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# Paroxetine Alters Cardiac Stress Markers in Rats with Aortic Regurgitation

**Ana Carolina Mieko Omoto[1], Leonardo Nazario Moraes[2], Geysson Javier Fernandez Garcia[2], Ivan José Vechetti-Júnior[2], Meliza Goi Roscani[3], Robson Francisco Carvalho[2] and Juliana Irani Fratucci De Gobbi[1*]**

[1]Department of Physiology, Institute of Bioscience of Botucatu, São Paulo State University, SP, Brazil

[2]Department of Morphology, Institute of Bioscience of Botucatu, São Paulo State University, SP, Brazil

[3]Department of Medicine, Federal University of São C arlos, SP, Brazil

[*]**Corresponding author:** Juliana Irani Fratucci De Gobbi, Department of Physiology, Institute of Bioscience of Botucatu, São Paulo State University, SP, Brazil, Tel: 55 14 38800319; E-mail: jdegobbi@ibb.unesp.br

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## Abstract

**Background:** Aortic regurgitation (AR) is a valvulopathy that causes volume overload to the heart leading to left ventricle dilation and eccentric hypertrophy. A common co-morbidity associated with cardiovascular disease is depression. We have previously shown that paroxetine, a selective serotonin re-uptake inhibitor widely prescribed as antidepressant, improves cardiac contractility by preserving fractional shortening (FS) of AR rat hearts.

In failing hearts, decrease of the ability of the myocardium to generate an effective shortness velocity is well correlated with the shift of myosin isoform distribution from α to β-MyHC expression. In order to understand the molecular mechanism involved in the improvement of FS after paroxetine treatment, we verified the expression of genes involved in heart contractility and hypertrophy.

**Methods and Findings:** Male Wistar rats were submitted to AR surgery, by retrograde puncture of the aortic valves leaflets, or sham surgery. Morphofunctional variables of the hearts were analyzed by echocardiograms at weeks 4 and 8 after the induction of AR.

At week 8 the animals were euthanized for tissue collection and posterior analysis of gene expression by RTq-PCR. Paroxetine treatment for 4 weeks in AR rats reduced the gene expression of β-MyHC and its myomiRs (miR-208 and miR-499). BNP, a molecular biomarker of hypertrophy, also showed their gene expression reduction after paroxetine treatment.

**Conclusion:** These results suggest that paroxetine treatment improves FS in AR hearts through reductions in the expression of several genes involved with cardiac contractility and hypertrophy.

**Keywords:** Volume overload; Paroxetine; Fractional shortening; Cardiac myosins; MicroRNAs

## Introduction

Cardiovascular diseases (CVDs) are responsible for millions of deaths worldwide [1]. Among these CVDs, aortic regurgitation (AR) continues to increase in underdeveloped countries as a consequence of rheumatic heart disease whereas in developed countries AR incidence is rising due to the increase in life expectancy [2-4]. AR is characterized by a diastolic reflux of the blood from the aorta to the left ventricle (LV) leading to a volume overload and eccentric hypertrophy, which is associated with a long asymptomatic period when the cardiovascular system starts a series of compensatory mechanisms [5-8].

There is no approved effective treatment to slow LV dilation, hypertrophy and loss of function in chronic AR [9,10]. Medical therapy with β1 adrenergic antagonists and angiotensin-converting enzyme inhibitors, such as atenolol and captopril, has been proposed because of its beneficial effects in avoiding LV hypertrophy and improving cardiac metabolism [6,8].

A bidirectional association between CVDs and depression is well established and the nature of this relationship seems to share a common pathophysiology and risk factors [11-13]. Several neurotransmitters are dysregulated in depressive states and the serotonin system, is the main one [14]. Selective serotonin reuptake inhibitors (SSRI) are among the most prescribed antidepressants and are safe to use in patients with CVD reducing the probability of arrhythmias and platelet aggregation [15-17]. It has been shown that treatment with an SSRI drug, paroxetine (Parox), in rats with subchronic AR preserved the FS of the heart and reduced sodium intake of these animals [18].

Decrease in the ability of the heart to generate an effective FS is one of the major problems in CVDs [1,6,9]. Studies conducted

to verify the influence of myosin-isoforms content in the control of cardiac muscle shortening demonstrated a linear correlation between the maximal speed of shortening and the ratio of α and β myosin heavy chain (MyHC) isoform expression [19-22]. During pathological hypertrophy mediated by pressure and volume overload a shift in the myosin isoform distribution occurs with an induction of β-MyHC at the expense of α-MyHC [21-23].

MyHC switching from α- to β-MyHC is controlled by post-transcriptional factors, miRNAs (microRNAs), which inhibit mRNA translation or promote mRNA degradation [24,25]. miR-208a, miR-208b and miR-499, also referred as MyomiRs, have the potential to indirectly control the expression of β-MyHC isoform gene by inhibiting validated transcriptional repressors of β-MyHC gene (myomiR's targets): Sox6, PurB and Thrap1 [24].

Together these myomiRs and transcriptional repressors give rise to a complex network that controls MyHC isoforms gene expression. Based on these studies we hypothesized that MyHC isoform ratio could be changed in AR hearts in order to to produce more of the slow isoform (β-MyHC) and Parox improves cardiac contractility by downregulating this isoform with both myomiRs and transcriptional repressors playing a role.

Another important question that we investigated is how the gene expression of ANP and BNP would be after Parox, once these peptides are involved in the pathophysiology of cardiac hypertrophies and their plasma levels are associated with cardiac patient's survival [26,27]. At molecular level, ANP and BNP are considered markers of pathological hypertrophy as well as α-skeletal actin [28]. In addition, the gene expression of both ANP and BNP is increased in the left ventricle of rat hearts with pressure and volume overload [29].

The purpose of this study is to understand the molecular mechanisms by which Parox improves the mechanical performance of AR rat hearts analyzing the gene expression of contractile proteins, MyomiRs, transcriptional repressors and the molecular markers of hypertrophy (ANP, BNP and α-skeletal actin).

## Methods

### AR and sham surgery

Male Wistar rats (280-300g) were used for AR or s ham surgery. Rats were anesthetized with intraperitonial injections of ketamine and xylazine (80 mg/kg and 20 mg/kg, respectively) and had the right internal carotid artery isolated and catheterized. Perforations in the aortic valve leaflets were made by retrograde puncture with a steel wire guided by the catheter [similar to 5,18]. All procedures were approved by the Institute of Biosciences Ethics Committee, São Paulo State University, Botucatu, SP, Brazil (protocol#454-CEUA)

### Paroxetine administration

Paroxetine chloride (10mg/kg of body weight, Pharma Nostra, Brazil) was dissolved in saline solution and administered subcutaneously three times a week for 4 weeks. The treatment started 4 weeks after the surgeries and continued until week 8. The study was divided into 4 groups: AR+Parox, AR+Saline, Sham +Parox and Sham+Saline.

### Echocardiogram

M-mode, 2D and Doppler echocardiogram (ECHO) was performed in the animals under anesthesia (ketamine 8 mg/kg and xilazyne 20 mg/kg) using a 11-Mhz probe of GE 6S echography to confirm the presence and severity of AR at week 1. The ECHOs exams were repeated for collecting morphofunctional variables at weeks 4 and 8. The criteria inclusion of the AR animals in the study groups was a ratio of regurgitate jet width to LV outflow diameter ≥ 50% of a retrograde holo-diastolic flow in the proximal descending aorta.

### Tissue collection

Rats were anesthetized with Sodium Thiopental (75 mg/rat; Cristália, Brazil) and had the heart perfused with phosphate buffered saline 0.1 M (pH-7.4, ~ 250 ml/rat) to remove possible contamination of blood residues in the samples. Following withdrawal of the hearts, they were weighed and the LV was isolated and kept at -80ºC until the molecular analyses were performed.

### RNA preparations

Total RNA was isolated using TRIzol reagent (Life Technologies, USA) as described by the manufacturer. Total RNA was solubilized in nuclease free-water and treated with DNA-free kit to eliminate genomic DNA contamination (Life Technologies, USA). Total RNA quantity was determined by the absorbance at 260 nm using nanoVue spectrophotometer (GE healthcare, USA) and the RNA purity was assessed by the A 260 nm/A 280 nm and A 260 nm/A 230 nm ratios (acceptable when both ratios were >1.8). RNA Integrity Number (RIN) was evaluated by using the RNA 6000 Nano kit (Agilent Technologies, USA) with the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). All RNA samples presented a RIN >8.

### Quantitative analyses of gene expression by real-time reverse transcription polymerase chain reaction (RT-qPCR)

The microRNAs miR-208a, -208b, and -499 were reversely transcribed using High Capacity RNA-to-cDNA master mix (Life Technologies, USA) and the quantitative PCR (qPCR) for the mature miRNAs analyses were performed with TaqMan® Master Mix (Life Technologies, USA), as described by the manufacturer.

The reactions were run on the Quantstudio™ 12K Flex Real Time PCR System (Applied Biosystems, USA). The raw data from each experiment was retrieved and imported into Expression Suite Software v1.0.3 (Life Technologies, USA). The small RNAs Y1 was selected as reference control gene to normalize the miRNA data based on previous analyses according to geNorm calculations [30], Relative quantification of miRNA expression was evaluated using the comparative quantification method [31].

Relative quantification of mRNA expression by RT-qPCR was carried out with GoTaq® qPCR Master Mix (Promega, USA), using specif ic primers (Data supplements) and cDNA of each sample of all groups, according to the manufacturer's instructions. Reactions were set up in a total volume of 20 µL and performe d in the Quantstudio™ 12K Flex Real Time PCR System. Relative quantification of mRNA expression by SYBR green I was assessed by using REST software 2009 v2.0.13, and the pair-wise fixed randomization test with 10,000 permutations [32], with PCR efficiency calculated by linear regression from fluorescence increase in the exponential phase in the program LinRegPCR v11.1 [33]. The genes were selected as reference control to normalize the miRNA data and genes Rplp0 and Ppib to further normalize mRNA data based on previous geNorm calculations [30].

### Statistical analysis

All graphs represent mean values ± SD. Echocardiographic data was compared by two-way ANOVA. The gene expression changes were compared using two-way ANOVA for repeated measures, with significance of $p \leq 0.05$, and Student-Newman-Keuls post-test.

## Results

### Paroxetine improved heart contractility in AR

Morphometric and functional parameters of the heart at weeks 4 and 8 are shown in **Tables 1 and 2**, respectively. AR groups after week 4 present a significant increase in left ventricle diastolic diameter (LVDD), sphericity index (SI), cardiac output (CO), left atrium systolic diameter (LA/Ao) and a decrease in fractional shortening (FS) compared with Sham groups. It is worth mentioning that AR groups showed similar parameters before the beginning of Parox. At the end of the protocol (week 8), there was no difference in body weight (BW) for all groups; LVDD, SI, CO and LA/Ao remained high in AR groups compared with sham as well as the heart weight normalized to BW. However, FS was preserved after 4 weeks of Parox in AR+Parox group compared with AR+Saline **(Figure 1).**

**Table 1.** Echocardiography morphometrical and functional parameters after AR surgery. Morphometrical and functional parameters analyzed by echocardiography at week 4 after AR surgery. LVDD, left ventricle diastolic diameter (mm); FS, Fractional Shortening (%); SI, Sphericity index of left ventricle; CO, cardiac output (ml/min); LA/Ao, left atrium systolic diameter normalized to aorta root diastolic diameter; BW, body weight (g).E/A, ratio of velocity waves of transmitral flow; RWT, relative wall thickness. Two way ANOVA was used for comparisons.* different from Sham ($p<0.05$).

| Morphofunctional variables | AR+Parox | AR+Saline | Sham+Parox | Sham+Saline |
|---|---|---|---|---|
| (Week 4) | n=6 | n=5 | n=8 | n=8 |
| LVDD (mm) | 8.18 ± 0.17* | 8.34 ± 0.43* | 6.7 ± 0.19 | 6.86 ± 0.09 |
| FS (%) | 44.13 ± 5.74* | 46.5 ± 1.65* | 53.67 ± 1.79 | 47.67 ± 3.41 |
| SI | 0.83 ± 0.02* | 0.84 ± 0.03* | 0.68 ± 0.02 | 0.64 ± 0.02 |
| CO (mL/mm) | 169.54 ± 40.75* | 179.51 ± 17.16* | 110.75 ± 12.15 | 86.12 ± 10.4 |
| LA/Ao | 1.54 ± 0.01* | 1.35 ± 0.17* | 1.23 ± 0.05 | 1.04 ± 0.02 |
| E/A | 1.39 ± 0.18* | 1.46 ± 0.11* | 2.07 ± 0.18 | 2 ± 0.17 |
| RWT | 1.56 ± 0.09 | 1.55 ± 0.04 | 1.45 ± 0.05 | 1.36 ± 0.08 |
| BW (g) | 384.1 ± 9.5 | 366.2 ± 17.2 | 390.1 ± 12.4 | 398.1 ± 12.4 |

**Table 2.** Echocardiography morphometrical and functional parameters after paroxetine treatment. Morphometrical and functional parameters analyzed by echocardiography at week 8 after AR surgery. LVDD, left ventricle diastolic diameter (mm); FS, Fractional Shortening (%); SI, Sphericity index of left ventricle; CO, cardiac output (ml/min); LA/Ao, left atrium systolic diameter normalized to aorta root diastolic diameter; BW, body weight (g).E/A, ratio of velocity waves of transmitral flow; RWT, relative wall thickness. Two way ANOVA was used for comparisons.* different from Sham ($p<0.05$); † different from AR+Saline ($p<0.01$).

| Morphofunctional variables | AR+Parox | AR+Saline | Sham+Parox | Sham+Saline |
|---|---|---|---|---|
| (Week 8) | n=6 | n=5 | n=8 | n=8 |
| LVDD (mm) | 9.06 ± 0.26* | 9.15 ± 0.58* | 7.35 ± 0.14 | 7.18 ± 0.28 |
| FS (%) | 45.67 ± 1.52*† | 31.97 ± 3.08* | 48.44 ± 1.84 | 52.25 ± 1.84 |
| SI | 0.85 ± 0.02* | 0.87 ± 0.04* | 0.70 ± 0.01 | 0.66 ± 0.01 |
| CO (mL/mm) | 186.69 ± 24.22* | 173.16 ± 19.43* | 122.96 ± 9.86 | 90.82 ± 13.43 |

| | | | | |
|---|---|---|---|---|
| LA/Ao | 1.50 ± 0.09* | 1.52 ± 0.05* | 1.20 ± 0.04 | 1.12 ± 0.01 |
| E/A | 1.52 ± 1.15 | 1.58 ± 0.3 | 1.83 ± 0.12 | 1.91 ± 0.15 |
| RWT | 1.62 ± 0.12* | 1.62 ± 0.14* | 1.34 ± 0.08 | 1.43 ± 0.04 |
| BW (g) | 438.62 ± 9.65 | 406.83 ± 20.58 | 433 ± 16.87 | 431.62 ± 13.20 |
| HW (g) | 0.44 ± 0.02* | 0.45 ± 0.03* | 0.34 ± 0.008 | 0.41 ± 0.03 |

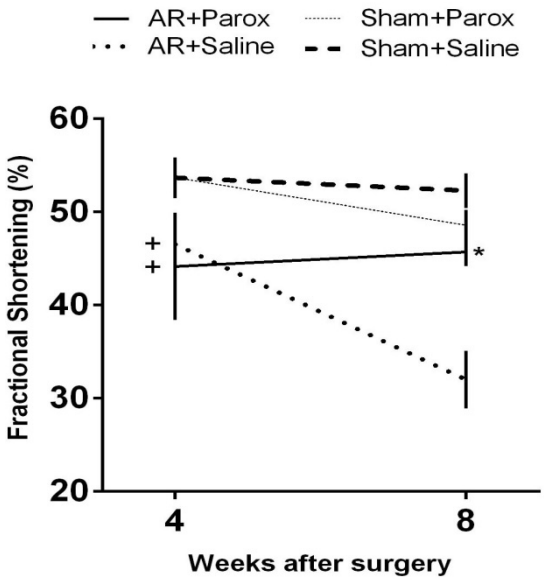


**Figure 1.** Paroxetine treatment preserve fractional shortening (FS) of AR rat hearts. Values (means ± SD, n=6/group) of FS before (week 4) and after (week 8) parox treatment are reported in percentage. FS(%)=LVDD-LVSD/LVDD x 100. LVDD=left ventricle diastolic diameter (LVDD); LVSD= left ventricle systolic diameter (LVSD). *different from AR+Saline, p<0.001.

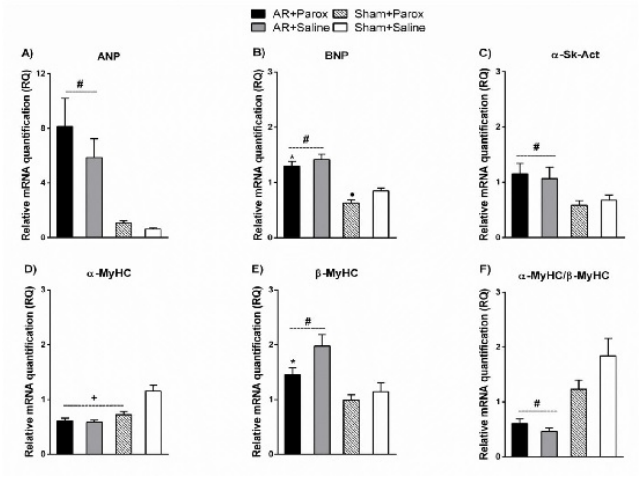


**Figure 2.** Cardiac stress markers gene expression. Real time quantitative RT-qPCR of LV mRNA levels of ANP (A), BNP (B), α-sk-act (C), α-MyHC (D), β-MyHC (E) and α-MyHC /β MyHC ratio (F) after week 8. Values (means ± SD, n=5/group) are reported in relative quantification (RQ). #different from sham, p<0.05; *different from AR+Saline, p<0.05; different from Sham+Saline, p<0.001+different from Sham+Saline, p<0.05.

## Paroxetine induced a change in the expression of the cardiac stress markers in AR

Pathological cardiac hypertrophy has been associated with upregulation/reactivation of fetal cardiomyocyte gene program, including α-SK-act, ANP, BNP, and β-MyHC, and the downregulation of α-MyHC, which is normally expressed at higher levels in the adult ventricle compared with the fetal ventricle [34,35].

The mRNA levels of these 5 genes were assessed in the left ventricles of AR+Parox, AR+Saline, Sham+Parox, and Sham +Saline by RT-qPCR **(Figure 2)**.

The results of this study showed that the expression levels of the cardiac stress markers α-SK-act, ANP, BNP and β-MyHC are up-regulated, and that α-MyHC is downregulated in AR animals compared with Sham, suggesting the development of pathological cardiac hypertrophy. Parox induced a change in these molecular markers; BNP and β-MyHC are decreased in AR +Parox hearts compared with AR+Saline group. However, the α-SK-act, ANP, and α-MyHC levels were not altered in AR+Parox compared with AR+Saline groups. Because of the changes in β-MyHC expression in AR, we searched for additional post-transcription control of the myosin transcripts that could be relevant for the Parox improvement in cardiac contractility.

## Paroxetine modifies the expression of the myomiRs miR-208b and miR-499 during AR

In order to study the miRNAs that might be involved in the regulation of myosin isoforms transition in AR induced by Parox, we evaluated the microRNAs miR-208a, -208b and -499 expression by RT-qPCR. The miR-208b was increased in AR animals and, interestingly, Parox reduced miR-208b transcript levels in AR+Parox compared with AR+Saline. Additionally, this microRNA expression analyses demonstrated that miR-499 is downregulated in AR+Parox compared to AR+Saline group. No

significant change was observed for miR-208a expression among the groups. Next, we evaluated the expression of transcriptional repressors known to regulate muscle gene expression and function including Sox6, Purβ and Thrap1, which are legitimate target of the microRNAs miR-208b and miR-499 **(Figure 3)**. However, no statistical difference was found in the expression levels of these transcriptional repressors.

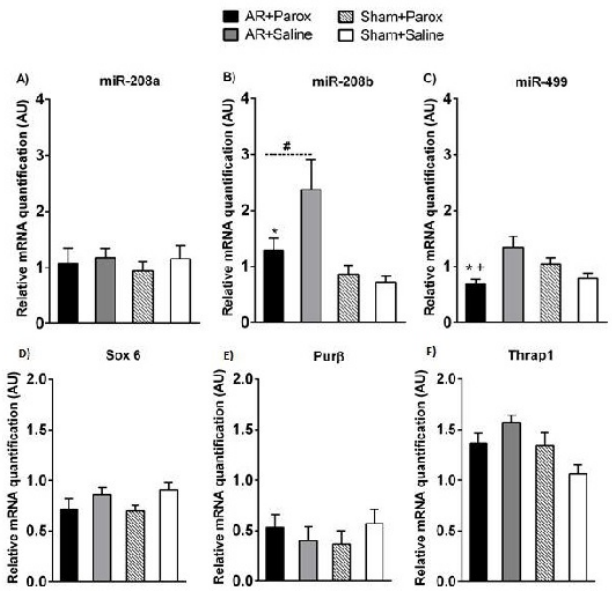


**Figure 3.** MyomiRs and myomiR's targets gene expression. Real time quantitative RT-qPCR of LV mRNA levels of miR-208a (A), miR-208b (B), miR-499 (C), Sox6 (D), Purβ (E) and Thrap1 (F) after week 8. Values (means ± SD, n=5/group) are reported in relative quantification (RQ). #different from sham, p<0.05; *different from AR+Saline, p<0.05.

## Discussion

The results of this study reveal that AR produced a volume overload and hypertrophy to the heart leading to increases in CO, SI, LA/Ao and decrease of FS **(Table 2)**. Parox, as showed previously, preserved FS [18]. In this study, novel findings are presented as to changes in the expression of genes involved with cardiac contractility and hypertrophy in AR hearts after Parox treatment. Parox showed an effect in the molecular regulation of contractile proteins that are responsible for the healthy heart performance. β-MyHC gene and myomiRs (miR-208b and -499) are downregulated by Parox. We also showed that gene expression of β-MyHC transcriptional repressors, Sox6, PurB and Thrap1, is not modified by this treatment suggesting the participation of other targets for miR-208b and -499. ANP, BNP and α-skeletal actin, molecular markers of cardiac hypertrophy, was up-regulated due to AR and Parox reduced BNP gene expression.

Natriuretic peptides ANP and BNP are highly expressed in cardiac hypertrophy due to cardiovascular diseases and are well known as molecular markers of hypertrophy [28]. According to the literature, our results show that volume overload caused by AR increased ANP and BNP expression [29]. Parox did not change ANP gene expression but was effective in reducing the expression of BNP gene. It is known that increased plasma levels of BNP were associated with bad prognostic of cardiac diseases [36]. Although we have not measured BNP plasma levels in our animals, downregulation of BNP gene expression in cardiac tissue could suggest some beneficial role in the myocardial contractility mechanisms.

Myosin is the main molecule responsible for cardiac and skeletal muscle contractility converting ATP energy into mechanical motion [21]. In cardiac myocytes, MyHC isoforms, α- and β-MyHC, are encoded respectively by Myh6 and Myh7 genes. Pathophysiological conditions causing hemodynamics and hormonal alterations could lead to upregulation of β-MyHC, which is considered energetically favorable but is associated with depressed contractile function promoting disease progression [22,23,37]. In the results presented, AR increased the expression of the gene that encoded β-MyHC isoform while Parox reduced it. It has been shown that decrease in β-MyHC expression in patients with idiopathic dilated cardiomyopathy is associated with improvement of the left ventricular function and mutation in such same gene which was identified in familial hypertrophic cardiomyopathy [38,39]. It has been observed that in human hearts but not in rodents' there is more β- than α-MyHC proportion [22] differently from rodent' hearts. Nevertheless, in a study in samples of human cardiac failure hearts it was observed a decrease in α-MyHC mRNA [23], similar to our present results in rodents. Taking this information into account it is important to highlight the effect of Parox in downregulating β-MyHC isoform expression.

MyomiRs targets, transcriptional repressors are involved in molecular regulation of β-MyHC gene expression [24]. Sox6, PurB and Thrap1 are considered important transcriptional repressors of β-MyHC mRNA translation; therefore we investigated if the gene expression of these transcriptional repressors were involved in AR and Parox. The results did not show alterations for any of the repressors analyzed so other transcriptional repressors might be involved in the regulation of β-MyHC gene expression in AR rats treated with Parox. On the other hand, our results displayed an important effect of Parox in downregulating miR-208b and -499. No difference was found for miR-208a expression. These miRNAs encoded by myosin genes are involved in regulating fiber transition from α- to β-MyHC in heart tissue. Van Rooij et al proposed a network of miRNAs within myosin genes that regulates myosin expression. In this network, miR-208a is encoded from Myh6 gene, related to the expression of α-MyHC isoform, which is necessary to inhibit transcriptional repressors of β-MyHC isoform, activating β-MyHC transcription. In the same network, miR-208b and -499 are encoded by Myh7 and Myh7b respectively, and are necessary for β-MyHC transcription as well as miR-208a through inhibition of transcriptional repressors Sox6, PurB and Thrap1 [24].

Interestingly, miRNAs have been pointed out as important biomarkers for several pathologies, mainly after the discovery that miRNAs are capable of circulating freely in the blood allowing its detection at plasma and serum with high sensitivity and specificity [40]. It was demonstrated in both humans and

animal models, that myocardial injuries increase miR-208b and -499 plasma levels [41-43]. Our results demonstrated that Parox might decrease the presence of biomarkers related to myocardial damage caused by AR.

In conclusion, we reveal for the first time that Parox in AR rats preserve FS of the heart possibly by preventing a wide expression of β-MyHC isoform and decrease expression of myomiRs such as miR-208b and -499. Taken together, our findings show the potential contribution of Parox treatment in attenuating cardiac contractility dysfunction during AR, which likely occurs through downregulation of cardiac stress markers and miRNAs involvement in the pathophysiology of this dilated cardiomyopathy.

## Acknowledgements

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## Competing Interests

There is no competing interest for the manuscript.

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