Veterinary Parasitology 196 (2013) 6–12

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Neutrophil dysfunction varies with the stage of canine visceral leishmaniosis

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ARTICLE INFO

Article history:
Received 26 September 2012
Received in revised form 5 February 2013
Accepted 17 February 2013

Keywords:
Polymorphonuclear
Leishmania spp.
Oxidative metabolism
Apoptosis

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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http://dx.doi.org/10.1016/j.vetpar.2013.02.016

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