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# Gene expression of endothelin receptors in replaced rheumatic mitral stenotic valves

***Expressão gênica de receptores de endotelina em valvas mitrais reumáticas estenóticas substituídas***

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*Abstract*

***Objectives:*** **Rheumatic fever is a highly prevalent disease in Brazil, and it poses a major public health problem. It is the leading cause of acquired heart disease in childhood and adolescence. The aim of this study was to evaluate the gene expression of ET-3 and its receptors, in replaced rheumatic mitral valves.**

***Methods:*** **We studied the gene expression of endothelin-3 (ET-3) and its receptors, endothelin receptor A and endothelin receptor B (ETr-A and ETr-B), in the rheumatic mitral valves of 17 patients who underwent valve replacement surgery. The samples also underwent a histological analysis.**

***Results:*** **Our data showed that almost all patients, regardless of individual characteristics such as gender or age, expressed the endothelin receptor genes, but did not express the genes for ET-3. In quantitative analysis, the ETr-A/GAPDH mean ratio was 33.04 ± 18.09%; while the ETr-B/GAPDH mean ratio was 114.58 ± 42.30%. Regarding histopathological individual features, the frequency of fibrosis is 100%, 88.23% of mononuclear infiltrate, 52.94% of neovascularization, 58.82% of calcification and absence of ossification.**

***Conclusion:*** **The presence of receptors ETr-A and ETr-B in rheumatic mitral valves suggests its interaction with the system of circulating endothelins, particularly ETr-B (known for acting in the removal of excess endothelin) detected in a greater proportion, which could explain the lack of expression of endothelin in rheumatic mitral valve, process to be elucidated.**

***Descriptors:*** **Rheumatic fever. Mitral valve stenosis. Endothelins.**

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| Abbreviations, Acronyms & Symbols | |
|---|---|
| bFGF | Basic fibroblast growth factor |
| ET-3 | Endothelin type 3 |
| ETr-A | Endothelins receptors type A |
| ETr-B | Endothelins receptors type B |
| ETr-C | Endothelins receptors type C |
| ETrs | Endothelins receptors |
| ETs | Endothelins |
| LAM | Laboratory of Molecular Anatomy |
| NO | Nitric oxide |
| PASP | Pulmonary artery systolic pressure |
| RF | Rheumatic fever |
| RT-PCR | Reverse transcription polymerase chain reaction |
| UFS | Universidade Federal de Sergipe |
| VEGF | Vascular endothelial growth factor |

***Resumo***

***Objetivos:*** **A febre reumática é uma doença altamente prevalente no Brasil, e representa um importante problema de saúde pública. É a principal causa de cardiopatia adquirida na infância e adolescência. O objetivo deste estudo foi avaliar a expressão gênica de ET-3 e seus receptores, em valvas mitrais reumáticas substituídas. Métodos: Estudamos a expressão gênica de endotelina-3 (ET-3) e de seus receptores, receptor da endotelina A e receptor da endotelina B (ETr-A e ETr-B), nas valvas mitrais reumáticas de 17 pacientes que se submeteram à cirurgia de troca valvar. As amostras também foram submetidas à análise histológica.**

***Resultados:*** **Nossos dados mostraram que praticamente todos os pacientes, independentemente de características individuais, como sexo ou idade, expressaram os genes de receptores de endotelina, porém não expressaram os genes para ET-3. Na análise quantitativa, a média da proporção ETr-A/GAPDH foi de 33,04 ± 18,09%; enquanto que a média da proporção ETr-B/GAPDH foi de 114,58 ± 42,30%. Em relação às características histopatológicas individuais, a frequência de fibrose foi de 100%, infiltrado mononuclear de 88,23%, neovascularização de 52,94%, calcificação de 58,82% e houve ausência de ossificação.**

***Conclusão:*** **A presença de receptores ETr-A e ETr-B em valvas mitrais reumáticas sugere sua interação com o sistema de endotelinas circulantes, particularmente ETr-B (reconhecido por atuar na remoção do excesso de endotelina), detectado em maior proporção, o que poderia explicar a ausência da expressão de endotelina em valva mitral reumática, processo a ser elucidado.**

***Descritores:*** **Febre reumática. Estenose da valva mitral. Endotelinas.**

## INTRODUCTION

Rheumatic fever (RF) is a serious public health problem and a strong indicator of poverty and poor health services in developing countries. It is a rheumatic and inflammatory disease with an autoimmune origin. It recurs in response to Streptococcus pyogenes (Group A beta-hemolytic Strep, GAS) infection. This agent is responsible for approximately 15.6 million cases of rheumatic heart disease annually across the globe, with 282,000 new cases and 233,000 deaths each year. As a result, health systems incur high costs, paying for the tests, surgeries and hospitalizations required to treat the complications of this condition. For instance, approximately 3 million patients per year are hospitalized due to congestive heart failure [1-4].We can divide the manifestations of rheumatic fever in acute and chronic. Acute rheumatic fever affects several sites such as the skin (erythema marginatum), the basal ganglia (chorea of Syndeham) and heart (rheumatic carditis). The involvement of cardiac valves (especially mitral valve) is extremely common in chronic rheumatic heart disease.

There are two types of valve dysfunction on rheumatic disease: stenosis and insufficiency. These two types of dysfunction are not mutually exclusive. A patient with mitral stenosis may remain asymptomatic for long periods of time despite the gradual decrease in cardiac output and increase in pulmonary vascular resistance, which may eventually lead to a vascular morphofunctional change [5-7]. The symptoms usually depend on the effective valve area involved and the tissue damage level (obtained via a regular echocardiogram), although this correlation is not always reliable. In advanced disease, all of these events result from inflammatory damage to the tissue that is accompanied by neovascularization and the calcification of the mitral apparatus, which was formerly an avascular structure [8].

Increased peripheral vascular resistance is a key event in the development of heart failure, and endothelin is one of the most potent vasoconstrictors involved in this disease [9], and stimulates the secretion also of inflammatory cytokines [10]. The endothelins (ETs) are potent peptides formed by a chain of 21 amino acids. Some of their properties are well known, such as vascular tone control in both vascular and non-vascular tissues. Currently, there are three identified isopeptides encoded by three different genes: ET-1, ET-2 and ET-3. The pharmacological effects of ETs indicate the existence of three subtypes of receptors

(ETrs): type A (ETr-A), type B (ETr-B) and type C (ETr-C) [11]. ETs and ETrs exhibit different affinities, and ETr-A acts as a vasoconstrictor in the smooth muscle layer (*tunica media*) of the arterial wall [12].

The importance of ETs and its receptors for the pathogenesis of many diseases has been the subject of intense research since its discovery [13-15]. Those diseases that involve excessive vasoconstriction and cell proliferation have been an especial focus. Over the last decade, a growing volume of research has been conducted on the actions of endothelin and its receptors, exploring its unique pharmacological response and its possible correlation with cardiovascular disease [16]. The correlation between the role of endothelin in the cardiovascular system and its pro-inflammatory activity has made it important to determine whether ETs participates in the pathophysiology of heart injury. However, severe pulmonary hypertension, either as a final outcome of those diseases that involve excessive vasoconstriction and cell proliferation or as perpetuating them, may also affect the influence of ETs [17].

Therefore, in this study we evaluated the expression of ET-3 and its receptors, ETr-A and ETr-B, in the rheumatic mitral valves of patients who underwent valve replacement surgery. Moreover, histological analysis in the valves was performed.

## METHODS

The University Hospital Ethics and Human Research Committee of the Federal University of Sergipe approved this project, which was assigned the number 0105.0.107.000-09. In addition, all experimental protocols were conducted according to Declaration of Helsinki and had signed the Informed Consent form. A histological analysis of the mitral valves was performed. The study group comprised seventeen patients (mean age 37.7 ± 13.7 years) with serious rheumatic mitral stenosis (mean valve area of 1.0 ± 0.28 $cm^2$ and mean pulmonary artery systolic pressure (PASP) of 45.82 ± 6.45 mmHg) (Table 1) who underwent surgical treatment between the months of June 2009 and March 2010. These patients were six male and eleven female adults. The most common clinical manifestations of them were dyspnea (94.11%), chest pain (35.29%), and tachypnea (47.05%) (Table 1).

Seventeen valves were collected from the Cardiothoracic Surgery Service at Cirurgia Hospital in Aracaju, Sergipe, Brazil. After the valves had been collected, samples were sent to the Laboratory of Molecular Anatomy (LAM) at the Morphology Department, Sergipe Federal University (Universidade Federal de Sergipe, UFS), where they were stored and subjected to a molecular and histological analysis.

Table 1. Echocardiographic data of patients.

| | Gender | Age | Dyspnea | Chest Pain | Tachypnea | Mitral Valve Area | PASP |
|---|---|---|---|---|---|---|---|
| LAM01 | F | 32 | x | 0 | x | 0.8 | 47 |
| LAM02 | M | 64 | x | x | 0 | 1.5 | 35 |
| LAM03 | F | 22 | x | 0 | x | 0.6 | 54 |
| LAM04 | F | 59 | x | x | 0 | 1.3 | 39 |
| LAM05 | F | 20 | x | 0 | x | 0.9 | 47 |
| LAM06 | F | 45 | x | 0 | 0 | 0.7 | 50 |
| LAM07 | F | 42 | x | 0 | 0 | 0.6 | 54 |
| LAM08 | M | 43 | x | x | x | 1 | 46 |
| LAM09 | M | 56 | 0 | 0 | 0 | 0.9 | 49 |
| LAM10 | M | 40 | x | x | 0 | 1.4 | 38 |
| LAM11 | F | 24 | x | 0 | x | 0.9 | 52 |
| LAM12 | M | 41 | x | 0 | 0 | 0.8 | 50 |
| LAM13 | F | 22 | x | 0 | x | 1.2 | 42 |
| LAM14 | F | 45 | x | x | 0 | 0.8 | 55 |
| LAM15 | F | 24 | x | 0 | x | 1.4 | 36 |
| LAM16 | F | 26 | x | 0 | 0 | 1 | 44 |
| LAM17 | M | 36 | x | x | x | 1.2 | 41 |
| Mean | | 37.70588 | | | | 1 | 45.82353 |
| Std Dev | | 13.70112 | | | | 0.285043856 | 6.454023 |

*F = female; M = male; PASP = Pulmonary Artery Systolic Pressure; Std Dev = standard deviation.*
*X represents presence of characteristic and 0 represents absence of it*

**Molecular analysis**

Immediately after removal, valve tissue obtained was fragmented into three roughly equal segments (named M1, M2 and M3), the first near the mitral annulus, second in the intermediate region and the third at the end in contact with the chordae tendineae. This division intended differentiation in regions that are macroscopically distinct as to their involvement by rheumatic disease. After that, each sample valve was submerged in RNAlater® stabilizer solution (Applied Biosystems/Ambion, Austin, Texas, USA) for 24 hours at room temperature (25°C). Next, the excess solution was discarded, and the samples were stored at -80°C. The mitral fragments (100 mg) were submitted to a total RNA extraction protocol using the SV RNA Purification Kit (Promega®, Madison, Wisconsin, USA). Then, each sample of total RNA was quantified by spectrophotometry (UV-1601-UV® Spectrophotometer Shimadzu Corporation). For this purpose, it was performed dilution of 4 µL de RNA in 196 µL of Milli-Q water totalizing 200 µL of solution. The samples were placed in an appropriate cuvette and then analyzed on the spectrophotometer. The absorbance values obtained were analyzed using the following formula: [RNA (µg/mL)] = 40 x A260 x dilution / 1000 [18]. A purity assessment was performed using the ratio of the absorbance values obtained at 260 nm and 280 nm (A260/A280); only values between 1.6 and 2.6 were considered viable.

The total cDNA was obtained from each analyzed sample via reverse transcription polymerase chain reaction (RT-PCR) analysis. We used the protocol provided by the manufacturer of the Reverse Transcriptase IMPROM-II™ Kit (Promega®, Madison, Wisconsin, USA). From the cDNAs obtained, we amplified the target fragment using PCR [19,20]. The primers used in this technique were ET-3 (Endothelin 3), ETr-A (receptor type A) and ETr-B (Receptor Type B) (Table 2). The constitutive gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was analyzed as a sample control (Table 2). The programs used for amplification were optimized using different annealing temperature combinations in accordance with mean temperature data supplied by Promega®. The amplification products were analyzed via electrophoresis on 2% agarose gel stained with ethidium bromide (1 mg/ml) and sample buffer (bromophenol blue) 6x. They were viewed using the photo documentation system (Kodak Gel Logic 100® Imaging System, Eastman Kodak Corporation, Rochester, NY, USA). Quantitative analysis of each sample was performed with software ImageJ (National Institute of Health, Bethesda, MD, USA). Quantification was performed by counting the average number of pixels of each sample (including GAPDH sample). Thereafter, it was performed a ratio between the average number of pixels in each sample by the amount found in GAPDH sample (ETr-A/GAPDH and ETr-B/GAPDH ratio).

Table 2. List of primers prepared for molecular analysis.

| Name of Oligonucleotide | Sequency |
|---|---|
| ET-3 Sense | 5' CCA AAC TCT GGA CGT CAG CAG 3' |
| ET-3 Antisense | 5' ATT TCC TGC ATG AAA CCG GAG 3' |
| ETr-A Sense | 5' TTC AGA CTT CGC CAG ACA GA 3' |
| ETr-A Antisense | 5' CAA GCA ACT GGA ACC TGA TGT 3' |
| ETr-B Sense | 5' AGA CAG GAC GGC AGG ATC T 3' |
| ETr-B Antisense | 5' GAA CAC AAG GCA GGA CAC AA 3' |
| GAPDH HUMAN Sense | 5' GCT CTC TGC TCC TCC TGT TC 3' |
| GAPDH HUMAN Antisense | 5' GTT GAC TCC GAC CTT CAC CT 3' |

**Histological analysis**

All of the excised, fragmented valves were formalin fixed in a neutral 10% solution (pH 7.0). They were then submitted to decalcification, embedded in paraffin and cut using a microtome (Hacker Edge SL-200® Microtome, Winnsboro, USA) at a 4 µm thickness. Hematoxylin and eosin staining was then performed. Each segment was examined for the presence of fibrosis, calcification, ossification, angiogenesis and mononuclear cells.

**Statistical analysis**

For statistical analysis, it was perfomed measures of central tendency and variance. We also examined the correlation between the results obtained for the mean ratio ETr-A/GAPDH and ETr-B/GAPDH. For correlation analysis, we used Pearson's correlation, with the significance level of 5% ($P$ value< 0.05).

## RESULTS

Regarding RNA extraction of mitral valves, the average concentration of nucleic acid was 7.20 ± 5.60 ng/µl. The absorbance value obtained at 260 nm was 0.18 ± 0.14 and absorbance at 280 nm was 0.09±0.06. Ratio between A260 and A280 (A260/280) was 1.81 ± 0.16 (Table 3). The expression of endothelin and its receptors in the mitral valves using the PCR of the cDNA is showed in the Figure 1. As shown in the Figure 1A, there was amplification of the 480 bp fragment corresponding with the expected ET-3 amplicon in only one sample. Figures 1B and 1C show the results for the ETr-A and ETr-B primers, respectively. Interestingly, we observed that for ETr-A and ETr-B,

Table 3. Quantification of total RNA and spectophotometry from mitral valves.

| Sample | Nucleic Acid Conc. (ng/µl) | A260 | A280 | A260/280 |
|---|---|---|---|---|
| LAM01 | 3.9 | 0.099 | 0.061 | 1.61 |
| LAM02 | 7 | 0.175 | 0.094 | 1.86 |
| LAM03 | 10.2 | 0.254 | 0.138 | 1.84 |
| LAM04 | 2.9 | 0.073 | 0.04 | 1.8 |
| LAM05 | 17.3 | 0.434 | 0.207 | 2.09 |
| LAM06 | 13.6 | 0.339 | 0.162 | 2.08 |
| LAM07 | 4.5 | 0.112 | 0.067 | 1.66 |
| LAM08 | 14.4 | 0.359 | 0.172 | 2.09 |
| LAM09 | 4 | 0.1 | 0.058 | 1.72 |
| LAM10 | 1.2 | 0.029 | 0.016 | 1.78 |
| LAM11 | 1.9 | 0.048 | 0.026 | 1.85 |
| LAM12 | 10.1 | 0.253 | 0.141 | 1.79 |
| LAM13 | 7.3 | 0.181 | 0.101 | 1.8 |
| LAM14 | 17.4 | 0.436 | 0.217 | 2.01 |
| LAM15 | 1.9 | 0.047 | 0.029 | 1.62 |
| LAM16 | 1.6 | 0.041 | 0.024 | 1.67 |
| LAM17 | 3.3 | 0.082 | 0.052 | 1.57 |
| Mean | 7.205882353 | 0.180117647 | 0.094412 | 1.814118 |
| Std. Dev | 5.601168471 | 0.140106603 | 0.066344 | 0.169524 |

*Std Dev = standard deviation*

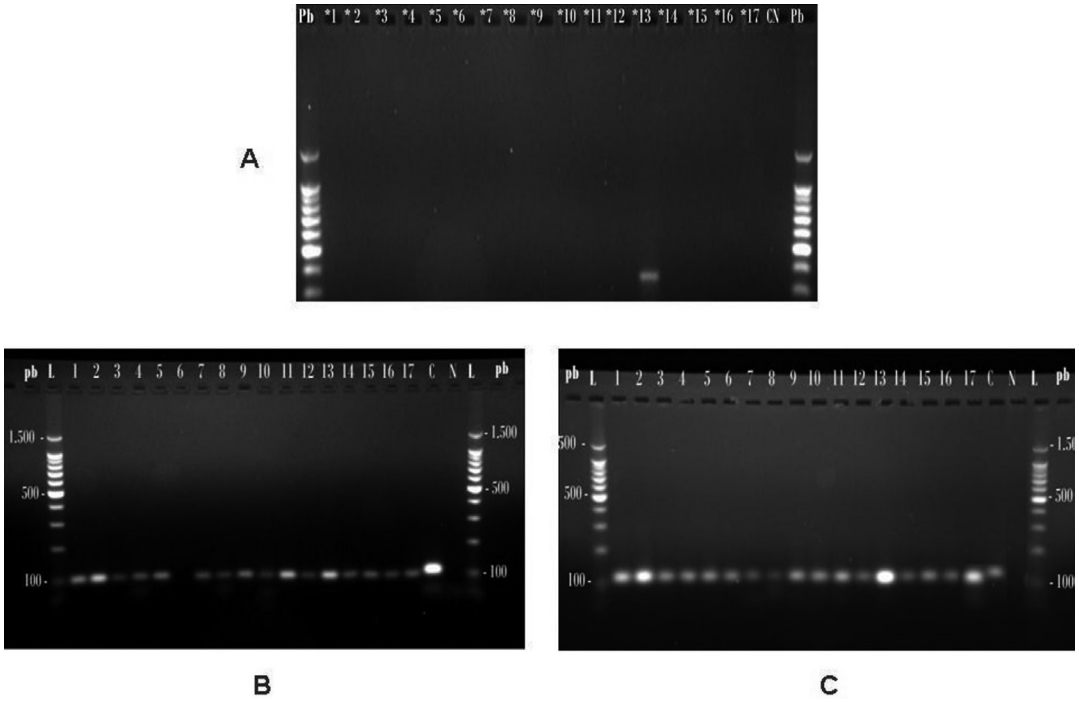


*Fig. 1 - Amplification of the ET-3, ETR-A and ETR-B encoding genes - Total RNA from 17 valve samples was reverse-transcribed to obtain cDNA and subsequent PCR using our primers for amplification of the genes analyzed. Figure 1A: ET-3; 1B: ETR-A; 1C: ETR-B*

Table 4. Densitometric analysis of the amounts of the amplified ETr-A and ETr-B genes after normalization with the GAPDH gene (ETr-A/GAPDH and ETr-B/GAPDH ratio).

| Number | ETr-A/GAPDH | ETr-B/GAPDH |
|---|---|---|
| LAM01 | 44.94% | 136.47% |
| LAM02 | 69.83% | 200.24% |
| LAM03 | 18.90% | 113.79% |
| LAM04 | 28.57% | 107.22% |
| LAM05 | 36.06% | 116.40% |
| LAM06 | 0.00% | 98.45% |
| LAM07 | 24.93% | 72.11% |
| LAM08 | 19.80% | 59.86% |
| LAM09 | 38.89% | 109.75% |
| LAM10 | 19.91% | 115.43% |
| LAM11 | 61.05% | 113.94% |
| LAM12 | 21.34% | 83.15% |
| LAM13 | 63.41% | 219.42% |
| LAM14 | 27.54% | 77.93% |
| LAM15 | 32.67% | 98.89% |
| LAM16 | 23.36% | 80.09% |
| LAM17 | 30.47% | 144.82% |
| Mean | 33.04% | 114.58% |
| Std. Dev | 18.09% | 42.30% |

*Std Dev = standard deviation*

the amplicons were present in 94.11% and 100% of the reactions, respectively, with different degrees of intensity. All experiments were performed in duplicate to confirm the reliability of the data obtained using this technique. In quantitative analysis, the ETr-A/GAPDH mean ratio is 33.04 ± 18.09%; while the ETr-B/GAPDH mean ratio is 114.58 ± 42.30% (Table 4). Pearson's correlation (R) between ETr-A/GAPDH and ETr-B/GAPDH of each sample is 0.73 (P= 0.0004), which is strongly positive.

In histological analysis on mitral valves, there were enormous amounts of fibrocytes, dense connective tissue, type I collagen (eosinophilic) and extracellular ground substance. The inflammatory process exhibited high cellularity, tissue vascularization with permeating capillaries, and transformed or neoformed collagen (Figure 2). It was also possible to observe fibroblasts, lymphocytes, and dystrophic calcification areas (tissue necrosis with deposits). Regarding histopathological individual features, fibrosis is found in all of the 17 samples (100%), mononuclear infiltrate in 15 samples (88.23%), neovascularization in nine samples (52.94%), calcification in ten samples (58.82%) and ossification in any sample (Table 5).

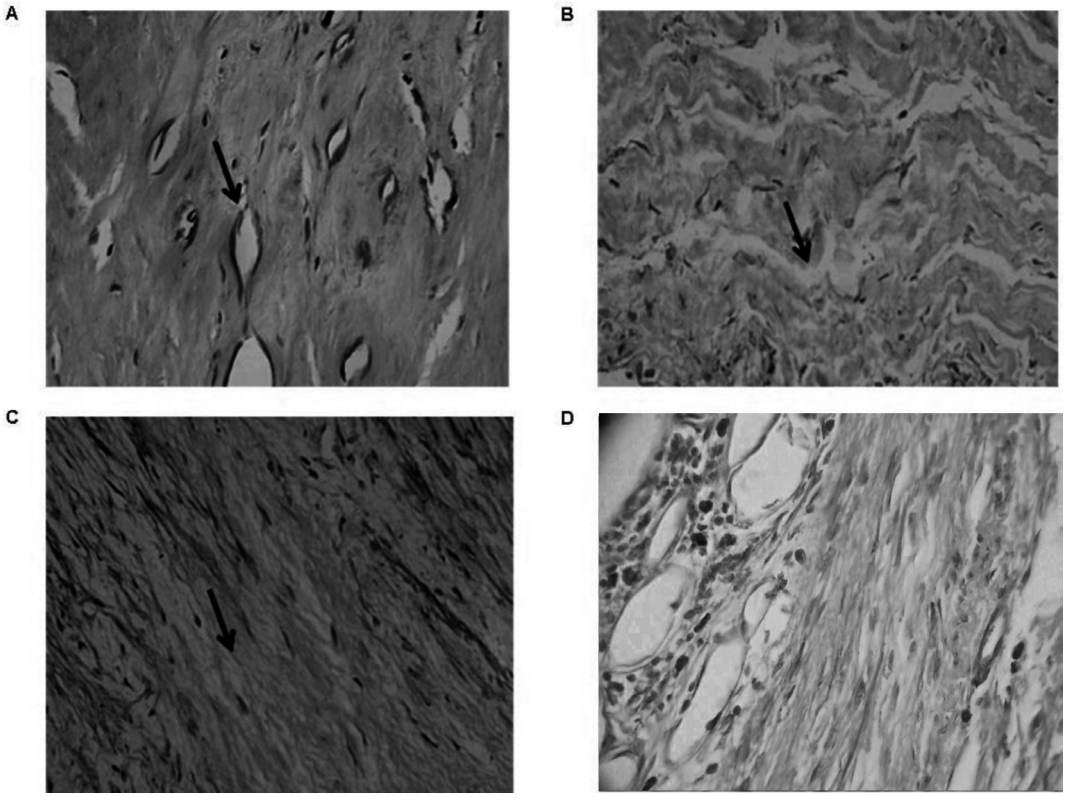


*Fig. 2 - Rheumatic mitral valves present neovascularization and an increase of mononuclear infiltrates - Longitudinal cuts were done and then fixed and stained with hematoxylin and eosin (HE) and analyzed by optical microscopy (40x). A: The arrows indicate neovascularization and tissue breakdown of collagen fibers. B and C: The arrows indicate the presence of fibroblasts. D: Photomicrograph showing inflammatory infiltrate, fibrosis and some neoformed vessels.*

Table 5. Histopathological individual features of 17 rheumatic mitral valves.

| | Fibrosis | Infiltrate | Neovascularization | Calcification | Ossification |
|---|---|---|---|---|---|
| LAM01 | x | x | x | x | 0 |
| LAM02 | x | x | 0 | x | 0 |
| LAM03 | x | x | x | 0 | 0 |
| LAM04 | x | 0 | 0 | x | 0 |
| LAM05 | x | x | x | 0 | 0 |
| LAM06 | x | x | x | x | 0 |
| LAM07 | x | x | x | 0 | 0 |
| LAM08 | x | x | x | x | 0 |
| LAM09 | x | x | 0 | x | 0 |
| LAM10 | x | x | x | x | 0 |
| LAM11 | x | x | 0 | x | 0 |
| LAM12 | x | x | 0 | 0 | 0 |
| LAM13 | x | x | x | 0 | 0 |
| LAM14 | x | 0 | 0 | 0 | 0 |
| LAM15 | x | x | 0 | x | 0 |
| LAM16 | x | x | x | 0 | 0 |
| LAM17 | x | x | 0 | x | 0 |

*X represents presence of characteristic and 0 represents absence of it*

## DISCUSSION

Our data suggest that the expression of ET-3 has no significant relation to rheumatic mitral valve disease in situ, because the amplification of the corresponding fragment was not visible in the gel. We also visualized the amplification of the ETr-A and ETr-B gene fragments in almost 100% of the samples. Moura et al. [21], studying 37 mitral valves, demonstrated that endothelin receptors (ETr-A and ETr-B) were present in this type of human tissue. However there were differences in the intensity of the amplified bands, which suggests that the receptors displayed different expression levels. The presence of ETr-A in the samples is interesting result but expected because in addition to being the predominant receptor in cardiac myocytes, this receptor is involved in inflammatory processes, mitogenesis and pathological vasoconstriction which are exuberant in rheumatic disease during the exudative and proliferative stages [16,21].

On the other hand, the presence of ETr-B in the samples in a higher proportion than ETr-A (114.58 ± 42.30% *vs*. 33.04 ± 18.09%) is not expected, because this receptor presents vasodilating properties, mediated by the release of nitric oxide (NO) and prostacyclin, which inhibits production of endothelin [16]. Beside vasodilator function, ETr-B, has an important role in removing excess of endothelin, being responsible for the maintenance of normal plasma levels of this peptide [22,23]. This paradoxical behavior of endothelin receptors is also found in some pathological conditions, such as chronic heart failure and myocardial ischemia [22].

Endothelin has some influence on histopathological features encountered in our seventeen mitral valves, such as neoangiogenesis (through the expression of ETR-A receptors that lead to increased vascular endothelial growth factor - VEGF) and calcification (by increasing the gene expression of osteocalcin and osteopontin) [24,25]. Regarding fibrosis, endothelin stimulates type I collagen production, inhibition of collagenase activity and abnormal production of extracellular matrix promoting a reactive fibrosis [25-27]. This mechanism can be mediated for basic fibroblast growth factor (bFGF), which up-regulates the expression of ETr-A and perhaps of ETr-B [22]. Endothelin also actives neutrophils, mast cells and stimulates monocytes to release some cytokines, such as TGF-beta and TNF-alfa [22].

The present data are preliminary but may be of great value because they may form the foundation for further investigation in this area, especially given the scarcity of studies of endothelins and its receptors in the context of rheumatic valve disease. In this study, the different patients may have different levels of intensity of expression of these receptors because they were experiencing different stages of rheumatic valve disease. Histological data are important indicators of the degree of valve involvement and suggest an interaction between neovasculogenesis and inflammatory molecular events. The presence of receptors ETr-A and ETr-B in rheumatic mitral valve suggests their interaction with the system circulating endothelins, particularly ETr-B (known for acting in the removal of excess endothelin), detected in a greater proportion, which could explain the absence of expression of endothelin in rheumatic mitral valve, process to be elucidated.

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