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Neutrophil dysfunction varies with the stage of canine visceral leishmaniosis

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ABSTRACT

Canine visceral leishmaniosis (CVL) causes a dependent-stage alteration in neutrophil oxidative metabolism. When production of reactive oxygen species (ROS) exceeds the antioxidant capacity of neutrophils, apoptosis is triggered, impairing the viability and function of these cells, which can predispose dogs to infection. However, the uremic condition observed in late-stage CVL can also alter the viability and function of human neutrophils. To more clearly understand this relationship, the apoptosis rate and oxidative metabolism of neutrophils from control dogs ($n=20$) were compared to dogs in moderate ($n=15$) and very severe ($n=15$) stage CVL, classified according to LeishVet Consensus. To assess neutrophil oxidative metabolism, superoxide production was measured using the nitroblue tetrazolium reduction test (NBT) in isolated neutrophils. The apoptosis rate of neutrophils was estimated using the morphological method. Moderate-stage dogs presented increased superoxide production, while dogs with very severe stage CVL presented decreased superoxide production and an increase neutrophil apoptosis rate. Leishmaniosis causes differential neutrophil dysfunction according to disease stage. In moderate stage CVL, increased superoxide production is observed with no change in neutrophil viability. However, in very severe stage CVL, decreased superoxide production and increased apoptosis occur associated with uremia.

1. Introduction

Neutrophils are the main effector cells in mammalian innate immunity, conducting the initial defense against invading microorganisms. Once recruited and activated by chemical mediators, neutrophils exert their microbicidal function through destroying invading pathogens following phagocytosis and the liberation of proteolytic enzymes and reactive oxygen species (ROS) produced from the superoxide anion (Borregaard et al., 2007). In addition, these cells link innate and adaptive immunity and participate in the resolution of inflammation (Nathan, 2006).

When adequately stimulated, neutrophils rapidly respond with ROS production, mainly primed cells (Hostetter, 2012). However, when ROS production exceeds the antioxidant capacity of the cell, cellular structures are oxidized and apoptosis is initiated due to oxidative stress. Once apoptosis is trigged, impairment of cell function occurs that diminishes the phagocytic capacity and oxidative metabolism of neutrophils (Anwar and Whyte, 2007).

Certain microorganisms can modify the oxidative metabolism and apoptosis of neutrophils and these mechanisms have been highlighted as a key component in the

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evasion of the immune system (Anwar and Whyte, 2007). Among these microorganisms, parasites like *Leishmania* spp. have been studied in human models due to their ability in inhibit the oxidative metabolism (Laufs et al., 2002) and apoptosis of parasitized neutrophils (Aga et al., 2002). As the first cells to arrive in infection site, neutrophils serve as "Trojan horses" in the initial phase of infection, sheltering the protozoan until monocytes arrive, so the parasite can enter in the target cell, without activating oxidative metabolism (Sunderkötter et al., 1993; Laskay et al., 2003).

Although the role of neutrophils in the establishment of infection is already known, studies concerning neutrophil function in canine visceral leishmaniosis (CVL) are scarce and contain contradictions. In vitro, *Leishmania* spp. is able to survive in nonlytic compartments of canine neutrophils and can also delay cellular apoptosis (Gueirard et al., 2008). In vivo, Brandonisio et al. (1996) and Vuotto et al. (2000) observed decreased oxidative metabolism, in which neutrophils from infected dogs showed a reduced capacity to respond to stimulus compared with neutrophils from uninfected dogs.

In contrast, Gómez-Ochoa et al. (2010) and Ciarlini et al. (2010) observed increased superoxide production in neutrophils from dogs with CVL using the nitroblue tetrazolium reduction test (NBT). It is believed that the increase in neutrophil oxidative metabolism of infected animals is related to the profile of inflammatory mediators produced by asymptomatic and resistant dogs (Pinelli et al., 1994).

Malafaia and Rezende (2009) highlighted the importance of evaluating neutrophil function in different stages of CVL. Thus, Gómez-Ochoa et al. (2010) suggested that alterations in neutrophil oxidative metabolism are dependent on the stage of disease, with increased superoxide production in early stages and a discrete decrease in the end stages of leishmaniosis, a factor that could predispose dogs with this condition to other infections.

The NBT is used to measure the metabolic activity of mammalian and microbial cells, such as neutrophils (Berridge et al., 2005). The NBT has been widely used to diagnose chronic granulomatous disease in humans. In this condition, neutrophils do not produce superoxide due to a failure in enzyme production, causing recurrent infections (Babior et al., 1973). Currently, NBT is being used in several species to assess not only the oxidative metabolism of neutrophils, but also its diagnostic potential for several infectious diseases, including leishmaniosis (Gómez-Ochoa et al., 2010, 2012; Hasegawa et al., 2005; Strasser et al., 2003).

Considering that the oxidative stress demonstrated in CVL (Bildik et al., 2004; Heidarpour et al., 2012) could be due to excessive production of ROS by neutrophils (Gómez-Ochoa et al., 2010) and this oxidative stress can cause neutrophil apoptosis, evaluation of neutrophil apoptosis is essential, since apoptotic neutrophils present functional impairment and this could predispose infected dogs to coinfections (Anwar and Whyte, 2007). Until now, studies evaluating the rate of neutrophil apoptosis in different stages of CVL have not yet been conducted.

It is believed that the apoptosis observed during oxidative stress is triggered by the activation of caspases (Curtin et al., 2002). Oxidative stress has also been demonstrated in humans with uremia (Johnson-Davis et al., 2011), a common condition in late-stage leishmaniosis, together with higher rates of neutrophil apoptosis in uremic patients (Cohen et al., 2001). The relationships between oxidative stress, superoxide production and neutrophil apoptosis in dogs with CVL require more thorough investigation.

Evaluation of neutrophil apoptosis can be performed following the incubation of cells using light microscopy, in which specific morphological changes in the process can be observed, such as vacuolated cytoplasm and densely condensed nuclei due to nuclear chromatin condensation and pyknosis, following the formation of apoptotic bodies. These alterations are not observed in mature and circulating neutrophils, making this a reliable method of assessing apoptosis (Savill et al., 1989).

In CVL, polysymptomatic late-stage dogs are frequently coinfected by other pathogens (Feitosa et al., 2000; Andreotti et al., 2006) and usually present a uremic condition due to chronic kidney disease (CKD), in contrast to early-stage dogs (Costa et al., 2003; Zatelli et al., 2003). Recent evidences demonstrated that uremic toxins compromise neutrophil function in dogs (Barbosa et al., 2010), a condition similar to that observed in dogs with late-stage CVL. Other authors have also reported neutrophil dysfunction in humans (Cendoroglo et al., 1999) and cats (Keegan and Webb, 2010) presenting a uremic condition, which could similarly compromise their innate immunity. To more clearly understand whether the uremic condition observed in the late stages of CVL compromise neutrophil function, the present study aimed to compare the apoptosis rate and oxidative metabolism of neutrophils from dogs with leishmaniosis presenting moderate and very severe stages of the disease.

2. Material and methods

The study was performed in accordance with the ethical principles concerning the use of experimental animals outlined by the Ethics Committee on Animal Experimentation of São Paulo State University (UNESP), under protocol no. FOA-9678/10.

According to clinical examinations, 50 adult mixed breed dogs (2–8 years) were selected and divided into three groups: Control, comprising 20 healthy dogs, 10 females and 10 males age from 2 to 4 years-old; Leish II ($n = 15$), comprising 8 males and 7 females, all with moderate stage leishmaniosis, presenting mainly onychogryphosis, lymphadenopathy, skin lesions and anemia with no alteration in renal function; Leish IV ($n = 15$), comprising 9 males and 6 females with very severe disease, presenting typical signs that included lymphadenopathy, skin lesions, evidence of bleeding, non-regenerative anemia, hyperglobulinemia, hypoalbuminemia and uremia due to CKD (stages III and IV according to IRIS guidelines; IRIS, 2006).

The dogs were classified as presenting moderate or very severe leishmaniosis according to the guidelines of the LeishVet Consensus (Solano-Gallego et al., 2009). The amastigote form of *Leishmania* spp. was detected in all dogs in the direct parasitological examination from popliteal lymph node aspiration and all animals were also positive in serology by the ELISA test (Lima et al., 2003). Healthy

dogs were negative in both parasitological examination of popliteal lymph node specimens and serological ELISA test (Lima et al., 2003) and showed no alteration in physical and laboratorial examinations (blood count, urinalysis and plasma biochemical profile). Dogs with other infections or recently treated with drugs capable of altering neutrophil function were not selected for the study.

Prior to blood collection the dogs were fasted for 8 to 12 h before 10 mL of blood were extracted via jugular venipuncture, 9 mL were collected in heparinized tubes for neutrophil isolation and to obtain plasma for biochemical analyzes. Another 1 mL of EDTA-treated blood was used for complete blood count.

Biochemical analyses were performed in an automated spectrophotometer using commercial reagents (BTS 370 Plus, Biosystems, Barcelona, Spain) that was previously calibrated with commercial calibrator. All reactions were monitored with control serum level I and II (Biosystems, Barcelona, Spain). Biochemical concentrations were determined as follows: plasma urea concentration using urease/glutamate dehydrogenase coupled with the UV enzymatic assay; plasma and urinary creatinine using the alkaline picrate kinetic assay; albumin using the bromocresol green assay; total protein using the biuret assay; cholesterol using the oxidase/peroxidase enzymatic assay; and urinary protein using the pyrogallol red assay. All biochemical tests were performed at 37 °C, in accordance with the manufacturer's recommendations.

Blood cell counts were performed in a veterinary automated cell count (BC-2800Vet, Shenzhen Mindray Bio-Medical Eletronics Co., Nanshan, China). Urine was collected by cystocentesis for urine examination and determination of the protein/creatinine ratio (UPC) in urinary supernatant. Urinary density was obtained by refractometry, chemical examination was performed using commercial reagent strips (Combur^{10} test®, Roche, Mannheim, Germany) and analysis of urinary sediment followed the criteria proposed by Osborne et al. (1995).

Neutrophils were isolated by the Histopaque-1077/1119 (Sigma–Aldrich Co., St. Louis, USA) double density gradient technique (Silva et al., 2013). To achieve this, 4 mL of heparinized blood were transferred to sterile polypropylene conical tubes containing a double gradient separation composed of equal volumes (3 mL) of Histopaque-1119® and 1077®. Following centrifugation at 340 *g* for 30 min, the layer of polymorphonuclear (PMN) cells was aspirated and washed twice with 0.14 M aqueous ammonium chloride for complete lysis of residual erythrocytes. Next, the sample was centrifuged at 100 × *g* for 5 min in Hanks' balanced salt solution (HBSS) (Sigma–Aldrich Co., St. Louis, USA) without Ca^{2+} and Mg^{2+} and 1 mL of RPMI 1640 (Sigma–Aldrich Co., St. Louis, USA) was added to the cell sediment. Cell concentration was determined in a hemocytometer and cell viability was estimated by the blue trypan dye exclusion method. The sample of isolated PMN was diluted in RPMI 1640 to obtain a final cell concentration of 10^6/mL, with the purity and viability of neutrophils equal to or more than 93 and 95%, respectively.

To evaluate neutrophil oxidative metabolism, superoxide production was estimated by the NBT according to the method of Poli et al. (1973) and modified by Ciarlini et al. (2010). Briefly, 100 μL of cell suspension (10^6/mL) were incubated with 50 μL of 0.2% saline NBT solution (Sigma–Aldrich Co., St. Louis, USA) in the presence or absence of 1 μL of a 16.2 μM solution of phorbol 12-myristate 13-acetate (PMA) to perform the stimulated assay. Following incubation at 37 °C and room temperature, both for 10 min, samples were cytocentrifuged and stained with hematological dye. The percentage of NBT-reducing neutrophils was established after counting at least 100 neutrophils. Positive cells were considered those that presented a blackish-blue precipitate typical of formazan formation.

To determine the neutrophil apoptosis rate, morphometric analysis of apoptotic neutrophils followed the criteria proposed by Savill et al. (1989) with modifications. To achieve this, 100 μL of cell suspension (10^6/mL) were incubated with 100 μL of RPMI 1640 (spontaneous assay) or 100 μL of a 310.8 μM solution of the inductor camptothecin (Sigma–Aldrich Co., St. Louis, USA) to perform the induction assay. After 4 h at 37 °C in a computerized thermal shaker (Thermomixer, Eppendorf, Mod. Comfort, Hamburg, Germany), samples were cytocentrifuged and stained with hematological dye. To quantify apoptotic cells, only cells that showed at least three of the following unique morphological characteristics of the process were considered: vacuolization of cytoplasm, nuclear condensation, nuclear fragmentation, cellular fragmentation and formation of apoptotic bodies. The apoptotic index was estimated as the percentage of apoptotic cells by counting at least 100 neutrophils.

After testing variables for normality and homoscedasticity, the Kruskal–Wallis test followed by Dunn's post hoc test (spontaneous and induced apoptosis, stimulated NBT) and ANOVA followed by the Tukey post hoc test (spontaneous NBT) were used to investigate differences between the groups. The tests were performed using statistical software (SAS Institute Inc. The SAS System, Release 9.2., Cary NC, 2008) and the results were considered statistically significant when $p < 0.05$.

3. Results

The main clinical signs and laboratory results of controls and dogs with visceral leishmaniosis are presented in Table 1.

The clinical and laboratory alterations of dogs with CVL are similar to those described by Baneth et al. (2008). Generally, dogs with moderate stage CVL presented lymphadenopathy, onychogryphosis and cachexia, while dogs with very severe stage CVL presented uremic halitosis and ulcers on the tongue and oral mucosa, clinical signs typical of uremic syndrome due to CKD. The laboratory findings most frequently observed were normocytic and normochromic anemia, hyperproteinemia with hyperglobulinemia and hypoalbuminemia. Very severe stage dogs mainly differed from moderate stage dogs regarding the signs of CKD, such as lower urinary density, persistent non-regenerative anemia and plasma creatinine levels compatible with stages III and IV of IRIS guidelines (IRIS, 2006).

Table 1
Frequency of clinical signs and laboratory results (mean ± standard deviation) of controls and dogs with leishmaniosis presenting moderate (Leish II) and very severe (Leish IV) disease stages.

Parameter	Control	Leish II	Leish IV
Clinical signs			
Lymphadenopathy	Absent	80%	53.3%
Onychogryphosis	Absent	73.3%	93.3%
Skin lesions	Absent	33.3%	40%
Periocular alopecia	Absent	53.3%	20%
Cachexia	Absent	40%	93.3%
Uremic halitosis	Absent	Absent	93.3%
Oral and tongue ulcerations	Absent	Absent	86.6%
Dehydration	Absent	20%	93.3%
Laboratory tests			
Blood count			
PCV (%)	47.7 ± 3.3	27.9 ± 7.5	21.5 ± 10.6
RBC (10^{12}/L)	6.5 ± 0.5	3.87 ± 1.35	3.02 ± 1.54
Hemoglobin (g/dL)	15.9 ± 1.0	9.3 ± 3.2	6.9 ± 3.2
MCV (fL)	73.2 ± 2.9	72.3 ± 3.7	71.6 ± 3.6
MCHC (%)	33.3 ± 0.2	33.5 ± 0.7	32.1 ± 2.41
WBC (10^9/L)	9.9 ± 2.6	13.0 ± 7.1	11.0 ± 6.23
Biochemical			
Urea (mg/dL)	32.6 ± 8.1	29.0 ± 10.8	263.0 ± 114.6
Creatinine (mg/dL)	1.07 ± 0.16	0.89 ± 0.25	4.43 ± 2.1
Total protein (g/L)	65.8 ± 6.3	79.9 ± 19.0	80.9 ± 17.1
Albumin (g/L)	31.0 ± 1.9	20.8 ± 7.2	22.9 ± 4.1
Globulin (g/L)	34.8 ± 6.0	59.1 ± 18.9	58.0 ± 18.0
Cholesterol (mg/dL)	178.3 ± 51.4	200.8 ± 46.6	256.7 ± 69.3
Urinalysis			
Urinary density	1.043 ± 0.003	1.040 ± 0.009	1.016 ± 0.003
UPC	0.2 ± 0.07	0.8 ± 0.6	3.6 ± 1.5

PCV, packed cell volume; RBC, red blood cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells; UPC, urinary protein/creatinine ratio.

Leishmaniosis altered both the oxidative metabolism and apoptosis of neutrophils from positive dogs depending on the stage of disease (Figs. 1 and 2). Spontaneously, neutrophils of dogs with moderate stage CVL (54.77 ± 15.9%) showed higher superoxide production, while those with very severe stage CVL (31.6 ± 12.2%) presented a decrease in superoxide production compared with control dogs (44.4 ± 13.6%; $p < 0.0001$) (Fig. 1). When stimulated with PMA, neutrophil superoxide production increased in both moderate (82.6 ± 21.4%) and very severe stage dogs (78.6 ± 30.5%), compared with control dogs (49.9 ± 24.5%; $p = 0.0027$) (Fig. 1).

The neutrophil apoptosis rate of dogs with very severe stage CVL (23.5 ± 7.3%; 46.6 ± 18.8%) was higher than that observed for neutrophils of control (15.6 ± 5.4%; 32.2 ± 6.4%) and moderate stage dogs (18.4 ± 11.3%; 37.2 ± 17%) in both spontaneous ($p = 0.0186$) and stimulated ($p = 0.0287$) trials (Fig. 2).

4. Discussion

In the present study was possible to establish that the alterations in oxidative metabolism and apoptosis of neutrophils were dependent on the stage of disease. Studies assessing the oxidative metabolism of neutrophils in dogs with leishmaniosis report different and conflicting results. Some authors have reported inhibition of neutrophil oxidative metabolism (Brandonisio et al., 1996; Vuotto et al., 2000), while others have described increased production of superoxide (Ciarlini et al., 2010; Gómez-Ochoa et al., 2010). In the present study, increased superoxide production in both spontaneous and stimulated trials was observed, similar to other studies that used NBT (Ciarlini et al., 2010; Gómez-Ochoa et al., 2010). However, other studies have found inhibition of oxidative metabolism using chemiluminescence (Brandonisio et al., 1996; Vuotto et al., 2000). In these studies, the stimulated production of superoxide in neutrophils of dogs with CVL was lower than for uninfected dogs, in disagreement with the results of the present study.

Different results in the assessment of neutrophil oxidative metabolism have been observed in humans and are probably due to the use of different methodologies (Cendoroglo et al., 1999). In addition to alternate methodologies, Vuotto et al. (2000) and Brandonisio et al. (1996) did not consider disease stage in their studies, which could also explain the difference between their results and those obtained in this study, in which inhibition of oxidative metabolism occurred in end-stage disease (Fig. 1). The decrease in oxidative metabolism observed in very severe stage CVL is likely due to the increased apoptosis also observed in this stage of the disease (Fig. 2).

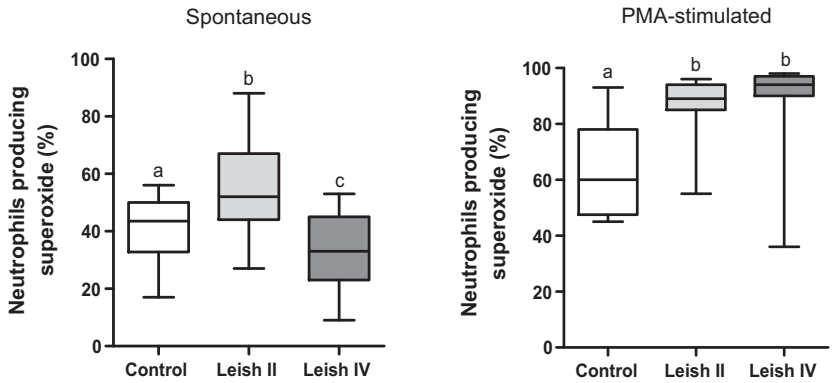

Fig. 1. Boxplot of neutrophils producing superoxide (%) in controls and dogs with leishmaniosis presenting moderate (Leish II) and very severe (Leish IV) disease stages spontaneously and in the presence of PMA-stimulus. Non-coincident letters indicate statistically significant differences ($p < 0.05$).

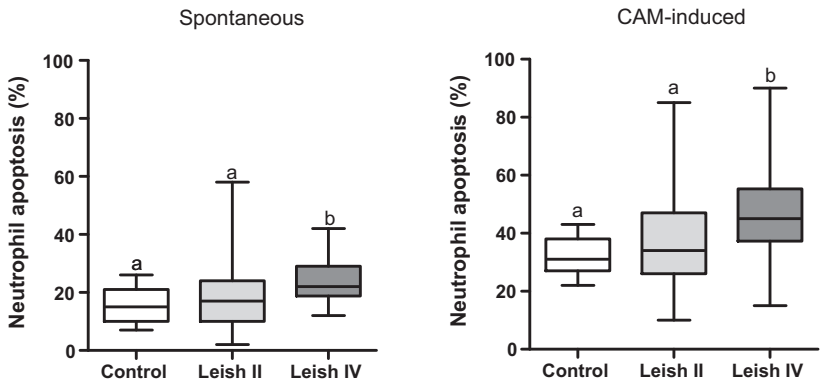

Fig. 2. Boxplot of neutrophil apoptosis (%) in control and dogs with leishmaniosis presenting moderate (Leish II) and very severe (Leish IV) disease stages spontaneously and in the presence of CAM-induction. Non-coincident letters indicate statistically significant differences ($p < 0.05$).

Similar to this study, Gómez-Ochoa et al. (2010) also verified decreased oxidative metabolism in neutrophils in end-stage CVL; however, the difference determined by their group was mild and nonsignificant. According to Gómez-Ochoa et al. (2010), the increase in oxidative metabolism during the initial stages of CVL could be related to greater production of chemotactic agents, such as cytokines, in asymptomatic dogs, which activate superoxide production in these animals, ensuring resistance to infection. However, in this study was observed that even symptomatic dogs with moderate stage disease showed an increase in oxidative metabolism. Thus, the increased production of cytokines associated with resistance to infection does not fully justify the increased production of superoxide.

Further investigation to evaluate the significance of activated neutrophils in the early stages of CVL is required. The increase in neutrophil oxidative metabolism observed in moderate stage CVL could strengthen the body's first line of defense, which would prevent the occurrence of coinfections. However, when neutrophil activation is prolonged, it can generate oxidative stress and induce neutrophil apoptosis, affecting the function of neutrophils and favoring coinfections that lead to worsening clinical signs of CVL (Kennedy and DeLeo, 2009).

Until now, there are no studies that have evaluated the apoptosis of neutrophils in dogs in different stages of CVL. In this study was possible to observe that the apoptosis rate of neutrophils increased in very severe stage CVL (Fig. 2), which occurs following the activation of oxidative metabolism in earlier stages of the disease (Fig. 1). It is reasonable to assume that the increased production of ROS resulting from the activation of neutrophil oxidative metabolism in early stage CVL contributed to the increased apoptosis rate observed in the very severe stage of the disease, as previously demonstrated (Kannan and Jain, 2000). In parallel, Bildik et al. (2004) showed that dogs with CVL exhibit oxidative stress with increased plasma lipid peroxidation. According to Anwar and Whyte (2007), when the production of ROS exceeds the antioxidant capacity of the cell, the process of apoptosis is triggered, and once initiated, the cell becomes less functional. In support of this hypothesis, treatments based on antioxidants have generated good results in human patients with leishmaniosis (Sen et al., 2000; Paula-Junior et al., 2006).

It is important to consider that increased apoptosis and decreased superoxide production of neutrophils from dogs presenting very severe stage CVL occurs concurrently with laboratory findings of uremia due to CKD (Table 1). Recently, studies have confirmed increased apoptosis (Silva et al., 2013) and inhibition of oxidative metabolism (Barbosa et al., 2010) in neutrophils from dogs with uremia. Considering only the uremic condition, without concomitant leishmaniosis, studies have demonstrated decreased oxidative metabolism in cats (Keegan and Webb, 2010) and humans (Cohen et al., 2011; Hirayama et al., 2001; Sardenberg et al., 2006). In uremia, the uremic toxins can induce spontaneous apoptosis in human neutrophils (Cohen et al., 2001; Jaber et al., 2001; Majewska et al., 2003). These findings strengthen the hypothesis that uremia present in very severe stage CVL contributed to the inhibition of oxidative metabolism and the increase in neutrophil apoptosis.

Cendoroglo et al. (1999) explained that the neutrophil dysfunction observed in uremia is due to increased oxidative metabolism observed in neutrophils in the early stages of CKD, in which the oxidizing substances exceed the antioxidant capacity of the cell. This would increase oxidative stress of the cell, injuring vital cellular structures and triggering the process of apoptosis, thus compromising the oxidative metabolism in advanced stages and predisposing patients with end-stage CKD to infections. This explanation would also serve to explain, at least in part, the increased rate of apoptosis observed in neutrophils of dogs with very severe stage CVL. During the moderate stage of the infection, activation of neutrophil oxidative metabolism occurs, leading to the depletion of antioxidant defenses and triggering neutrophil apoptosis, as observed by the higher rate of apoptosis and decreased superoxide production in very severe stage CVL.

It is reasonable to assume that the neutrophil dysfunction observed in this study is due to the sum of oxidative stress caused by infection and the uremic condition that characterizes very severe stage CVL. Regardless of the mechanisms involved, this inhibition of oxidative metabolism and increased apoptosis of neutrophils seems

to damage the first line of defense of the organism, thus favoring the occurrence of coinfections in later stages of CVL.

5. Conclusion

The function of neutrophils is altered according to the stage of canine visceral leishmaniosis. In moderate stage CVL, increased superoxide production occurs without any change in neutrophil viability. While in very severe stage CVL, decreased superoxide production and increased apoptosis occur in association with uremia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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