Fasting/Refeeding Cycles Prevent Myocardial Dysfunction and Morphology Damage in the Spontaneously Hypertensive Rats

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Abstract

Background: Caloric restriction is known to impair the cardiac function and morphology in hypertrophied hearts of spontaneously hypertensive rats (SHR); however, the influence of fasting/refeeding (RF) is unknown.

Objective: To investigate the fasting/refeeding approach on myocardial remodeling and function. In addition, the current study was designed to bring information regarding the mechanisms underlying the participation of Ca\(^{2+}\) handling and \(\beta\)-adrenergic system.

Methods: Sixty-day-old male SHR rats were submitted to food ad libitum (C), 50\% food restriction (R\(_{50}\)) or RF cycles for 90 days. Cardiac remodeling was assessed by ultrastructure analysis and isolated papillary muscle function. The level of significance considered was 5\% (\(\alpha = 0.05\)).

Results: The RF rats presented lower cardiac atrophy than R\(_{50}\) in relation to C rats. The C rats increased weight gain, R\(_{50}\) maintained their initial body weight and RF rats increased and decreased weight during RF. The RF did not cause functional impairment because the isotonic and isometric parameters showed similar behavior to those of C. The isotonic and isometric cardiac parameters were significantly elevated in RF rats compared to R\(_{50}\) rats. In addition, the R\(_{50}\) rats had cardiac damage in relation to C for isotonic and isometric variables. While the R\(_{50}\) rats showed focal changes in many muscle fibers, the RF rats displayed mild alterations, such as loss or disorganization of myofibrils.

Conclusion: Fasting/refeeding promotes cardiac beneficial effects and attenuates myocardial injury caused by caloric restriction in SHR rats, contributing to reduce the cardiovascular risk profile and morphological injuries. Furthermore, RF promotes mild improvement in Ca\(^{2+}\) handling and \(\beta\)-adrenergic system. (Arq Bras Cardiol. 2018; [online].ahead print, PP0-0)

Keywords: Rats; Hypertension; Myocardial/dysfunction; Chronic Disease; Fasting; Refeeding; Caloric Restriction

Introduction

The major causes of chronic non-communicable diseases (NCD)-attributable mortality are cardiovascular disease, cancers, chronic respiratory disease and diabetes.1 These conditions share a small number of behavioral risk factors, which aggravate the NCD and include unhealthy diet, which is closely related to hypertension.

Caloric restriction (CR) has been recognized throughout history for promoting several beneficial effects.2,3 Nevertheless, although CR may prevent cardiac damage in hypertrophied hearts of spontaneously hypertensive rats (SHR),4 it is common to note body weight fluctuations typically referred to as the "yo-yo syndrome" while on a regimented diet, and these fluctuations have shown deleterious cardiovascular effects.5,6 Researches from our laboratory and others have shown that, when dietary restriction is severe, it can promote morphological injuries and impairment of cardiac function in normal or SHR rats.7-12

Recently, intermittent fasting or fasting/refeeding has also shown to extend lifespan and have beneficial health effects as compared to ad libitum food consumption.3,13,14 as it enhances cardiovascular function and improves several risk factors for cardiovascular diseases.15,16 This dietary approach also implies a protective effect against oxidative stress, lower rates of kidney disease,17 prolongation of reproductive function,18 and leads to the normalization of resting energy expenditure and protein synthesis recuperation, but can cause many metabolic disturbances.15,20

In normotensive rats submitted to food restriction, chronic refeeding decreased the incidence of cardiac arrhythmia and reversed the depletion of heart proteins.21,22 Food restriction caused cardiac function disturbances that were almost completely reversed back to normal after chronic refeeding in the isolated rat heart.23 In our laboratory, we observed that fasting/refeeding cycles reversed the mechanical dysfunction...
and attenuated the structural injuries in papillary muscles caused by CR in normotensive rats. Nevertheless, it is not yet clear whether fasting/refeeding cycles are able to promote similar effects and/or reverse the cardiac damage induced by food restriction in SHR rats. Thus, the objective was to investigate the fasting/refeeding approach on myocardial remodeling and function. In addition, the current study was designed to bring information regarding the mechanisms underlying the participation of Ca²⁺ handling and β-adrenergic system. Our hypothesis is that fasting/refeeding condition would attenuate the myocardial injury caused by food restriction and would contribute to normal cardiac remodeling in SHR rats without alterations in the Ca²⁺ handling and β-adrenergic system.

Methods

Animal model and experimental protocol

Sixty-day-old male SHR were distributed into three groups: control (C, n = 7); food-restriction (RF, n = 7); and fasting/refeeding cycles (RF, n = 7). The C group was fed Labina rat chow containing 7.0% fat, 20.55% protein, 62.95% carbohydrate, 5.0% fiber and 4.5% moisture (Agribands, Brazil), and water was provided ad libitum. All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and were approved by the ethics committee of Botucatu School of Medicine, UNESP, São Paulo, Brazil.

Table 1 – Ingredient composition of the Labina rat experimental diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>(g/kg)</th>
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<tbody>
<tr>
<td>Starch</td>
<td>307.5</td>
</tr>
<tr>
<td>Dextrinized Com Starch</td>
<td>132.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>629.5</td>
</tr>
<tr>
<td>Casein</td>
<td>200.0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>Protein</td>
<td>205.5</td>
</tr>
<tr>
<td>Soy oil</td>
<td>20.0</td>
</tr>
<tr>
<td>Fat</td>
<td>7.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>7.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

Systolic blood pressure

Systolic blood pressure evaluation was assessed by the non-invasive tail-cuff method with a Narco BioSystems Electro-Sphygmomanometer (International Biomedical, Austin, TX, USA) at the beginning and after the end of the experimental protocol. The average of two pressure readings was recorded for each animal.

Isolated muscle performance

Cardiac intrinsic contractile performance was evaluated by studying isolated left ventricular (LV) papillary muscle as described previously. Isometric contraction parameters, including peak of developed tension (DT, g/mm²), defined as peak isometric tension minus resting tension, resting tension (RT, g/mm²), time to peak tension (TP, ms), peak isometric tension development rate (+dT/dt, g/mm²/ms) and maximum tension decline rate (-dT/dt, g/mm²/ms), time from peak tension to 50% relaxation (RT, g/mm²) were measured. The isotonic parameters were percentage of shortening (PS, %), time to peak shortening (TPS, ms), maximum shortening velocity (-dL/dt, ML/s) and maximum relaxation velocity (+dL/dt, ML/s).

The mechanical behavior of the papillary muscle was evaluated under baseline conditions at 1.25 mM [Ca²⁺] and after the following inotropic maneuvers: increase in extracellular Ca²⁺ concentration from 0.625 to 1.25, 2.5 and 5.2 mM, and β-adrenergic stimulation with 0.01, 0.1 and 1.0 µM isoproterenol. The parameters used to characterize papillary muscle were as follows: length (mm), weight (mg) and CSA (mm²). Muscle length (ML) at peak DT was defined as Lmax in vitro and measured with a Gartner catheter (Chicago, IL, USA). To compare the mechanical function between different muscle lengths, isometric and isotonic parameters were normalized to CSA and Lmax.

Morphological study

For the ultrastructural study (three animals per group), small pieces of the LV papillary muscle were fixed in Karnowsky’s fixative in 0.12 M phosphate, pH 7.2, for 1-2 hours and were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 hours. After dehydration in a graded ethanol series, the samples were embedded in epoxy resin. Ultrathin sections were cut from selected areas with a diamond knife, double-stained with uranyl acetate and lead citrate, and examined using a Philips EM 301 electron microscope. The LV myocyte CSA was measured using a compound microscope attached to a computerized imaging analysis system (Image-Pro Plus 3.0, Media Cybernetics, Silver Springs, MD, USA).

Statistical analysis

Statistical analyses were performed using SigmaStat 3.5 software (SYSTAT Software Inc., San Jose, CA, USA). Normally distributed variables from general characteristics and
Results

General and morphological characteristics of rats

Significantly higher values of FBW, LVW, RVW, LVW/FBW and RVW/FBW were found in C compared to R_{50} and RF rats (Table 2). After 12 weeks, fasting/refeeding cycles promoted a substantial elevation of FBW and food consumption that were significantly greater than those in the R_{50} group. In relation to cardiac parameters, the RF and R_{50} groups presented different behavior. Specifically, the LVW (RF: 12.12% and R_{50}: 48.5%; p < 0.05), RVW (RF: 19.04% and R_{50}: 47.62%; p < 0.05), LVW/FBW (RF: 6.64% and R_{50}: 19.2%; p < 0.05) and RVW/FBW (RF: 12.06% and R_{50}: 18.96%; p < 0.05) were reduced significantly in RF rats as compared to C rats. Nevertheless, the fasting/refeeding cycles presented lower cardiac atrophy than R_{50} rats in relation to C rats.

In addition, C rats experienced increasing weight gain, while R_{50} rats maintained their IBW after 12 weeks of experimental protocol (Figure 1). On the other hand, RF rats gained weight dependent on food intake, with body weight increasing and decreasing during refeeding and fasting, respectively (Figure 1).

Isolated muscle performance

Fasting/refeeding cycles did not cause functional impairment (Tables 3 and 4). Nevertheless, the isotonic [-dL/dt, TPS, and RT_{ps}) and isometric parameters (TPT, +dT/dt, -dT/dt, RT_{dp}) were significantly elevated in RF rats compared to those in the R_{50} group, indicating that fasting/refeeding cycles preserve the contraction and relaxation phase of cardiac function. Furthermore, the R_{50} rats presented cardiac damage in relation to the C group for isotonic and isometric variables. In addition, the papillary muscle CSA showed no difference among groups.

Calcium stimulation

After baseline condition, the increases in extracellular Ca^{2+} concentrations from 0.625 to 5.2 mM resulted in a positive inotropic effect in myocytes from all groups (Figures 2A-F). However, the results shown in Figures 2B, C and E indicate that extracellular Ca^{2+} (1.25 and 2.5 mM) induced a greater response in +dT/dt (RF: 99.1 ± 23.6; 132.1 ± 36.2 g/mm^2/s vs. R_{50}: 63.2 ± 12.8; 91.5 ± 22.0 g/mm^2/s; p < 0.05, respectively), -dT/dt (RF: 30.6 ± 5.9; 35.9 ± 5.8 g/mm^2/s vs. R_{50}: 22.0 ± 4.4; 28.5 ± 6.1 g/mm^2/s; p < 0.05, respectively) and -dL/dt (RF: 2.19 ± 0.45; 2.77 ± 0.51 ML/s vs. R_{50}: 1.47 ± 0.24; 1.99 ± 0.31 ML/s; p < 0.05, respectively) in the RF rats than in the R_{50} rats. In addition, -dT/dt and -dL/dt were significantly diminished in the R_{50} myocardium at Ca^{2+} concentration of 5.2 mM when compared to those in the RF group. When submitted to inotropic maneuvers, DT, PS and +dT/dt were similar between RF and R_{50}. In relation to the cardiac function of C rats after Ca^{2+} stimulation, the fasting/refeeding cycles presented similar behavior (Figures 2A-F). The only significant result between C and R_{50} was noted in the highest

Table 2 – General characteristics of rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>IBW (g)</td>
<td>247 ± 15</td>
</tr>
<tr>
<td>FBW (g)</td>
<td>366 ± 14</td>
</tr>
<tr>
<td>FC (g/week)</td>
<td>159 ± 23</td>
</tr>
<tr>
<td>SBP initial (mmHg)</td>
<td>177 ± 8</td>
</tr>
<tr>
<td>SBP final (mmHg)</td>
<td>163 ± 13</td>
</tr>
<tr>
<td>LVW (g)</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>RVW (g)</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>LVW/FBW (mg/g)</td>
<td>2.71 ± 0.05</td>
</tr>
<tr>
<td>RVW/FBW (mg/g)</td>
<td>0.58 ± 0.04</td>
</tr>
</tbody>
</table>

C: control group; R_{50}: animals with food restriction of 50%; RF: animals with alternation between food restriction of 50% and refeeding; IBW: initial body weight; FBW: final body weight; FC: food consumption; SBP: systolic blood pressure; LVW: left ventricle weight; RVW: right ventricle weight. Values are means ± SD (n = 7).

* significant at p < 0.05 vs. C; † p < 0.05 vs. R_{50}. One-way ANOVA and post hoc Tukey’s test.
Changes in body weight after 90 days of treatment. Control (C; closed squares, n = 7), animals with food restriction of 50% (R_{50}; closed triangles, n = 7) and animals with alternation between food restriction of 50% and refeeding (RF; open circles, n = 7). Values are means ± SD; * significant at p < 0.05, R_{50} vs. C; † p < 0.05, RF vs. R_{50}; ‡ p < 0.05, C vs. RF. Repeated measures two-way ANOVA; post hoc Bonferroni’s test. Source: Research team.

Myocardial morphology

The C group rats showed normal morphological characteristics, with myofibrils filling the entire sarcoplasm, well-defined sarcomeres, mitochondria with lamellar cristae, sarcoplasmic membranes with regular aspect, sarcoplasmic reticulum among myofibrils and nuclei with uncondensed chromatin (Figures 4A and B). The R_{50} group presented focal changes, including disorganization or absence of myofibrils, some polymorphic mitochondria with a decreased number of cristae and areas of sarcoplasmic reticulum dilation (Figures 4C, D and E). In RF rats, the only change observed was a loss of mitochondrial cristae in some organelles. Most of the fibers had normal morphology (Figures 4F and G).

Discussion

Interestingly, little information is available on the relationship between cardiac function and morphology during fasting/feeding in SHR hypertrophied hearts. Within this context, this dietary regimen has become the subject of

| Table 3 – Isotonic contraction of groups at baseline condition |
|------------------|------------------|------------------|
|                  | Groups           |                  |
|                  | C               | R_{50}           | RF               |
| PS (%)           | 19 ± 3          | 18 ± 3           | 20 ± 2           |
| -dL/dt (ML/s)    | 1.89 ± 0.40     | 1.60 ± 0.36      | 2.19 ± 0.45†     |
| TPS (ms)         | 168 ± 26        | 205 ± 14‡        | 161 ± 14‡        |
| +dL/dt (ML/s)    | 4.28 ± 1.26     | 4.13 ± 1.11      | 4.79 ± 0.86      |
| RT_{50} (ms)     | 55 ± 10         | 76 ± 12‡         | 53 ± 9‡          |
| CSA (mm²)        | 0.95 ± 0.22     | 0.85 ± 0.17      | 0.91 ± 0.18      |

C: control group; R_{50}: animals with food restriction of 50%; RF: animals with alternation between food restriction of 50% and refeeding; PS: percentage of shortening; -dL/dt: maximum shortening velocity; TPS: time to peak shortening; +dL/dt: maximum relaxation velocity; RT_{50}: time from peak tension to 50% relaxation; CSA: muscle cross-sectional area. Values are means ± SD (n = 7) at basal calcium concentration (1.25 mM); * significant at p < 0.05 vs. C; † p < 0.05 vs. R_{50}. One-way ANOVA and post hoc Tukey’s test.

Ca^{2+} concentration (5.2 mM); -dL/dt was significantly lower in R_{50} than in C group (C: 2.66 ± 0.35 vs. R_{50}: 2.18 ± 0.33 ML/s, p < 0.05) (Figure 2E).
considerable scientific interest for weight loss and improving cardiometabolic health. Thus, the main finding of this study was that fasting/refeeding attenuated the damage caused by CR. The results reveal that fasting/refeeding showed increased isotonic and isometric parameters at baseline, as well as improved the myocardial inotropic response to calcium and isoproterenol. In addition, fasting/refeeding prevented cardiac atrophy and morphological injuries.

Less body weight gain was observed in the RF group than in the C group (Table 1, Figure 1), but more body weight gain than in the R_{50} group. According to literature, body weight reduces approximately 13% when the animals are submitted
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This result appears to be mediated by hormones, such as leptin that acts regulating appetite and weight gain. A rapid inhibition of ob gene expression in the white adipose tissue occurs in fasting, and this effect can be reversed by refeeding. 25,26

Cardiac hypertrophy, a major pathological process involved in cardiac remodeling, initially serves as a compensatory mechanism to preserve cardiac output. 27 Cardiac remodeling may be regarded as a first step in the sequence of adaptive responses of the heart to stress caused by a large number of physiological and pathological conditions, such as changes in volume and pressure loads and/or metabolic alterations. 28

Current study revealed that fasting/refeeding induced cardiac atrophy visualized by reduced total heart and left ventricle, as well as in the LVW/FBW. A decrease in left ventricle weight relative to body weight is very common in small animals submitted to food restriction 22 and fasting/refeeding. 29

Inhibition of myocardial protein synthesis and reduction in average protein half-lives are possible explanations for reduced cardiac mass under starvation. 30 Protein synthesis, an anabolic process, is required for cardiac hypertrophy. Two major pathways regulating protein synthesis are inhibited by AMPK, a primary regulator of metabolic pathways, which plays an essential role in a wide variety of cellular processes to protect against cardiac hypertrophy. 31 Therefore, cardiac atrophy could be regulated by the common signaling pathway of AMPK in the hypothalamus.

In the ultrastructural analysis, food restriction caused focal morphological damage in most papillary muscle fibers. The same alterations were less intense in the intermittent refeeding condition. Intermittent refeeding seems to aid in the attenuation of the mechanisms responsible for this damage and seems to act by enhancing protein anabolism and retarding protein degradation. Recent findings suggest that the beneficial effects of refeeding result from a reduction in oxidative injury and an increase in cellular stress resistance. 2,32 One possible mechanism for our result may be linked to the expression of atrogin-1, an E3 ubiquitin ligase also known as muscle atrophy F-box (MAFbx). E3-ligases are part of the ubiquitin proteasome pathway utilized for protein degradation during muscle atrophy. The literature has shown that atrogin-1/MAFbx expression results in muscle atrophy during catabolic condition. 33,34

The isolated papillary muscle analysis showed that food restriction promotes cardiac dysfunction, but refeeding condition prevents the state. These stimuli provide evidence that the improvement of myocardial function assigned to fasting/refeeding cycles was related to changes in intracellular Ca²⁺ handling, mainly in the recapture and/or extrusion of

Figure 3 – Effects of isoproterenol stimulation on myocardial function in papillary muscles from control (C = black bars), animals with food restriction of 50% (R 50 = gray bars) and animals with alternation between food restriction of 50% and refeeding (RF = white bars). Isoproterenol stimulation experiment: 7 animals each group. Isometric parameters: A: DT (peak developed tension normalized per cross-sectional area); B: +dT/dt (peak isometric tension development rate normalized per cross-sectional area); C: -dT/dt (maximum tension decline rate normalized per cross-sectional area). Isotonic parameters: D: PS (percentage of shortening); E: -dL/dt (maximum shortening velocity at L max); F: +dL/dt (maximum relaxation velocity at L max). L max: muscle length at peak DT. Values are means ± SD; * significant at p < 0.05 vs. C; † p < 0.05 vs. R 50 . Repeated measures two-way ANOVA and post hoc Tukey’s test. Source: Research team.
cytosolic Ca\(^{2+}\), and β-adrenergic system. Nevertheless, the lower response of food restricted rats to the increase of extracellular Ca\(^{2+}\) concentration can be related to changes in the general mechanisms involved in Ca\(^{2+}\) cycling such as sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger, sarcolemmal L-type channel, sarcoplasmic reticulum (SR), ryanodine receptor, SR Ca\(^{2+}\) uptake pump, and the myofilament Ca\(^{2+}\) sensitivity. In relation to RF, this process may be faster and more balanced, but no study was found to support this statement and show the activity and protein expression of Ca\(^{2+}\) handling regulatory proteins.

Another explanation could be related to the role of cytokine in intermittent fasting mediated cardioprotection. The influx of inflammatory cells and production of pro-inflammatory mediators contribute to myocardial injury. Nevertheless, adiponectin can protect myocardial cells against ischemic injury by activating the cyclic AMP-dependent protein kinase - Akt pathway, being the latter mediated, in part, by caloric restriction. Thus, the beneficial effects of fasting/refeeding may function through anti-inflammatory cytokine pathways.

Few studies have evaluated the β-adrenergic components in experimental models of fasting/refeeding. Some studies have shown that cardiac function impairment is related to β-adrenergic system changes, while other researchers have not reported reduced β-adrenergic response. The literature shows that a decrease in cardiac β-receptor number has been reported in several hypertensive models known to be associated with an increase in sympathetic nerve activity, including SHR. Thus, the association between increased sympathetic activity and cardiac β-receptor downregulation is sufficiently close to suggest that the finding of decreased β-receptor number after starvation and refeeding is indicative of persistently elevated cardiac sympathetic drive. However, in the current study, there is no damage of β-system in the RF rats, since the cardiac function was similar to that of the C group. The present data tend to support the hypothesis that isoproterenol stimulation reveals that the β-adrenergic system and cAMP phosphorylation of proteins related to Ca\(^{2+}\) handling were preserved in refeeding rats.

Thus, fasting/refeeding cycles have become the subject of considerable scientific interest as a potential dietary approach for weight-loss and improving cardiometabolic health.
The beneficial effects of the intermittent fasting result from at least two mechanisms: the oxidative stress and the stress resistance hypothesis. According to literature, during the intermittent fasting, there are fewer free radicals produced in the mitochondria of cells and, therefore, less oxidative damage to the cells. Another hypothesis is the resistance to stress that is associated with increased resistance of cells in many different tissues to injury induced by oxidative, genotoxic and metabolic insults. The conservation of stress resistance responses to intermittent fasting across a range of species provides strong evidence that this mechanism contributes to the lifespan-extending action of dietary restriction.

It is worth noting that according to studies in rodents and humans, intermittent food restriction is capable of promoting weight loss and/or favorably influence an array of cardiometabolic health indices, with equal or greater efficacy than conventional continuous energy restriction approaches, such as food restriction. Fasting/refeeding cycles increase cardiac tolerance to ischemic injury and can affect the development of cardiovascular disease, preventing postinfarct cardiac remodeling, and impeding chronic heart failure. Comparing the two dietary approaches, studies show that caloric restriction may exert its beneficial effects primarily by reducing oxidative stress, whereas RF may act primarily by a stress resistance mechanism, which can have a cardioprotective effect.

Study limitations

The study did not investigate the activity and protein expression of Ca\(^{2+}\) handling regulatory proteins known to affect myocardial contraction and relaxation. In addition, the current study did not evaluate the involvement of anti-inflammatory cytokines, free-radical production and cellular stress response, which could help and consolidate the beneficial effects of intermittent fasting.

Conclusion

We demonstrated that fasting/refeeding promotes cardiac beneficial effects and attenuates myocardial injury caused by CR in SHR rats, contributing to the reduction of cardiovascular risk profile and morphological injuries. Furthermore, RF promotes mild improvement in the Ca\(^{2+}\) handling and \(\beta\)-adrenergic system.

Author contributions

Conception and design of the research: Pinotti MF, Cicogna AC, Leopoldo AS; Acquisition of data: Pinotti MF, Matias AM, Sugizaki MM, Nascimento AF, Pai MD, Leopoldo APL; Analysis and interpretation of the data: Pinotti MF, Sugizaki MM, Nascimento AF, Pai MD, Leopoldo APL; Statistical analysis: Sugizaki MM, Nascimento AF, Pai MD, Leopoldo APL; Obtaining financing: Cicogna AC; Writing of the manuscript: Pinotti MF, Matias AM, Cicogna AC, Leopoldo AS; Critical revision of the manuscript for intellectual content: Matias AM, Pai MD, Leopoldo APL, Cicogna AC, Leopoldo AS.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

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Study Association

This study is not associated with any thesis or dissertation work.

Ethics approval and consent to participate

This study was approved by the Ethics Committee on Animal Experiments of the Faculdade de Medicina de Botucatu, UNESP under the protocol number 439/2004.

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