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Heart Zebrafish (*Daniorerio*) - quantification of tissue constituents

FabieniTiemy Menezes Okiyama, André Santos-Leonardo, Kaique Nogueira, Anna Clara dos Santos, André Rodrigues da Cunha Barreto-Vianna, Eduardo Maurício Mendes de Lima

Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília (UnB), ICC Sul, Campus Universitário Darcy Ribeiro, Cx. Postal 4508, Brasília, DF 70910-970, Brazil.

Address For Correspondence:
FabieniTiemy Menezes Okiyama, Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília (UnB), ICC Sul, Campus Universitário Darcy Ribeiro, Cx. Postal 4508, Brasília, DF 70910-970, Brazil.
E-mail-limaemm@unb.br

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ABSTRACT

Problems relating to injuries and heart failure are worrying as well as morphological changes inherent in this condition. We use 10 fishes *Daniorerio*, of both sexes, and quantify the density of cardiomyocytes, connective tissue and collagen type I and type III fibers by Sirius Red staining. There was statistical difference between all parameters when compared atrium and ventricle, probably due to the size difference between the chambers. The atrium of males and females together (GT) showed higher density of cardiomyocytes in the atrium (6.93% ± 0.51) than in the ventricle (3.84% ± 0.20) and higher density of myocardium in the ventricle (68,77% ± 1,25) than in the atrium (60.30% ± 2.44). The ventricle (8.02 ± 0.61%) showed a lower density of connective tissue than the atrium (24.50 ± 1.35%). The density of the collagen fiber type I in the atrium was lower compared to the fibers of type III (4.28% ± 1.20 and 5.68 ± 1.90%, respectively) and higher in the ventricle (3.22%± 0.89% and 0.14 ± 0.05, respectively). Based on these results opens up the range within these parameters for future studies related to the specie studied.

INTRODUCTION

The high prevalence of heart failure and the high cost associated with this condition demonstrate the importance of establishing experimental models for research and improving the methods of diagnosis and treatment for this condition. The use of zebrafish in biomedical research has been increasing due to several factors including the ease of administration and rapid absorption of most drugs; visible embryonic development; morphological and molecular basis of embryogenesis similar to that of other vertebrates, including humans; small size, easy breeding and management; and low production costs compared to other models. (Zhu, J.J. *et ai*., 2013) Seventy-two hours after fertilization, the cardiovascular system of zebrafish is completely functional and is easily visualized, which facilitates studies associated with the heart and its ability to regenerate. (Poss, K.D. *et ai*., 2002)

Similarly to other bony fish, zebrafish heart has only one atrium and one ventricle. Both structures are formed primarily by cardiomyocytes and connective tissue. Collagen is the main constituent of this tissue, and five types can be found, namely, collagen type I, III, IV, V and VI, of which collagen type I is the predominant type. (Mill, J.G., D.V. Vassallo, 2001)

In this species, the heart fully regenerates in only two months; therefore, it is a great model for cardiovascular plasticity. However, the exact mechanism of heart regeneration has not yet been elucidated. (Parente, V. *Et ai*., 2013) This process does not occur in the human myocardium. However, it has been

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suggested that mammals have the stem cells required to optimize myocardial regeneration but that they are inactivated. (Lepilina, A. *et ai*., 2006)

This study aimed to provide morpho-quantitative parameters for the heart tissue structures of *Daniorerio* that will be used as a basis for further studies.

MATERIALS AND METHODS

The study followed the standards of the Ethics Committee on Animal Use of the University of Brasilia protocol #127542-2013. A total of 10 adult fish (*Daniorerio*) aged three months old that were obtained from a *fish* hatchery (Psicultura Tropical Maeda, Brasília/DF, Brazil) were used. The animals underwent a 10-*day acclimation period* in 26-L tanks. These tanks had a closed circulation system, constant temperature controlled by thermostat, and biological, chemical and mechanical filters. The pH was checked daily with a pH test kit (pH Tropical®, LabconTest) and adjusted using a pH-adjusting agent (Alcali,Labcon®). The fish were subjected to a 14/10 h photoperiod and fed twice a day with commercial feed through an automatic feeder(Auto Feeder AF-2009D RESUN®).

An 0,2% aqueous MS22 solution (Finquel, Argent Laboratories Group®) followed by immersion in cold water (4ºC) for 15 minutes was used to *euthanize* the animals. Then, the fish were fixed whole in aqueous Dietrich's solution for 72 hours. Standard histological technique was used. The blocks were cut at 3-µm thickness using a manual microtome (Leica RM 2125RT), and on average, 10 sections were obtained for each fish. The sections were then stained with (BX51 Olympus®) Sirius Red.

The images were obtained using a light microscope (BX51 Olympus®) that was coupled to a camera and to image acquisition and analysis software (ProgRes® Capture Pro 2.5). Images (TIFF, 36-bit color, 1280x1024 pixels) were acquired from 15 random fields of each fragment using areas with longitudinal cardiac fibers as a standard. To quantify the cardiomyocytes, the connective tissue and the types of collagen, the area without tissue and/or with erythrocytes and satellite cells was disregarded.

To quantify the area occupied by cardiomyocytes, a *stereological software (*STEPanizer®-http://stepanizer.com/) with 100 points (100x objective) was used for which each point corresponds to an area of 13.2 µm². This procedure was used to obtain the mean area of each analyzed tissue. (Mandarim-de-Lacerda, C.A., 2003)

The slides of the animals were separated in groups (GT- males and females, GM- only males and GF- only females) and the cardiomyocyte density, myocardial density, connective tissue density and type I and type III collagen fibers density were quantified. The number of points corresponding to the *cardiomyocyte nuclei* and the atrium and ventricle myocardium were counted; the difference between the mean area of the myocardium and the mean area of the connective tissue corresponded to the mean area occupied by cardiomyocytes (N° cardiomyocyte = average area of myocardium - average area of connective tissue).

Polarized light microscopy and Image-Pro Plus 6 image analysis software were used to differentiate between and calculate the percentage of type I and type III collagen fibers.[7] The software and light microscopy were also used to quantify the connective tissue.

Descriptive statistics were used, followed by the Kolmogorov-Smirnov normality test, t-test and Mann Whitney *post hoc* test with the program GraphPad Prism®6. The data are expressed as the mean ± standard error of the mean, and $P \leq 0.05$ was considered significant.

Results:

The analysis revealed that the atrium (AT) is thin, with thin trabeculae, an atrioventricular valve, muscular wall and some areas with a higher density of connective tissue, especially on the edges. The ventricle (VT) has a bulboventricular valve, its wall is thicker than the atrial wall, and it comprises an inner layer with several trabeculae, with some of them exhibiting connective tissue, and an outer compact layer of cardiac muscle where the connective tissue is more abundant (Figure 1).

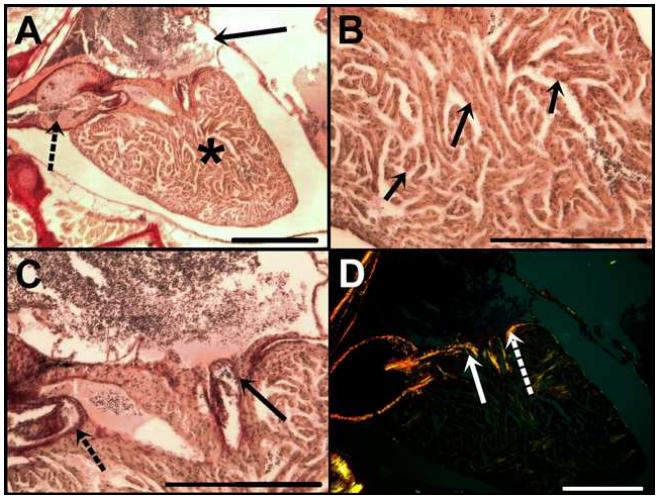

Fig. 1: (A) Lateral view of the cardiac region where are visualized the atrium (arrow), bulb (segmented arrow) and ventricle (asterisk); **(B)** Trabeculae of the ventricle (arrows); **(C)** Atrioventricular valve (arrow) and bulboventricular valve (arrow segmented); and **(D)** Collagen type I (segmented arrow) and collagen type III (arrow). Bar: 50µm. Picrosirius red, light microscopy (A, B and C) and polarized microscopy.

Regarding the GT, the cardiomyocyte nuclei were more abundant in the atrium than in the ventricle (AT: 6.0% ± 0.41 and VT: 3.84 ± 0.20), while myocardium (AT: 60.30%± 2.44 and VT: 68.77 ± 1.25) was more abundant in the ventricle (Figure 2A and 2B). A greater density of both the cardiomyocyte nuclei (GM: 6.93% ± 0.51 and GF: 6.38 ± 0.47) and myocardium (GM: 60.18% ± 2.77 and GF: 53.03 ± 2.19) was observed in the GM (Figure 2C, 2D, 2E and 2F).

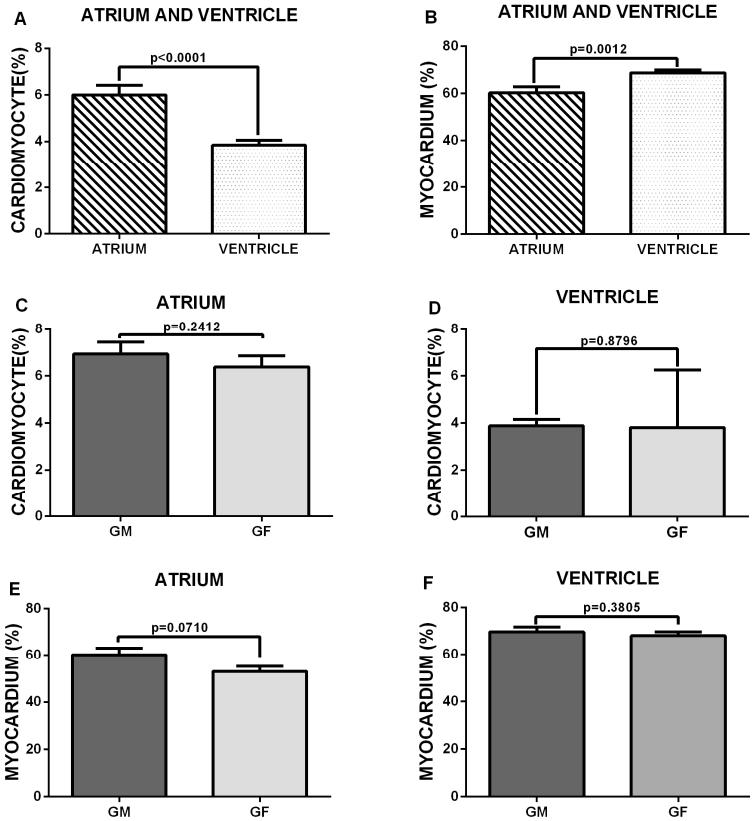

Fig. 2: (A) Comparison of density of cardiomyocytes between the atrium and ventricle of the GT; **(B)** Comparison of the density of myocardium in the atrium and ventricle of the GT; **(C)**Comparison of density of cardiomyocyte in the atrium between males and females; **(D)** Comparison of ventricular density of cardiomyocytes between males and females; **(E)** Comparison of ventricular density of myocardium between males and females; **(F)** Comparison of density of myocardium and cardiomyocytes between males and females. Graphical representation of the mean ± SEM. $P \leq 0.05$ by paired t test(n=10).

Regarding the quantification of the connective tissue of GT, the ventricle (8.02% ± 0.61) exhibited a lower density than the atrium (24.50% ± 1.35) when the two chambers were compared (Figure 3A). The atrium (GM: 22.80% ± 1.74 and GF: 27.96% ± 1.97) of GM showed a lower density of connective tissue while the ventricle (GM: 13.47% ± 1.08 and GF: 4.63% ± 0.29) showed a greater density compared to the atrium and ventricle of the GF (Figure 3B and 3C). Regarding the types of collagen in the GT, type I collagen fibers (3.61% ± 0.73) were more abundant than type III collagen fibers (3.13% ± 1.05) when the atrium and ventricle where analyzed together (Figure 4A). In this group, the type I collagen fibers (4.28% ± 1.20) were less abundant than type III collagen fibers (5.68% ± 1.90) in the atrium (Figure 4B), while type I collagen fibers (3.22% ± 0.89) were more abundant than type III collagen fibers (0.14% ± 0.05) in the ventricle (Figure 4C). Regarding the GM, the total density of type I collagen fibers (AT: 7.65% ± 2.20 and VT: 5.33% ± 2.05) in both the atrium and ventricle was higher compared to the GF (AT: 0.92% ± 0.40 and VT: 2.67% ± 0.99). The density of type III collagen fibers was greater in the atrium of the GM group (10.85% ± 3.58) compared to the GF group (0.51% ± 0.25). Conversely, the density of type III collagen fibers was lower in the ventricle of the GM (0.09% ± 0.05) compared to the GF (0.16% ± 0.06) (Figure 4D and 4E).

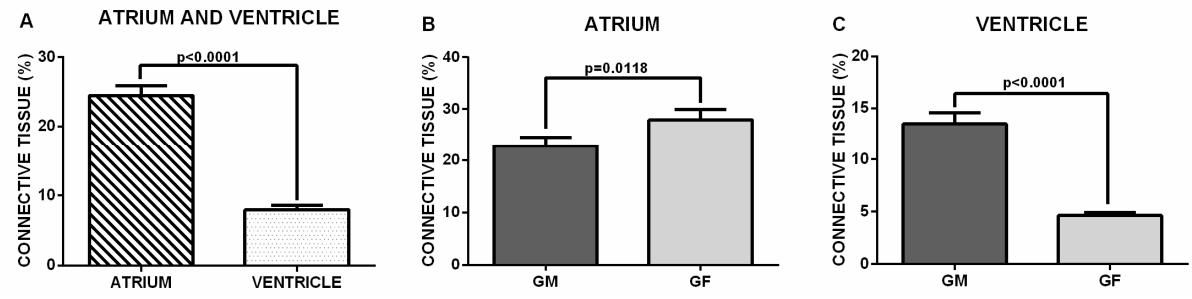

Fig. 3: (A) Comparison of density of connective tissue between the atrium and ventricle of the GT; **(B)** Comparison of the density of connective tissue in the atrium between GM and GF; **(C)** Comparison of the density of connective tissue in the ventricle between GM and GF. Graphical representation of the mean ± SEM. P ≤ 0.05 by paired t test(n=10).

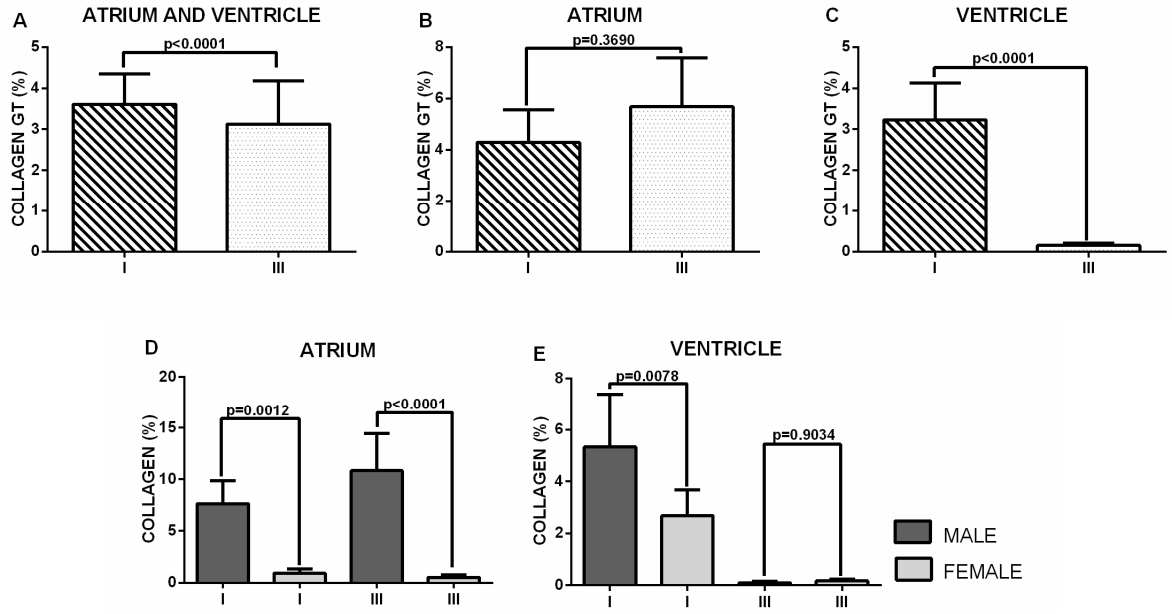

Fig. 4: (A) Comparison of density of collagen type I and type III between the atrium and ventricle of the GT; **(B)** Comparison of density of collagen type I and type III in the atrium of GT; **(C)** Comparison of density of collagen type I and type III in the ventricle of GT; **(D)** Comparison of density of collagen type I and type III in the atrium between GM and GF; **(E)** Comparison of density of collagen type I and type III in the ventricle between GM and GF. Graphical representation of the mean ± SEM. P ≤ 0.05 by paired t test(n=10).

When the total area of the myocardium was adjusted by the difference of the connective tissue areas, the following areas of cardiomyocytes/µm² were obtained:

Atrium: GT- 45.53, GM- 46.46 and GF- 38.20; and ventricle: GT- 63.25, GM- 60.26 and GF- 64.84.

Discussion:

The zebrafish is able to regenerate heart tissue, even after extensive tissue loss, (Poss, K.D. *et ai*., 2002) but the mechanisms involved in this process are still unclear. Because the process of zebrafish heart regeneration is still unknown, the density of the cardiomyocytes, connective tissue and type I and type III collagen fibers of the heart of these animals were quantified in the present study.

The ventricle exhibits stratified muscle layers, which favor contractile efficiency and ventricular pressure. (Hu, N. *et ai*., 2001). In the present study, the differences between the ventricle and atrium were statistically significant in the analyzed animals; the ventricle exhibited a stratified wall and a thick middle layer, which emphasizes the functional difference between the heart chambers. (Cerra, M.C. *et ai*., 2004) Although it showed distinct functional aspects, the density of cardiomyocyte tissue in the atrial and ventricular chambers was not significantly different between males and females. These data suggest that unlike other species, gonadal hormones do not affect the density of cardiomyocytes between the walls of the atrium and ventricle. (Fliegner, D. *et ai*., 2010)

The density of connective tissue in males was higher in the ventricle and lower in the atrium compared to that of females. This finding may be associated with the risk of heart disease, which is lower among women compared to men in the same age group (Vaccarino, V. *et ai*., 2011) and possibly occurs due to the protective action of estrogen that prevents collagen deposition in the heart. (Cabanelas, L.A. *et ai*., 2012) Therefore, it is suggested that there may be a distinguishing factor that is pre-determined by gender.

The quantitative data for collagen fibers found in the atrium and ventricle of zebrafish are crucial to elucidate their structural role in anchoring the cardiomyocyte layers and in supporting the vascular and nerve structures. The density of collagen fibers observed in the myocardium of zebrafish is higher than that observed in the myocardium of tilapia. (Waldemarin, K.C.A. *et ai*., 2012) Studies in eel (*Anguilla anguilla L*.) have shown that collagen can increase the stiffness of the compact layer and improve the resilience of the organ, which can contribute to improving the mechanical performance of the heart. (Cerra, M.C. *et ai*., 2004). The concentration of the two collagen fibers needs to be different, and this difference needs to be maintained, (Souza, R.R., 2002) because this feature basically ensures the maintenance of cardiac contractility. However, despite the predominance of type I collagen fibers, it is clear that they work together with the other tissues present in the interstitium.

Regarding gender, collagen was more abundant in the atrium of females, whereas it was more abundant in the ventricles of the males; the connective tissue exhibited the same pattern. A study demonstrated that intact rats exhibited a higher proportion of cardiac collagen compared to ovariectomized rats undergoing estrogen replacement therapy, suggesting that females exhibit greater cardiovascular protection due to the female hormones. (Cabanelas, L.A. *et ai*., 2012) The increase in myocardial type I collagen may also contribute to decreasing the elasticity of the ventricle with increasing age, impairing the normal functioning of the myocardium. (Souza, R.R., 2002) The female zebrafish likely exhibits this protective effect conferred by gonadal hormones because both connective tissue and type I collagen fibers are found in lower concentrations in the ventricle.

The amounts of cardiomyocytes, connective tissue and collagen are greater in the ventricle than in the atrium of females, whereas the amounts of connective tissue and type I collagenfibers are higher in the ventricles of males. This study describes the components of the young adult zebrafish heart. These data will be useful for gaining a better understanding of the normal and abnormal mechanisms of the morphology and functioning of the heart and will provide the basis for further studies on this topic.

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