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PERFIL INFLAMATÓRIO EM CÃES COM DOENÇA MIXOMATOSA DA VALVA MITRAL

(Inflammatory profile in dogs with myxomatous mitral valve disease)

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RESUMO: Substâncias inflamatórias como interleucinas (IL), fator alfa de necrose tumoral (TNF-α) e proteína-C reativa (CRP) desempenham papel importante na progressão da insuficiência cardíaca. Os objetivos desse estudo foram dois: investigar a concentração de citocinas circulantes em cães com doença mixomatosa da valva mitral (DMVM) em diferentes estágios, e acessar a relação entre essas substâncias e a morfologia e função cardíacas. Com esse intuito, doze cães com DMVM (7 em estágio B2; 5 em estágio C) e nove controles saudáveis foram submetidos à ecocardiografia transtorácica e tiveram as concentrações séricas de IL-1β, 4, 6, 10, TNF-α e CRP quantificadas. Os dados foram submetidos ao teste de normalidade de Shapiro-Wilk, os grupos foram comparados por meio da ANOVA ou Kruskal-Wallis de acordo com a distribuição, e os coeficientes de correlação obtidos pelo teste de Spearman. A concentração de IL-1β diferiu (P: 0,0056) entre os grupos. Ademais, houve correlação positiva entre IL-1β e o diâmetro ventricular esquerdo ao final da diástole (R: 0,56), relação átrio esquerdo aorta (R: 0,63) e parâmetros indicadores de congestão. Similarmente, a IL-4 exibiu correlação positiva de média a forte com parâmetros de remodelamento e congestão cardíacos, enquanto a IL-6 apresentou correlação negativa com a fração de encurtamento. Como conclusão principal, o nível de IL-1β aumenta em cães com DMVM e valores ainda maiores são observados nos animais sintomáticos.

Palavras chave: inflamação, insuficiência mitral, interleucinas, ecocardiografia, insuficiência cardíaca

ABSTRACT: Inflammatory substances such as interleukins (IL), tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP) have been demonstrated to play a role in heart failure progression. The aims of this study were two-fold: to investigate the concentration of circulating cytokines in dogs with myxomatous mitral valve disease (MMVD) in different stages, and to assess the relationship between these substances and cardiac morphology and function. For this purpose, twelve dogs with MMVD (7 stage-B2; 5 stage-C) and nine healthy controls underwent a transthoracic echocardiogram and had their serum concentrations of IL-1β, 4, 6, 10, TNF-α and CRP assessed. Data was submitted to Shapiro-Wilk normality test, groups were compared with ANOVA or Krukal-Wallis test according to distribution, and correlation coefficients were obtained with Spearman test. The IL-1β levels were different (P: 0.0056) between groups. Also, a positive correlation existed between IL-

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1β and left ventricular end-diastolic diameter (R: 0.56), left atrium-to-aorta ratio (R: 0.63) and echocardiography congestion surrogates. Similarly, IL-4 exhibited moderate-to-strong positive correlation with parameters of cardiac remodeling and congestion, while IL-6 was negatively correlated with the shortening fraction (R: -0.49). As the main conclusion, IL-1β levels increases in dogs with MMVD, and even higher levels are observed in overtly symptomatic animals.

Key words: echocardiography, mitral insufficiency, heart failure, inflammation, interleukins

INTRODUCTION

Myxomatous mitral valve disease (MMVD) is widely accepted as the most common acquired cardiac disease in dogs. The impaired leaflets coaptation ultimately leads to blood regurgitation from the left ventricle (LV) to the left atrium (LA) during ventricular systole (BORGARELLI et al., 2004; ATKINS et al., 2009).

Blood regurgitation decreases cardiac output and triggers the activation of neuroendocrine mechanisms, leading to parasympathetic decline and upregulation of the sympathetic tone, which causes both LA and LV volume overload and clinical signs ascribed to congestive heart failure. Interestingly, many inflammatory substances, like endothelin (ET), interleukins (IL), tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP) have been demonstrated to play a role in heart failure pathogenesis as well (FILIPPATOS et al., 2003; CHEN et al., 2008; SLUPE et al., 2008). Those inflammatory mediators are able to induce cardiomyocyte hypertrophy, activation of metalloproteinases, degradation of extracellular matrix, fibrosis, besides having negative inotropic effects (LEVINE et al., 1990; DUTKA et al., 1993; ORAL et al., 1995).

The ET activates the necrosis factor, a transcription agent that increases the production of pro-inflammatory substances (MULLER et al., 2003). In dogs affected by MMVD it was observed an augmentation in ET receptors expression on mitral valve leaflets (MOW et al., 1999; LU et al., 2015). Similarly, a large population-based study, the ¨InCHIANTI study¨ demonstrated that increased serum levels of interleukin-1β (IL-1β) in patients with heart failure correlates with its severity irrespective of its etiology (DI IORIO et al., 2003), and similar results were found in dogs (KICZAK et al., 2008). Moreover, it was also demonstrated that interleukin-8 (IL-8) is upregulated in dogs with MMVD, increasing according to its severity (MAVROPOULOU et al., 2016).

The CRP is a major acute phase protein in the dog, that is released from hepatic cells after stimulation by cytokines early in the course of the inflammatory process, and increased serum values have been recorded in dogs with acquired (RUSH et al., 2006; CUNNINGHAM et al., 2012; POLIZOPOULOU et al., 2015) and congenital (SAUNDERS et al., 2009) cardiac diseases.

In chronic cardiac diseases such as MMVD, where clinical changes can be unapparent, periodic evaluation of biomarkers has been proposed as a complementary method for clinical and therapeutic monitoring and prognosis assessment in dogs (LU et al., 2015a; POLIZOPOULOU et al., 2015). Therefore, the aims of this study were two-fold: to investigate the concentration of circulating cytokines in dogs with MMVD in different stages, and to assess

the relationship between these substances and cardiac morphology and function assessed by echocardiography.

MATERIAL AND METHODS

This study had a prospective, cross-sectional and observational design, and was conducted at a veterinary teaching facility. All procedures were previously approved by the institutional Animal Use Committee (protocol #4628/13). The inclusion criteria included dogs with the diagnosis of MMVD based on both physical examination and a complete transthoracic echocardiogram. The exclusion criteria were altered laboratory analysis, including complete blood count (CBC) and biochemistry profile, as well as the diagnosis of MMVD with no cardiac remodeling and/or evidence of cardiac diseases other than MMVD. Once enrolled, dogs were categorized in groups that represented the disease stages, according to the American College of Veterinary Internal Medicine consensus statement on canine valvar disease (ATKINS et al, 2009). Furthermore, normal age-matched dogs were recruited from the institution's experimental kennel as healthy controls.

The echocardiogram was performed with the dogs restrained in right and left lateral recumbencies, using an ultrasound machine equipped with 5.0-7.5 MHz phased-array transducer (Siemens X300 Premium Edition, United States). Images were acquired and stored for offline measurements. The echocardiographic parameters aimed at assessing systolic function and cardiac morphology included the left ventricular end-diastolic (LVd) and end-systolic (LVs) diameters, the left atrium-to-aorta ratio (LA/Ao), and the fractional shortening (FS), which were all documented from transverse images obtained from the right parasternal window. Diastolic assessment included the ratio between left ventricular rapid and slow filling velocities (Em/Am), isovolumetric relaxation time (IVRT), and Em-to-IVRT ratio, which were measured from either apical 4- or 5-chamber images obtained from the left parasternal window. Also, tissue doppler imaging was used to acquire mitral annular velocity at early (ETDI) and late (ATDI) diastole, which allowed the calculation of Em-to-ETDI ratio. All echocardiographic data represented the mean of at least three consecutive cardiac cycles, and were obtained in accordance with the guidelines described elsewhere (BOON, 2011).

Blood samples were drawn aseptically from the jugular vein and stored in tubes without anticoagulant, which were immediately taken to the laboratory to undergo centrifugation. Several aliquots of serum were obtained and stored at -80°C until the batch analyses were performed. Immediately before the procedures, the aliquots were thawed and laboratory processing followed the kit manufacturer's instructions.

Enzyme-linked immunosorbent assays were used to determine the serum concentrations of IL-1β (SEA563Ca, USCN®, China), IL-4 (SEA077Ca, USCN®, China) and C-reactive protein (CYT296, Millipore®, USA). Once the reactions were over, the software MultiCalc (MultiCalc®, Florianópolis, Brazil) was used to calculate the substance's concentration from the intensity of color documented in an ELISA reader (Stat Fax 2600, Awareness®, Palm City, USA). The serum concentrations of IL-6, IL-10 and TNF-α were determined with an automatic analyzer (Luminex 200, Luminex Corporation, Austin, USA) using a commercially-available multiplex immunoassay (CCYTO-90K Milliplex MAP, Millipore®, USA). The minimum detectable concentrations of IL 1β, IL 4, IL 6, IL 10, TNF-α and CRP were at

least 5.8 pg/mL, 6.0 pg/mL, 1.98 pg/mL, 0.9 pg/mL, 0.7 pg/mL and 3.6 µg/mL, respectively. All results represent the average of the concentrations obtained from two duplicate samples.

Data were submitted to the Shapiro-Wilk normality test to investigate the distribution. Whenever a normal distribution was attained, Student's T test was used to compare control and MMVD dogs. Also, the analysis of variance (ANOVA) followed by the post-hoc Tukey test was used to further compare healthy and MMVD dogs divided according to stages. Whenever data was not normally distributed, normal and diseased animals were compared with the Mann-Whitney test, and further comparison between healthy dogs and MMVD animals separated in stages was performed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Finally, correlations between the serum concentration of the inflammatory substances and the echocardiographic data were investigated using the Spearman test. All analyses were done with the software Prism Windows 3.0, (GraphPad®, San Diego, USA), and significance was defined as $P<0.05$.

RESULTS

Twelve adult dogs (3.0-17.3 kg) with MMVD and remodeled hearts were recruited at the end of the study. Cardiac dilatation was based on the documentation of an increased LA/Ao ratio (>1.4) on the echocardiogram. Five dogs (3.0-14.0 kg) had overt clinical signs attributable to heart failure (stage C), while seven animals (10.2-14.0 kg) were still asymptomatic (stage B2). Also, nine adult dogs (10.2-17.3 kg) that presented no murmur on auscultation nor exhibited any signs that could be ascribed to heart failure were recruited as healthy controls. No differences in body weight existed between groups (P: 0.40).The serum concentration of IL-1β was considered different between control (0.40 pg/mL), stage B2 (10.47 pg/mL) and stage C (17.31 pg/mL) groups (P: 0.0015) (figure 1).

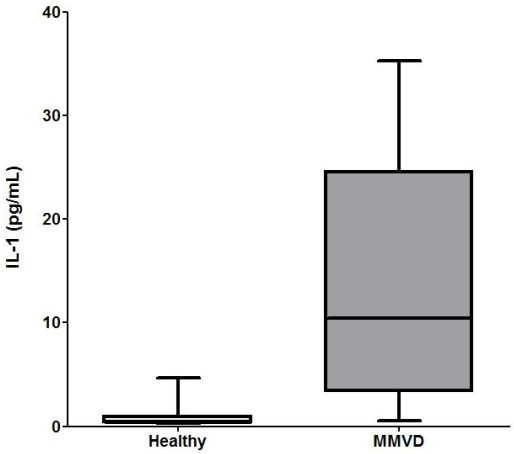

Figure 1: Boxplots illustrating the statistically different plasma concentration of interleukin 1β in healthy dogs and dogs with myxomatous mitral valve disease. Medians, interquartile ranges and minimum-maximum values are shown.

Concentrations of IL-4, 10 and CRP were considered similar among groups, even though there is a clear trend in increasing levels according to MMVD severity. On the other hand, values of IL-6 and TNF-α seems to decrease with the progression of the disease, in spite of no statistical difference being recorded between groups. Results are summarized in table 1.

Table 1. Inflammatory profile obtained from serum samples of either healthy or MMVD dogs. Either medians (IQR) or means (SD) are shown.

	Group			*P*
	Healthy (n=9)	**Stage B2 (n=7)**	**Stage C (n=5)**	
IL-1β (pg/mL)	0.40 (0.39-0.91) [A]	10.47 (3.72-13.34) [B]	17.31 (2.65-33.04) [B]	0.0056
IL-4 (pg/mL)	6.00 (6.00-6.00)	17.48 (6.00-51.77)	47.98 (6.00-124.2)	0.0792
IL-6 (pg/mL)	88.50 (31.64-540.0)	91.64 (4.68-145.0)	16.78 (9.73-65.66)	0.3245
IL-10 (pg/mL)	0.90 (0.90-70.85)	2.62 (0.90-11.91)	6.81 (3.28-25.10)	0.7562
TNF-α (pg/mL)	21.51 (5.87-106.80)	19.97 (0.77-33.77)	0.77 (0.77-27.88)	0.2056
CRP (µg/mL) *	2.09 (0.61)	2.22 (0.87)	2.66 (0.87)	0.4156

IQR: interquartile range; SD: standard deviation; IL-1β: interleukin 1β; IL-4: interleukin 4; IL-6: interleukin 6; IL-10: interleukin 10; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; * parametric data.

IL-1β attained a moderate-to-strong correlation with the echocardiographic parameters. A positive correlation was documented between IL-1β and LVd, LA/Ao, Em/IVRT and Em/ETDI, whereas a negative correlation existed with IVRT. Also, the concentration of IL-4 was positively correlated with LA/Ao (R: 0.47) and Em/ETDI (R: 0.70), while a negative correlation was documented between IL-6 and FS (R: -0.49). Scatterplots showing all significant correlations with their respective identity lines, 95% confidence bands and correlation coefficients are represented in figure 2.

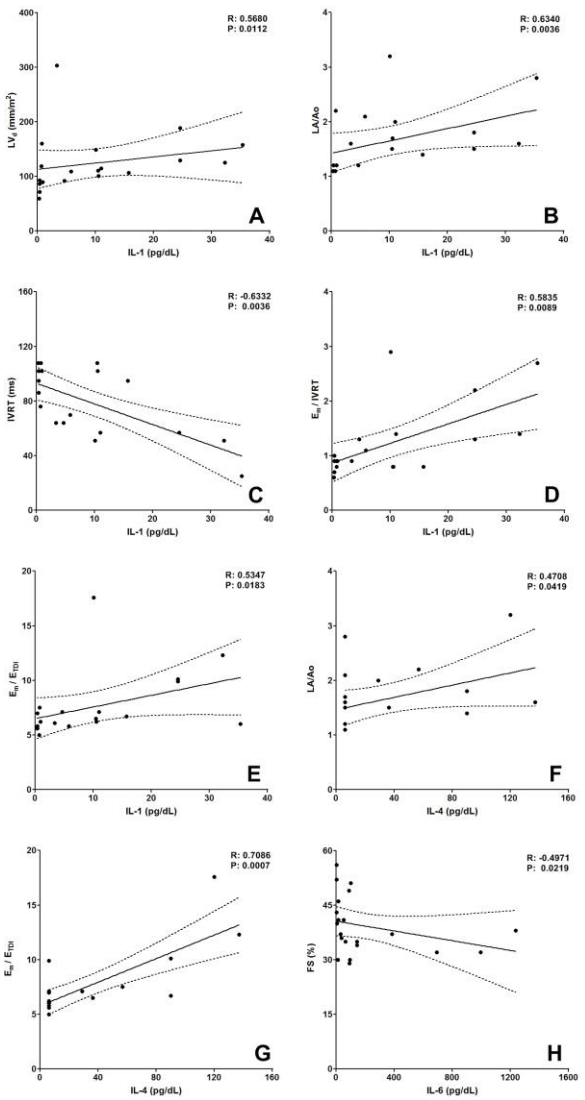

Figure 2. Scatterplots showing the moderate-to-strong correlations between the echocardiographic data and the serum concentration of interleukins 1β, 4 and 6. Best-fit lines and their 95% confidence intervals are shown. (A) interleukin 1β versus the left ventricular internal diameter in diastole; (B) interleukin 1β versus the left atrium-to-aorta ratio; (C) interleukin 1β versus the isovolumic relaxation time; (D) interleukin 1β versus the left ventricular rapid filling velocity-to-isovolumic relaxation time ratio; (E) interleukin 1β versus the left-ventricular rapid filling velocity-to-mitral annular velocity at early diastole; (F) interleukin 4 versus the the left atrium-to-aorta ratio; (G) interleukin 4 versus the left-ventricular rapid filling velocity-to-mitral annular velocity at early diastole; (H) interleukin 6 versus the left ventricular fractional shortening.

DISCUSSION

It was already demonstrated that IL-1β mRNA expression elevates even in early stages of cardiac diseases in dogs, with gradual increases until end-stage left ventricle dysfunction (KICZAK et al., 2008, 2015). Our results demonstrate a progressive

augmentation in IL-1β along MMVD stages. Also, the diseased groups (B2 and C) had higher levels in comparison with healthy dogs.

The moderate-to-strong positive correlation coefficients obtained between IL-1β and echocardiography surrogates of volume overload (LVd; LA/Ao) and congestion (Em/TRIV; Em/ETDI), suggest a relationship among higher levels of that cytokine and the progression of mitral insufficiency towards heart failure. According to previously published studies, the biological effects of pro-inflammatory cytokines, such as IL-1β, results in cardiomyocyte hypertrophy and atrophy, leading to cardiac fibrosis and inflammatory infiltrates (THAIK et al., 1995; DINARELLO et al., 1996; BARTH et al., 2000; KICZAK et al., 2008). Moreover, IL-1β was shown to significantly upregulate the brain natriuretic peptide (BNP) transcription - a neurohormone released by ventricles in response to volume or pressure overload, thus being used as biomarker of cardiac damage - in atrial and ventricular cardiomyocytes (HE et al., 1999).

The interleukin-4 has multiple biological effects as well, promoting tissue fibrotic remodeling in diseases involving lungs (WALFORD et al., 2013), skin (ZUBER et al., 2006) and liver (SHI et al., 1997). Studies have shown a positive correlation between circulating IL-4 levels and cardiac fibrotic remodeling in both human beings (ROSELLÓ-LLETÍ et al., 2007; CATAPANO et al., 2008) and laboratory animals (LEVICK et al., 2009; CIESLIK et al., 2011; PENG et al., 2016). Aside from the positive correlation among echocardiographic data of volume overload (LA/Ao) and congestion (Em/ETDI) and IL-4 levels, we observed a clear trend of increase in IL-4 according to the progression of MMVD, in spite of no statistical difference being documented between disease stages.

Owing to MMVD progression, the organism activates neuroendocrine compensatory mechanisms, in an attempt to maintain the stroke volume and arterial blood pressure within the normal range. The autonomic dysfunction caused by heart failure enhances the sympathetic tone, which increases the circulating levels of norepinephrine (HÄGGSTRÖM; KVART; PEDERSEN, 2005). Also, the activation of the renin-angiotensin-aldosterone system (RAAS) promotes sodium and water reabsorption, leading to preload improvement (HÄGGSTRÖM; KVART; PEDERSEN, 2005). However, these two compensatory actions trigger the Franking-Starling mechanism, resulting in increased fraction of shortening (FS) thereafter. The moderate negative correlation obtained between IL-6 levels and FS was expected, once advanced stages of MMVD dogs usually exhibit higher FS, and lower levels of IL-6 were detected in stage C. However, the relationship between IL-6 and progression of canine cardiac diseases remains unclear, and studies are conflicting. Although the IL-6 plasma levels increased in heart failure dogs when compared with control group in one study (TANAKA-ESPOSITO et al., 2014), Zois et al. (2012) did not found difference between IL-6 levels in MMVD dogs according to stages.

The small number of dogs according to MMVD stages, and mostly important, the absence of animals in stage D, may have a negative impact in our results, once some inflammatory substances, such as IL-4, IL-10 and TNF-α, showed a trend of variation but no statistical difference was actually found among groups. Moreover, a longitudinal study design could probably better elucidate the role of IL-6, IL-10, TNF-α and CRP in the pathogenesis of heart failure. The results of this research

should be interpreted in context of its limitations.

CONCLUSION

Our major findings are as follows. (1) The serum levels of interleukin 1β increases in dogs with myxomatous mitral valve disease, and even higher levels are observed in overtly symptomatic animals; (2) there is an important correlation between cardiac remodeling and congestion as assessed by echocardiography and the circulating levels of IL-1β and IL-4; (3) the serum levels of interleukin-6 and the left-ventricular shortening fraction are negatively correlated.

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