Aging and Cardiac, Biochemical, Molecular and Functional Changes: an Experimental Study

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Abstract

Background: Aging involves physical and psychological changes that reduce the elderly’s ability to adapt themselves to society, which is the leading risk factor for cardiovascular diseases.

Objective: To investigate changes in the cardiovascular system resulting from the aging process in rats.

Methods: Murinometric/nutritional, echocardiographic and hemodynamic parameters were determined in 1, 5 and 12-month aged male rats. The expression of proteins that are critical to intracellular calcium dynamics and leptin signaling, as well as cardiac ATPase activity, was investigated in cardiac homogenates of rats. Data were expressed as mean ± standard error and analyzed by ANOVA one-way test (* p <0.05 vs. one month and #p <0.05 vs. 5 months).

Results: Whereas the body mass index increased (0.46±0.01 g/cm²; 0.75±0.01 g/cm²*, 0.78±0.01 g/cm²*), the food efficiency ratio (0.431±0.013; 0.359±0.003*; 0.364±0.001*), maximum speed during maximal exercise stress testing (3.36±0.34 km/h; 1.38±0.04 km/h*; 1.20±0.13 km/h*) and heart rate (410.2±5.9 bpm; 375.9±7.6 bpm*; 376.6±3.3 bpm*) decreased with age. Left ventricular hypertrophy and diastolic dysfunction along with reduced leptin receptor expression (2.1±0.4; 1.9±0.2; 0.8±0.2*) and SERCA-type calcium pump activity (1981±77 nmol Pi/mg protein/h; 2385±205 nmol Pi/mg protein/h; 1148±152 nmol Pi/mg protein/h#) were observed in the hearts.

Conclusions: Aging process is related to cardiometabolic risk, with cardiac leptin receptor downregulation and reduced cardiac SERCA2 calcium pump activity presumably being mechanisms underlying the left ventricular diastolic dysfunction and consequent exercise intolerance.

Keywords: Aging; Left ventricular dysfunction; Calcium signaling; Anthropometry; Nutritional physiological phenomena

Introduction

People get older and so do countries. The 1970’s were marked by an epidemiological change in the mortality profile of Brazilian population, when the infectious and parasitic diseases became second to cardiovascular diseases as the leading cause of mortality. Mortality profile emerged then to that of the “first world” countries. Since then, an unstoppable increase has been observed in cardiovascular diseases, attributed to urbanization, change in food habits, sedentary lifestyle and aging.

Population aging is a global phenomenon. Reduced mortality and fertility rates have provided a quantitative and proportional increase in the elderly group in society. Aging is a continuous process, during which both physical and psychological changes occur, causing elderly to lose ability to adapt themselves to society.

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Aging is the leading risk factor for cardiovascular diseases\textsuperscript{5}. Furthermore, anthropometric measures are an important nutritional component in clinical practice and in large epidemiologic studies\textsuperscript{4}. Many studies relate anthropometric measures to the risk of cardiovascular diseases\textsuperscript{5,6}.

Health-oriented educational actions are designed to build individuals’ autonomy and responsibility to take care of their own health and that of the whole community, through the transformation of knowledge and allowing citizenship to be practiced\textsuperscript{7}. Health-oriented education is one of the most promising ways for both individuals and families to adopt healthy behaviors and change health-damaging ones\textsuperscript{8}.

Despite the existence of a number of studies in the area of cardiovascular sciences that encompass both young and old humans, many questions remain unanswered as to how aging-regulating pathways in experimental models impact on the cardiovascular system aging. Literature lacks studies correlating the molecular and biochemical aspects of cardiac calcium homeostasis during the aging process directly to cardiac function and anthropometric/nutritional parameters within a single experimental model evaluated at the same ages\textsuperscript{9}.

Zhou and Gal\textsuperscript{9}, in their review manuscript, published in 2010, compare data from echocardiographic studies conducted in mice to findings on humans, using the aging process, in which the systolic function is preserved to the detriment of the diastolic function, and the exercise capacity is reduced. However, inferences drawn from the molecular mechanisms underlying diastolic dysfunction, involving calcium signaling pathway in hearts, were based on studies conducted in rabbits and rats, using different experimental protocols, not necessarily related to the aging process.

Lima and Menezes\textsuperscript{10} discuss in their work about the scarce production of knowledge on the long-lived elderly. This finding elicits the need to encourage practitioners and investigators to correlate the subjects of their studies with the social reality of aging populations and the remarkable increase in the number of long-lived elderly in the population. Despite not standing out as the country with the highest life expectancy rate, Brazil has the highest prevalence of studies on the long-lived population. However, a concentration of studies basically focused on clinical and curative approach is noticeable, with few significant studies on the longevity phenomenon and the peculiarities of the long-lived elderly. The results obtained evidence the need to promote greater visibility to the long-lived elderly and longevity phenomenon, considering the peculiarities that emerge at this stage of life. This process requires the development and implementation of government’s social and health care policies aimed at this sector of the population, as well as encouraging the production of knowledge on it.

This study aims, therefore, to evaluate possible biochemical, molecular, structural and functional changes in the cardiovascular system, as well as the cardiometabolic risk resulting from the aging process. Animal models enable a mechanistic parallel with human populations, which gets the benefits of interdisciplinary teams’ education, prevention and treatment actions into perspective in order to foster aging and well-being.

**Methods**

**Animals’ murinometric and nutritional profile evaluation**

Wistar male and female rats, provided by the animal housing room of the Núcleo de Animais de Laboratório (NAL) of Universidade Federal Fluminense (UFF) were used as parent stock for the experimental animals. All rats (parent stock and offsprings) were kept in the animal housing room of the Instituto Biomédico, at UFF, and were given free access to the commercial animal feed Nuvilar\textsuperscript{\textregistered} (standard feed used in the animal housing room) and water. The animals were maintained in a 12-hour, light-dark cycle and at a temperature of 21-25 °C. Wistar female rats were placed in boxes with male rats at a 2:1 ratio, and the offspring, after birth, was adjusted to six male offsprings per mother rat. The male animals were evaluated at the age of one (n=11), five (n=11) and 12 (n=4) months.

The following murinometric and nutritional parameters were evaluated\textsuperscript{11}:

(a) Body weight – body weight was monitored twice a week until the end of the experimentation, always at the same weighing time for all analysis days;

(b) Body length – nose-to-anus length (NAL) was measured with a measuring tape, after the animal was intraperitoneally anesthetized with thiopental (100 mg/kg);

(c) Food intake – after weaning, animal’s feed intake was monitored twice a week until the end of the
experimentation period. The amount of feed consumed is equal to the difference between the weight of the feed that was left in the cage and the total amount originally served. These data were used to calculate the daily feed intake according to the formula: feed intake (g)/number of days;

(d) Food efficiency ratio (FER) – the ratio between weight gain in a given period and the amount of feed consumed in the same period, expressed by FER = (FBW - IBW) / TF, where FBW is the animal’s final body weight (g) during the monitoring period; IBW is the animal’s body weight (g) at the beginning of the period, and TF the total amount of feed (g) consumed during the monitoring period. The food efficiency ratio was calculated from the weaning period to the 1st month of age (1 month), from the 1st to the 5th month of age (5 months) and from the 5th to the 12th month of age (12 months);

(e) Body mass index (BMI) – it is defined by the body weight (g) to NAL (cm$^2$) ratio.

**Functional tests**

Animals at the age of one (n=11), five (n=11) and 12 (n=4) months were submitted to a maximal exercise stress test, echocardiographic studies and tail plethysmography. After a five-day adaptation period, rats were submitted to treadmill exercise in order to reach a maximum stress level. The initial speed was 0.3 km.h$^{-1}$, followed by progressive increments of 0.3 km.h$^{-1}$ every 3 minutes until animals were exhausted.

After a minimum 72-hour period of maximal exercise stress testing, rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) and underwent echocardiography, with measurement of the ejection fraction, left atrium to aortic ratio, left ventricular mass and mitral deceleration time, in addition to the relative wall thickness, interventricular septum wall thickness, left ventricular posterior wall thickness and left ventricular internal dimension at diastole.

According to Wonders et al., cardiac function has been fully recovered within 72 hours after the maximal exercise stress testing. In order for mice’s systolic blood pressure and heart rate to be measured, they were placed in an acrylic cylindrical tube with an opening for the tail. A blood pressure cuff and a sensor that captures the signals to be recorded on the computer were adjusted in the proximal portion of the tail, attached to a sphygmmomanometer and a signal amplifier (PowerLab 8/30, ADInstruments, Dunedin, New Zealand). This equipment has been connected to a computer for storage and analysis of data (LabChart7, ADInstruments, Dunedin, New Zealand). Measurements were carried out in awake animals, always in the morning, considering only the values obtained in the absence of spontaneous movement of the animals’ tails. The final value of systolic blood pressure represented an average of six consecutive measurements. Before such measurement values were obtained, rats had been exposed to a three day adaptation period so that they could become accustomed to the procedure and stress interfering with the measurement could be minimized.

**Biochemical and molecular testing**

Rats at the age of one (n=3-6), five (n=4-6) and 12 (n=4-6) months were euthanized with an intraperitoneally administered, lethal dose of thiopental (100 mg/kg), then their hearts were removed in order to obtain homogenates. Rats were weighed and their hearts removed, dissected and rinsed with solution I (320 mM sucrose, 1 mM EDTA and 5 mM imidazole-HCl buffer, pH 7.2), dried in filter paper and weighed. Next, their organs were homogenized with the solution I plus 0.1 mM PMSF (phenylmethylsulfonyl fluoride) using Ultraturrax, followed by gauze filtration and ultracentrifugation. A crude preparation, more suitable for measurement and comparison between molecular entities within tissues in plasticity conditions for a reproducible and high recovery of protein, was used. The protein concentration of the biological preparations was determined by the Lowry method.

P-type ATPase activity was assessed by the colorimetric method of Fiske & Subbarow, using cardiac homogenates of rats at the age of one (n = 3), five (n = 4) and 12 (n = 4) months. For the Na$^+$/K$^+$ ATPase activity to be assessed, the preparation was incubated at 37 °C, for 2 hours, in a medium containing 84 mM NaCl; 3 mM KCl; 3 mM MgCl$_2$; 1.2 mM ATPNa$_2$; 2.5 mM EGTA; 10 mM sodium azide and 20 mM Tris-maleic acid buffer, pH 7.4, in the presence or absence of 1 mM ouabain. The specific enzyme activity is the difference between total ATPase activity and activity measured in the presence of 1 mM ouabain.

For the Ca$^{2+}$-ATPase activity to be measured, rat heart homogenates were incubated at 37 °C, for 1 hour, in a medium containing 50 mM Tris/HEPES, pH 7.4; 10 mM NaN$_3$; 0.3 mM EGTA; 5 mM ATPNa$_2$; 4 mM MgCl$_2$; 60 mM KCl; 5 uM A23187; with or without 10 mM of free Ca$^{2+}$, in the presence or absence of 3 uM thapsigargin. The Ca$^{2+}$ ATPase activity was determined by measuring the activity in the presence of Ca$^{2+}$ (Ca$^{2+}$-Mg$^{2+}$ ATPase...
activity) subtracted from the basic activity determined in the absence of this ion (Mg\(^{2+}\) ATPase activity). Thapsigargin-sensitive activity attributed to the sarcoplasmic reticulum Ca\(^{2+}\) pump (SERCA) was obtained by subtracting the Ca\(^{2+}\) ATPase activity measured in the presence of thapsigargin from Ca\(^{2+}\) ATPase activity observed in the absence of this substance\(^{20}\).

The expression of these proteins that are critical to the Ca\(^{2+}\) dynamics and the cardiac leptin receptor were assessed by Western blot analysis\(^{16,21}\), using heart homogenates of rats at the ages of one (n=6), five (n=6) and 12 (n=6) months. Samples were separated on polyacrylamide gels and transferred to nitrocellulose membrane. After the transfer, membranes were incubated in 5% (w/v) non-fat dried skimmed milk powder in 0.1% TBST (Tris-buffered saline plus Tween 20). Then, the membranes were incubated at room temperature with primary antibodies (anti-ObR, anti-SERCA or anti-Na\(^{+}\)/K\(^{+}\) ATPase). After washing, membranes were exposed to the peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence, using Hyperfilm ECL.

**Statistical analysis**

Data were expressed as mean ± standard error of the mean, and the differences among the three studied age groups were evaluated using the one-way analysis of variance (ANOVA), followed by post-hoc Bonferroni test (p<0.05* #). The study was approved by the Animal Research Ethics Committee at Universidade Federal do Rio de Janeiro, under no. CEPA / UFF00123-09.

**Results**

The animals’ weight and feed intake increased significantly with age (Table 1). However, as weight gain during aging increased more than feed intake in the same periods (data not shown), rats’ food efficiency ratio decreased with age (Table 1). Additionally, as the animals’ body weight increased more than the nose-to-anus length during aging, three and 12-month aged rats showed a BMI higher than that shown at the age of one month. Although these rats’ BMI was lower, the chest-to-abdominal circumference ratio did not vary significantly with age (Table 1).

Echocardiographic study data are shown in Table 2. The ejection fraction exceeded 50% in all age groups. However, left ventricular mass, interventricular septum wall thickness, left ventricular posterior wall thickness and left ventricular internal dimension, all measured at diastole, besides the mitral deceleration time, increased from five months of age. The relative wall thickness and left atrium to aortic ratio, in their turn, increased only at the age of 12 months.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1 month n=11</th>
<th>5 months n=11</th>
<th>12 months n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>109.0±2.3</td>
<td>386.9±7.7*</td>
<td>502.1±15.7*#</td>
</tr>
<tr>
<td>Feed intake (g/day)</td>
<td>18.6±0.6</td>
<td>25.3±0.2*</td>
<td>35.8±0.1*#</td>
</tr>
<tr>
<td>Food efficiency ratio</td>
<td>0.43±0.013</td>
<td>0.035±0.003*</td>
<td>0.003±0.001*#</td>
</tr>
<tr>
<td>Nose-to-anus length (cm)</td>
<td>16.0±0.4</td>
<td>23.9±0.2*</td>
<td>25.4±0.2*#</td>
</tr>
<tr>
<td>BMI (g/cm(^2))</td>
<td>0.46±0.01</td>
<td>0.75±0.01*</td>
<td>0.78±0.01*</td>
</tr>
<tr>
<td>Chest-to-abdominal circumference ratio</td>
<td>1.13±0.01</td>
<td>1.12±0.01</td>
<td>1.12±0.02</td>
</tr>
</tbody>
</table>

BMI – body mass index
* p<0.05 vs. one month; # p<0.05 vs. 5 months
The one-month aged animals (n=11) reached a maximum speed of 3.36±0.34 km/h during the maximal exercise stress testing, whereas the ones at the ages of five (n=11) and 12 months (n=4) reached a significantly lower maximum speed of 1.38±0.04* km/h and 1.20±0.13* km/h respectively (*p<0.05 vs. 1 month).

No significant differences were observed in systolic blood pressure in the different age groups (Table 3). Reduced heart rates, however, were observed from the age of five months.

Western blot analysis with rat heart homogenates of the three age groups did not reveal any change in neither expression of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase pump and the sodium-potassium ATPase in the plasma membrane (Figure 1). However, a significant reduction in leptin receptor expression at the age of 12 months was observed.

Although no difference was found in protein expression, a reduced activity of the cardiac SERCA calcium pump at the age of 12 months was observed (Table 4). Cardiac sodium-potassium ATPase pump remained unchanged, and so did its expression.

### Table 2
**Echocardiographic parameters during aging**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1 month  n = 11</th>
<th>5 months n = 11</th>
<th>12 months n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction (%)</td>
<td>93.83±0.87</td>
<td>88.06±1.65*</td>
<td>89.20±2.33</td>
</tr>
<tr>
<td>Relative wall thickness (cm)</td>
<td>0.70±0.01</td>
<td>0.70±0.05</td>
<td>0.62±0.02*</td>
</tr>
<tr>
<td>Left ventricular mass (g)</td>
<td>0.78±0.01</td>
<td>1.44±0.04*</td>
<td>1.78±0.13*#</td>
</tr>
<tr>
<td>Interventricular septum wall thickness (cm)</td>
<td>0.133±0.003</td>
<td>0.206±0.005*</td>
<td>0.249±0.011*#</td>
</tr>
<tr>
<td>Left ventricular posterior wall thickness (cm)</td>
<td>0.136±0.003</td>
<td>0.215±0.007*</td>
<td>0.244±0.015*</td>
</tr>
<tr>
<td>Left ventricular internal dimension (cm)</td>
<td>0.390±0.013</td>
<td>0.681±0.020*</td>
<td>0.707±0.027*</td>
</tr>
<tr>
<td>Left atrium to aortic ratio</td>
<td>1.02±0.01</td>
<td>1.02±0.02</td>
<td>1.63±0.15*#</td>
</tr>
<tr>
<td>Mitral deceleration time (ms)</td>
<td>61.67±2.21</td>
<td>85.82±3.19*</td>
<td>86.67±1.76*</td>
</tr>
</tbody>
</table>

* p<0.05 vs. one month; # p<0.05 vs. 5 months

### Table 3
**Hemodynamics measures during aging**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1 month  n = 11</th>
<th>5 months n = 11</th>
<th>12 months n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>118.3±0.3</td>
<td>117.4±0.9</td>
<td>118.6±0.6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>410.2±5.9</td>
<td>375.9±7.6*</td>
<td>376.6±3.3*</td>
</tr>
</tbody>
</table>

* p<0.05 vs. one month
**Discussion**

Obesity in rats can be estimated using BMI. According to Novelli et al.\(^1\), BMI values ranging from 0.45 \(\text{g/cm}^2\) to 0.68 \(\text{g/cm}^2\) are associated with adult male Wistar rats considered to be eutrophic. Thus, the data obtained from this study indicate that obesity starts from the age of five months, as the values found were above the eutrophic threshold limit for this species. Changes in this index are also associated with the dyslipidemic profile, oxidative stress and suggest increased risk for cardiovascular diseases\(^1,2\).

The increase in food efficiency ratio due to the higher weight gain as compared to the increase in feed intake observed in this study suggests that the energy consumption is reduced by the aging process. Toste et al.\(^2\), in their work, mention that leptin, a protein produced by the obese gene and mainly secreted by adipocytes, decreases food intake and increases energy expenditure through a pathway involving hypothalamic leptin receptors. Resistance to leptin, due to, for example, a decreased expression of leptin hypothalamic receptors, is associated with obesity\(^2\) and can explain both the age-related increase in the food efficiency rate and in the BMI.

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**Table 4**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age</th>
<th>1 month (n=3)</th>
<th>5 months (n=4)</th>
<th>12 months (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoplasmic reticulum calcium-ATPase – SERCA (nmol Pi/mg protein/h)</td>
<td>1981±77</td>
<td>2385±205</td>
<td>1148±152(#)</td>
<td></td>
</tr>
<tr>
<td>Sodium-potassium ATPase pump (nmol Pi/mg protein/h)</td>
<td>2555±37</td>
<td>1586±248</td>
<td>2586±566</td>
<td></td>
</tr>
</tbody>
</table>

* * p<0.05 vs. 5 months
Echocardiographic studies showed that the left ventricular ejection fraction exceeded 50% for the three age groups studied. These data suggest that systolic function remained preserved in these animals. An increased left ventricular mass observed from the age of five months, and the increase in other echocardiographic parameters at diastole, such as the interventricular septum wall thickness and posterior wall thickness, are indicative of left ventricular hypertrophy associated with the aging process. The increase in the relative wall thickness only at the age of 12 months suggests that the eccentric hypertrophy observed at the age of five months has evolved into concentric hypertrophy. All these morphological and functional findings associated with increased left ventricular internal diameter and left atrium-to-aorta ratio are indicative of diastolic dysfunction and consistent with Zhou and Gal’s description in their review manuscript, published in 2010. Both ventricular dilation and progressive mitral deceleration time observed in these animals may reflect an attempt to normalize filling pressure. In 1993, Yamamoto et al. showed that mitral deceleration time and heart rate are inversely related, which was also observed in this study, as the increase in that echocardiographic parameter was accompanied by reduced heart rate in animals from the age of five months. In 1991, Ferrari et al. showed that aging increases the response of cardiac muscarinic receptors to acetylcholine, a neurotransmitter that stimulates the parasympathetic activity, which may explain the findings as to the mitral deceleration time and heart rate.

Cardiorespiratory capacity can be inferred by the maximal exercise stress testing. Literature provides a linear relationship between rats’ maximum running speed and their oxygen consumption rate. Thus, the data obtained from this study suggest a reduced tolerance to exercise from five months of age, in addition to cardiac dysfunction demonstrated by echocardiography.

Recent evidence suggests that leptin may also have an impact on cardiac function by stimulating cardiac leptin receptors. In this study, data on leptin receptor downregulation at the age of 12 months, when most of the morphological and functional changes have already occurred, are consistent with those of literature. The absence of leptin signaling in mice, obtained with deficiency of this peptide or its receptor, was associated with age-related left ventricular hypertrophy, as observed in this study.

Reduced calcium uptake by the sarcoplasmic reticulum is associated with slowing of heart muscle relaxation rate during aging. Although this study did not reveal any change in neither the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase pump expression, nor the sodium-potassium ATPase expression, the SERCA calcium pump activity was significantly reduced. These findings, according to Xu and Narayanan, indicate that changes in intrinsic functional properties of the SERCA pump and/or in its phosphorylation-dependent regulation may contribute to the relaxation impairment associated with diastolic dysfunction observed in this study.

The reported difference in size between the experimental groups from among the murinometric, nutritional and functional studies was due basically to logistical issues, including the animal housing room infrastructure still under development and the difficulty in housing animals at the age of 12 months for the purpose of observation. For biochemical and molecular testing, the size of experimental groups was more homogeneous to one another and appropriate to the type of measure obtained, although reduced when compared to murinometric, nutritional and functional studies. Reported differences do not compromise data analysis and quality of the information obtained.

**Conclusions**

Aging process is related to cardiometabolic risk. Data obtained suggest that downregulation of cardiac leptin receptors and reduced cardiac sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} ATPase (SERCA) pump activity are presumably some of the molecular and biochemical mechanisms underlying the left ventricular diastolic dysfunction and the resulting age-related exercise intolerance.

**Potential conflicts of interests**

No relevant conflicts of interest.

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**Academic association**

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