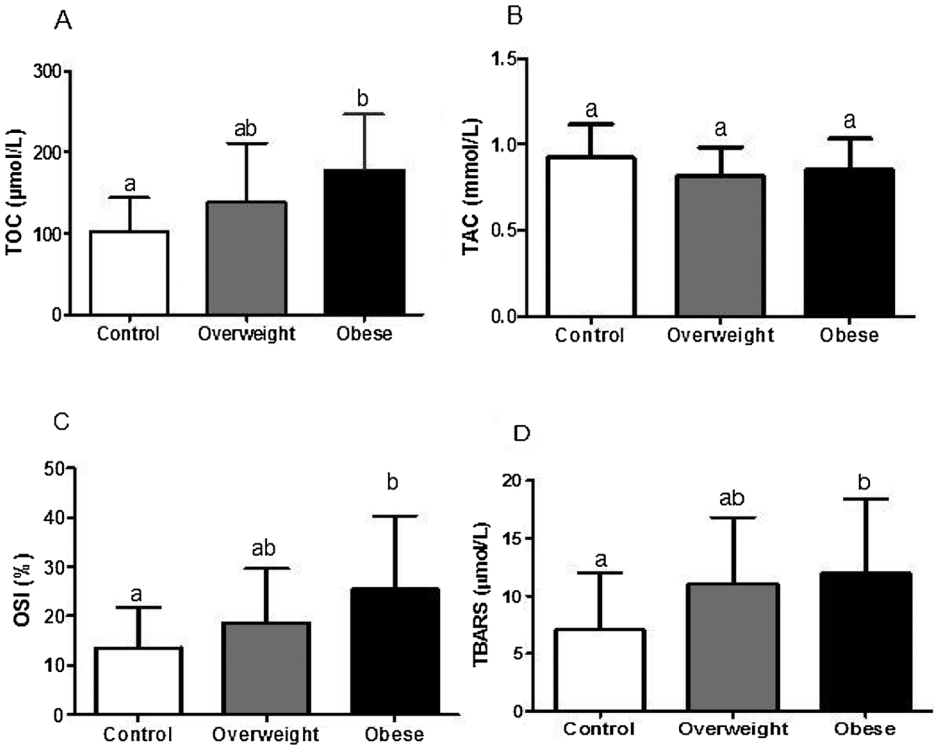


**Fig. 1.** Plasma concentration of oxidative stress markers: total oxidant capacity (TOC, **A**), total antioxidant capacity (TAC, **B**), oxidative stress index (OSi, **C**) and lipid peroxidation determined by thiobarbituric acid reactive substances (TBARS, **D**) of dogs according to body condition score: control (BCS 4–5, n = 24), overweight (BCS 6–7, n = 25) and obese (BCS 8–9, n = 27). The graphs represent the mean and standard deviation. Differential letters indicate statistically significant differences ($p < 0.05$) by the ANOVA test with Tukey post-test (A and B) or the Kruskal-Wallis test with Dunn post-test (C and D).

Plasma adiponectin levels of obese dogs were lower than those of control dogs (11.79 ± 6.40 vs. 10.27 ± 2.86 pg/mL), but this difference was not statistically significant (p = 0.7607) (Fig. 2).

### 3.3. Activation of oxidative metabolism and viability of neutrophils in obese dogs

Spontaneous superoxide production in obese (14.48 ± 3.31) and overweight dogs (15.46 ± 5.16) did not differ (p = 0.3670) from dogs with normal body score (13.79 ± 3.78). However, under PMA stimulation, neutrophil superoxide production of obese dogs (215.62 ± 63.87) was higher (p = 0.0349) than that of control dogs (173.80 ± 53.01) (Fig. 3). A positive correlation was observed between PMA-stimulated superoxide production and BCS (r = 0.2745; p = 0.0164; $r^2 = 0.0636$).

The mean spontaneous hydrogen peroxide production of neutrophils from obese (18.78 ± 16.22), overweight (18.56 ± 11.28) and control (18.25 ± 8.33) dogs did not differ significantly (p = 0.6864) (Fig. 3). In the presence of PMA stimulation (Fig. 3), this difference was also not significant (p = 0.3870) among obese (61.31 ± 28.39) and overweight dogs (52.49 ± 19.89) and those with a normal body weight score (48.91 ± 18.84).

Obese dogs showed lower neutrophil viability and higher neutrophil apoptosis than dogs with normal body weight scores (Table 3). These differences were not observed in the presence of the inducer CAM (Table 3). A positive correlation was also observed between the rate of spontaneous apoptosis and BCS (r = 0.3939, p = 0.0005).

### 3.4. Relation between hyperleptinemia and the activation of neutrophil oxidative metabolism and systemic oxidative stress in dogs

Hyperleptinemia (> 13.09 ng/mL) occurred in 44.4% of overweight dogs and in 73.7% of obese dogs. Spontaneous neutrophil superoxide production was not significantly higher in dogs with hyperleptinemia (14.92 ± 3.94 vs. 15.20 ± 2.93, p = 0.8026), however, following activation with PMA (159.50 ± 46.00 vs. 216.30 ± 70.13, p = 0.0094), a significant increase was observed in dogs with high leptin levels (Fig. 4). In addition, considering the BCS of all dogs, a positive correlation was determined between leptin and PMA-stimulated superoxide production (r = 0.2990, p = 0.0226, $r^2 = 0.0759$).

Dogs with hyperleptinemia presented higher TOC (97.16 ± 45.49 vs. 155.70 ± 64.92 µmol/L, p = 0.0070), OSi (11.24 ± 5.27 vs. 20.23 ± 12.19%, p = 0.0114) and lipid peroxidation (6.08 ± 3.63 vs. 10.13 ± 5.57 µmol/L, p = 0.0228) than dogs presenting lower PL levels, however, no change was observed in TAC (0.89 ± 0.20 vs. 0.87 ± 0.19 mmol/L, p = 0.8170) (Fig. 5). PL also showed a positive correlation with TOC (r = 0.3314, p = 0.0451, $r^2 = 0.0133$), OSi (r = 0.4109, p = 0.019, $r^2 = 0.0337$) and lipid peroxidation

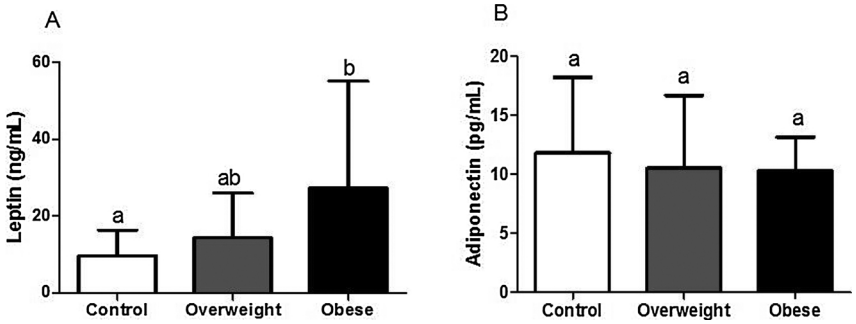


**Fig. 2.** Plasma concentration of leptin (**A**) and adiponectin (**B**) of dogs according to body condition score: control (BCS 4–5, n = 18), overweight (BCS 6–7, n = 18) and obese (BCS 8–9, n = 19). The graphs are represented by mean and standard deviation. Differential letters indicate statistically significant differences ($p < 0.05$) by the Kruskal-Wallis test with Dunn post-test.

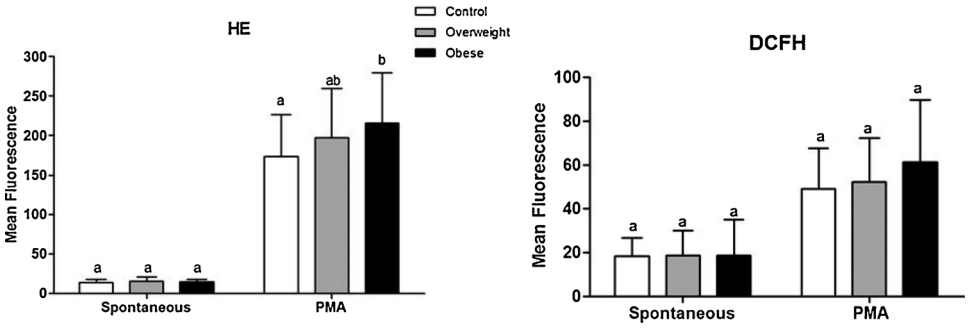


**Fig. 3.** Neutrophil superoxide production quantified by the probe hydroethidine (HE) and hydrogen peroxide quantified by the probe diacetate dichlorofluorescein (DCFH) of dogs according to body condition score: control (BCS 4–5, n = 24), overweight (BCS 6–7, n = 25) and obese (BCS 8–9, n = 27) in the absence (spontaneous) and presence of stimulus with phorbol myristate acetate (PMA). The graphs represent the mean and standard deviation. Differential letters indicate statistically significant difference ($p < 0.05$) by the Kruskal-Wallis test with Dunn post-test.

**Table 3**
Viability and apoptosis (mean and standard deviation) of neutrophils non-stimulated (NS) or stimulated with camptothecin (CAM) measured by Annexin V-PE using flow cytometry, according to body condition score (BCS): control (BCS 4–5, n = 24), overweight (BCS 6–7, n = 24) and obese (BCS 8–9, n = 26).

| | Control | Overweight | Obese | p-value |
|---|---|---|---|---|
| Viability NS (%) | 98.82 ± 0.57[a] | 98.49 ± 0.94 [ab] | 98.01 ± 1.39[b] | 0.0289 |
| Viability CAM (%) | 79.52 ± 6.46 [a] | 80.85 ± 7.36 [a] | 79.64 ± 8.36 [a] | 0.5633 |
| Total apoptosis NS (%) | 0.62 ± 0.34 [a] | 0.87 ± 0.72[ab] | 1.34 ± 1.14 [b] | 0.0094 |
| Total apoptosis CAM (%) | 18.59 ± 8.04 [a] | 16.41 ± 7.20 [a] | 19.54 ± 8.28 [a] | 0.4363 |

Different letters indicate significant statistical difference ($p < 0.05$) by the Kruskal-Wallis test with the Dunn post-test.

($r = 0.3543$, $p = 0.0398$, $r^2 = 0.0197$).

## 4. Discussion

Hematological and biochemical examinations of dogs from the control group (Table 1) corresponded to normal values for dogs (Thrall et al., 2004). The changes in laboratory exams observed in obese dogs, such as increased ALP, ALT and triglycerides, were similar to those described previously (Tribuddharatana et al., 2011; Rafaj et al., 2016).

PL levels is considered a non-subjective index of dog adiposity, regardless of age and breed (Ishioka et al., 2007), and has a direct correlation with BCS (Park et al., 2014). PL is considered a pro-inflammatory adipokine (Paz-Filho et al., 2012) and hyperleptinemia has been associated with increased oxidative stress in humans (Blanca et al., 2016). Thus, we quantified some systemic oxidative stress markers in dogs with different BCS. Higher values of TOC, lipid peroxidation and OSi confirmed systemic oxidative stress in obese dogs. The intermediate values of these markers in overweight dogs suggest that systemic oxidative stress is probably progressive and dependent on increases in adiposity over time.

Uric acid accounts for 60% of the TAC of human plasma (Boban and Darko, 2010). The increase in uric acid reported in human (Abdul-Majeed, 2009) and rodent obesity (Tsushima et al., 2013) also occurred in obese dogs. It seems probable that this higher concentration of uric acid contributed to maintaining the TAC within normal range, however it insufficient to prevent systemic oxidative stress in obese dogs.

Lipid peroxidation was estimated by the plasma concentration of thiobarbituric acid reactive species (TBARS), which is a widely used methodology for evaluating systemic oxidative stress (Liu et al., 1997). Increased lipid peroxidation has been described in human (Ozata et al., 2002) and rat obesity (Beltowski et al., 2000). In contrast, lipid peroxidation did not increase in dogs submitted to fattening in a short-term study (13 weeks) (Van de Velde et al., 2012). Higher plasma lipid peroxidation in obese dogs suggests that systemic oxidative stress could contribute in the long term to other systemic changes associated with obesity.

Adiponectin is secreted by adipose tissue and has anti-inflammatory effects (Park et al., 2014). In humans, via regulation of NADPH oxidase, it inhibits neutrophil superoxide generation *in vitro* (Magalang et al., 2006). The decrease in adiponectin reported in obese dogs (Park et al., 2014) was not observed in this study. Compared to adiponectin, leptin has an inverse action, activating the oxidative metabolism of neutrophils (Caldefie-Chezet et al., 2001) and potentially contributing to oxidative stress (Blanca et al., 2016). Analysis of the results obtained determined a positive correlation between the degree of canine obesity, systemic oxidative stress and the increase in PL. To our knowledge, this is probably the first evidence that hyperleptinemia in dogs is positively associated with the degree of obesity and systemic oxidative stress.

There is evidence that hyperleptinemia in humans plays a key role in the pathogenesis of complications associated with obesity, including chronic inflammation (Wisse, 2004). The mechanism by which leptin activates ROS production has yet to be fully elucidated, though it is known that leptin stimulates mitochondrial oxidation of fatty acids (Konukoglu et al., 2006) and increases the expression of the p67phox/p47phox subunits of NADPH oxidase, which induces the generation of superoxide anions (Blanca et al., 2016). However, little is known about the effect of PL on neutrophils. An *in vitro* study observed that neutrophil oxidative metabolism is activated in the presence of PMA at leptin concentrations varying from 50 to 500 ng/mL (Caldefie-Chezet et al., 2001).

Reports on the effect of obesity on neutrophils remain scarce. In

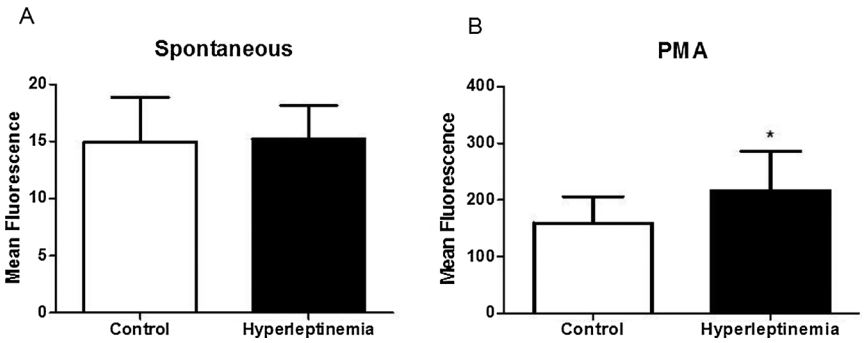


**Fig. 4.** Neutrophil superoxide production quantified by the probe hydroethidine (HE) with and without phorbol myristate acetate (PMA) stimulation of dogs with normal (Control, n = 15) and increased plasma leptin levels (Hyperleptinemia, n = 22). The graphs represent the mean and standard deviation. (*) Indicates statistically significant differences ($p < 0.05$) by the unpaired *t*-test.

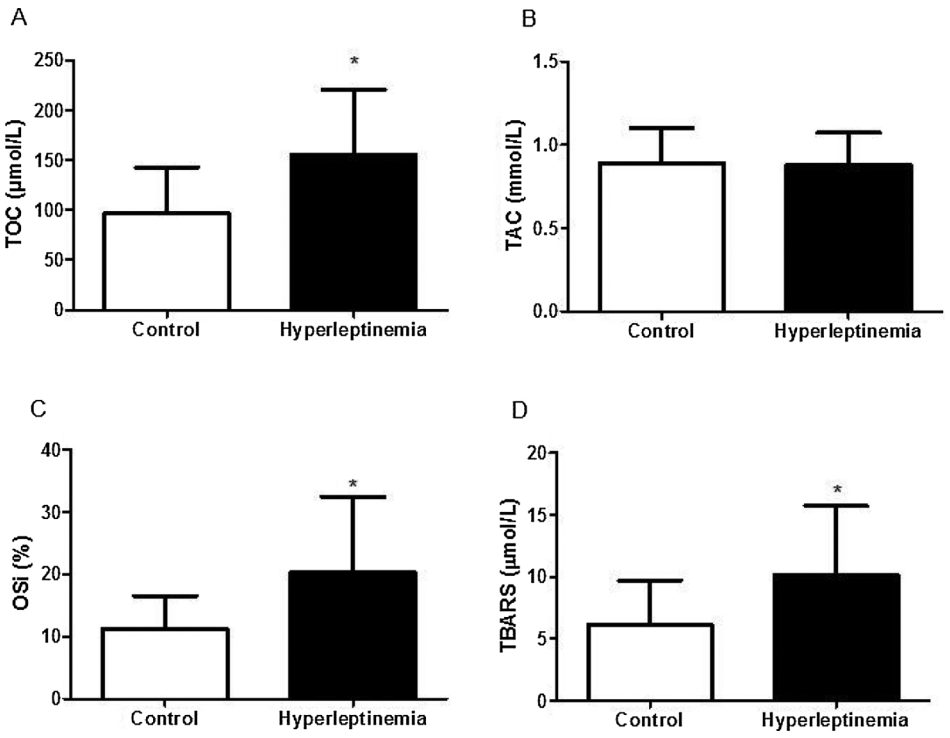


**Fig. 5.** Plasma concentration of oxidative stress markers: total oxidant capacity (TOC, **A**), total antioxidant capacity (TAC, **B**), oxidative stress index (OSi, **C**) and lipid peroxidation determined by thiobarbituric acid reactive substances (TBARS, **D**) of dogs with normal (Control, n = 15) and increased plasma leptin levels (Hyperleptinemia, n = 22). The graphs represent the mean and standard deviation. (*) Indicates statistically significant differences ($p < 0.05$) by the unpaired *t*-test (B and C) or the Mann Whitney test (A and D).

humans, neutrophils increase superoxide production in the absence and presence of oxidative metabolism activators, such as zymosan and fMLP, suggesting that neutrophil activation could contribute to obesity-related diseases (Brotfain et al., 2015). In this study, spontaneous neutrophil production of superoxide and hydrogen peroxide did not differ between the groups; however, a large increase in superoxide in obese dogs occurred following stimulation with PMA, confirming the primed state of this polymorphonuclear cell. It is likely that PL promotes a stimulus capable of preactivating neutrophils of obese dogs. These primed neutrophils produce greater amounts of superoxide over time and this increase can lead to oxidative stress (Farah et al., 2010). Strengthening this hypothesis, a positive correlation was established between PL, BCS and superoxide production of primed neutrophils in obese dogs.

Little is known about the implications of this increase in neutrophil oxidative metabolism in the innate immune response in obesity. In obese humans, an increase in blood M30 has been described, a marker used to quantify epithelial cell apoptosis (Trellakis et al., 2012). We observed lower viability and a higher rate of neutrophil apoptosis in obese dogs. This is probably the first evidence that canine obesity can compromise innate immune response, similar to reports on humans (Milner and Beck, 2016). It is known that oxidative stress is an inducer of cellular apoptosis (Kannan and Jain, 2000; Yamamoto et al., 2002), but the primed state of neutrophils indicates that its bactericidal function is preserved.

We conclude that hyperleptinemia and systemic oxidative stress in canine obesity are associated with each other and with the preactivation of neutrophil oxidative metabolism. The need to better understand these changes and their clinical implications in order to guide new therapeutic and nutritional strategies for controlling systemic oxidative stress in obese dogs is now evident.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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