Obesity does not Lead to Imbalance Between Myocardial Phospholamban Phosphorylation and Dephosphorylation

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Abstract

**Background:** The activation of the beta-adrenergic system promotes G protein stimulation that, via cyclic adenosine monophosphate (cAMP), alters the structure of protein kinase A (PKA) and leads to phospholamban (PLB) phosphorylation. This protein participates in the system that controls intracellular calcium in muscle cells, and it is the primary regulator of sarcoplasmic reticulum calcium pump activity. In obesity, the beta-adrenergic system is activated by the influence of increased leptin, therefore, resulting in higher myocardial phospholamban phosphorylation via cAMP-PKA.

**Objective:** To investigate the involvement of proteins which regulate the degree of PLB phosphorylation due to beta-adrenergic activation in obesity. In the present study, we hypothesized that there is an imbalance between phospholamban phosphorylation and dephosphorylation, with prevalence of protein phosphorylation.

**Methods:** Male Wistar rats were randomly distributed into two groups: control (n = 14), fed with normocaloric diet; and obese (n = 13), fed with a cycle of four unsaturated high-fat diets. Obesity was determined by the adiposity index, and protein expressions of phosphatase 1 (PP-1), PKA, PLB, phosphorylated phospholamban at serine16 (PPLB-Ser16) were assessed by Western blot.

**Results:** Obesity caused glucose intolerance, hyperinsulinemia, hypertriglyceridemia, hyperleptinemia and did not alter the protein expression of PKA, PP-1, PLB, PPLB-Ser16.

**Conclusion:** Obesity does not promote an imbalance between myocardial PLB phosphorylation and dephosphorylation via beta-adrenergic system. (Arq Bras Cardiol. 2014; [online].ahead print, PP .0-0)

**Keywords:** Obesity; Phosphorylation; Rats; Leptin; Adyposity.

Introduction

The beta-adrenergic system (BAS) modulates cardiac performance via beta receptor, G protein, adenylyl cyclase, and cyclic adenosine monophosphate (cAMP). The cAMP alters protein kinase A (PKA), thus releasing the catalytic subunit and activating the phosphorylation of myocardial proteins, which are involved in calcium (Ca^{2+}) transport - Figure 1.

Phospholamban (PLB) participates in the control of intracellular calcium in the myocardium; it is the protein that regulates the activity of Ca^{2+} pump of the sarcoplasmic reticulum (SERCA2a); the dephosphorylated PLB forms the complex PLB-SERCA2a, which inhibits the pump and does not allow the transfer of the cytosolic Ca^{2+} to the sarcoplasmic reticulum; phosphorylation uncouples the complex PLB-SERCA2a, therefore increasing the calcium recapture by SERCA2a.

The connection PLB-SERCA2a is controlled by cycles of phosphorylation and dephosphorylation, by the action of PKA and phosphatase 1 (PP-1), respectively. The prevalence of PLB phosphorylation, site of serine 16, occurs with the activation of PKA. Simultaneously, phosphorylates the inhibitory protein (I-1), thus forming the complex I-1/PP-1 and preventing PLB dephosphorylation caused by PP-1. Dephosphorylation is prevalent when PKA is deactivated, therefore there is no PLB and I-1 phosphorylation; when phosphate is not added to I-1, the formation of I-1/PP-1 is not possible, which allows PP-1 to dephosphorylate PLB, in its active state (Figure 2).

Obesity - excessive fat tissue in relation to lean mass produces adipokines, which interfere in biological processes, including the activation of BAS by leptin. The BAS stimulation phosphorylates the myocardial PLB via cAMP-PKA. There are no studies analyzing the balance between PLB phosphorylation
and dephosphorylation via BAS in obesity. Relling et al\textsuperscript{11} used obese rats for 12 weeks and showed increased PLB expression and decreased phosphorylated PLB (pPLB). Lima-Leopoldo\textsuperscript{12} verified decreased pPLB via cAMP in serine 16 in obese rats for 15 weeks. These authors did not evaluate kinase and phosphatase proteins in the animals.

The inexistence of papers analyzing PLB activation and deactivation in obesity induced the investigation concerning the involvement of proteins that regulate PLB phosphorylation via BAS. The hypothesis of this study is that obesity promotes the imbalance between phospholamban phosphorylation and dephosphorylation, with prevalent phosphorylation.

**Methods**

**Animals and experimental protocol**

Twenty-seven male 30-day old Wistar rats were used, coming from the bioterium of the Medical Clinic Department at the Medical School of Botucatu (SP) — Unesp, under the following conditions: individual polypropylene cages with chrome wire tops covered with sterilized pine wood shaving; room temperature of 24°C and 12-hour light cycles. All of the procedures were conducted according to the Guide for the Care and Use of Laboratory Animals\textsuperscript{13}, being afterwards approved by the Committee on Animal Research and Ethics of the Medical School of Botucatu (Unesp, Botucatu), protocol number 765.

Animals were randomized into two groups: control (C) and obese (Ob). Animals in C (n = 15) were fed with a normocaloric diet, RC Focus 1765, Agroceres\textsuperscript{®}, Rio Claro, São Paulo, Brazil (22% protein, 42.7% carbohydrate, 4% fat, 9% minerals, 8% fibers, 12% humidity, 1.5% calcium, 0.8% phosphorus); animals in group Ob received a cycle of four hyperlipidic diets Agroceres\textsuperscript{®}, Rio Claro, São Paulo, Brazil (20% protein, 26.4% carbohydrate, 20% fat, 10% minerals, 9% fibers, 12.5% humidity, 1.4% calcium, 0.7 phosphorus), which were rotating for a 15-week period. The profile analysis of fatty acids in the diet showed that unsaturated ones correspond to 80%, and saturated ones, to 20%. The food intake of animals was measured daily, and the intake of water, \textit{ad libitum}. Animals were weighed weekly, with the digital scale Mettler\textsuperscript{®}, model Spider 2. After 15 weeks of treatment, all
of the animals were anesthetized with pentobarbital sodium (50 mg/kg/ip; Cristália® Produtos Químicos Farmacêuticos Ltda., Itapira, São Paulo, Brazil) and euthanized by decapitation.

Constitution of control and obese groups

In the biological testing, especially in experimental trials, even at similar laboratory conditions, the response homogeneity is not certain. In this sense, rats submitted to standard and hyperlipidic diets may present characteristics in common, in higher or lower scales, such as adiposity index. A study published previously showed that this fact may lead to classification errors, that is, animals submitted to standard diets could be classified as control, when in fact they exhibit aspects of obese animals, and vice-versa. Therefore, it is necessary to establish criteria to separate the animals in two distinct groups, according to the adiposity index. With that purpose, a 95% confidence interval (CI) was established for the average adiposity level in control and obese rats. The adopted separation point (SP) stood between the mean and the upper limit point in group C and the lower limit point of group Ob; considering that point, animals with adiposity index higher than the SP were excluded from group C, and those with adiposity index lower than the SP were excluded from group Ob.

Nutritional profile of the animals

In order to assess if obesity had altered the nutritional profile, food consumption was analyzed, as well as caloric intake, dietary efficiency, body mass, body fat and adiposity index. Food intake was daily calculated from individual leftovers. Caloric intake was calculated by the following formula: weekly food consumption multiplied by the energetic value of each diet (g x kcal). With the objective of analyzing the capacity of converting the consumed food energy into body weight, dietary efficiency was calculated by dividing the total body weight gain of the animals (g) by the total energy intake (kcal).

Characterization of obesity

The characterization of obesity, at the end of the 15-week period, was established by the adiposity index. The deposits of epididymal, retroperitoneal and visceral fat in the animals were dissected in order to quantify body fat. The adiposity index was measured by the sum of fat deposits normalized by final body weight multiplied by 100. This method allows a consistent analysis of body fat deposits.

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**Figure 2** - A. The prevalence of phosphorylation occurs when PKA is activated while it phosphorylates I-1, thus preventing PLB dephosphorylation. B. The prevalence of dephosphorylation occurs when PKA is not activated. There is no PLB and I-1 phosphorylation, therefore PP-1 maintains its active state. cAMP: 3’,5’ cyclic adenosine monophosphate; I-1: inhibitory protein 1; P: phosphate; PKA: protein kinase A; pPLB: phosphorylated phospholamban; PLB: dephosphorylated phospholamban; PP-1: phosphatase-1; Serca2a: Ca^{2+} pump.
Comorbidities associated with obesity

Since obesity can lead to cardiovascular, metabolic and hormonal comorbidities, such as systemic arterial hypertension, glucose intolerance, systemic resistance to insulin, dyslipidemia, hyperglycemia, hyperinsulinemia, and hyperleptinemia, the following variables were analyzed:

a) Systemic blood pressure

Blood pressure was assessed by measuring the systolic arterial pressure (SAP). SAP was measured by plethysmography, using the electronic sphygmomanometer, Narco Bio-System®, model 709-0610 (International Biomedical, Austin, TX, United States). The rats were previously warmed, at a temperature of 40°C for five minutes, in a wooden box (50 × 40 cm), covered with sterilized wood shaving, with the objective of producing the vasodilatation of the caudal artery. Afterwards, the cuff was connected to a pulse transducer placed around the animal’s tail and insufflated to 200 mmHg; then, it was uninsufflated. The arterial pulses were recorded with a Gould RS 3200 polygraph (Gould Instrumenta Valley View, Ohio, United States).

b) Glucose tolerance test

Animals were submitted to a six-hour fasting period. Blood collection in the caudal artery was conducted in basal condition and after the intraperitoneal administration of 25% glucose (Sigma®-Aldrich, Saint Louis, MO, United States), equivalent to 2.0 g/kg. Blood samples were collected in moments 0 (basal condition), at 15, 30, 60, 90 and 120 minutes. The ACCU-CHEK GO glucose monitor kit (Roche Diagnostic Brazil Ltda., São Paulo, Brazil) was used to measure the glycemic index.

c) Hormonal profile: insulin and serum leptin

The serum concentrations of these hormones were determined by the ELISA method, using specific kits (Linco Research Inc, St. Louis, MO, United States). A microplate reader was used for the analysis (Spectra MAX 190, Molecular Devices, Sunnyvale, CA, United States).

d) Glycemic and lipid profiles

The lipid and glycemic profiles were assessed by analyzing serum glucose, triacylglycerol, total cholesterol, and high and low density lipoprotein and non-esterified fatty acids (NEFA). Animals fasted for 12 to 15 hours, and they were anesthetized with pentobarbital sodium (50 mg/kg/ip; Cristália® Produtos Químicos Farmacêuticos Ltda., Itapira, São Paulo, Brazil) and euthanized by decapitation. The heart of the animals was removed and dissected, and the following determinations were made: total weight of the heart, of the left and right ventricles, and the atrium, and their respective relations with body weight and tibial length at the time of euthanasia. These analyses may indicate the presence of cardiac remodeling at atrial and ventricular levels.

Characterization of cardiac remodeling

Since obesity can lead to cardiac remodeling, it was studied by the structural post mortem evaluation and by analyzing the expression of kinase and phosphatase proteins, which regulate the level of PLB phosphorylation resulting from the beta-adrenergic activation of the myocardium.

a) Cardiac structural analysis

Animals were submitted to fasting from 12 to 15 hours, being afterwards anesthetized with pentobarbital sodium (50 mg/kg/ip; Cristália® Produtos Químicos Farmacêuticos Ltda., Itapira, São Paulo, Brazil) and euthanized by decapitation. The heart of the animals was removed and dissected, and the following determinations were made: total weight of the heart, of the left and right ventricles, and the atrium, and their respective relations with body weight and tibial length at the time of euthanasia. These analyses may indicate the presence of cardiac remodeling at atrial and ventricular levels.

b) Protein expression analysis

The protein expression of total PLB, pPLB (ser-16), PKA and PP-1 was conducted by the Western Blot technique.

The Western Blot technique

a) Protein extraction

Fragments of the left ventricle were rapidly frozen in liquid nitrogen and stored in a freezer at −80°C. The frozen sample was homogenized in a Polytron device (Ika Ultra Turrax™ T25 Basic, Wilmington, United States) with hypotonic lysis buffer (potassium phosphate 50 mM pH 7.0, sucrose 0.3 M, DTT 0.5 mM, EDTA 1 mM pH 8.0, PMSF 0.3 mM, NaF 10 mM and phosphatase inhibitor). The process was performed three times for 10 seconds at 4°C, with 20-second intervals. The product of homogenization was centrifuged (Eppendorf 5804R, Hamburg, Germany) at 12.000 rpm for 20 minutes at 4°C, and the supernatant was transferred to Eppendorf tubes and stored in a freezer at −80°C. The protein concentration was analyzed by the Bradford method15, using the curves in the BSA Protein Standard (Bio-Rad, Hercules, CA, United States) as a pattern.

The protein samples were diluted in a Laemmli buffer (Tris-HCL 240mM, SDS, 0.8%, 40% glycerol, 0.02% bromophenol blue and 200 mM beta-mercaptoethanol) and separated by electrophoresis using the Mini-Protean 3 Electrophoresis Cell system (Bio-Rad, Hercules, CA, United States). Electrophoresis was conducted with biphasic stacking (Tris-HCL 240mM pH 6.8, 30% polyacrylamide, APS and Temed) and resolution gel (Tris-HCL 240mM pH 8.8, 30% polyacrylamide, APS and Temed), with concentrations of 6% to 12%, depending on the molecular mass of the analyzed protein. In the first gel well, one molecular mass pattern was applied,
with the Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA, United States), in order to identify the size of the bands. Electrophoresis was made at 120 V (Power Pac HC 3.0A, Bio-Rad, Hercules, CA, United States), for approximately three hours, with loading buffer (Tris 0.25M, glycine 192 mM and 1% SDS). Afterwards, proteins were transferred to a nitrocellulose membrane in a Mini-Trans Blot system (Bio-Rad, Hercules, CA, United States), by using the transfer buffer (Tris 25 mM, glycine 192 mM, 20% methanol and 0.1% SDS). Membranes were washed twice with a TBS buffer (Tris-HCl 20mM pH 7.6 and NaCl 137mM). The non-specific binding sites of the primary antibody to the membrane were blocked by incubation, with a 0.5% skimmed milk powder solution dissolved in a TBS-T buffer, pH 7.4 (Tris-HCl 20mM, NaCl 137mM and 0.1% Tween 20 detergent) for 120 minutes at room temperature under constant agitation. Afterwards, the membrane was washed three times in TBS-T buffer (Tris 1M pH 2.8, NaCl 5M and Tween 20) and incubated with the primary antibody diluted in the blocking solution, under constant agitation for 12 hours. After the incubation with the primary antibody, the membrane was washed three times in TBS-T buffer and incubated with the secondary antibody in a blocking solution for two hours under constant agitation. In order to remove the excessive secondary antibody, the membrane was washed three times in TBS-T buffer. Finally, immunodetection was performed by the chemiluminescence method, according to the manufacturer’s instructions (Enhancer Chemi-Luminescence, Amersham Biosciences, NJ, United States). The nitrocellulose membranes were exposed to radiographic films X-Omat AR (Eastman Kodak Co., Maryland, United States), which is a free software available at: http://www.scioncorp.com/

Statistical analysis
All of the variables were submitted to the test of normality Kolmogorov-Smirnov. The nutritional profile, the comorbidities associated with obesity, the anatomical data and the cardiac protein expression were analyzed by the Student’s t-test for independent samples. The glucose tolerance test was examined by the analysis of variance (ANOVA) for the model of repeated measures in two independent groups, being complemented by the Bonferroni test. The Sigma Plot 3.5 for Windows was used for statistical analyses (Systat Software Inc., San Jose, CA, United States). Data were presented as mean ± standard-deviation. The 5% significance level was considered for all of the variables.

Results

Composition of control and obese groups
After the criterion established to compose the experimental groups was applied, 27 animals remained in the study and constituted the control (C, n = 14) and the obese group (Ob, n = 13).

Nutritional profile
Table 1 shows the nutritional profile of animals in C and Ob. Final body weight, weight gain, deposits of epididymal, retroperitoneal and visceral fat, total body fat and adiposity index were higher in the obese group in relation to the control group. Animals in the Ob consumed less food than those in the C group. There was no difference between both groups with regard to caloric intake.

Analysis of comorbidities

a) Hormonal profile and systolic arterial pressure
Figure 3 illustrates the result of serum insulin values (A) and leptin (B); obesity leads to increasing concentrations of these hormones. The result of the final systolic arterial pressure (C) did not present any significant differences between groups.

b) Glucose tolerance test
Figure 4 shows the results of the glucose tolerance test performed in groups C and Ob. Glycemic levels were similar at the baseline between groups. After the intraperitoneal administration of glucose, glycemia was high in the Ob group and in moments 15, 30, 60 and 90 in comparison to group C.

c) Glycemic and lipid profile
Table 2 shows the serum biochemical analyses of animals in groups C and Ob. The plasma concentrations of glucose, cholesterol, HDL and NEFA were not different between treatments; the triglyceride concentration was significantly higher in Ob than in C.

Cardiac remodeling

a) Macroscopic structure of the heart
Table 3 shows the post mortem macroscopic structure of the heart of rats in C and Ob. After 15 weeks of obesity, there was a significant difference concerning the weight of the atria.
b) Protein expression analysis

According to Figure 5, we did not observe significant differences in protein expressions of PLB (A), pPLB Ser-16 (B), PKA (C) and PP-1 (D) between the control and obese groups.

Discussion

The main finding in this study was that obesity induced by an unsaturated high-fat diet did not lead to changes in the balance between phosphorylation and dephosphorylation in the heart; the behaviors of kinase and phosphatase proteins were similar in both analyzed groups.

The diet-induced obesity is similar to that found in the human population, and it has been used to reproduce possible molecular, structural, metabolic, and functional changes in different organs of the human body. In this experiment, which was enough to promote obesity among rats, was a result of the high content of unsaturated fats. In this study, results showed that the adiposity index was significantly higher among obese rats (control = 4.61 ± 0.85; obese = 7.53 ± 1.36; *p < 0.005) in relation to the ones in the control group. This result is in accordance with studies (conducted with rodents) that classify obesity using this index.

Obesity has been characterized by several comorbidities, such as glucose intolerance, insulin resistance, systemic arterial hypertension, dyslipidemia, hyperinsulinemia and hyperleptinemia. In this study, obese animals presented the following comorbidities: glucose intolerance, hyperinsulinemia, hypertriglyceridemia, and hyperleptinemia. Glucose intolerance associated with increasing serum insulin showed that animals were resistant to the action of insulin. The increasing levels of insulin in obese rats were not able to maintain the homeostasis of carbohydrates facing the supplementation of this substrate in obese animals. The increasing levels of triglycerides in obese rats can be a consequence of the high capture of triglycerides in the form of chylomicrons and/or the decreasing absorption of triglycerides by peripheral tissues. The increased leptin levels were caused by larger fat deposits, since there is correlation between the levels of leptin and the fat tissue. Since leptin is a hormone that derives from the fat tissue, it participates
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Table 2 – Glycemic and lipid profile

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>C (n = 14)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>125 ± 16</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>43.3 ± 10.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>62.4 ± 11.5</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>23.5 ± 3.0</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.42 ± 0.10</td>
</tr>
</tbody>
</table>

HDL: high-density lipoprotein; NEFA: non-esterified fatty acids. Data expressed as mean ± standard deviation. Student’s t-test for independent samples, * p <0.05 × C.

Figure 4 – Glucose tolerance test in control (n = 14) and obese animals (n = 13). Data expressed as mean ± standard deviation. Analysis of variance (ANOVA) for the model of repeated measures in independent groups, complemented by the Bonferroni test. * p <0.05 × C.

in energy balance, thus regulating the food intake and the oxidation of lipids\(^{27,28}\). The reduced food intake by obese rats suggests that the increasing levels of leptin were effective for appetite control. Data concerning comorbidities observed in this study are in accordance with other studies that induced obesity experimentally\(^{14,17,29,30}\).

The most important observation in this study was that diets induced by unsaturated fat did not change the pPLB-ser16 expression, and proteins in charge of balancing phosphorylation and dephosphorylation, PKA and PP-1, respectively. Since the beta-adrenergic via is in charge of phosphorylation in the site of serum-16 of the PLB, we can infer that this system was not sufficiently stimulated to lead to changes in the myocardial PLB phosphorylation or that another system was opposed to such an activation. The behavior of pPLB-ser16 in this study is not in accordance with a previous study conducted in our laboratory\(^{12}\), in which decreased PLB was found in its phosphorylated state in serum 16 among obese rats treated with the same diet used in this study. We could not find an explanation for these different results; such a divergence could be related to the adiposity index in obese animals, which was 16% higher in the study conducted by Lima-Leopoldo\(^{12}\). No studies in literature analyzed the relationship between proteins that interfere in myocardial PLB phosphorylation and dephosphorylation in obese rats submitted to a hyperlipidic diet.
Table 3 – Macroscopic structure of the heart and tibia

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>C (n = 14)</td>
</tr>
<tr>
<td>Tibia (cm)</td>
<td>4.30 ± 0.07</td>
</tr>
<tr>
<td>LV (g)</td>
<td>0.79 ± 0.06</td>
</tr>
<tr>
<td>RV (g)</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>AT (g)</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Heart total (g)</td>
<td>1.11 ± 0.09</td>
</tr>
<tr>
<td>LF/tibia (g/cm)</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>RV/tibia (g/cm)</td>
<td>0.050 ± 0.005</td>
</tr>
<tr>
<td>AT/tibia (g/cm)</td>
<td>0.020 ± 0.002</td>
</tr>
<tr>
<td>Heart/tibia (g/cm)</td>
<td>0.30 ± 0.02</td>
</tr>
</tbody>
</table>

C: control; Ob: obese; AT: atrial mass; RV: right ventricle mass; LV: left ventricle mass; AT/tibia: AT to tibia length ratio; RV/tibia: RV to tibia length ratio; LV/tibia: LV to tibia length ratio; Data expressed in mean ± standard-deviation. Student’s t-test for independent samples. *p< 0.05 vs C.

Figure 5 – Expressions of PLB (A), pPLB-ser16 (B), PKA (C) and PP-1 (D) normalized by beta-actin. PKA: protein kinase A; PLB: dephosphorylated phospholamban; pPLB-ser16: phosphorylated phospholamban in serin-16; PP-1: phosphatase-1. Control (n = 6) and obese (n = 6). Data are expressed as mean ± standard-deviation. Student’s t-test * p<0.05 vs C.

**Conclusion**

The initial hypothesis of this study was not confirmed. Obesity does not promote imbalance between myocardial PLB phosphorylation and dephosphorylation by the beta-adrenergic via.

**Author contributions**

Conception and design of the research: Freire PP, Lima-Leopoldo AP, Leopoldo AS, Silva DCT, Campos DHS, Cicogna AC; Acquisition of data: Freire PP, Alves CAB, Deus AF, Campos DHS; Analysis and interpretation of the data: Freire PP, Alves CAB, Deus AF, Lima-Leopoldo AP, Leopoldo AS, Silva DCT, Tomasi LC, Campos DHS, Cicogna AC; Statistical analysis: Freire PP, Alves CAB, Lima-Leopoldo AP, Leopoldo AS, Silva DCT, Tomasi LC, Campos DHS, Cicogna AC; Obtaining financing: Freire PP, Lima-Leopoldo AP, Leopoldo AS, Cicogna AC; Writing of the manuscript: Freire PP, Tomasi LC, Cicogna AC; Critical revision of the manuscript for intellectual content: Freire PP, Alves CAB, Deus AF, Lima-Leopoldo AP, Leopoldo AS, Silva DCT, Tomasi LC, Cicogna AC.
Potential Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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