Research in Veterinary Science 100 (2015) 12–17

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Serum biomarkers of oxidative stress in cats with feline infectious peritonitis

F. Tecles [a], M. Caldín [b], A. Tvarijonaviciute [a], D. Escribano [a], S. Martínez-Subiela [a], J.J. Cerón [a,*]

[a] Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Veterinary School, Campus of Excellence Mare Nostrum, University of Murcia, 30100 Espinardo, Murcia, Spain
[b] San Marco Veterinary Hospital, 35141 Padova, Italy

ARTICLE INFO

Article history:
Received 14 July 2014
Accepted 13 February 2015

Keywords:
Cat
Feline infectious peritonitis
Inflammation
Oxidative stress
Paraoxonase
Total antioxidant capacity

ABSTRACT

The purpose of this study was to elucidate the possible presence of oxidative stress in cats naturally affected by feline infectious peritonitis (FIP) by investigating two antioxidant biomarkers in serum: paraoxonase-1 (PON1) and total antioxidant capacity (TAC). PON1 was measured by spectrophotometric assays using three different substrates: *p*-nitrophenyl acetate (pNA), phenyl acetate (PA) and 5-thiobutil butyrolactone (TBBL), in order to evaluate possible differences between them. The PA and TBBL assays for PON1 and the assay for TAC were validated, providing acceptable precision and linearity although PA and TAC assays showed limit of detection higher than the values found in some cats with FIP. Cats with FIP and other inflammatory conditions showed lower PON1 values compared with a group of healthy cats with the three assays used, and cats with FIP showed significant decreased TAC concentrations. This study demonstrated the existence of oxidative stress in cats with FIP.

1. Introduction

Oxidative stress can be defined as an imbalance between the oxidant and antioxidant system, with an advantage toward the oxidant system (Suresh et al., 2009). In this situation the organism is unable to detoxify reactive oxygen species, which accumulate, producing a harmful effect on the functional and structural integrity of biological tissues (Yilmaz, 2012). Cats seem to be more susceptible to oxidative stress and damage, probably influenced by the particular spleen structure of this species (Christopher et al., 1995; Harvey and Kaneko, 1977), and a situation of oxidative stress has been demonstrated in various diseases in this species such as diabetes mellitus (Webb and Falkowski, 2009), chronic renal failure (Keegan and Webb, 2010) and feline immunodeficiency virus (FIV) infection (Webb et al., 2008). However, to the author's knowledge there are no studies about oxidative stress in feline infectious peritonitis (FIP), a viral disease resulting from feline coronavirus (FCoV) infection.

Inflammation plays a major role in FIP infection, since a major inflammatory response is presented during the course of FIP which is involved in the pathogenesis, producing fibrinous serositis, with accumulations of highly proteinaceous fluid within body cavities, disseminated pyogranulomatous formation, hypergammaglobulinemia, and the development of immune complexes (Gunn-Moore et al., 1998; Pedersen, 2014a). Increases in acute phase proteins (APPs), which are markers of inflammation, such as alpha-1 glycoprotein or serum amyloid A can be used as a diagnostic aid in this disease. Knowing the relation between inflammation and oxidative stress (Montorfano et al., 2014), it could be postulated that oxidative stress could be present in cats affected by FIP.

Several enzymes and non-enzymatic molecules are included within the antioxidant system (Delmas-Beauvieux et al., 1996). Paraoxonase 1 (PON1) is a serum enzyme that has a protective role against oxidation (James, 2007). In humans, a reduction of PON1 activity has been reported in several pathologic conditions, including bacterial and viral infections (Farid and Horii, 2012). In addition, PON1 is associated with inflammation, being considered as a negative acute phase protein in several species. Feingold et al. (1998) reported a reduced hepatic synthesis of this enzyme in hamsters during the acute phase response. Decreases have been also described in cattle (Bionaz et al., 2007), laboratory animals (Franco-Pons et al., 2008), humans (Novak et al., 2010), horses (Turk et al., 2011) and dogs (Tvarijonaviciute et al., 2012a, 2012b). Owing to the wide spectrum of species on which PON1 is reduced in inflammation, to study this molecule also in cats may be important.

Lactones are considered as the natural substrates of PON1 (Billecke et al., 2000), and other artificial substrates can also be used for measurement of this enzyme as paraoxon, phenyl acetate (PA)

* Corresponding author. Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Veterinary School, Campus of Excellence Mare Nostrum, University of Murcia, 30100 Espinardo, Murcia, Spain. Tel.: +34 868 88 4722; fax: +34 868 88 4147.
E-mail address: jjceron@um.es (J.J. Cerón).

http://dx.doi.org/10.1016/j.rvsc.2015.02.007
0034-5288/

or *p*-nitrophenyl acetate (pNA; Ceron et al., 2014). However, use of paraoxon as substrate would not be optimal due to its toxicity (Camps et al., 2009) and the possibility of analyzer contamination (Mogarekar and Chawhan, 2013). Divergences in the diagnostic performance between different substrates have been described in certain diseases (Dantoine et al., 1998; Keskin et al., 2009). Therefore, comparative studies in which various substrates are used for PON1 measurements would be recommended when this enzyme is evaluated in a new disease.

Serum total antioxidant capacity (TAC) considers the cumulative effect of all antioxidants present in the blood (Nagy et al., 2006) and provides an integrated index of the oxidative status (Ghiselli et al., 2000). The most widely used colorimetric methods are based on oxidation of a colorless molecule, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), to a blue–green $ABTS^{+}$ (Erel, 2004). Decreased TAC has been reported in human patients with HIV infection and was negatively correlated with lipid peroxidation, suggesting the presence of oxidative stress in these individuals (Suresh et al., 2009).

We hypothesize that oxidative stress could be present in the cats affected by FIP, probably influenced by the major inflammatory reaction which is associated with this disease, and therefore changes in serum oxidative biomarkers may be present. To test this hypothesis, serum PON1 activity and TAC in a group of cats naturally affected by FIP were measured and compared with a group of healthy cats and cats with other inflammatory diseases. PON1 activity was measured using three different substrates: pNA, PA and 5-thiobutil butyrolactone (TBBL) to evaluate the possible differences in the values of PON1 in FIP depending on the substrate. Assays for measurement of PON1 using PA and TBBL, and an assay for TAC determination, were validated for use in cats.

2. Materials and methods

2.1. Animals

In this retrospective study, the serum samples from cats were selected from the authors' veterinary hospitals database (2011–2012). The serum samples corresponded to diagnoses made based on physical examination, hematological and biochemistry evaluations, urinalyses, radiography and ultrasonography, cytology, serology and immunohistochemistry, depending on each clinical case. Based on the results of the different diagnostic approaches, the serum samples were selected and separated into 3 groups.

Serum of 18 clinically healthy cats presented for routine health screening examinations was the control group. These cats had no history of illness and no clinical signs on physical examination; all were serologically negative for FCoV infection using indirect fluorescence antibody (VMRD), FIV and feline leukaemia virus (FeLV) infections by using commercially available ELISA tests (ViraCHEK, Synbiotics). Serology was considered positive when titers were >1/80 in case of FeLV and FIV tests and when titers were >1/1600 in case of FCoV tests. These cats did not show any clinical sign compatible with FIP 1 year after sample collection.

The second group consisted of serum from 19 cats naturally infected with FCoV that presented clinical signs consistent with FIP. All these cats had negative serology to FIV and FeLV. Hematological changes included low packed cell volume in 7/19 cats, neutrophilia in 8/19 cats and lymphopenia in 12/19 cats. FIP diagnosis was confirmed in all 19 cats by necropsy, histology and immunohistochemistry (Licitra et al., 2013). Fourteen cats had effusive FIP and 5 cats had the non-effusive or dry form.

The third group consisted of serum from cats with other inflammatory diseases. This group was comprised of 13 cats that were serologically negative for FCoV, FeLV and FIV infections, but had high serum amyloid A (SAA) concentrations (median 42.9 μg/ml; interquartile range 25.8–53.7 μg/ml) consistent with inflammatory disease. Diagnoses in this group included feline lower urinary tract disease, bone fracture, cholangiohepatitis ($n = 2$ each), chronic renal insufficiency, aseptic pneumonia, gastroenteritis, cranial trauma, dehiscence of a surgical stitches, chronic interstitial nephritis and pyometra ($n = 1$ each). One cat with cholangiohepatitis, the cat with chronic renal insufficiency and the cat with chronic interstitial nephritis died in a period of less than 1 year after collecting the samples and post mortem examination rule out the presence of FIP. The cats that survived did not presented signs compatible with FIP 1 year after collecting the samples.

The serum samples were kept at –80 °C until analyses for PON1, TAC and SAA. This study was approved by the Ethics Committee of the University of Murcia (Spain).

2.2. PON1 analyses

2.2.1. Serum PON1 activity measured with pNA

This activity was measured following a previously described method (Tvarijonaviciute et al., 2012b). Three hundred microliters of the working reagent consisted of 50 mM Tris (Tris [hydroxymethyl] aminomethane, Sigma-Aldrich), pH 8.0, with 1.0 mM $CaCl_2$ (calcium chloride dihydrate, Sigma-Aldrich) was added together with 2 μl of the serum sample. After an incubation period of 325 s at 37 °C, 72 μl of the start reagent consisting of 2.5 mM pNA (Sigma-Aldrich) in water was added. The rate of formation of *p*-nitrophenol was determined at 405 nm after 250 s in an automated chemistry analyzer (Olympus 2700). The nonenzymatic hydrolysis of pNA, which was based on the hydrolysis rate in the absence of serum, was subtracted from the total hydrolysis rate. The activity, expressed in U/ml, was based on the molar absorptivity (14,000/M/cm) of *p*-nitrophenol at 405 nm. This method has been previously validated in cats (Tvarijonaviciute et al., 2012c).

2.2.2. Serum PON1 activity measured with PA

In this method, PON1 activity was analyzed by measuring the hydrolysis of PA into phenol as described elsewhere (van Himbergen et al., 2005). The assay was performed in a 96-well microplate. The sample buffer consisted of 50 mM Tris and 1 mM $CaCl_2$ (pH 8.0). The serum sample was diluted in sample buffer to 1:40 ratio, and 5 μl of the diluted sample was added to the wells. Then, 200 μl of the freshly made substrate reagent containing 1 mM PA (Sigma-Aldrich) in sample buffer was added. The reaction was monitored for 5 min at 260 nm and 37 °C in a microplate reader (PowerWave XS, Bio-Tek Instruments). The nonenzymatic hydrolysis of PA, which was based on the hydrolysis rate in the absence of serum, was subtracted from the total hydrolysis rate. PON1 activity was expressed as U/ml of serum. The molar extinction coefficient used to calculate the rate of hydrolysis was 1310/M/cm.

2.2.3. Serum PON1 activity measured with 5-thiobutil butyrolactone (TBBL)

The method involves the use of a chromogenic lactone that structurally resembles the proposed natural lipolactone substrates (Marsillach et al., 2009). The assay was performed in a 96-well microplate. The sample buffer consisted of 50 mM Tris and 1 mM $CaCl_2$ (pH 8.0). The method comprised of four pippeting steps: 1 μl of a chromophore solution containing 100 mM 5,5′-dithio-bis-2-nitrobenzoic acid (Sigma-Aldrich Co) in dimethyl sulfoxide (Sigma-Aldrich Co) was added to the wells, then followed by 45 μl of 4% acetonitrile (Mulrisolvent HPLC grade ACS, Sharlau Chemie SA, Sentmenat, Spain) solution in sample buffer. In the third step, 55 μl of diluted serum sample at 1:200 ratio in sample buffer was added. Finally, 100 μl of the freshly made substrate containing 0.4 mM TBBL (provided by Dr. Khersonsky, Weizmann Institute of Science, Israel) in sample buffer was added. Two minutes after TBBL addition, the