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Heart structure, serum cholesterol, and adiposity of rats treated with a hypercaloric diet: effectiveness of *Citrus sinensis* (L.) Osbeck and swimming

Estrutura cardíaca, colesterol sérico e adiposidade de ratos submetidos á dieta hipercalórica: eficácia da *Citrus sinensis* (L.) Osbeck e da natação

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Abstract
This study evaluated the effects of the herbal medicine red orange (*Citrus sinensis* (L.) Osbeck) and swimming for 84 days on the animal, heart, and abdominal fat weight and the histomorphometric aspects of heart and total cholesterol of Wistar rats. The rats were divided into seven experimental groups of 12 animals each, consisting of a normocaloric diet (Dn), hypercaloric diet (Dh), normocaloric diet and herbal medicine (DnH), hypercaloric diet and herbal medicine (DhH), normocaloric diet and swimming (DnS), hypercaloric diet and swimming (DhS), and hypercaloric diet, swimming, and herbal medicine (DhSH). The data were analyzed statistically by the Tukey test and considered significant when p<0.05. Groups treated with the normocaloric diet had lower abdominal fat weight. The normocaloric diet and herbal medicine (DnH) provided the smallest thickness of the right ventricle. The hypercaloric diet (Dh) reduced the number of cardiomyocytes and the perimeter of cardiac muscle fibers. Swimming and the red orange extract acted synergistically by reducing the deleterious effects of the hypercaloric diet and increasing the thickness of the cardiac chambers and the number of cardiomyocytes. Only the supplementation with the red orange extract did not reduce abdominal fat in rats treated with a hypercaloric diet. Therefore, red orange alone did not promote beneficial changes in the studied data, but its association with swimming increased the number of cardiomyocytes and thickness of muscle fibers, which could contribute to preventing cardiovascular diseases and maintaining health, as well as the regular swimming and a normocaloric diet, which provided less adiposity.
Keywords: cafeteria diet; aerobic exercise; herbal medicine.

Resumo
Este estudo avaliou os efeitos do fitoterápico *Citrus sinensis* (L.) Osbeck e da natação durante 84 dias sobre o peso dos animais, do coração e da gordura abdominal e aspectos histomorfométricos do coração e colesterol total de ratos Wistar. Sete grupos experimentais, de 12 animais cada, que consistiram em dieta normocalórica (Dn), hipercalórica

(Dh), dieta normocalórica e fitoterápico (DnF), dieta hipercalórica e fitoterápico (DhF), dieta normocalórica e natação (DnN), dieta hipercalórica e natação (DhN), e dieta hipercalórica, natação e fitoterápico (DhNF). Os dados foram analisados estatisticamente pelo teste de Tukey e considerados significativos quando p<0,05. Os grupos tratados com dieta normocalórica tiveram menor peso da gordura abdominal. A dieta normocalórica e fitoterápico (grupo DnF) proporcionou menor espessura do ventrículo direito. A dieta hipercalórica (Dh) reduziu o número de cardiomiócitos e o perímetro das fibras musculares cardíacas. A natação e o extrato de laranja agiram de forma sinérgica ao reduzir os efeitos deletérios da dieta hipercalórica e aumentou a espessura das câmaras cardíacas e o número de cardiomiócitos. Somente a suplementação com extrato de laranja vermelha não reduziu a gordura abdominal em ratos tratados com dieta rica em calorias. Conclui-se que a laranja vermelha sozinha não promoveu alterações benéficas nos dados estudados, entretanto, associada com a natação, promoveu aumento no número de cardiomiócitos e espessura das fibras musculares, o que poderia contribuir com a prevenção de doenças cardiovasculares e a manutenção da saúde, assim como a prática regular de natação e dieta normocalórica, que proporcionaram uma menor adiposidade.
Palavras-chave: dieta de cafeteria; exercício aeróbio; fitoterápico.

Introduction

One of the biggest public health problems in the world is obesity. According to the Brazilian Association for the Study of Obesity and Metabolic Syndrome,[1] more than 50% of the Brazilian population is overweight. As in humans, obesity in dogs and cats has become the most common nutritional disorder, affecting domestic animals.[2]

The consumption of processed foods, rich in carbohydrates, lipids, and sugars, is one of the main factors related to the increase in body fat tissue. A high incidence of cardiovascular changes is the consequence of fat accumulation since the adipose tissue synthesizes and secretes adipocytokines, which contribute to systemic and vascular inflammation, directly or indirectly regulating pathophysiological processes such as arterial hypertension, endothelial dysfunction, and vascular remodeling, which promote disease development.[3]

The excess adipose tissue overloads the circulation, increasing blood volume and cardiac output, which generates higher tension and dilation of the ventricle wall due to the increase in the venous return resulting from an increase in blood volume. According to Halpern *et al.*,[4] 2–3 mL of blood is needed to perfuse 100 g of adipose tissue in a human. Therefore, an individual with 100 kg of excess body fat would require a 3 L/min increase in blood in cardiac output. Thus, systolic volume increases according to body weight and overloads cardiac functioning.[5] Left ventricular (LV) hypertrophy is due to increased vascular resistance, which results from changes such as fibrosis and cardiomyocyte hypertrophy, while the right ventricle may be hypertrophic due to changes in the left ventricle (LV).[6]

Cardiac tissue degeneration and inflammation can occur in individuals with excess adipose tissue, which leads to myocardial fibrosis and contributes to heart failure,[7] which is the result of the death of cardiomyocytes due to the development of fibrosis, dilation of the ventricle, and increased peripheral resistance.

The change in cardiac structure due to obesity is often irreversible, in contrast to cardiac remodeling resulting from the practice of physical activity.[8] Individuals with excess adipose tissue are more likely to develop changes in the heart, whose weight reduction improves cardiac function.[9]

In addition to structural cardiac changes, the high-fat diet can increase total cholesterol in rats compared to animals that receive a balanced diet.[10] Therefore, the cafeteria diet has been used in experimental models to increase body fat due to excess energy and saturated fatty acids and carbohydrates.

Several alternatives have been proposed to reduce the excess adipose tissue, such as natural agents from plants, known as herbal medicines.[11,12] Red orange (*Citrus sinensis* (L.) Osbeck) is rich in phenolic compounds (flavonoids and hydroxycinnamic acids) and vitamin C, which act as potent antioxidant agents by inhibiting lipid peroxidation and modulating the inflammation generated by excess adipose tissue.[13–15]

This fruit has a characteristic color due to a pigment belonging to the anthocyanin class, and the Moro variety has the highest amount of this pigment.[16] Some fruits rich in antioxidants, such as black raspberry (*Rubus* sp.),[17] sweet cherry (*Prunus avium*),[18] and blueberry (*Vaccinium myrtillum*),[19] have been effective in reducing adipose tissue due to the presence of these compounds. Red orange, especially the Moro variety, has been used to control body weight.[20]

Some of the measures to reduce adipose tissue are based on the association of physical exercises and a balanced diet. Swimming, as an aerobic activity, promoted lipolysis and decreased adipose body mass in Wistar rats with a high percentage of body fat,[21] and reduced total cholesterol levels in humans.[22]

Considering possible harmful effects of the hypercaloric diet on the cardiovascular system and the regular physical activity as a strategy for health promotion and prevention or treatment of many chronic diseases,[23] this research was carried out to evaluate the effects of herbal medicine red orange (*Citrus sinensis* (L.) Osbeck) and swimming for 84 days on the animal, heart, and abdominal fat weight and the histomorphometric aspects of heart and total cholesterol of Wistar rats treated with a hypercaloric diet.

Material and methods

The experiment was carried out in the vivarium of the Department of Animal Morphology and Physiology of FCAV/UNESP – Jaboticabal Campus, after approval of the project by the Ethics Committee for the Use of Animals (CEUA) under the protocol 5848/15.

Purina® Presence ration (3.8 kcal/g) was used for animals of the control group (normocaloric diet), composed of 40% carbohydrates, 26% proteins, 3.8% lipids, and 4.5% fibers. A cafeteria diet (5.4 kcal/g), consisting of 40% Purina® ration, 20% Sadia® solid fat (lard), 3% Marvigel® emulsifier, 10% chocolate powder, 8% condensed milk, 3% starch, 5% chocolate wafer, 5% ON® whey protein, 4% table cream, and 2% vegetable oil, was used to promote the accumulation of adipose tissue. This diet consisted of 50% carbohydrates, 26% proteins, 9.3% lipids, and 4.5% fibers. These homogenized ingredients were placed inside a PVC pipe and pushed out to develop a pellet shape, remaining under refrigeration before being fed to the animals.

Twenty-one-day old, male Wistar rats (*Rattus norvegicus*) (n = 84) from the vivarium were distributed through a completely randomized design in seven groups of 12 animals, remaining in cages with three animals each, with water and food supplied ad libitum, in an environment with a temperature of 22 °C and a light/dark cycle of 12 hours. The Moro orange dry extract (*Citrus sinensis* (L.) Osbeck) was supplied after 55 days of adaptation to the cafeteria diet. The extract was obtained from the pharmaceutical industry Galena®, and each 100 g of dry extract is composed of 4.5% ascorbic acid, 1% hydroxycinnamic acids, 2.2% flavanones, and 0.9% anthocyanins. The used dose was 7 mg/kg, according to the weight of each animal. The dose was diluted in 1 mL of distilled water for each animal and administered by gavage, once a day, for 84 days.

The treatments consisted of groups with a normocaloric diet (Dn), hypercaloric diet (Dh), normocaloric diet and herbal medicine (DnH), hypercaloric diet and herbal medicine (DhH), normocaloric diet and swimming (DnS), hypercaloric diet and swimming (DhS), and hypercaloric diet, swimming, and herbal medicine (DhSH).

The groups submitted to swimming went through six days of adaptation, with a gradual increase in the period of physical activity (five minutes on the first day, 10 minutes on the second day, 15 minutes on the third day, 20 minutes on the fourth day, 25 minutes on the fifth day, and 30 minutes on the sixth day).[24] The rats were placed in pools with a 95 cm long × 58 cm wide × 58 cm high smooth surface filled with water heated at 32 °C, changed daily. The adaptation period to physical exercise was carried out before the beginning of the 84-day treatment. The animals had a mark on the tail to identify both the individual and the group to which they belonged. Swimming was carried out in the vivarium of the Department of Morphology and Physiology where they were reared, avoiding stress due to displacement.

The animals were euthanized at the end of the experiment with the use of volatile general anesthetic (isoflurane), and then weighed.

After certification of anesthesia, the hearts were removed and weighed for macroscopic evaluation as to color, contour, consistency, and wall thickness in the middle region of the right and left ventricles and right atrium, obtained using a Digimess® digital caliper. Abdominal fat from the viscera was removed and weighed to calculate the relative weight, according to the following formula: relative organ weight = (organ weight/live weight) × 100.

A volume of 1.5 mL of blood was taken from each rat by cardiac puncture at the time of euthanasia. After collection, the blood was left to rest to obtain the serum, which was stored in Eppendorf tubes, identified, and frozen at −20 °C. After thawing, the samples were centrifuged for 10 minutes at 3000 G for colorimetric enzymatic analysis using the commercial kit Cholesterol Liquiform®. The serum with the enzymatic reagent was inserted in the Labquest device (Labtest Diagnóstica S.A, Lagoa Santa, Minas Gerais, Brazil).

After removal, the organs were immediately fixed in 4% buffered formaldehyde. Subsequently, they were cross-sectioned (3 mm thick) in two regions: height of the caudal vena cava insertion in the right atrium and the middle third of the ventricle. The two fragments obtained from each animal were processed for inclusion in paraffin. The slides underwent dehydration and hydration processes and were immediately stained with hematoxylin and eosin (HE) to determine the thickness of the left ventricle, the number of cardiomyocytes, area, and perimeter and diameter of the cardiac muscle fibers, using the software Cellsens, Olympus.

The data were evaluated using the Kolmogorov-Smirnov test to determine the distribution pattern. The analysis of variance and the Tukey test at a 5% significance were performed. The General Linear Models (GLM) procedure of the software SAS was used.

Results and discussion

Only the type of diet influenced body weight and abdominal fat. Heart weight and cholesterol did not differ between groups (p>0.05). The control group (DhS) showed a higher average weight than the normocaloric diet groups, which did not differ from the DhSH group (Table 1).

Table 1. Mean and standard deviation of the final body (g), heart (g), and abdominal fat (g) weight and cholesterol of rats.

Group	Final body weight	Heart	Abdominal fat	Cholesterol
Dn	496.64±43.69^{b}	1.72±0.09	12.50±4.23^{c}	74.09±16.61
DnH	515.42±69.99^{b}	1.62±0.32	14.25±7.87^{bc}	81.88±7.47
DnS	505.80±42.32^{b}	1.73±0.13	13.30±5.30^{bc}	65.19±21.09
Dh	540.92±61.46^{ab}	1.64±0.23	25.23±12.84^{a}	72.93±9.25
DhH	515.33±55.11^{b}	1.61±0.11	24.03±16.15^{ab}	74.73±8.57
DhS	602.00±88.11^{a}	1.66±0.18	23.42±10.00^{abc}	76.22±7.45
DhSH	548.36±47.14^{ab}	1.74±0.14	17.24±9.03^{abc}	73.75±13.55

Means followed by distinct letters in the column differ from each other (p≤0.05) by the Tukey test. Treatments: normocaloric diet (Dn), hypercaloric diet (Dh), normocaloric diet and herbal medicine (DnH), hypercaloric diet and herbal medicine (DhH), normocaloric diet and swimming (DnS), hypercaloric diet and swimming (DhS), and hypercaloric diet, swimming, and herbal medicine (DhSH).

Body weight showed no statistical difference between the groups that received a normocaloric diet. However, among the groups with a hypercaloric diet, those submitted to swimming (DnS) presented higher body weight than the group submitted to herbal medicine (DnH) (Table 1). Other authors have not observed a significant difference in the weight of rats treated with normocaloric and cafeteria diets.(25,26) Titta *et al.*(27) observed that the use of Moro orange juice, associated with a hypercaloric diet for 12 weeks, reduced body weight gain.

Heart weight did not differ between groups (p>0.05) (Table 1). However, Gupte *et al.*(28) found a higher absolute heart weight in rats that received a lipid-rich diet for 385 days, suggesting that, in the long term, the hyperlipidic diet can cause changes in the weight of this organ.

Abdominal fat showed no difference (p>0.05) between groups that received the normocaloric diet and between groups of the hypercaloric diet, but the Dh group was statistically superior to all groups of the normocaloric diet (Table 1). Other studies(29,30) have observed that Wistar rats treated with a hypercaloric diet significantly increased abdominal fat. The use of Moro orange juice, associated with a high-fat diet for 12 weeks, reduced the size of adipocytes and lipid accumulation.(27)

Among groups with the same type of diet, the use of herbal medicine and swimming did not change the adipose tissue weight (Table 1). Kaume *et al.*(31) observed that anthocyanins from black raspberry (in the form of juice or dry extract) did not reduce the body fat accumulation induced by a high-fat diet (60% energy) in mice. Zambon *et al.*(32) demonstrated that intermittent swimming reduced abdominal fat. Motta *et al.*(33) observed that rats fed a high-carbohydrate diet for 18 weeks increased abdominal fat, while its association with running on a treadmill three times a week for the same period decreased abdominal fat, demonstrating that the physical exercise has beneficial effects regardless of the diet used.

Cholesterol showed no differences between groups (p>0.05), with values similar to those found by Dantas *et al.*(34) and Gomez-Smith *et al.*(35) Zanchet *et al.*(36) observed no differences regarding the total cholesterol levels between groups of the normocaloric and hypercaloric diets.

The relative heart weight of the DhS group was statistically lower than the Dn and DnS groups, not differing from each other (Table 2). According to Krames & Liere,(37) groups made up of heavier animals had lower relative heart weight.

Abdominal fat showed no difference (p>0.05) between groups that received the normocaloric diet and between groups of the hypercaloric diet, but the Dh and DhH groups were statistically superior to all groups of the normocaloric diet (Table 1). Malafaia *et al.*(38) found a higher retroperitoneal fat weight in the group that received a sucrose-rich diet for three months than the control group.

Groups that practiced swimming showed higher LV wall thickness (p<0.0001) (Table 2). The DhS and DhSH groups showed higher RV thickness compared to the Dn, DnH, and DhH groups, indicating that a hypercaloric diet associated with swimming can promote higher thickness of the right ventricle. Swimming can generate eccentric hypertrophy due to volume overload, which increases the thickness of the left ventricular wall in a compensatory way.[39] According to Haskell *et al.*,[40] a larger left ventricle improves the diastole phase and reduces heart rate.

Table 2. Mean and standard deviation of the relative heart (%) and abdominal fat weight (AF) (%), and left ventricle (LV) (mm), right atrium (RD) (mm), and right ventricle thickness (mm) (RV), of Wistar rats.

Group	Heart	AF	LV	RD	RV
Dn	0.35±0.04ᵃ	2.58±0.86ᵇᶜ	3.09±0.32ᶜ	1.12±0.15ᵇ	1.37±0.15ᶜᵈ
DnH	0.31±0.05ᵃᵇ	2.24±1.67ᶜ	3.27±0.25ᵇᶜ	1.14±0.09ᵇ	1.27±0.08ᵈ
DnS	0.34±0.03ᵃ	2.58±0.98ᵇᶜ	3.74±0.33ᵃ	1.27±0.12ᵃᵇ	1.57±0.19ᵃᵇ
Dh	0.30±0.06ᵃᵇ	4.08±2.17ᵃ	3.15±0.34ᵇᶜ	1.25±0.20ᵃᵇ	1.53±0.35ᵃᵇᶜ
DhH	0.32±0.04ᵃᵇ	3.78±3.32ᵃ	3.42±0.22ᵇ	1.22±0.19ᵇ	1.40±0.10ᵇᶜᵈ
DhS	0.28±0.05ᵇ	3.60±1.32ᵃᵇ	3.80±0.40ᵃ	1.31±0.36ᵃᵇ	1.59±0.27ᵃᵇ
DhSH	0.31±0.02ᵃᵇ	3.11±1.65ᵃᵇᶜ	3.97±0.46ᵃ	1.42±0.20ᵃ	1.67±0.21ᵃ

Means followed by distinct letters in the column differ from each other (p≤0.05) by the Tukey test. Treatments: normocaloric diet (Dn), hypercaloric diet (Dh), normocaloric diet and herbal medicine (DnH), hypercaloric diet and herbal medicine (DhH), normocaloric diet and swimming (DnS), hypercaloric diet and swimming (DhS), and hypercaloric diet, swimming, and herbal medicine (DhSH).

The DhSH group had a higher thickness of the right atrium wall (RA) ($p<0.05$) than the Dn, DnH, and DhH groups (Table 2). Oliveira Júnior *et al.*[41] observed that genetically hypertensive rats that received a hyperlipidic diet presented higher thickness of the right and left atria than animals that received a normocaloric diet.

The group treated with a hypercaloric diet (Dh) had a lower amount of cardiomyocytes (Table 3), as described by Okere *et al.*,[42] who reported that the fatty acids in the high-fat diets caused the loss of cardiomyocytes. Schipke *et al.*[43] observed a lower number of cardiomyocytes in obese mice.

The DhSH group presented a higher number of cardiac cells than rats that did not practice swimming, indicating that aerobic exercises cause cardiac hypertrophy, as observed by Zazycki & Gomes.[39]

The area and diameter of cardiac muscle fibers of the DhS group were higher only to the DnS group, and the other groups were similar to each other. Regarding the perimeter, the DhS group was superior only to the Dh group (Table 3). The association of a high-lipid diet with swimming promotes changes in cardiac mass compared to the normocaloric diet and swimming. Barreti *et al.*[44] observed an increase in left ventricular mass in sedentary obese Zucker rats and obese rats that practiced swimming, but the hypertrophy was lower in the group that practiced physical activity compared to sedentary obese rats, indicating that aerobic exercise attenuates changes in cardiac structure. Leite *et al.*[(45)] found no statistical difference regarding cardiac mass and left ventricular mass in sedentary and exercised Wistar rats, which received a normocaloric diet, a fat-rich diet.

Table 3. Mean and standard deviation of the number of left ventricular cardiomyocytes and area (μm²), perimeter (μm), and diameter (μm) of muscle fibers of Wistar rats.

Group	Cardiomyocytes	Area	Perimeter	Diameter
Dn	56.77±9.57^{d}	402.81±136.85^{ab}	91.21±19.37^{ab}	21.50±3.71^{ab}
DnH	60.73±8.64^{d}	382.05±178.55^{ab}	87.14±22.08^{ab}	20.90±4.76^{ab}
DnS	76.04±4.69^{bc}	330.64±87.90^{b}	83.93±13.30^{ab}	19.45±2.79^{b}
Dh	33.64±10.23^{e}	349.59±98.56^{ab}	81.71±15.02^{b}	20.31±3.08^{ab}
DhH	66.58±8.88^{cd}	422.35±131.02^{ab}	93.91±15.76^{ab}	21.96±3.51^{ab}
DhS	92.33±5.29^{a}	455.45±169.50^{a}	99.74±24.46^{a}	22.70±4.37^{a}
DhSH	81.97±7.14^{ab}	412.38±116.86^{ab}	94.65±19.34^{ab}	21.79±3.09^{ab}

Means followed by distinct letters in the column differ from each other ($p \leq 0.05$) by the Tukey test. Treatments: normocaloric diet (Dn), hypercaloric diet (Dh), normocaloric diet and herbal medicine (DnH), hypercaloric diet and herbal medicine (DhH), normocaloric diet and swimming (DnS), hypercaloric diet and swimming (DhS), and hypercaloric diet, swimming, and herbal medicine (DhSH).

Conclusions

The hypercaloric diet alone affected the cardiac structure, causing a reduction in the number of cardiomyocytes and the perimeter of the cardiac muscle fibers in Wistar rats. Swimming and red orange extract had a synergistic action, reducing the deleterious effects of the hypercaloric diet, causing an increase in the thickness of the cardiac chambers and an increase in the number of cardiomyocytes.

The diet rich in fats, carbohydrates, and sugars promoted an increase in adiposity, generating an accumulation of abdominal fat, but only the supplementation with red orange extract was not efficient in reducing abdominal fat in rats treated with high levels of carbohydrates and fats in the diet. There is a need to associate a regular practice of swimming and an adequate diet, which would provide less abdominal adiposity and could contribute to the prevention of cardiovascular diseases and the maintenance of health.

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