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IL-10 and ET-1 as biomarkers of rheumatic valve disease

IL-10 e ET-1 como biomarcadores de doença valvar reumática

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Abstract

Objective: **To evaluate the immunological profile and gene expression of endothelin-1 (ET-1) in mitral valves of patients with rheumatic fever originated from a reference service in cardiovascular surgery.**

Methods: **This was a quantitative, observational and cross-sectional study. Thirty-five subjects (divided into four groups) participated in the study, 25 patients with chronic rheumatic heart disease and ten control subjects. The mean age of the sample studied was 34.5 years. Seventeen of them (48.58%) were male and 18 (51.42%) were female. Inflammatory cytokines (TNF-α, IL-4 and IL-10) were measured and ten mitral valves of patients who underwent first valve replacement were collected for determination of gene expression of endothelin-1 by real time PCR.**

Results: **Among the groups studied (patients vs. controls), there was a statistically significant difference in IL-10 levels (*P*=0.002), and no differences in other cytokines. Expression of endothelin-1 was observed in 70% of samples. Quantitatively, average of ET-1 expression was 62.85±25.63%.**

Conclusion: **Inflammatory cytokine IL-10 participates in the maintenance of chronicity of rheumatic fever in patients who underwent valve replacement and those who are undergoing medical treatment. The expression of endothelin-1 in heart valve lesions in patients undergoing mitral valve replacement confirms its association with inflammatory activity in rheumatic fever.**

Descriptors: **Interleukin-10. Interleukin-4. Receptors, Tumor Necrosis Factor. Endothelin-1. Mitral Valve Stenosis.**

Resumo

Objetivo: **Avaliar o perfil imunológico e a expressão gênica de endotelina-1 em valvas mitrais de pacientes com febre reumática, originados de um serviço de referência em cirurgia cardiovascular.**

Métodos: **Este foi um estudo quantitativo, observacional e transversal. Trinta e cinco indivíduos (divididos em quatro grupos) participaram do estudo, 25 deles com doença cardíaca reumática crônica, além de 10 controles. A média de idade da amostra estudada foi de 34,5 anos. Dezessete (48,58%) dos indivíduos eram homens, e 18 (51,42%) eram mulheres. Foram medidas algumas citocinas inflamatórias (TNF-α, IL-4 e IL-10) e coletadas 10 valvas mitrais de pacientes que se submeteram a primeira troca valvar para determinação da expressão gênica de endotelina-1 pelo PCR real-time.**

Resultados: **Entre os grupos estudados (pacientes e controles), observou-se diferença estatisticamente significante em rela-**

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Abbreviations, acronyms & symbols	
RF	Rheumatic fever
CRHD	Chronic rheumatic heart disease
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3

ção aos níveis de IL-10 (*P*=0,002), sem diferenças nas outras citocinas. Em relação à endotelina-1, foi observada sua expressão em 70% das amostras. Quantitativamente, a expressão média de endotelina-1 foi de 62,85±25,63%.

***Conclusão:* A citocina inflamatória IL-10 participa da manutenção da cronicidade da febre reumática em pacientes que se submeteram a troca valvar e naqueles que estão em tratamento médico. A expressão de endotelina-1 nas lesões em valvas cardíacas de pacientes que foram submetidos à troca valvar mitral confirma sua relação com a atividade inflamatória na febre reumática.**

***Descritores:* Interleucina-10. Interleucina-4. Receptores do Fator de Necrose Tumoral. Endotelina-1. Estenose da Valva Mitral.**

INTRODUCTION

Rheumatic fever (RF) represents a serious public health problem. It is a rheumatic and inflammatory disease of autoimmune origin, which occurs in response to an infection by group A streptococcus (*Streptococcus pyogenes*). On a global scale, this agent is responsible for approximately 15.6 million annual cases of rheumatic heart disease, with 282,000 new cases and 233,000 deaths each year. From this perspective, health systems face higher expenses with clinical exams, surgeries and frequent hospitalizations due to congestive heart failure [1-4].

The pathogenesis of RF involves a complex network of genetic, environmental and immunological interactions. Genetic factors predispose individuals to developing autoimmune reactions [5]. Cytokines are proteic molecules, glycosylated or not, that send a range of stimulatory, modulatory or inhibitory signals to the various cells of the immune system. Studies indicate that the inflammatory response in acute RF on cardiac tissues is generated by antigenic mimicry of the protein M leading to an abundant infiltration of CD4+ T cells [5-7]. This leads to production of inflammatory cytokines (e.g., TNF-α, IL-2, and IL-10), which have a decisive influence on the immune response of patients with rheumatic fever. It is also known that increased levels of Th1 inflammatory cytokines (TNF-α and IFN-γ) and lower levels of Th2 and regulatory cytokine IL-4 lead to maintenance and progression of rheumatic valvulopathy [8-12].

Endothelin is a highly potent vasoconstrictor peptide. This peptide is composed of 21 amino acids and has three isoforms. The three isoforms are called endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) [13,14]. Endothelin-1 is the subtype predominantly produced by cardiac endothelium. Some studies show gene expression of endothelin in heart valves of patients who underwent surgical valve replacement [15,16].

Cardiac involvement in acute RF characterizes the most serious and most important of all manifestations of the disease because of the possibility of progressing to chronic rheumatic valvular disease or death. The most common rheumatic mitral valvulopathy is a dual unbalanced dysfunction, i.e, insufficiency and stenosis in different stages of development, which may lead to an indication of surgical repair or replacement of the damaged valve in children and young people in productive age [17,18].

The aim of this study was to compare the levels of some interleukins (TNF-alpha, IL-4 and IL-10) among different patients with RF. In addition, we sought to assess gene expression of endothelin-1 in native replaced mitral valves.

METHODS

A quantitative, field, observational and cross-sectional study was performed after obtaining approval by the Ethics Committee for Human Research of the Federal University of Sergipe (CAAE 2344.0.000.107.10) and written informed consent from participants. Socio-epidemiological data and peripheral venous blood of 35 individuals, 25 patients with RF and chronic rheumatic heart disease (CRHD) originated from a cardiovascular surgery service in the city of Aracaju and 10 control subjects were collected. The different groups of RF patients were divided as follows: G1 (ten patients with RF /CRHD who underwent first valve replacement); G2 (five patients with CRHD who underwent second valve replacement); G3 (ten patients with RF in clinical treatment and regular medical monitoring, without indication of valve replacement). The control group (G4) consisted of healthy individuals without evidence of any autoimmune disease and who did not use antibiotics at the time of data collection. The exclusion criteria were adults aged over 65 years or older, pregnant women and patients with autoimmune disease. All participants answered a clinical and socio-epidemiological questionnaire.

The sample size was determined from the amount of surgeries performed where this research was conducted: 107 surgical valve replacements in 2009 (75 native valve replacements and 32 second valve replacements). Using a confidence level of 95% and a level of heterogeneity of 99%, we arrived at 13 patients for the first group and 11 patients for the second group. There were difficulties related to the composition of group 2 (second valve replacement) due to the natural progression of disease (death before the second valve replacement).

Epidemiological profile

Of the total number of subjects, 17 (48.57%) were male and 18 (51.43%) were female. Of the 25 patients with RF/CRHD, 13 (52%) were female and 12 (48%) were male. Mean age was 34.5±2.56 years. Among the different groups, G1 had a mean age of 43.7± .85 years, G2 had a mean age of 40 ± 8.91 years, and the average age of patients with RF/CRHD without indication of surgical replacement (G3) was 33.70±2.56 years. The control group (G4) had an average age of 21.6±0.52 years (*P*=0.0005).

Regarding the frequency of symptoms, dyspnea was the most prevalent symptom (68%), followed by chest pain (16%), palpitations (8%) and edema in the legs (8%). Echocardiographic data showed mitral valve involvement in 64% of the patients, followed by the aortic and mitral double lesion in 24% of the patients. There was no involvement of the pulmonary and tricuspid valves. Regarding the type of valvular involvement, we observed reflux in 80% of the sample, followed by stenosis (68%), calcification (40%), and prolapse of the chordae (4%).

Determination of endothelin-1 by real-time PCR

We collected ten mitral valves of patients that underwent first valve replacement surgery (G1). These valves were stored in RNA stabilization solution at -20ºC. For the extraction of total RNA from the valves, we used 30-40 µg of valve tissue, manually macerated in the presence of liquid nitrogen, in accordance with the protocol recommended by Mini Kit RNeasy Fibrous (QIAGEN®). Total RNA was quantified by spectrophotometry in Nanodrop® (Thermo Scientific™). The quantification was performed in duplicate, obtaining the average RNA concentration in ng/µL. Absorbance values obtained were analyzed according to the following formula: [RNA (µg/ml)] = 40 x A260 x diluition/ 1000 (Maniatis). Purity was assessed by the ratio of absorbance values obtained at 260 nm and 280 nm (A260/A280), and samples with between 1.8 and 2.0 were considered viable. cDNA from the valves was obtained by reverse transcriptase reaction (RT). To obtain cDNA, we used 38.4 to 82.5 ng of total RNA from each sample in accordance with the protocol recommended by QuantiTect Reverse Transcription Kit (QIAGEN®). The cDNAs were quantified on Nanodrop® (Thermo Scientific™).

cDNA Control 1 (CT1) was used to generate a calibration curve for efficiency ET1 and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) primers. The cDNA sample was diluted in 5x and 10x dilutions, and PCR reactions in real time were subsequently performed using the primers for ET-1 and GAPDH. By using the results of the slope and the number of cycles required to increase the amount of molecules 10x, we can calculate the efficiency of reactions for both primers with the formula: efficiency = $10^{(-1/slope)} - 1$. The expression of the mRNA of target genes of ET-1 and GAPDH primers was quantified with real-time PCR using QuantiTect Primer Assay 10x (QIAGEN®). Reactions were carried out with 15 µl of QuantiFast SYBR Green PCR kit (Qiagen®). After that, a dissociation curve was run to verify the specificity of each pair of primers.

Data from real-time PCR were tabulated and analyzed by the CFX96 Real Time System (BIORAD®) device and calculations of relative expression were performed by the Delta Ct method (Pfaffl, 2001) [19], according to the formula:

$$\text{ratio} = \frac{(\text{Etarget})^{\Delta Ct\ target\ (control\text{-}treated)}}{(\text{Eref})^{\Delta Ctref\ (control\text{-}treated)}}$$

Cytokines

We collected 10 ml of peripheral blood, which was centrifuged and stored at -80ºC, to determine TNF-alpha, IL-4 and IL-10 by sandwich ELISA immunoenzymatic assays (eBioscience). Measurements of these cytokines followed the instructions provided by the manufacturer. Wells of polystyrene distributed into strips were used in the adsorption of specific monoclonal antibodies for each cytokine (100 µL/well) at the appropriate concentration. This step for sensitization was performed overnight at 4ºC and completed after five washes of the wells with the wash solution provided by the manufacturer. Subsequently, blockade of residual free sites was done with 200 µL/ well of diluent for one hour at room temperature. The wells were again washed five times and then incubated overnight at 4ºC with 100 µL/ well of patterns and samples corresponding to each cytokine.

A new washing cycle was processed, followed by the addition of 100 µL/well of biotin-conjugated antibody for detection, and incubation for one hour at room temperature. Following new washes, the wells were incubated with 100 µL/well of conjugate formed by peroxidase-labeled streptavidin for 30 minutes at room temperature. After a new round of washes, the reactions were developed with 100 µL/ well of substrate (tetramethylbenzidine solution containing hydrogen peroxide) for 15 minutes at room temperature. After stopping the reaction with 2N of HCl, the absorbance was read at 450 nm-570 nm in an ELISA reader. Cytokine concentrations were determined in serum pg/ml, using the previously established pattern curves with known quantities of cytokines.

Statistical analysis

For distribution of continuous variables, we used D'Agostino, Pearson and Kruskal-Wallis tests. We considered statistically significant the results of the analysis with $P<0.05$. Statistical analyzes were performed using Graph Pad Prism 5.0 (GraphPad Software Inc., USA).

RESULTS

Determination of endothelin-1 by real-time PCR

The average amount of RNA in the samples was 65.75±19.72 ng/ul (Table 1). The mean concentrations of nucleic acid (total RNA), and cDNA were 20.60±26.84 ng/µl and 615.31±77.20 ng /µl, respectively (Table 1). Mean values of absorbance at 260 nm and 280 nm were 0.51±0.66 UA (A260) and 0.25±0.31 UA (A280), respectively. The A260/A280 ratio was 1.79±0.26 (Table 1).

In the real-time PCR reactions, it was observed that the slope for ET-1 appeared in -3.272 (R2=0.944), resulting in an efficiency of 102.1%; and the slope of GAPDH was in -3.286 (R2=0.996), resulting in an efficiency of 101.5%. According to these calculations, reactions with both primers have adequate efficiency. After generating the calibration curve and calculating the efficiency of the reactions, we plotted dissociation curves for both primers, showing that both have specificity.

Based on standardized protocol for the calibration curve, we performed amplifications for the reactions with ET-1 and GAPDH primers. We observed the expression of ET-1 in seven of the ten samples collected. Quantitatively, the average gene expression relative to ET-1 was 62.85± 25.63% (Figure 1).

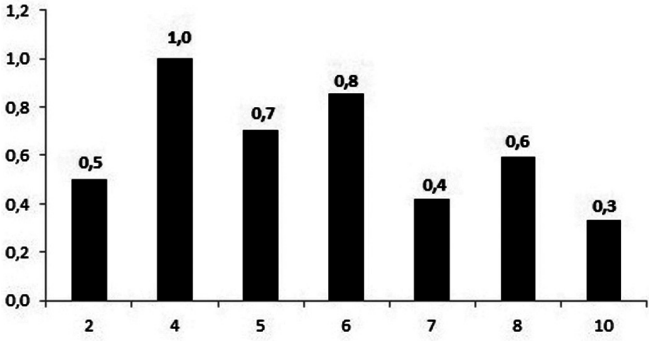

Fig. 1 – Graph showing the relative expression of endothelin 1 (ET-1) in mitral valves samples 2, 4, 5, 6, 7, 8 and 10

Cytokines

Patients submitted to the first (G1) and second valve replacement (G2) had an average concentration of cytokine IL-4 of 2.39±4.37 pg/ml and 15.71±34.66 pg/ml, respectively, whereas in RF patients (G3) and in the control group, the values were 16.66±51.81 pg/ml and 0.32±0.64 pg/ml (P=0.56), respectively (Figure 2A). Regarding the levels of IL-10, there was a mean concentration of 7.30±8 pg/ml in the first group, 8.07±2.26 pg/ml in the second group, 6.97±1.68 pg/ml in the third group (RF patients) and 0.77±1.68 pg/ml in the control group (P=0.002) (Figure 2B). The dosage of TNF-alpha in the first and second groups was 4.25±11.87 pg/ml and 2.67±5.09 pg/ml, respectively. In RF patients group and in the control group, levels of TNF-alpha were, respectively, 1.43±4.54 pg/ml and 4.04±12.61 pg/ml (P=0.91) (Figure 2C).

Table 1. Quantification of total RNA, cDNA and spectophotometry from heart valves.

Samples	Mean Nucleic Acid Conc. (ng/ml)	Total cDNA	A260	A280	A260/A280
GAM01	7.5	724.8	0.178	0.097	1.835
GAM02	66	659.3	1.686	0.82	2.056
GAM03	4.5	573.9	0.123	0.087	1.413
GAM04	75	565.4	1.805	0.878	2.055
GAM05	18	594	0.435	0.212	2.051
GAM06	11	760.5	0.253	0.139	1.82
GAM07	3.2	617	0.076	0.05	1.52
GAM08	3.3	547.1	0.111	0.072	1.54
GAM09	3.5	588.6	0.115	0.072	1.597
GAM10	14	522.5	0.349	0.167	2.089
Mean	**20.6**	**615.31**	**0.5131**	**0.2594**	**1.7976**
Std Dev	**26.84995345**	**77.20521643**	**0.6600568**	**0,3148898**	**0,26153997**

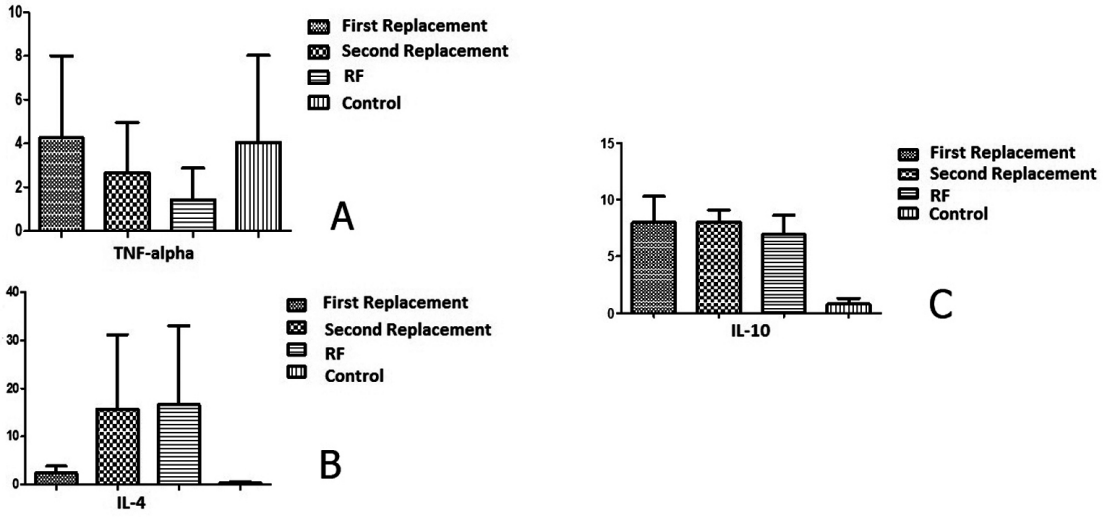

Fig. 2 – Graph showing the mean expression for different interleukins (A: TNF-alpha; B: IL-4 and C: IL-10). Legends-TNF-alpha: Tumor necrosis factor alpha; IL-4: Interleukin-4; IL-10: Interleukin-10

DISCUSSION

From the results presented, serum levels of TNF-alpha as well as the levels of IL-4 and IL-10 were shown to be reduced when compared to a previous study [20]. When analyzing the group of patients with RF/ CHRD (compared to the control group), we identified lower serum concentrations of TNF-alpha as well as elevated serum levels of IL-4 and IL-10. Considering that the patients with RF/CRHD had had rheumatic disease for over a decade and they had not been submitted to surgical replacement of the mitral valve, it suggests that decreased levels of TNF-alpha (which is a pro-inflammatory cytokine) can correspond to a possible immunological control of disease in this group [6,8,12]. In addition, serum levels of TNF-alpha were similar in patients who had replaced the native heart valve and in the control group indicating pro-inflammatory immune response.

There were high levels of IL-4 in patients who have replaced the bioprosthetic valve and in patients with RF in clinical treatment. In patients that underwent native valve replacement, there were low levels of IL-4, and in the control group, production of this interleukin was insignificant, as expected. There were large variations in the data concerning IL-4, leading to a non-significant *P*-value. Guilherme et al. [6,9] showed that lower production of IL-4 by the infiltrating cells of valvular tissue can lead to persistence and progression of rheumatic valvular disease. There are more cells producing IL-4 in the myocardium, hence, rheumatic myocarditis healing occurs after a few weeks [9]. There were higher levels of IL-10 in patients who have replaced the native mitral valve and in patients with RF without surgical treatment. Their levels were decreased in the control group, as expected. Since the IL-10 is an anti-inflammatory cytokine, results show the immune response to control the inflammatory process that triggers valvular lesions [12].

Situating endothelin-1 in rheumatic fever, several studies have reported high serum levels of this peptide in patients with rheumatic disease, associated with mitogenesis, fibrosis and inflammatory activity [14]. Chen et al. [21] reported increased serum levels of endothelin-1 in patients with rheumatic mitral stenosis. In our study, there is gene expression of endothelin-1 in damaged heart valves in patients that underwent mitral valve replacement, resembling those seen in other samples of the Brazilian population. In this sense, Moura et al. [1] found that 40.7% of mitral valves (fibrosed and stenosed) replaced in patients with RF presented gene expression of ET-1 and, in our previous study [22], we observed expression of both endothelin receptors (ETrA and ETrB) in replaced rheumatic mitral valves.

Chang [12] showed that TNF-alpha induces the increase of ET-1mRNA expression. In another study, Patel et al. [23] reported that TNF stimulates the release of endothelin-1 and its vasoconstrictor activity. This finding was confirmed by Wagner [24], who exposed endothelial cell cultures to high concentrations of TNF, finding a considerable increase of the secretion of ET-1 accompanied by a correspondingly increase in the levels of the pre-pro-ET-1 mRNA transcription. Despite the connection between production of TNF-alpha and endothelin-1, in this study, we did not find a difference between the levels of TNF-alpha in the different groups. Moreover, a limitation of this study was the small sample

size, which may have been responsible for the lack of statistical significance in some comparisons.

We conclude that inflammatory cytokine IL-10 participates in maintaining the process of chronicity of RF in patients that underwent valve replacement and in those who are undergoing medical treatment. Additionally, the presence of gene expression of endothelin-1 was observed in most of the valvular fragments studied.

Authors' roles & responsibilities	
SCL	Translation and editing of the article
MRML	Data collection; original writing of the article and final revision of the article
HMN	Data analysis
SOS	Data collection and analysis
TMAR	Original writing and final revision of the manuscript

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