Molecular evidence of tissue remodeling in an animal model of heart failure

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Summary. Heart failure (HF) is the final common pathway of many cardiovascular diseases. Metalloproteinases and their inhibitors, such as MMP9 and TIMP-1, assist in maintaining the extracellular matrix, leading to tissue remodeling observed after HF. Previous studies have shown that L-Arginine (LA) appears to have beneficial effects for the treatment of HF, contributing to vasodilation, the reestablishment of the endothelial function and an increase in muscle contractile force. This study analyzed heart tissue remodeling in an animal model of HF induced by aortocaval fistula (ACF) and submitted to LA treatment. After 4 weeks of ACF, animals were treated with LA for 4 weeks (SHAM-LA, HF-LA) or for 8-12 weeks with saline (SHAM, HF8, HF12). Rats were euthanized and the hearts removed for histological processing. The samples were stained with Hematoxylin-Eosin (HE), Masson's Thichome (MT), or submitted to immunohistochemistry (IHC) for MMP9 and TIMP-1. Light microscopy analysis showed cardiac striated muscle without fibrosis in all experimental groups. Immunostaining of MMP9 and TIMP-1 were positive for all experimental groups. LA administration significantly reduced MMP9 content after HF. These data indicate molecular changes in metalloproteinases expression prior to tissue remodeling and point out LA as an adjuvant therapy to pharmacological treatment of patients with HF.

Key words: Aortocaval fistula, Histochemistry, Metalloproteinases

Introduction

Heart failure (HF) is a major public world health problem, with high rates of hospitalization and mortality. It is estimated that 6.4 million people are affected by this disease in Brazil (Albuquerque et al., 2015).

Organic nitrates such as nitroglycerin have been successfully administered for the treatment of HF even before the knowledge that the underlying mechanisms were partially due to the release of nitric oxide (NO). However, the therapeutic benefits of organic nitrates are limited by the development of tolerance. L-Arginine is an important precursor of NO and acts on cardiac physiology, with a significant improvement in vasodilation and hemodynamic reestablishment of the endothelial function and increase in muscle contractile force (Loscalzo, 2000). Furthermore, the mitochondria of cardiomyocytes in failing hearts exhibited decreased uptake of L-Arginine, which is suggestive that L-Arginine might be a useful therapeutic approach in HF (Williams et al., 2014).

Additionally, the structural integrity of the heart is maintained by extracellular matrix (ECM), which acts directly on the control of vascular remodeling and repair

(Lindsey and Zamilpa, 2012). Tissue remodeling can be defined as changes in size, geometry, shape, composition and function after heart attack. Hypertrophy is characterized by an increase in cardiomyocyte size, high protein synthesis and changes in the extracellular matrix, generating an increase of collagen, connective tissue and fibrosis (Bregagnollo et al., 2007). Such changes are due to extracellular matrix degradation of matrix metalloproteinases (MMPs) (Abassi et al., 2011; Lindsey and Zamilpa, 2012).

The MMP family includes soluble enzymes including collagenases (MMP-1, -13) that digest structural or fibrilar collagens (types I to III) and gelatinases (MMP-2, -9) that digest denatured collagen (gelatin) and types IV and V collagen (Levick and Brower, 2008). Such specific substrates and functions are finely tuned through the expression of tissue inhibitors of metalloproteinases (TIMPs). Therefore, changes in the homeostasis of MMPs and their endogenous TIMPs are often observed in cardiovascular disease (Albuquerque et al., 2015). Moreover, MMP-9 and TIMP-1 are increased in heart failure and positively correlated to the severity of the disease (Morishita et al., 2017).

L-Arginine can improve metabolic processes such as fibroblast proliferation (Fujiwara et al., 2014), angiogenesis and collagen synthesis, mediated by NO, leading to changes in the ECM (Garcia et al., 2016). Thus, we hypothesized that in an animal model of HF induced by aortocaval fistula, the histopathological changes in myocardium ECM, specifically evaluated by the expression of MMP9 and TIMP-1, may be prevented by chronic treatment with L-Arginine, decreasing tissue remodeling during the HF.

Materials and methods

Animals and treatment

Male Sprague Dawley rats (280-300g; 3 months old; N=25), provided by the Center for Biological Research Multidisciplinary (CEMIB) of the University of Campinas (UNICAMP; Campinas, SP, Brazil) were used in this study. The animals were kept in the Central Animal Laboratory at the São Francisco University (USF; Bragança Paulista, SP, Brazil) at 24°C in 12h light/dark cycles and free access to food and water. The experimental protocols were approved by the Ethical Commitee for Animal Experimentation (protocol 004.06.11). The animals were housed in plastic cages (2 per cage) and divided into SHAM-operated or heart failure (HF) induced by aortocaval fistula (ACF), receiving or not L-Arginine (LA) according to the five experimental groups as follow: 1- Sham-operated (SHAM; n=5); 2- Sham-operated receiving LA (SHAM-LA; n=5); 3) ACF-operated 8 weeks (HF 8; n=5); 4) ACF-operated 12 weeks (HF12; n=5); 5) ACF-operated 8 weeks receiving LA (HF-LA; n=5).

Animals subjected to LA treatment started receiving LA 4 weeks after SHAM operation or ACF and the treatment lasted 4 weeks, totalling 8 weeks (groups SHAM-LA and HF-LA). L-Arginine was diluted in drinking water in a concentration of 1.25 g/L, which corresponds to a daily dose of approximately 62.5 mg/rat/day.

Induction of heart failure (HF) by aortocaval fistura (ACF)

ACF was conducted as previously described (Brower and Janicki, 2001). Briefly, rats were anesthetized with an intraperitoneal injection (i.p.) of xylazine (7 mg/kg) and ketamine (62 mg/kg) for surgical procedures. After trichotomy and ventral laparotomy, the abdominal aorta and vena cava were exposed and a short-bevel 18 gauge needle was inserted into the abdominal aorta below the renal arteries, towards the vena cava, crossing the medial wall to create a shunt. The needle was removed and the site of the abdominal aorta puncture was sealed with cyanoacrylate. Shunting of aortic blood into the vena cava was visually evident and indicated successful creation of the fistula. The musculature and skin incisions were closed by standard techniques with absorbable- and non-absorbable suture, respectively. Postoperative analgesia was provided by buprenorphine HCl (0.05 mg/kg) at the time of surgery.

Histopathological analysis

Histochemistry - Hematoxylin-Eosin (HE) and Masson's Trichrome (MT)

At the end of the treatment rats were euthanized and the left ventricle was removed for histological processing. Briefly, fragments of the left ventricle were fixed in 4% paraformaldehyde for 24 hours and processed for paraffin embedding. Samples were dehydrated in ethanol (70%, 80%, 90% and 100%) and ethanol:xylene (1:1); cleared in xylene 100%, embedded in paraffin:xylol (1:1) and paraffin pure. The paraffin blocks were sectioned and the sections (5μm) collected on glass slides. Slides were deparaffinized in xylene, hydrated in a descending series of ethanol (100%, 90%, 80%, 70%) and water, and then stained with Hematoxylin (H) for 5 minutes and Eosin (E) for 3 minutes. Then, the slides were dehydrated in an increasing ethanol series and xylene pure, and mounted with synthetic Canada balsam.

For collagen analysis, the Masson's Trichrome (MT) technique was used. As described for HE staining, slides were also deparaffinized and hydrated, maintained in Bouin solution for 1 hour, in iron-hematoxylin for 5 minutes, in Biebrich Scarlet solution for 3 minutes, in phosphomolybdic acid for 8 minutes and in aniline blue for 3 minutes. After the staining process, the slides were washed and dehydrated, and mounted with Synthetic Canada balsam, as described above.

Immunohistochemistry - IHC

Slides were deparaffinized in xylene, hydrated in descending series of ethanol (100%, 90%, 80%, 70%) and water. Sections were permeabilized with 0.3% Triton for 15 min, washed in 0.05M PBS (3X for 5 minutes), treated with peroxidase (for 30 minutes), washed again in 0.05 M PBS (3X for 5 minutes), treated with PBS-BSA 1% solution (for 1 hour), and finally washed in 0.05M PBS (3X for 5 minutes).

Then, the sections were dried with filter paper and incubated with specific primary antibodies [MMP9 (sc-13520) and TIMP-1 (sc-21734); 1:50 dilution in PBS:BSA 1%], "overnight" at 4°C. After this, the sections were washed in 0.05M PBS (3X for 5 minutes) for incubation with anti-mouse secondary antibody [(a9044); 1:200 dilution in PBS: BSA1 %], for 2 hours at room temperature. The slides were washed, dried and incubated with DAB (1 minute).

The slides were finally rinsed, counterstained with hematoxylin (for 30 seconds), washed, dehydrated in ethanol series (70%, 80%, 95%, for 1 minute each, 100% 2x for 5 minutes) and xylene 100% (2x for 5 minutes), and mounted with synthetic Canada balsam, as described above for histochemical techniques. The negative control was obtained by the omission of the primary antibody.

Quantification

The slides were observed at 10X objective. Image processing was assisted by computer and the number of pixels in each field was transformed into percentage values. For each histological section three different fields were analyzed. The average of these measurements was considered the ultimate measure of immunostaining content. Positive and negative controls have been adopted to ensure the quality of the measurements. The quantification of the protein expression was obtained from the image analysis progam NIS (for Windows), as previously described by other researchers (Martinez et al., 2011).

Statistical analysis

ANOVA was used for comparison between MMP9 and TIMP-1 proteins. Spearman correlation test was used to evaluate the association between both metalloproteinaes in the different groups. Data are expressed as means ± S.E.M. of n experiments. A $p<0.05$ was assumed to be statistically significant.

Results

The histomorphological analysis of the left ventricle of all experimental groups exhibited the same heart morphology (Fig. 1A-E), characterized by elongated cardiac striated muscle fibers with transverse and longitudinal striations, intact intercalated disks and central nuclei (Fig. 1F).

In addition, the distribution of connective tissues, particularly collagen fibers, showed typical longitudinal distribution of mature collagen, without signs of fibrosis or inflammation (Fig. 2).

The immunostaining of MMP9 (Fig. 3A-E) and TIMP-1 (Fig. 4A-E) were positive for all experimental groups.

Regarding MMP9, SHAM and SHAM-LA groups presented similar immunostaining values, without significant differences. The imuno content was higher in HF8 and HF12 when compared with HF-LA and SHAM, respectively, and significant differences were observed between these groups. Quantification of MMP9 revealed a trend to progressively increase in HF8 group, being significantly higher in HF12 group. HF-LA animals displayed lowest content of MMP9. Administration of LA significantly prevented the increase in the MMP9 content in HF-LA group (Fig. 3F).

However, considering TIMP-1 immunostaining, there were no significant differences among groups. SHAM and SHAM-LA groups presented similar immunostaining values, without significant differences. The imuno content was progressively higher in HF12, HF8 and HF-LA. Quantification of TIMP-1 showed that there was a tendency of increase in TIMP-1 content in all groups subjected to ACF (HF8, HF12 and HF-LA groups). Treatment with LA did not affect TIMP-1 content in both SHAM and HF groups (Fig. 4F).

The ratio of MMP9 to TIMP-1 was increased in HF8 and HF12 groups compared with SHAM. However, after L-Arginine treatment, it was observed an inverse relationship between MMP9 and TIMP-1; while TIMP-1 expression remained at the same levels, MMP9 started decreasing (Fig. 5).

Discussion

HF is a public health problem in Brazil, with large numbers of hospitalizations, and cause-consequence of other comorbidities; heart disease, vascular disease, hypertension (Bierman, 2004; Fung et al., 2004; Dubeau et al., 2009; Mangini et al., 2008; BRAZIL, 2015). In 2015 there were 27,434 deaths due to heart failure, generating a high cost to the national public health (BRAZIL, 2015).

The main cause of chronic HF is ischemic heart disease associated with hypertension. Changes in blood pressure (diastolic, systolic or both) (Bocchi et al., 2012), increased blood volume and contractility, hyperactivation of the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS) (Chatterjee, 2005) lead to structural changes in the myocardium, such as hypertrophy. The myocardium remodeling process consists of myocyte apoptosis and the dynamic synthesis and breakdown of ECM (Tyagi, 1997; Antonov et al., 2018), particularly MMP9 (Toyoshima and Hunter, 1994; Fujimoto et al., 2000;
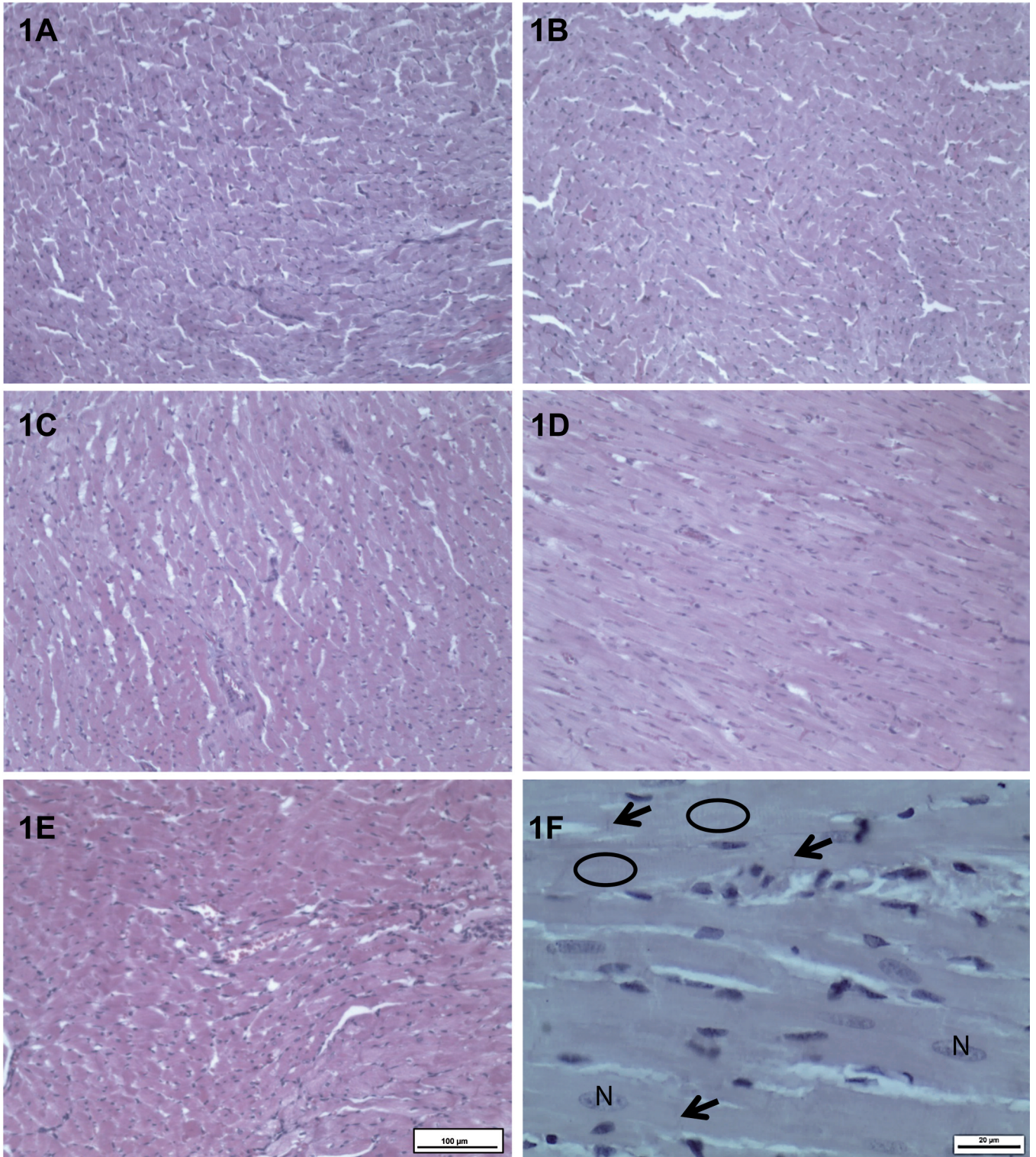
Fig. 1. Transversal **(A-C, E)** and longitudinal **(D, F)** histological sections of the left ventricle of rats submitted to HE staining. An unchanged myocardium pattern was observed in all experimental groups: SHAM **(A)**, SHAM-LA **(B)**, HF 8 **(C)**, HF-LA **(D)** and HF 12 **(E)**. **F.** Observe the unaltered histology of cardiac fibres, with intact sarcomeres (ellipse), central nuclei (N) and intact intercalary discs (arrow). A-E, x 100; F, x 400.
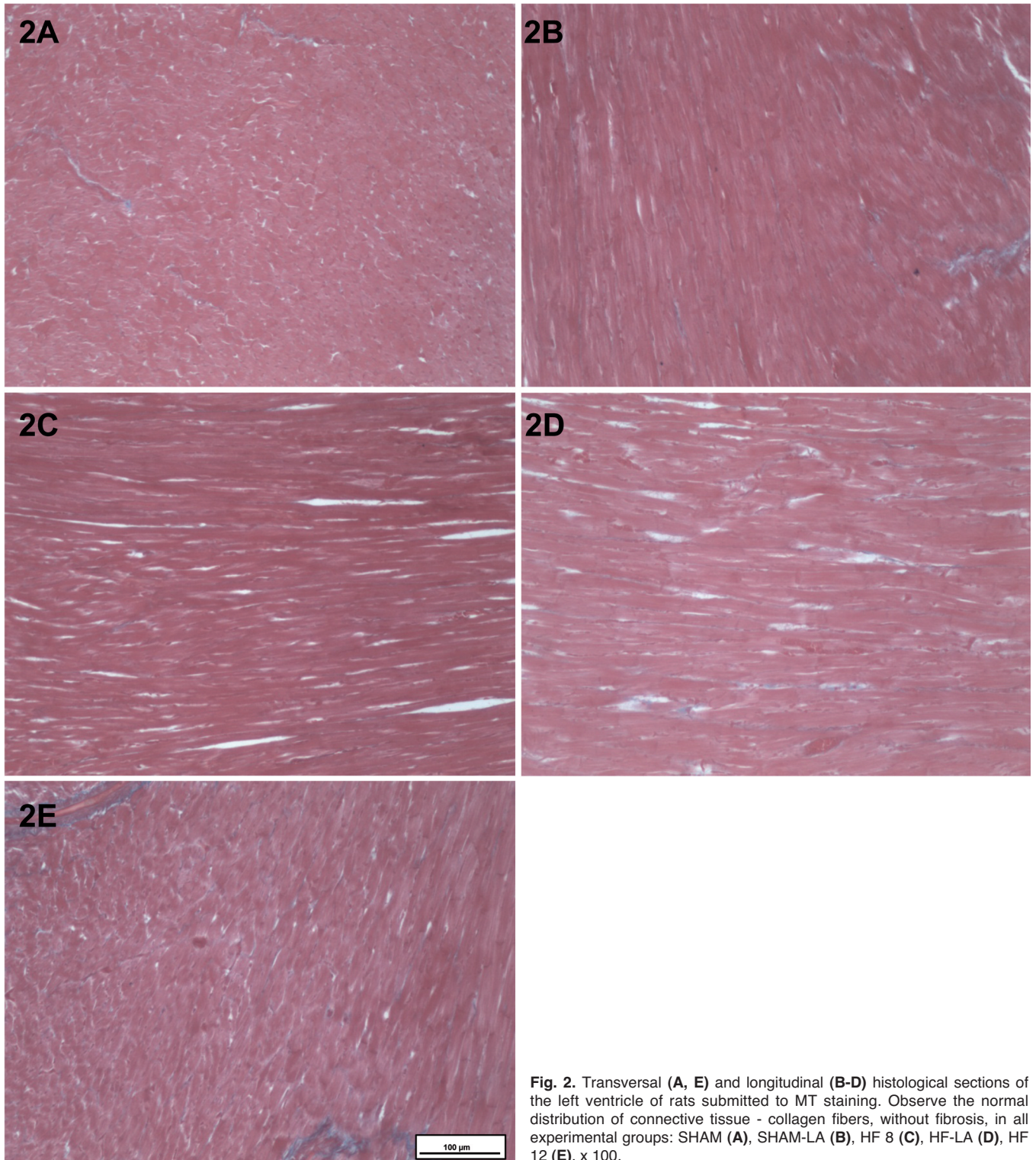
Fig. 2. Transversal **(A, E)** and longitudinal **(B-D)** histological sections of the left ventricle of rats submitted to MT staining. Observe the normal distribution of connective tissue - collagen fibers, without fibrosis, in all experimental groups: SHAM **(A)**, SHAM-LA **(B)**, HF 8 **(C)**, HF-LA **(D)**, HF 12 **(E)**. x 100.
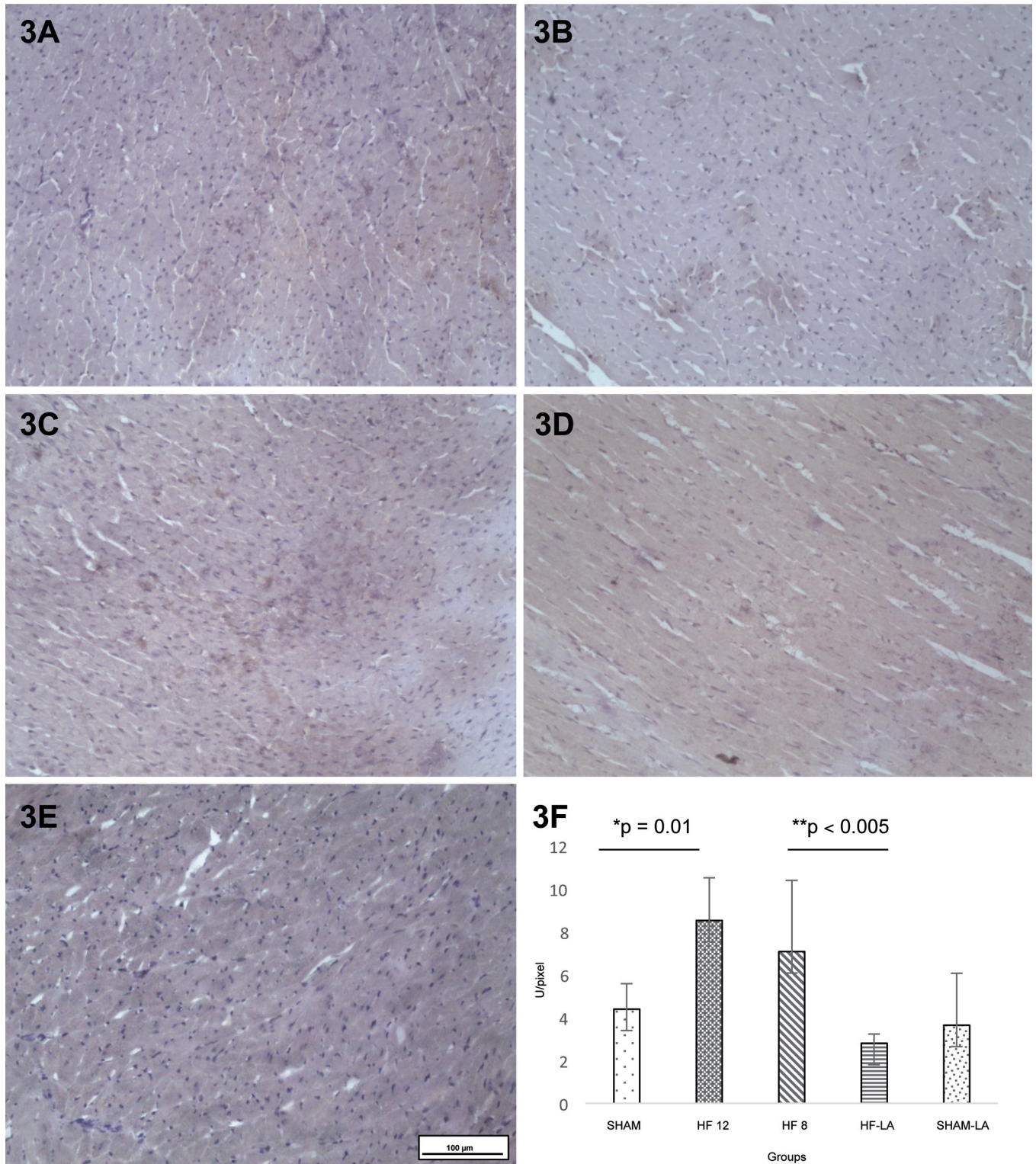
Fig. 3. Transversal **(A-C, E)** and longitudinal **(D)** histological sections of the left ventricle of rats submitted to immunohistochemistry for MMP9. A positive labelling was observed in all experimental groups: SHAM **(A)**, SHAM-LA **(B)**, HF 8 **(C)**, HF-LA **(D)**, HF 12 **(E)**. Immunostaining quantification of MMP9. There are significant differences between the SHAM and HF 12, and HF 8 and HF-LA groups. * p=0.01 and ** p<0.005 (F). x 100.
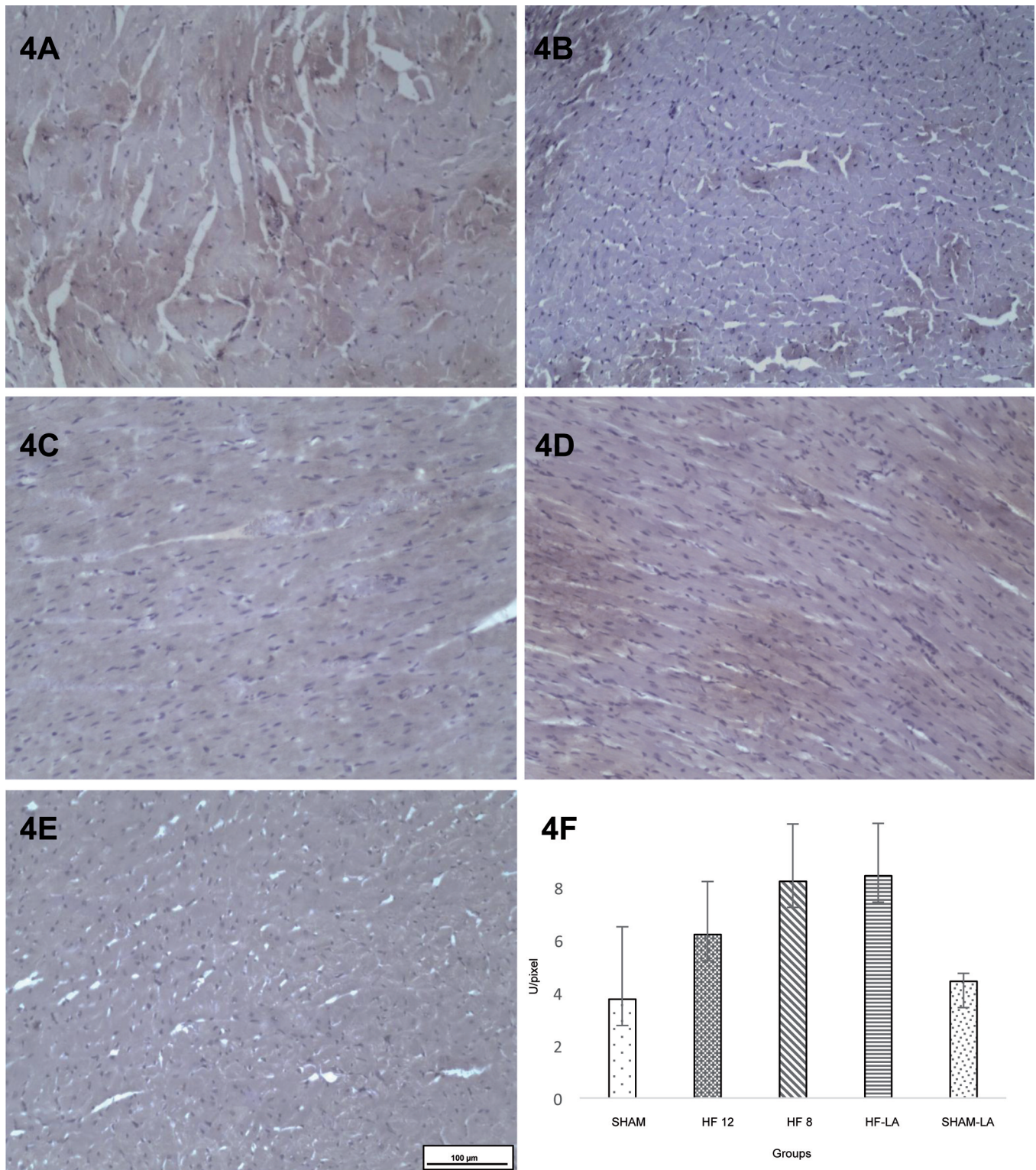
Fig. 4. Transversal **(A, B, E)** and longitudinal **(C, D)** histological sections of the left ventricle of rats submitted to immunohistochemistry for TIMP-1. A positive labelling was observed in all experimental groups: SHAM **(A)**, SHAM-LA **(B)**, HF 8 **(C)**, HF-LA **(D)**, HF 12 **(E)**. Immunostaining quantification of TIMP-1. There were no significant differences among groups **(F)**. x 100.

Bohnsack and Hirschi, 2004; Neganova and Lako, 2008).

The histochemical data show that there was no structural/morphological damage after 8 and 12 weeks of HF induction by ACF. As reported by Brower and Janick (2001) animals which undergo ACF present, after 10 weeks, an increase in cardiac output and, after 21 weeks, damage to the myocardium, with hypertrophy signals. These data corroborate with the present study since 8 and 12 weeks were not enough to lead to morphological changes in the myocardium.

In animal models of HF, MMP levels such as MMP9 are increased, indicating the relationship of these proteins with cellular and structural changes in cardiac striated muscle (Wang et al., 2013; Mayer et al., 2018). We observed a significant increase in MMP9 content in HF animals after 8 and 12 weeks, compared to SHAM animals. These data indicate that, even without morphological damage, molecular changes can already be detected.
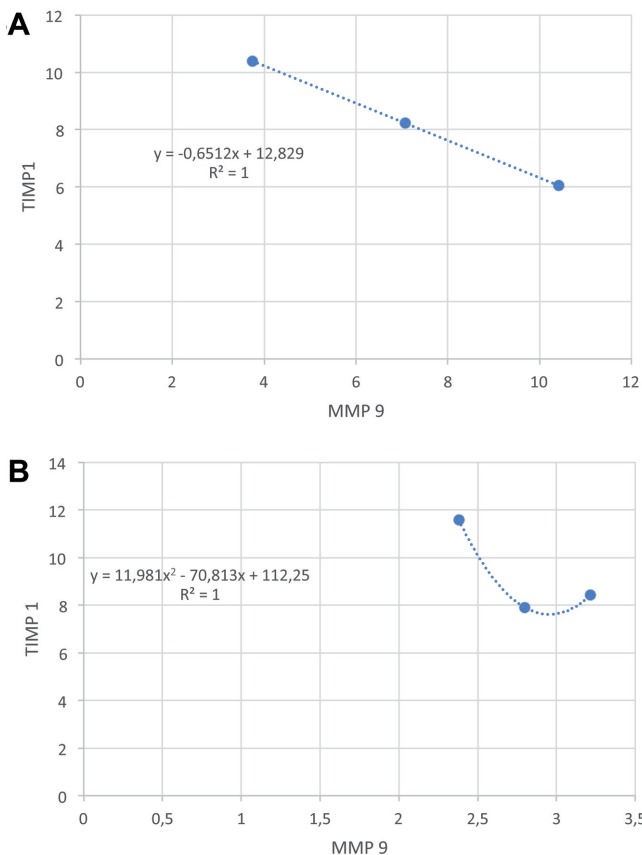
The expression of TIMP during physiological tissue remodeling mantains the metabolic balance and structural ECM. In the case of cardiovascular diseases where there is an uncontrolled ECM renewal, there is an imbalance between the expression of MMP9 and TIMP-1 (Batle et al., 2007; Kitaoka et al., 2010; Lindsey and Zamilpa, 2012), indicating ventricular remodeling. In this study, immunohistochemical marking TIMP-1 appeared positive in all groups with trends to increased expression in HF groups although without significant differences between them.

The pharmacological treatment of HF aims to reduce the overload in the ventricular myocardium and the pathological ventricular remodeling (Granger et al., 2003). However, it is important to note that all of them are administered once the morphological effects of the disease are already established.

Among other changes in animal model of HF, a reduction in NO bioavailability is also observed (Nogueira et al., 2010). The NO (Fujiwara et al., 2014) and L-Arginine (Shi et al., 2003; Barbul, 2008) may act increasing collagen synthesis. Endogenously, Arginine synthesis is sufficient for the maintenance of connective and muscular tissue, although the metabolic stress makes it insufficient (Barbul, 2008; Fujiwara et al., 2014), resulting in low collagen synthesis and modifications of the ECM (Raynaud-Simon et al., 2012). According to the current literature data, oral supplementation with L-Arginine promotes significant improvement in vasodilation, hemodynamics, reestablishment of the endothelial function and muscle strength increasing contractility (Melo et al., 2013).

Patients with HF presented an increase in collagen synthesis and deposition and their MMP-1:TIMP-1 ratio, for example, was higher when compared to normotensive subjects (López et al., 2006). In our study, the ratio of MMP9 to TIMP-1 was higher in HF than in SHAM groups. However, an inverse relation was observed between HF-LA and SHAM-LA animals. The highest content of TIMP-1 levels was observed in HF-LA group which exhibited the lowest content of MMP-9, suggesting that L-Arginine administration induced changes in the expression of MMP9 and TIMP-1, playing a protective role to the ECM.

Altogether, our results indicate that under a pathological condition such as HF, oral supplementation with L-Arginine significantly reduces content of MMP9 and can thus prevent ventricular remodeling, pointing MMP9 and TIMP-1 as early biomarkers in HF and L-Arginine as a potential adjuvant to the current pharmacological therapeutic treatment for HF, avoiding the installation of morphological changes.

A $y = -0,6512x + 12,829$
$R^2 = 1$

B $y = 11,981x^2 - 70,813x + 112,25$
$R^2 = 1$

Fig. 5. Comparative analyse of MMP9 and TIMP-1 protein expression between HF 8 **(A)** and HF-LA **(B)** groups. An imbalance in the expression of both proteins was observed, showing a decrease of TIMP-1 expression and an increase of MMP9 expression in HF 8 group **(A)**. After L-Arginine treatment TIMP-1 expression increase, while MMP9 remained at the same level **(B)**.

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