

Serum endothelin-1 measurement in healthy Maine Coon cats

Mensuração de endotelina-1 sérica em gatos saudios da raça Maine Coon

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Abstract

The plasma concentration of endothelin (ET)-1 increases in human patients with congestive heart failure and is correlated with the degree of hemodynamic and functional changes. An increase in plasma ET-1 concentration is also correlated with cell proliferation, vasoconstriction, hypertrophy of cardiomyocytes and activation of cardiac fibroblasts, all of which are associated with clinical manifestations of heart failure (HF) and pathological remodeling of the heart. Hypertrophic cardiomyopathy (HCM) is the most commonly diagnosed heart disease in cats and is characterized by increased cardiac mass associated with concentric left ventricular hypertrophy (LVH), which is associated with mutations in the cardiac myosin binding protein C (MYBPC3) gene in Maine Coon (MC) cats. ET-1 serum concentrations were measured in 38 healthy MC cats using the human ELISA assay validated for the feline species. Unfortunately, the ET-1 values could not be assessed in the present study because they were above the maximum levels of sensitivity of the test used. ET-1 serum measurements without aprotinin in healthy MC cats is greater than values ET-1 plasma concentration with aprotinin reported by literature in healthy cats using the Biomedica Endothelin (1-21) diagnosis kit. Using this kit for serum ET-1 measurements without aprotinin in MC cats the dilution of samples is necessary.

Key words: Feline, cardiac markers, biomarkers, ET-1, congestive heart failure

Resumo

A concentração plasmática de endotelina (ET)-1 aumenta em pacientes humanos com insuficiência cardíaca congestiva, sendo correlacionada com o grau de alteração hemodinâmica e funcional e, ainda, com proliferação celular, vasoconstrição, hipertrofia de miócitos cardíacos e ativação de fibroblastos cardíacos, que se associam às manifestações clínicas da insuficiência cardíaca (IC) e ao remodelamento patológico do coração. A cardiomiopatia hipertrófica (CMH) é a doença cardíaca mais diagnosticada em gatos e caracterizada por aumento da massa cardíaca associado à hipertrofia concêntrica do ventrículo esquerdo (HVE) que, em gatos da raça Maine Coon (MC), está associada à mutação do gene da β -miosina ligada à proteína C (MYBPC3). Foram mensuradas concentrações séricas de ET-1 em 38 gatos saudios da raça MC utilizando ensaio ELISA humano, validado para a espécie felina. Infelizmente, não foi possível determinar os valores de ET-1 no presente estudo, pois estes se encontravam acima dos níveis de sensibilidade do ensaio utilizado. A mensuração sérica de ET-1 sem adição de aprotinina em gatos saudáveis da raça MC são superiores aos valores de concentração plasmática de ET-1 com adição de aprotinina em gatos saudáveis como reportado pela literatura com o kit diagnóstico Biomedica

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Endothelin (1-21). Ao utilizar esse kit diagnóstico para mensuração sérica de ET-1 sem aprotinina em gatos da raça MC é necessária a diluição das amostras.

Palavras-chave: Felino, marcadores cardíacos, biomarcadores, ET-1 e insuficiência cardíaca congestiva

Introduction

Endothelin is a peptide consisting of 21 amino acids, which was identified, isolated and recognized as the most potent vasoconstrictor (YANAGISAWA et al., 1988). The endothelin family consists of three peptides: ET type 1 (ET-1), ET type 2 (ET-2) and ET type 3 (ET-3). ET-1 causes prolonged constriction of medium and large-sized arteries, and the other isoforms also cause vasoconstriction albeit with less potency. ET-1 is synthesized as a pre-prohormone and is cleaved into a 30-amino acid peptide termed big ET-1. Upon release into the bloodstream, big ET-1 is converted by endothelin converting enzyme into the 21-amino acid active peptide called “mature” ET-1 or simply ET-1 (LEVIN, 1995).

ET-1 is the predominant form of endothelin produced by endothelial cells and cardiomyocytes and has a broad spectrum of action given the presence of receptors of different subtypes including ET receptor subtype A (RET-A), ET receptor subtype B (RET-B) and ET receptor subtype C (RET-C). Vasoconstriction of smooth muscles, increased myocardial contractility and aldosterone secretion are the main effects mediated by RET-A. Thus, chronic stimulation of RET-A and a persistent increase in ET-1 levels can cause proliferation and vascular smooth muscle hypertrophy of cardiomyocytes, as ET-1 acts as a mitogen responsible for the pathological remodeling of vessels and the heart in response to chronic hypertension and heart failure (HF) (LEVIN, 1995).

ET-1 plasma concentration increases in human patients with chronic heart failure (CHF) and is correlated with the degree of hemodynamic and functional changes (MCMURRAY et al., 1992). Some studies have suggested that endothelin may be a better prognostic indicator than natriuretic peptides (SELVAIS et al., 2000), which are also

found at elevated levels in patients with CHF (MCMURRAY et al., 1992). One study comparing the amino acid sequence of ET-1 in dogs and cats concluded that ET-1 is identical in dogs, humans, mice and rats; however, there is a single amino acid change at position seven in ET-1 of cats (BIONDO et al., 2003). The application of a human ELISA assay in cats, which uses an amino acid sequence that is identical in both species and shows differences in ET-1 plasma values between healthy cats, cats suffering from hypertrophic cardiomyopathy (HCM) and cats suffering from hypertrophic cardiomyopathy (HCM) and CHF, was validated in 2004 (PROSEK et al., 2004).

HCM is a disease characterized by left ventricular hypertrophy albeit without dilation of the ventricular chamber in the absence of another cardiac or systemic disease, which, by itself, causes obvious hypertrophy (FOX; LIU; MARON, 2005). Maine Coon cats may be affected by familial HCM (KITTLESON et al., 1999), whose transmission is related to cardiac myosin binding protein C (MYBPC3) gene mutations (MEURS et al., 2005). Few studies exist in the literature analyzing ET-1 in felids. Currently, further studies in feline patients are required to clarify the true meaning of the increased plasma concentration of endothelin in cats suffering from HCM (BOSWOOD, 2004). The aim of this study was to assess the ET-1 values in healthy Maine Coon cats.

Materials and Methods

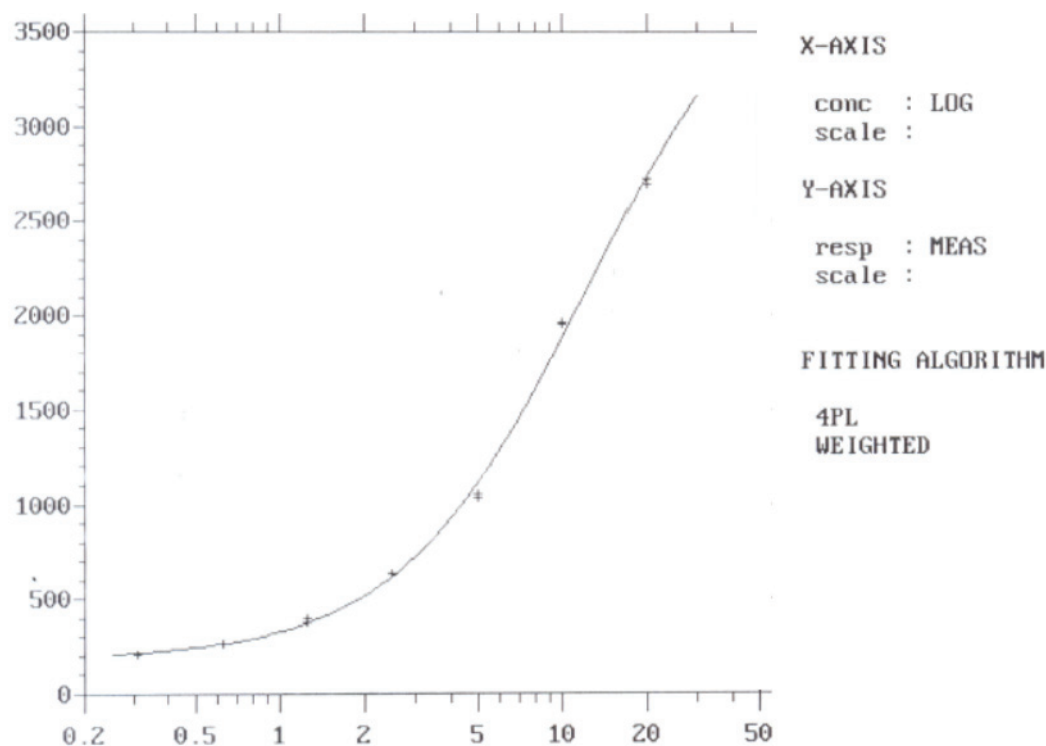
This study was performed between March 2011 and November 2011 at the Cardiology Unit (*Serviço de Cardiologia*) in the Department of Internal Medicine (*Departamento de Clínica Médica, VCM*) of the Veterinary Hospital (*Hospital Veterinário, HOVET*) at the School of Veterinary Medicine and

Animal Science of the University of São Paulo (*Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, FMVZ-USP*), at the Clinical Laboratory of the VCM/HOVET/FMVZ/USP, at Genese Institute of Scientific Analysis (*Instituto Gênese de Análises Científicas*), at PROVET Laboratory of Hormone Testing – Institute of Diagnoses and Specialities (*Laboratório de Dosagens Hormonais PROVET - Instituto de Diagnósticos e Especialidades*) and at Veterinary Cardiac Genetics Laboratory at Washington State University. The Maine Coon cats used in this study were originally from breeders informed about the research study proceedings, who signed a consent and commitment form. The study was approved by the Ethics Committee on animal use at FMVZ-USP.

The animals were selected regardless of gender and age considering the following exclusion criteria: medication used including angiotensin-converting enzyme inhibitors (ACEi), calcium channel blockers and diuretics; total T_4 (T_4) and plasma creatinine concentrations above 3.9 $\mu\text{g}/\text{dL}$ (SAMPEDRANO et al., 2009) and 2.17 mg/dL (REYNOLDS et al., 2010), respectively; systolic blood pressure (SBP) measurements were performed by ultrasonic Doppler method (Vascular Doppler DV610B, Medmega, Franca, SP) with a forelimb cuff (BROWN et al., 2007) and the normal range of SBP in cats was below 160 mmHg (STEPIEN, 2011); positive animals for mutations in the MYBPC3 gene; and presence of congenital heart disease and/or cardiac hypertrophy diagnosed by conventional echocardiography. Left ventricular hypertrophy was assessed through M-mode echocardiographic examination where the thickness of the left ventricular free wall and/or the ventricular septum in diastole was greater than 6 mm (FOX; LIU; MARON, 1995).

Blood samples were collected from animals fasting for eight to 12 hours by jugular venipuncture and were stored in tubes with BD gel separator. Immediately after collection, the blood tubes were left standing for approximately 20 minutes on ice in a Styrofoam box (internal temperature of approximately $+20^\circ\text{C}$) and then centrifuged at 3,000 rpm at $+4^\circ\text{C}$ for eight minutes (Sorvall Legend RT refrigerated centrifuge). The samples showing lipemia and/or hemolysis were discarded. Sera were aliquoted into 600 μL Axygen microcentrifuge tubes and were stored in a freezer at a temperature of approximately -80°C for up to eight months. Serum creatinine and T_4 quantifications were performed on blood from the sample collection.

Microcentrifuge tubes were transported in Styrofoam boxes containing dry ice because the endothelin assays were performed by a third-party laboratory at the Genese Institute of Scientific Analyses (*Instituto Gênese de Análises Científicas*). Samples were thawed and kept at room temperature immediately prior to performing the assay using the Endothelin (1-21) ELISA kit for humans (Biomedica Gruppe, Vienna, Austria). The assay was performed following instruction provided by the manufacturer then aprotinin was not utilized in the present study. The choice of kit was based on the validation performed by Prosek et al. (2004). The plate readings were performed using automatic reader (Awareness Technology Inc. Stat Fax 2100). The ET-1 detection range was between 0.31 and 20.00 fmol/mL with a threshold of 0.02 fmol/mL. The analytical standard curve for each assay was generated by using the ET-1 standard provided in the kit but adding two other dilutions based on a 1:1 dilution of the first concentration and a doubling of the final concentration (0.3125/ 0.625/ 1. 25/ 2.5/ 5/ 10/ 20 fmol/mL) (Figure 1).

Figure 1. Curve analysis pattern designed for testing the Biomedica Endothelin (1-21) diagnosis kit- Sao Paulo, 2012.

Source: Genese Institute of Scientific Analysis (*Instituto Gênese de Análises Científicas*).

The echocardiogram was performed by using the Vivid 7 Expert echocardiograph (Vivid 7, GE Vingmed Ultrasound AS, Horten, Norway). Echocardiographic examinations were performed according to the American Society of Echocardiography (ASE) recommendations (BOON, 1998). The M-mode parameters evaluated were as follows: thickness of the interventricular septum in diastole (IVSd); thickness of the left ventricular free wall in diastole (LVWd); internal diameter of the left ventricular cavity in diastole (LVDD); internal diameter of the cavity of the left ventricle in systole (LVDs); fractional shortening (FS); heart rate (HR); aortic root (Ao); left atrium (LA); ratio between the aortic root and left atrium measurements (Ao/LA). Three measurements were performed for each echocardiographic parameter, and the mean of the three values was considered for analysis. Furthermore, we attempted to minimize interference caused by breathing, motion of the

heart within the thorax and changes in cardiac filling pressure (BOON, 1998).

A statistical summary of the mean, standard deviation (SD), median (Med) and interquartile range (IQR) of the echocardiographic measurements as well as the levels of T_4 , creatinine, SBP and ET-1 was compiled (MINITAB® Release 14.12.0).

Results

A total of 38 healthy MC cats were evaluated, 13 males (34.2%) and 25 females (65.8%) including seven castrated males (53.85%) and 17 spayed females (68%). The mean age of the animals was 40.6 ± 21.59 months (ranging from 9 to 114 months). The mean weight of the animals was 6.18 ± 1.52 kg (ranging from 3.4 to 7.5 kg). Results about M-mode echocardiographic values, SBP, T_4 and creatinine serum levels are show with mean, SD, Med and IQR as well as their reference rangers are shown in Table 1.

Table 1. Mean, standard deviation (SD), median (Med) and interquartile range (IQR) of echocardiographic measurements, T₄, creatinine and SBP - Sao Paulo, 2012.

Parameter	Values	Parameter	Values
IVSd (cm)		HR (bpm)	
Mean (SD)	0.423 (0.052)	Mean (SD)	205.92 (26.64)
Med (IQR)	0.425 (0.38;0.463)	Med (IQR)	203.5 (189;226.5)
LVWd (cm)		Ao (cm)	
Mean (SD)	0.43 (0.058)	Mean (SD)	1.04 (0.12)
Med (IQR)	0.43 (0.40;0.47)	Med (IQR)	1.01 (0.95;1.13)
Septum/wall ratio		LA (cm)	
Mean (SD)	0.989 (0.095)	Mean (SD)	1.32 (0.14)
Med (IQR)	0.989 (0.933;1.051)	Med (IQR)	1.28 (1.19;1.46)
LVDD (cm)		Ao/LA	
Mean (SD)	1.55 (0.22)	Mean (SD)	0.79 (0.09)
Med (IQR)	1.54 (1.38; 1.74)	Med (IQR)	0.784 (0.75;0.84)
LVDs (cm)		SBP (mmHg)	
Mean (SD)	0.738 (0.192)	Mean (SD)	128.4 (15)
Med (IQR)	0.700 (0.625;0.845)	Med (IQR)	129.0 (118.5;140)
LVEF (%)		T₄ (ug/dL)	
Mean (SD)	85.64 (7.25)	Mean (SD)	1.79 (0.52)
Med (IQR)	85.97 (81.54;92,11)	Med (IQR)	1.67 (1.42; 2.09)
FS (%)		C (mg/dL)	
Mean (SD)	52.79 (8.88)	Mean (SD)	1.48 (0.26)
Med (IQR)	51.77 (46.83;60.36)	Med (IQR)	1.5 (1.28; 1.7)

Source: Elaboration of the authors.

ET-1 serum concentration in 38 healthy MC cats was 21.4 ± 9.6 fmol/mL. The values for the

measurements of ET-1 for each animal are shown in Table 2.

Table 2. Serum concentrations of ET-1 from each animal – Sao Paulo, 2012.

Animal	Serum concentrations of ET-1 (fmol/mL)	Animal	Serum concentrations of ET-1 (fmol/mL)
1	23.316	20	26.685
2	21.437	21	26.878
3	29.575	22	31.217
4	7.443	23	28.271
5	30.507	24	29.35
6	31.582	25	30.683
7	11.939	26	28.565
8	31.732	27	27.727
9	33.913	28	5.316
10	9.639	29	13.169
11	6.997	30	10.384
12	7.167	31	25.853
13	20.347	32	8.622
14	20.981	33	27.705
15	31.722	34	32.224
16	12.124	35	4.15
17	19.62	36	9.685
18	29.944	37	20.254
19	16.625	38	29.417

Source: Elaboration of the authors.

Discussion

Unfortunately, the ET-1 values could not be assessed in the present study because lower ET-1 values were expected based on the results reported by another study (PROSEK et al., 2004). However, the ET-1 values of 21.4±9.6 fmol/mL for the mean and SD and 24.58 fmol/mL (11.55; 29.67) for the med and IQR were derived based on the analytical standard curve (Figure 1). These values are beyond the mean value of the ET-1 plasma concentration reported by Prosek et al. (2004) in healthy cats, which was 0.777 fmol/mL.

No serine protease inhibitor enzymes such as aprotinin were used in the present study based on the instructions by the manufacturer (Biomedica), which is in contrast to the report by Prosek et al. (2004). However, the ET-1 values in our study were higher than the ET-1 values found by Prosek et al. (2004), and our ET-1 values were higher than

the assay sensitivity threshold. The values were approximately one decimal point above those described by Prosek et al. (2004). Upon request, the manufacturer indicated that the antibody used in our study was identical to the one used in the study by Prosek et al. (2004), although technical changes had occurred, which could explain the difference in assay sensitivity. The use of serine protease inhibitors such as aprotinin remains controversial among researchers even for the measurement of natriuretic peptides such as N-terminal prohormone of brain natriuretic peptide (NT-proBNP). According to Ettinger et al. (2012), the analysis of NT-proBNP requires a mix of protease inhibitors, whereas Wess et al. (2011) do not use protease inhibitors. Mueller et al. (2004) performed a study comparing the serum concentration of brain natriuretic peptide (BNP) and NT-proBNP at various times after freezing the samples without using serum protease inhibitor

and concluded that freezing considerably affects the concentrations assessed when using BNP, while there was no significant difference in NT-proBNP concentrations before and after freezing and thawing at -20°C for up to four months. The use of aprotinin does not appear to be relevant for preserving ET-1, which is similar to NT-proBNP because the ET-1 serum levels found in the present study are higher than the plasma values found by Prosek et al. (2004).

In the present study, cat serum was obtained from blood centrifugation in tubes with a gel separator, whereas Prosek et al. (2004) used plasma obtained from centrifugation in tubes containing ethylene diaminetetraacetic acid (EDTA). We decided to use serum because the manufactory instruction refers that measurement maybe done using serum, plasma, urine or cell culture supernatants. This difference in protocol could be another explanation for the discrepancy in ET-1 values. Although the National Academy of Clinical Biochemistry (WU et al., 1999) recommends performing cardiac marker measurement using plasma, some studies have shown higher cardiac troponin measurements in serum fractions relative to plasma fractions from the same blood samples (GERHARDT et al., 2000). The relationship between troponins and heparin remains unclear. Although the formation of a complex that impairs the adhesion of antibodies to commercial assays appears to occur in human blood (ERIKSSON et al., 2003), the gel separator does not seem to affect the adhesion of troponins (GERHARDT et al., 2000). A similar fact may occur with endothelin based on our results, which could explain the higher ET-1 values we found.

In the present study we used just serum samples without aprotinin for ET-1 measurements, so we can not confirm that high values we found can be justified by difference between the samples nature (serum/ plasma) or by absence of use of aprotinin, although, currently, the use of aprotinin is not indicated by manufacturer assay instruction.

Further studies on ET-1 are understandably required based on the results found because ET-1 theoretically has great potential as a cardiac marker (BOSWOOD, 2004). The method of sample collection for ET-1 analysis should first be understood regarding the use of serum protease inhibitors as well as differences between the serum and plasma concentrations of ET-1. However, ET-1 quantification in serum from Maine Coon cats using the Biomedica Endothelin (1-21) diagnosis assay requires a 1:20 dilution of samples considering the fact that the ET-1 values in cats with HCM and/or CHF may be up to five times the values found in healthy cats according to Prosek et al. (2004).

Conclusions

ET-1 serum measurements (21.4 ± 9.6 fmol/mL) without aprotinin in healthy MC cats is greater than values ET-1 plasma concentration with aprotinin reported by Prosek et al. (2004) in healthy cats (0.777 fmol/mL), using the same Biomedica Endothelin (1-21) diagnosis kit. Using this kit for serum ET-1 measurements without aprotinin in MC cats the dilution of samples is necessary.

Ethics Committee

This study was approved by the Ethics Committee on animal use at the School of Veterinary Medicine and Animal Science at the University of São Paulo (Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo) under protocol No. 2010/2010, on 09/29/2010.

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