Original Paper

High-Fat and Combined High-Fat and Sucrose Diets Promote Cardiac Oxidative Stress Independent of Nox2 Redox Regulation and Obesity in Rats

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Key Words
Oxidative stress • Nox2 • 1 angiotensin receptor • Hypercaloric diets • Cardiac

Abstract
Background/Aims: Oxidative stress is associated with cardiometabolic alterations, and the involvement of excess glucose and fatty acids has been demonstrated in this process. Thus, the aim of this study was to investigate the effects of different hypercaloric diets on cardiac oxidative stress. **Methods:** *Wistar* rats were randomized into four groups: control (C), high-sucrose (HS), high-fat (HF), and high-fat with sucrose (HFS). Nutritional assessment, food profiles, histological analysis, comorbidities, and cardiovascular characteristics were determined. Cardiac oxidative stress was analyzed by malondialdehyde (MDA) and carbonylated proteins, and the cardiac protein expression levels of type 1 angiotensin receptor (AT-1), nicotinamide adenine dinucleotide phosphate oxidase 2 (Nox2), superoxide dismutase (SOD 1 e 2), glutathione peroxidase (GPX), and catalase (CAT) were determined by western blot. **Results:** The HF group showed an increase in adiposity; however, it did not present adipocyte hypertrophy and comorbidities. Cardiac MDA and carbonylated protein levels were higher in the HF and HFS compared with the C group. The levels of oxidant and antioxidant proteins showed no difference between the groups. **Conclusion:** HF and HFS dietary interventions promoted cardiac oxidative stress, in the presence and absence of obesity, respectively. However, this process was neither mediated by the pro-oxidants AT1 and Nox2, nor by the quantitative reduction of antioxidant enzymes.

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Introduction

Food consumption has been modified over the past few decades, so that different dietary patterns modulate cardiovascular risk factors [1]. Excessive intake of saturated fat, simple carbohydrate or the combination of both, show alterations in body composition with concomitant adipokine dysregulation, sympathetic nervous activity, pathological cardiac remodeling, systolic dysfunction, and increased myocardial stiffness [2-5]. Among all the mechanisms that may be involved, the production of reactive oxygen species (ROS) is one of the most investigated, although it has been widely believed that electron leakage from the mitochondrial electron transport chain is the primary source of oxidative stress in heart injury, increasing lines of evidence suggest that enzymes which produce ROS may also contribute to it [6].

NADPH oxidases are transmembrane enzymes dedicated to producing superoxide ($O2^{\bullet-}$) by transferring an electron from NAD(P)H to molecular oxygen [7]. At the cardiovascular level, the isoform NADPH oxidase 2 (Nox2) stands out as one of the most important [8], and its activation occurs mainly by the hormonal action of angiotensin II. This hormone triggers a variety of intracellular signaling by protein kinase C (PKC) activation, tyrosine kinases, and transcription factors, with a consequent increase in the production of $O2^{\bullet-}$ radicals [9].

Under conditions of positive energy balance, excess calories are stored in the form of triglycerides in adipose tissue, which leads to organ expansion through cellular adaptations such as adipocyte hypertrophy and hyperplasia [10]. These processes are physiologically treated; the increase in triglycerides in the cytoplasm of these cells compromises their function, causing adipose tissue to respond by increasing the secretion of substances such as inflammatory mediators, ROS, and pro-angiotensin II, which may favor an oxidizing scenario for the organism defiling the heart, and toxic metabolites can accumulate in cardiomyocytes leading to cardiac redox imbalance, impairing homeostasis and cardiac function [11, 12].

The consumption of high-calorie diets is indirectly related to the exacerbated production of angiotensin II, as it is associated with excessive expansion of adipose tissue, which secretes bioactive molecules generated in the renin-angiotensin system from its precursor angiotensinogen, such as the inflammatory hormone angiotensin II, thus inducing the production of ROS mainly through its regulation of Nox2 activation [5, 12].

However, knowing the particularities of each nutrient, the literature is scarce regarding studies that point to the effects of fat and sucrose consumption specifically on cardiac tissue, not providing sufficient bases to define whether high levels of ROS are related to the kind of nutrients offered per se or are due to obesity. In addition, there is a lack of consistent information related to possible signaling pathways involved in this process that can be stimulated by hypercaloric diets. It is suggested that the excess of nutrients is related to the greater formation of free radicals and the accumulation of adipose tissue, contributing to the increase in the hormonal production of angiotensin II, which, when bound to its type 1 angiotensin receptor (AT1), activates NADPH oxidase and causes the formation of ROS [9, 12]; however, antioxidant defenses, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) would not be sufficient, resulting in lipid and protein damage and cellular dysfunction. In this context, this study aims to investigate the effects of different hypercaloric diets on cardiac oxidative stress, testing the hypothesis that these diets promote cardiac oxidative stress, resulting in increased lipid peroxidation products and protein carbonylation. This process will be mediated by greater expression of AT1 and Nox2, as well as by the reduction of the enzymes SOD, CAT, and GPX, and will be more evident in the high-fat hypercaloric diet with sucrose.

Materials and Methods

Animal care and experimental design

Twenty male *Wistar* rats, 30 days of age, were obtained from the Central Biotério of Federal University of Espirito Santo (UFES), Vitória, Espírito Santo, Brazil. The experimental procedures were carried out in accordance with the Manual of Care and Use of Laboratory Animals and approved by the Animal Use Ethics Committee (CEUA) of UFES under number 52/2019.

Rats were housed on a 12/12 dark/light cycle with controlled temperature (24 ± 2°C) and humidity (55 ± 5%), allowed to acclimatize for 7 days, and then randomly distributed into four dietary groups: a Control (C) that received a standard chow diet (n = 5; 3.55 kcal/g); high-sucrose (HS): fed with a high-sucrose diet and water with sucrose (300 g/L) in alternate weeks (n = 5; 4.25 kcal/g); high-fat (HF): fed a high-fat diet with lard (n = 5; 4.59 kcal/g); and high-fat with sucrose (HFS): fed a high-fat diet with lard *plus* sucrose (n = 5; 4.49 kcal/g). The duration of the experimental protocol was 20 weeks (Fig. 1).

All animals had free access to water and chow (40 g/day), and daily food consumption was measured. The feed efficiency (FE) was calculated by dividing the total weight gain of the animals (g) by the total ingested energy (kcal) [13, 14]. Caloric intake was calculated by daily food consumption multiplied by the caloric value of each diet (g x kcal) [15]. To calculate the caloric intake of the HS group, the energy from the intake of water with sucrose was also quantified (1.2 kcal/mL).
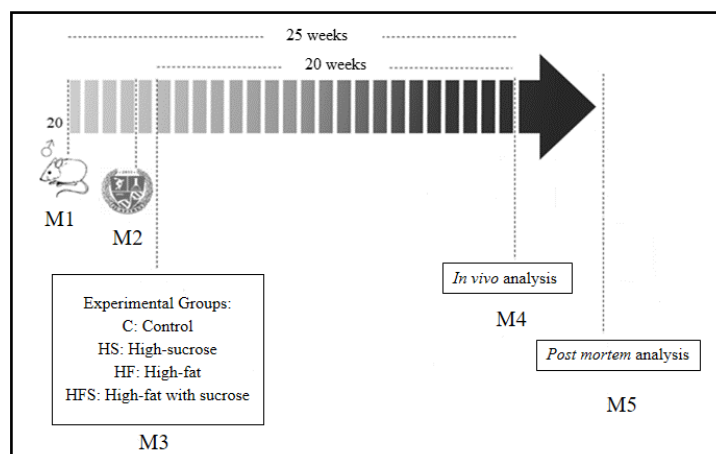
The diets used in the current study were formulated by Nutriave Alimentos®, Vitória, Espírito Santo, Brazil [16]. The composition (g/kg) and macronutrients for each experimental diet are described in Table 1.

At the end of the 20th week, the animals were fasted for 12–15 hours, heparinized (500U/kg/intraperitoneal; Hepamax-S®, Blau Farmacêutica SA, Cotia, São Paulo, Brazil), and anesthetized with ketamine hydrochloride (50 mg/kg/intraperitoneal; Dopalen®, Sespo Indústria e Comércio Ltda., Vetbrands Division, Jacareí, São Paulo, Brazil) and xylazine hydrochloride (10 mg/kg/intraperitoneal; Anasedan®, Sespo Indústria e Comércio Ltda., Vetbrands Division, Jacareí, São Paulo, Brazil). When the animal did not have an adequate anesthetic plan for the surgical intervention, a single higher dose (20%–30% of the initial dose of anesthetics) was administered [17]. After euthanasia, the animals were submitted to median thoracotomy for blood and tissue sample collection.

Nutritional assessment

Body weight was measured weekly, and body fat (BF) amount was determined from the dissection of the epididymal, mesenteric, and retroperitoneal fat pads. The adiposity index (AI) was calculated using the following formula: AI = (total BF/final body weight) × 100 [18, 19].

Fig. 1. Schematic representation of the 20-week experimental protocol. The weeks are represented by individual bars that form the figure in the shape of an arrow. Moment 1 (M1): birth of the animals (Central biotério - UFES). Moment 2 (M2): acclimatization period at our laboratory. Moment 3 (M3): start of the experimental protocol and randomization of the groups: control (C), high-sucrose (HS), high-fat (HF) and high-fat with sucrose (HFS) (week 0). Moment 4 (M4): *in vivo* analysis (glucose tolerance test and tail plethysmography). Moment 5 (M5): end of the experimental protocol, euthanasia of the animals and performance of *post mortem* analysis (biochemical, tissue collection, determination of oxidative stress and western blot).

Histological analysis

Histological analyses were performed on mesenteric adipose tissue fixed for 24 hours in 4% paraformaldehyde with 0.1 M phosphate buffer (pH = 7.4). After dehydration in ethanol and clearing in xylol, the tissue was embedded in paraffin to form blocks. Sections 5 μm thick were obtained using a LEICA RM2125 microtome (LEICA Biosystems Inc., Richmond, Illinois, USA) and stained with hematoxylin-eosin (H&E). Images were captured with a video camera (Evolution, Media Cybernetics, Inc., Bethesda, MD) coupled to an optical microscope (Eclipse 400, Nikon) under 40× magnification. Measurements were performed using the specific software (ImageJ Pro-Plus®, Media Cybernetics, Silver Spring, Maryland, USA). The cell area (μm^2) was obtained, and the examiner was blinded to the experimental groups.

Comorbidities

Arterial blood pressure was assessed indirectly using the tail plethysmography method, coupled to a data acquisition system (IITC INC, Life Science, Woodland Hills, CA, USA), and the mean of three recordings was obtained for each animal.

Table 1. Composition and macronutrient values of diets. C: standard chow diet; HS: high-sucrose diet; HF: high-fat diet; HFS: high-fat diet with sucrose. *Mineral and Vitamin mix: selenium, iron, copper, manganese, iodine, zinc, cobalt, calcium, phosphorus, vit. A, vit. D3, vit. E, vit. K3, vit. C, B complex, choline. **Inert material, without nutritional value and calories

Components (g/kg)	Diets			
	C	HS	HF	HFS
Corn	200	200	180	80
Rice	200	200	200	200
Bone meal	120	120	120	120
Sucrose	-	100	-	100
Soy oil	75	75	-	-
Lard	-	-	200	200
Gluten	200	200	200	200
Salt	3.5	3.5	3.5	3.5
Mineral mix*	35	35	35	35
Vitamin mix*	16.5	16.5	16.5	16.5
Inert material**	150	50	45	45
Total	1000	1000	1000	1000
Macronutrient (%)				
Protein	24.8	21.8	17.8	19.2
Carbohydrate	49.6	52.3	44.6	43.4
Lipids	25.6	25.9	37.6	37.4

Glucose intolerance was determined by the glucose tolerance test after 6 hours of fasting. Glycemic levels were analyzed under baseline conditions (time 0) and after intraperitoneal overload of 25% glucose (Sigma-Aldrich®, St Louis, MO, USA), equivalent to 2 g/kg [20] after 30, 60, 90, and 120 minutes and were evaluated by the area under the curve (AUC) for glucose.

The presence of dyslipidemia was assessed by triglycerides (TG), total cholesterol (T- Chol), and high-density lipoprotein (HDL) concentrations using specific kits (Bioclin Bioquímica®, Belo Horizonte, Minas Gerais, Brazil, and Synermed do Brasil Ltda., São Paulo, Brazil).

Cardiac characteristics

The cardiac remodeling process was assessed by means of macroscopic structural analysis by determining the weights of the heart, left ventricle (LV), right ventricle (RV), and total atrium (AT), and relationships with tibia length [21]. In addition, markers of cardiac injury were added by means of creatine kinase (CK), creatine kinase fraction MB (CK-MB), and lactate dehydrogenase (LDH) using specific kits (Bioclin Bioquímica®, Belo Horizonte, Minas Gerais, Brazil) and analyzed by the BS-200 automated biochemical equipment (Mindray do Brasil - Trade and Distribution of Medical Equipment Ltda., São Paulo, Brazil).

Cardiac oxidative stress markers

Fragments of LV (100 mg) were homogenized in 1:10 sodium phosphate buffer and transferred to Eppendorf microcentrifuge tubes. Then, the samples were centrifuged at 3000 rpm for 10 minutes, and the supernatant was used for measuring thiobarbituric acid reactive substance (TBARS) and carbonylated protein.

To evaluate the peroxidation of membrane lipids, the TBARS method was used. For this purpose, 250 μL of cardiac tissue homogenate was placed in a test tube and 750 μL of 10% trichloroacetic acid was added. After vortexing, the samples were centrifuged at 3000 rpm for 5 minutes, in test tubes, 500 μL of the supernatant and 500 μL of 0.67% thiobarbituric acid (TBA) were placed. Then, the samples were heated in a water bath at 100 °C for 15 minutes. The malondialdehyde (MDA) reacted with the TBA in a 1:2 MDA-TBA

ratio. After cooling in an ice bath, the reading at 535 nm was performed on the Spectra Max 190 microplate reader (Molecular Devices®, Sunnyvale, CA, USA). The MDA concentration was obtained by the molar extinction coefficient (1.56×10^5 M^{-1} cm^{-1}) and the absorbance of the samples, the results being expressed in nmol/g of protein [22-23].

Carbonylated proteins were measured using the method adapted from Mesquita et al. [24], 100 μL of cardiac tissue homogenate were used for 100 μL of the derivatizer 2, 4-dinitrophenyl-hydrazine. The samples were incubated for 10 minutes at room temperature, and 50 μL of NaOH (6 M) were added and incubated again for 10 minutes. The reading was performed at 450 nm in a Spectra Max 190 microplate reader (Molecular Devices®, Sunnyvale, CA, USA), and the result was obtained (nmol/mg of protein) through the absorbance of the samples and the molar extinction coefficient (22 000 M^{-1} cm^{-1}).

Oxidizing and Antioxidizing Signaling Pathways

The oxidant and antioxidant signaling pathways were analyzed using the western blot technique, with determination of the oxidant proteins AT-1 and Nox2 and antioxidants SOD-1, SOD-2, CAT, and GPX, using the β-actin protein for normalization.

Homogenization of frozen cardiac tissues was performed in plastic tubes containing lysis buffer (Tris-HCL 10 mM pH = 7.4, 1 mM NaVO$_3$, 1% SDS, 0.05 mM DTT, 5 mM EDTA, 1 mM PMSF, 10 mM NaF) with protease inhibitor (1: 100) added in the proportion 1 mL/100 mg. The samples were centrifuged for 20 minutes at 14 000 rpm at 4 °C. The pellet formed was discarded and the protein concentration of the supernatant quantified by the method of Bradford [25]. The absorbance was measured at 595 nm using a spectrophotometer.

Then, aliquots were prepared containing the necessary volume for a 50 μg protein load, in addition to the sample buffer (Laemmli 4×). All aliquots were standardized to a final volume of 15 μL, completing the volume of each one with deionized water. The samples were heated at 95 °C for 10 minutes and then loaded onto 10% SDS-polyacrylamide gels, and then electrophoresis was performed. Subsequently, the protein was transferred electrophoretically to a polyvinylidene fluoride membrane (Bio-Rad, CA, USA).

At the end of the transfer, the membranes were incubated for 1 hour, at room temperature, with 5% bovine serum albumin blocking solution. Then, the membranes were incubated overnight at 4 °C, under agitation, with the primary antibodies AT1 receptor through the AT-1R anti-mouse monoclonal antibody (1:500, Santa Cruz Biotechnology) and Nox 2 by the antibody monoclonal anti-mouse for Nox 2 (Gp91phox) (1:500, Santa Cruz Biotechnology), SOD, by the monoclonal anti-mouse antibody for the SOD-1 isoform (1:1000, Santa Cruz Biotechnology) and the polyclonal anti-rabbit for SOD-2 (1:500 Sigma-Aldrich), CAT through monoclonal anti-mouse antibody for CAT (1: 2000, Sigma-Aldrich) and GPX with the antibody polyclonal anti-rabbit for GPX-1 (1:3000 Sigma-Aldrich). The binding of the primary antibody was detected with secondary antibodies conjugated with peroxidase anti-mouse IgG (1:10000, Santa Cruz Biotechnology) or an anti-rabbit IgG (1: 10000, Santa Cruz Biotechnology), and the detection of the bands of the proteins of interest was carried out using the ECL chemiluminescence detection reagent (GE Healthcare, UK).

To quantify the density of the bands, the software ImageJ (National Institute of Health, NIH, USA) was used and the results calculated using the relationship between the density of the proteins of interest corrected by the intensity of the protein used as a control (β-actin: 1: 1000, Cell Signaling).

Statistical Analysis

Data were expressed using the mean ± standard error of the mean (SEM) and subjected to analysis of variance (ANOVA) one way (diet factor) for independent samples. When significant differences were found ($p < 0.05$), a Tukey *post hoc* test was carried out. The level of significance was 5%.

Results

The nutritional assessment demonstrated no statistical difference between the groups regarding initial body weight, final body weight, and body weight gain after the end of the experimental protocol (Table 2). Only the HF group showed a significant increase in retroperitoneal fat pad (97.8%; p = 0.03) and total body fat (69.2%; p = 0.04), as well as in the adiposity index (33.8%; p = 0.02), relative to the C group. Considering the other parameters, all dietary interventions showed similar results to the C.

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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Cell Physiol Biochem Press GmbH&Co. KG

623

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

The total body fat (109%; p < 0.001) as well as the mesenteric (92%; p = 0.02) and retroperitoneal (156%; p = 0.08) fat pads were significantly elevated in the HF when compared to the HS group, consequently reflecting a higher adiposity index (62%). This index was also significantly higher in the HFS group when compared to the HS (45.4%; p = 0.01); however, it was not followed by significant changes in fat pads and total body fat. The HF and HFS groups showed similar behavior for all evaluated adiposity parameters (Table 2).

Regarding the food profile, food consumption in grams (g/day) in the HS (29.5%; p = 0.009) and HFS (26%; p = 0.02) groups was lower than in the C group; however, there were no changes in caloric intake. In addition, food consumption (p = 0.37) and caloric intake were similar between groups C and HF (p = 0.61). However, the results show that the HF and HFS groups had a significant increase in feed efficiency compared with the C group, reflecting in an elevation of 14.8% (p = 0.007) and 17.4% (p = 0.002), respectively. In relation to HS, all groups showed significantly higher feed efficiency. There was no significant difference between the HF and HFS groups for all food profile parameters evaluated (Table 2).

The histological results regarding the mesenteric adipose tissue show that the adipocyte areas were similar between the experimental groups (p = 0.31) (Fig. 2).

Table 2. Nutritional assessment and food profile. Values expressed as mean ± SEM of five animals per group; C: control; HS: high-sucrose; HF: high-fat; HFS: high-fat with sucrose. IBW: Initial body weight; FBW: Final body weight. One way ANOVA followed by Tukey's post hoc test. p < 0.05; * vs. C; # HS vs. HF; & HS vs. HFS

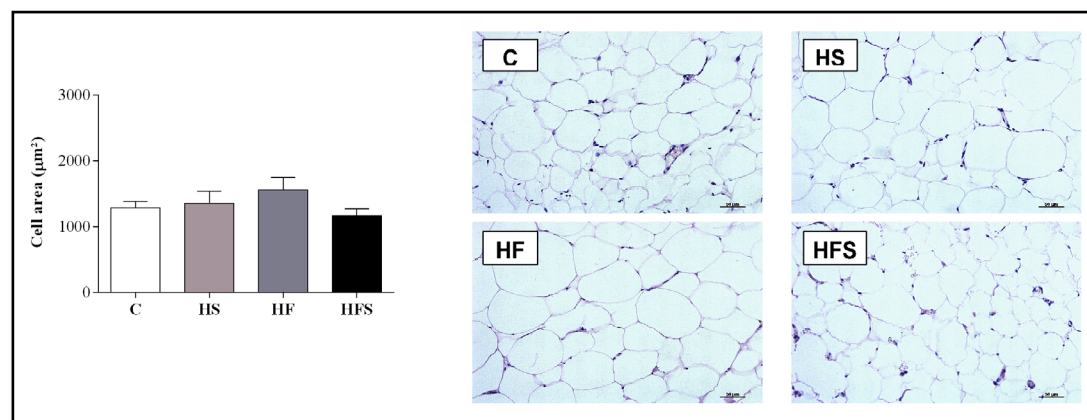| Variables	Groups			
	C	HS	HF	HFS
IBW (g)	110 ± 6	113 ± 5	119 ± 5	117 ± 7
FBW (g)	575 ± 29	559 ± 28	712 ± 73	626 ± 32
Body Weight Gain (g)	464 ± 32	446 ± 29	592 ± 69	509 ± 26
Epididymal Fat (g)	11.9 ± 0.8	9.7 ± 0.8	13.7 ± 1.8	13.9 ± 1,5
Mesenteric Fat (g)	10.9 ± 0.9	9.6 ± 1.1#	18.4 ± 2.9	15.2 ± 2.0
Retroperitoneal fat (g)	23.0 ± 1.5	17.8 ± 0.4#	45.5 ± 9.3*	31.9 ± 4.3
Total Body Fat (g)	45.8 ± 2.4	37.1 ± 2.0#	77.5 ± 13.1*	61.0 ± 7.1
Adiposity Index (%)	8.0 ± 0.5	6.6 ± 0.2#&	10.7 ± 0.8*	9.6 ± 0.6
Food consumption (g/day)	23.2 ± 1.4	16.4 ± 0.9*	20.1 ± 1.8	17.2 ± 0.9*
Caloric intake (kcal/day)	82.4 ± 4.9	93.9 ± 4.0	92.2 ± 8.4	77.1 ± 3.9
Feed efficiency (%)	3.9 ± 0.06	3.3 ± 0.11*#&	4.5 ± 0.14*	4.6 ± 0.10*

Fig. 2. Effect of diet intake on the size of the mesenteric adipocyte. Representative histological images of mesenteric adipose tissue stained with hematoxylin-eosin (H&E). Scale bar: 50 µm. C: control (n = 5); HS: high-sucrose (n = 5); HF: high-fat (n = 5); HFS: high-fat with sucrose (n = 5). Data are expressed as mean ± SEM. One-way ANOVA followed Tukey's *post hoc* test.

There was no statistical difference in systolic blood pressure between the groups (p > 0.05); however, the AUC (mg/dL/min) of the HFS group was higher than that in the C group, representing an increase of 21% in blood glucose (Table 3). It is noteworthy that there was no significant difference in glycemic behavior between the other groups (p > 0.05) in relation to the C group, nor were there any differences in comparisons between other groups. In addition, the results showed no significant differences in relation to lipid profiles (Table 3).

There was no difference between the groups regarding the heart, left ventricle, right ventricle, and atrium weight, and normalization of these variables by tibia length did not change the result. The results demonstrate an absence of cardiac remodeling (Table 4). Similarly, cardiac injury markers also showed no difference between the groups; however, there was an increase in CK-MB of 35.6% in the HS group, 22.8% in the HF, and 76.7% in the HFS in relation to the C group (C = 236 ± 6.65 *vs.* HS = 320 ± 46.9; HF = 290 ± 10.4; HFS = 417 ± 82.4, p = 0.17), while LDH increased by 19.8%, 170.1%, and 175% respectively (C = 484 ± 26.1 *vs.* HS = 580 ± 112; HF = 1308 ± 323; HFS = 1332 ± 518, p = 0.16) (Fig. 3).

The levels of MDA (nmol/mg protein), a marker of lipid peroxidation, were significantly higher in the HF and HFS groups, representing an increase of approximately four times when compared with the C (C = 136 ± 17 *vs.* HF = 591 ± 103, p = 0.003; HFS = 545 ± 104, p = 0.007). The same behavior was observed for carbonylated protein, which increased significantly in the HF and HFS groups in relation to the C (C = 9.95 ± 2.13 *vs.* HF = 24.6 ± 3.5, p = 0.01; HFS = 27.0 ± 3.8, p = 0.004) (Fig. 4), indicating the occurrence of oxidative stress from the supply of a high-fat diet and a high-fat diet with sucrose. However, markers of cardiac oxidative stress were similar between the C and HS groups.

Table 3. Comorbidities in the experimental groups. Values expressed as mean ± SEM. SBP: systolic blood pressure C: control (n = 5) HS: high-sucrose (n = 5); HF: high-fat (n = 4); HFS: high-fat with sucrose (n = 4). Area under the glycemic curve (AUC). C: control (n = 4); HS: high-sucrose (n = 5); HF: high-fat (n = 5); HFS: high-fat with sucrose (n = 5). TG: triglycerides; T-Chol: total cholesterol; HDL: high-density lipoprotein; C: Control (n = 3); HS: High-sucrose (n = 5); HF: High-fat (n = 4); HFS: High-fat with sucrose (n = 4); One way ANOVA followed by Tukey's post hoc test. p < 0.05; *vs. C

Variables	Groups				p-value
	C	HS	HF	HFS	
SBP (mmHg)	147 ± 4.6	171 ± 7.8	163 ± 3.4	176 ± 12.5	0.07
AUC (mg/dL/min)	1212 ± 17.5	1332 ± 57.9	1431 ± 84.3	1465 ± 47.6*	0.04
TG (mg/dL)	28.0 ± 10.4	22.9 ± 7.7	44.8 ± 4.7	32.8 ± 9.9	0.30
T-Chol (mg/dL)	94.5 ± 4.5	69.9 ± 4.5	82.1 ± 7.1	71.6 ± 6.4	0.06
HDL (mg/dL)	32.0 ± 1.6	23.8 ± 1.2	32.2 ± 6.2	24.4 ± 3.2	0.23

Table 4. Cardiac morphological characteristics. Values expressed as mean ± SEM of five animals per group; C: control; HS: high-sucrose; HF: high-fat; HFS: high-fat with sucrose. LV: Left ventricle; RV: Right ventricle AT: Atrium. One way ANOVA followed by Tukey's post hoc test

Variables	Groups			
	C	HS	HF	HFS
Heart weight (g)	1.24 ± 0.10	1.18 ± 0.04	1.44 ± 0.15	1.31 ± 0.06
LV (g)	0.91 ± 0.08	0.88 ± 0.03	1.07 ± 0.11	0.98 ± 0.05
RV (g)	0.21 ± 0.01	0.21 ± 0.01	0.27 ± 0.03	0.23 ± 0.02
AT (g)	0.11 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.10 ± 0.01
Heart weight /Tibia (g/cm)	0.29 ± 0.02	0.27 ± 0.01	0.33 ± 0.03	0.30 ± 0.01
LV/Tibia (g/cm)	0.22 ± 0.02	0.20 ± 0.01	0.24 ± 0.02	0.22 ± 0.01
RV/Tibia (g/cm)	0.05 ± 0.003	0.05 ± 0.003	0.06 ± 0.007	0.05 ± 0.003
AT/Tibia (g/cm)	0.03 ± 0.003	0.02 ± 0.001	0.03 ± 0.003	0.02 ± 0.002

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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625

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

Fig. 3. Cardiac injury markers. A) Total creatine kinase (CK). B) Creatine kinase MB fraction (CK-MB). C) Lactate dehydrogenase isoenzymes (LDH). C: control (n = 3); HS: high-sucrose (n = 5); HF: high-fat (n = 4); HFS: high-fat with sucrose (n = 4). Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's *post hoc* test.
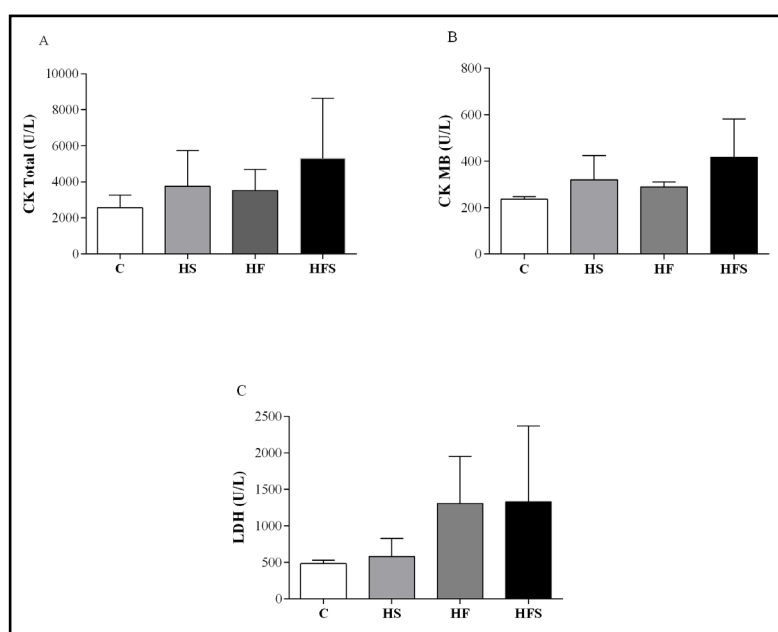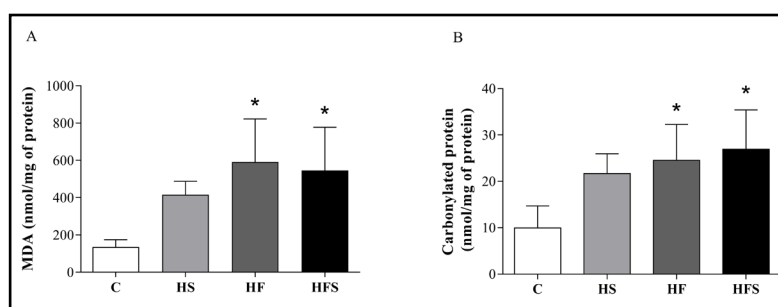

Fig. 4. Cardiac Oxidative Stress. A) MDA: Malondialdehyde. B) Carbonylated Protein. Five animals per group; C: control; HS: high-sucrose; HF: high-fat; HFS: high-fat with sucrose. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's *post hoc* test. p<0.05; * *vs*. C.
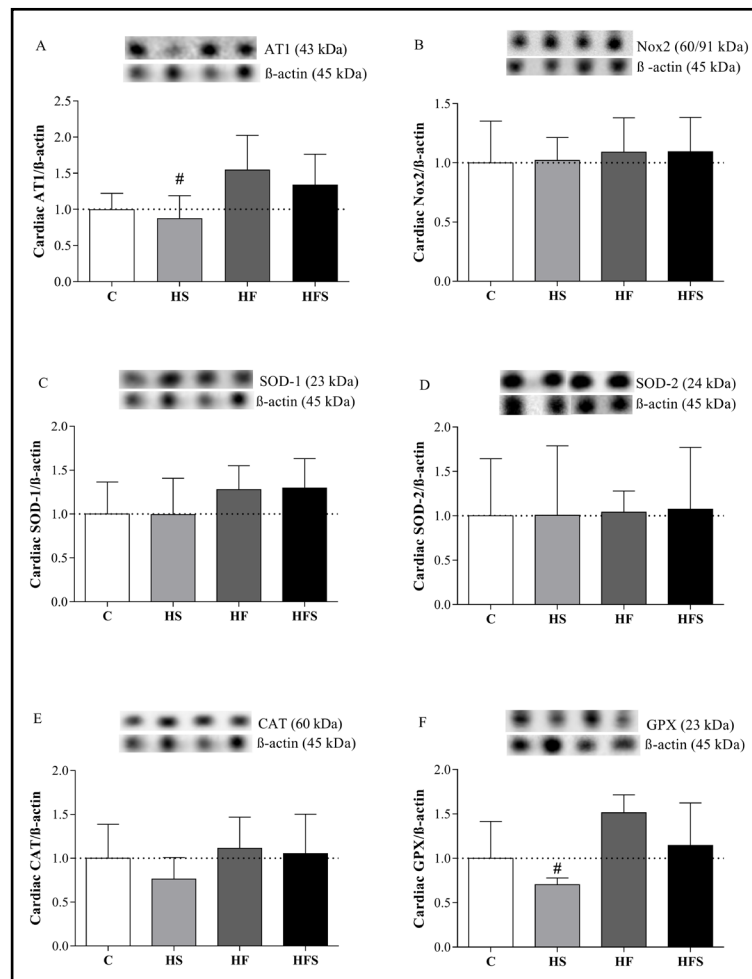

The expression of oxidant proteins AT1 and Nox2 and antioxidants SOD-1, SOD-2, GPX, and CAT did not present statistical differences between the groups when compared with the C group. However, the HS group obtained lower levels of the AT1 receptor (HS = 0.88 ± 0.14 *vs*. HF = 1.55 ± 0.21, p = 0.04) and GPX (HS = 0.705 ± 0.03 *vs*. HF = 1.51 ± 0.09, p = 0.007) when compared with the HF. Considering the HS and HFS groups, as well as the HF and HFS groups, the results were similar for all evaluated pathways (Fig. 5).

Discussion

The aim of this study was to investigate whether different types of hypercaloric diets in the presence and absence of obesity would promote an increase in the levels of cardiac oxidative stress biomarkers, as well as to identify the participation of the pro-oxidant signaling pathways AT1 and Nox 2 and the antioxidants SOD, CAT, and GPX in that process. The main findings were that only the high-fat dietary intervention (HF) was able to cause an increase in adiposity; however, it did not present adipocyte hypertrophy and associated comorbidities. In addition, the HF and HFS dietary interventions caused cardiac oxidative stress visualized by the increased oxidation of lipids and proteins; however, there was no involvement of the pro-oxidant signaling pathway mediated by the AT1 receptor and the Nox2 enzyme, suggesting that other pathways may be involved. The quality of the diet,

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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626

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

Fig. 5. Cardiac expression of oxidant and antioxidant signaling pathways after the 20-week experimental protocol. A) AT1: Angiotensin type 1 receptor. B) Nox 2: Nicotinamide adenine dinucleotide phosphate oxidase 2. C) SOD-1: Superoxide dismutase 1. D) SOD-2: Superoxide dismutase 2. E) CAT: Catalase. F) GPX: Glutathione peroxidase. Five animals per group; C: control; HS: high-sucrose; HF: high-fat; HFS: high-fat with sucrose. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's *post hoc* test. $p < 0.05$; # HS *vs.* HF.

depending on the energetic substrate, in this case fat, can increase beta oxidation, generating ROS, oxidizing more fat, and raising the levels of MDA and carbonylated protein [26]. It is important to emphasize that antioxidant pathways were not quantitatively reduced in dietary interventions when cardiac oxidative stress occurred (HF and HFS).

Models of diet-induced obesity have been frequently applied, relating to the accumulation of adipose tissue and associated metabolic disorders [27, 28]. In our study, the dietary intervention rich in fat (HF) promoted obesity, visualized by the increased deposition of adipose tissue, but without changes in body mass gain. Previous work carried out in our laboratory confirmed the efficiency of the high-fat diet model for the promotion of obesity [3, 16, 29]. These findings are associated with the high obesogenic effect of this diet, which leads to a reduction in the rate of lipid oxidation as opposed to a high intake, favoring lipogenesis, increasing the oxidation of proteins and contributing to the reduction of energy expenditure [30, 31].

In our study, feed intake was reduced in both groups that consumed diets containing sucrose; however, animals that received water supplemented with sucrose compensated this reduction by increasing their intake of the sugary drink (data not shown), an observation also reported by Sheludiakova et al. [32]. Caloric intake did not differ between the groups; however, the feed efficiency in the interventions of the HF and HFS groups was high, confirming the greater use of the lipids from lard for fat deposition. A lower feeding efficiency was observed in the HS group, reflected in a consequent absence of weight gain in these animals as demonstrated by Vileigas et al. [33] and Matias et al. [16].

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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Cell Physiol Biochem Press GmbH&Co. KG

627

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

However, despite the high lipid concentrations in the diet, which are associated with promotion of adipocyte hypertrophy, triggering the lipogenesis process and contributing to an increase in fat accumulation capacity [34], it should be noted that the experimental HF model triggered obesity without adipocyte hypertrophy, demonstrating that the increase in adiposity was mild.

The association between diet and increased adiposity has been related to the appearance of several comorbidities, which imply an increased risk for the development of cardiovascular diseases [4, 28]. However, in our study there were no changes in systemic blood pressure or lipid profiles, corroborating other authors [5, 29, 35-38]. It is noteworthy that the animals evaluated in the present study did not show any gain in body mass or accumulation of mesenteric adipose tissue that would justify the development of dyslipidemia or arterial hypertension. Furthermore, lard contains 41.2% monounsaturated fatty acids, which may have a protective effect [39, 40]. Regarding the glycemic profile, the HFS group showed a possible glucose intolerance, corroborating the findings of Apaijai et al. [41]. The authors attribute this to the decrease in the function of protein kinase B (AKT), caused by the addition of simple carbohydrates to a high-fat diet, which would lead to a marked increase in insulin resistance in these animals, a fact not evaluated in the present study.

Cardiac remodeling is associated with excess adiposity due to the increased metabolic demand imposed by this condition, which results in increased cardiac output and volume overload, with dilation of the ventricular cavity, leading to eccentric hypertrophy. On the other hand, when pressure overload occurs, there is an increase in the thickness of the ventricular wall called concentric hypertrophy [42]. Thus, when structural myocardial disorders like left ventricular hypertrophy, cardiomyocyte hypertrophy, increased perivascular and interstitial fibrosis, capillary rarefaction, and functional abnormalities like systolic and diastolic dysfunction occur in a sufficient way to cause a heart abnormality, the condition is characterized as cardiomyopathy [43].

Our findings demonstrate that dietary interventions have not been able to promote hypertrophy and cardiac damage. One possible explanation may be related to the absence of volumetric and pressure overload in the evaluated groups. According to Sharma et al. [44], food intake of carbohydrates and lipids largely determines the exposure of the heart to insulin, leading to activation of AKT and mammalian target of rapamycin (mTOR) protein or peroxisome proliferator activated receptors (PPARs) related to the development of hypertrophy. However, in our study, dietary interventions were not able to produce sufficient metabolic changes to activate these pathways, which would induce cardiac remodeling.

Considering the dosages of CK, CK-MB, and LDH, the literature evidences the occurrence of cardiac damage with the consumption of hypercaloric diets. Fouad (2020) [45], when evaluating *Wistar* rats fed a high-fat diet rich in cholesterol, showed an increase in CK-MB, related to structural and functional changes in the cardiac muscle and disruption of the integrity of the cell membrane; the author also reports that this event may be secondary to lipid peroxidation in cardiac membranes. Similar data were found by Wang et al. [46], who attributed the increase in CK-MB and LDH to the inflammatory effect of saturated fatty acids. However, the data from the present study, in turn, contradict these findings, since there was no significant increase in CK-MB, the most expressed isoform in the heart, and in LDH. However, despite the absence of changes in these cardiac damage markers, it is noteworthy that an important percentage increase was observed in the studied dietary interventions, which may be indicative of the initial triggering of cardiac injury.

Regarding the occurrence of cardiac oxidative stress, studies by several authors have demonstrated the relationship between the consumption of hypercaloric diets and the increase in the production of free radicals, ROS and RNS [41, 47-51]. In our study, cardiac oxidative stress occurred markedly in the HF and HFS dietary interventions, promoting a substantial increase in protein carbonylation and lipid peroxidation. Our findings agree with Apaijai et al. [41] using a high-fat (59.28% of lard and cholesterol) and high-fat diet plus sugar (43.3% of lard and cholesterol, 32.4% of fructose syrup and sweetened condensed milk). Similarly, Emelyanova et al. [52], Ma and Xie (2017) [53] and Yu et al. [54] verified,

after intervention with a high-fat diet, an increase in cardiac MDA as well as Noeman et al. [55] who also showed an increase in the levels of carbonylated proteins. It is worth mentioning, however, that despite the lack of studies, some authors have found divergent results [56, 57] relating the absence of oxidative stress to the lack of ectopic accumulation of lipids or inflammatory signaling in cardiac tissue [56].

A possible explanation for the oxidative stress observed in the HF and HFS dietary interventions may be related to the increase of the lipid substrate in the myocardium, which becomes a major target for oxidation by free radicals and mitochondrial utilization, resulting in a greater leakage of electrons in the chain transport and increased production of $O_2^{\bullet-}$ [55]. These mitochondrial changes and cardiac oxidative damage have been reported by Sverdlov et al. [48], Yu et al. [54], and Jiménez-González et al. [58]. Thus, the high intake of fats in the present study resulted in an increase in adipose tissue in the HF group and a possible intolerance to glucose in the HFS group, both situations that could contribute to greater uptake of fatty acids by the heart.

Lard, the main component of these diets, contains about 39% saturated fatty acids, 23.8% of which is palmitic acid, which has been identified as the main cause of oxidative stress induced by diet [39, 59]. Although saturated fatty acids are less oxidizable, the rest of the unsaturated fatty acids contained in the lard may have promoted lipoperoxidation. However, it is worth mentioning that saturated fatty acids increase ROS, which may enhance lipid peroxidation [60]. In situations of high plasma concentrations of free fatty acids, they are transported to cardiomyocytes, leading to greater formation of ceramides and diacylglycerol, in addition to promoting an increase in unoxidized palmitate in the cytoplasm of cells, suggesting that not only lipid oxidation but also excess lipid accumulation are involved in cardiac lipotoxicity [11, 57, 61].

In disagreement with our initial hypothesis, the HS group did not show an increase in the oxidative markers evaluated, an observation in line with that verified by Szűcs et al. [62] in animals with a hypercaloric diet rich in fructose (60%). In this sense, sucrose was not able to produce oxidative damage in animals on a HS diet, nor aggravate it in the HFS group, since there was no difference in the levels of cardiac oxidative stress between the HF and HFS groups. These findings differ from the results assessed by Apaijai et al. [41], who showed greater cardiac oxidative damage in animals fed a high-fat diet plus sucrose and fructose. However, it is important to note that isolated cardiac mitochondria produce 23% more hydrogen peroxide (H_2O_2) when they oxidize fatty acids than mitochondria that use glucose as an energy substrate via the glycolytic pathway [11, 63].

The literature still lacks consistent information about the signaling pathways that may be related to the process of cardiac oxidative stress promoted by hypercaloric diets. Within this context, the excess of lipids and sucrose would act through the metabolic overload caused by the increase of free fatty acids and glucose in the plasma, which, when internalized by cardiomyocytes and transported to the mitochondria, would lead to the formation of ROS with consequent PKC stimulation, which in turn activates Nox2. In addition, the caloric excess contributes to the development of obesity, which can increase sympathetic activity, with greater production of angiotensin II, which binds to the membrane receptor AT1 in the cardiac cell, and this, also via PKC, activates Nox2, which causes an increase in $O_2^{\bullet-}$ [9, 64, 65].

In our initial hypothesis, we suggested a possible participation of the pro-oxidant pathway involving the AT1 and Nox2. However, no increase in cardiac expression of these pro-oxidant proteins was observed in dietary interventions. This fact may be associated with the absence of hyperactivity of the renin angiotensin system, given that the animals evaluated did not present arterial hypertension. Among the adipokines released by adipose tissue, angiotensin II, a hypertensive hormone generated from the renin angiotensin system, is associated with the pathogenesis of oxidative stress; angiotensinogen, a precursor of angiotensin II, from adipose tissue, contributes about one third of circulating angiotensinogen in rodents and plays an important role in the function of this tissue through the modulation of adipogenesis and lipid metabolism [12, 66, 67]. The activity of angiotensin II is mediated through its connection with its plasma membrane receptors, among them, the AT1 receptor

is the most related to its harmful actions in heart, including the increase in the production of ROS by stimulating NADPH oxidase [68]. In our study, the low levels of adiposity may reflect that there was no hypertrophy, which would justify the lower expression of the AT1 receptor, since angiotensin II is produced in excess by hypertrophied adipose tissue.

In addition, the ROS resulting from this activation can enter the mitochondria, making it dysfunctional, while the organelle is producing more ROS due to the increase in the oxidation processes of nutrients, which in turn activate the PKC which in a cyclic way increases plus the activity of NADPH oxidase, resulting in overproduction of ROS [69]. The involvement of this enzyme in the oxidative stress process was confirmed by Bhatti and Li (2020) [47], who reported Nox2-isoform-dependent higher production of $O_2^{\bullet-}$, in addition to greater phosphorylation of AKT and Extracellular Signal Regulated Protein Kinase (ERK) 1/2, which are involved in hypertrophy processes in C57BL/6J WT mice that received a high-fat diet; these changes are significantly reduced or absent in Nox2 knockout animals. Gamez-Mendez and collaborators (2015) [70] also showed an increase in peroxynitrite $(ONOO^-)$ and $O_2^{\bullet-}$ in mice fed a high-fat diet; however, when offering the same diet plus apocynin, an antioxidant inhibitor of NADPH oxidase, they observed that the vascular generation of these molecules was significantly lower.

However, according to our findings, there was no evidence of involvement of the AT1/Nox 2 pathway in the development of cardiac oxidative stress, which points to the participation of other mechanisms as responsible for the increase in oxidative markers observed in the HF and HFS groups; in particular, in addition to the oxidative phosphorylation carried out by the mitochondria, the activation of other enzymatic pathways, such as xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome p450 enzymes, and uncoupled nitric oxide synthase (eNos), as well as other isoforms of the NADPH oxidase enzyme that can also be found in the heart, especially Nox4, could promote oxidative stress in these animals [6, 12].

In addition, Drosatos and Schulze (2013) [63] propose that saturated fatty acids, especially palmitate, can inhibit AMP-activated protein kinase (AMPK), which increases levels of malonyl-CoA and inhibits carnitine palmitoyl transferase 1 (CPT-1), causing accumulation of fatty acids and lipotoxicity. Other than that, an increase in diacylglycerol induces over-regulation of PKC, which in turn inhibits the insulin signaling pathway by blocking the insulin substrate 1 receptor (IRS-1), which may exacerbate the accumulation of lipids in the heart, increasing the uptake of fatty acids. This chronic supply of lipids to the heart increases excessive ROS production, contributing to damage to various organelles and macromolecules via oxidative changes [71].

In relation to antioxidant defenses, in disagreement with our initial hypothesis, in which a reduction in the amount of defense enzymes in cardiac tissue was expected, under the effect of HF and HFS dietary interventions, which showed an increase in oxidative stress, no differences were found in protein expression of SOD-1, SOD-2, CAT, and GPX. However, studies have shown that in addition to quantitative determination, the evaluation of enzyme activity plays an important role in the oxidative stress process [50, 54]. In the present study, this assessment was not carried out; however, it is observed that the levels of SOD, CAT, and GPX in the HF and HFS groups were not sufficient to promote redox balance, suggesting a possible decrease in activity. Thus, it is noted that, even though there was no significant difference between the groups, there was an increase in the levels of SOD-1 (27.9% in the HF group and 29.6% in the HFS group), and GPX (51.4% in the HF group and 14.4% in the HFS group), possibly as an attempt to compensate for the reduced capacity of these enzymes to prevent oxidative damage, possibly due to an increase in the transcription of antioxidant enzyme genes, mediated by regulatory systems such as nuclear factor 2 related to erythroid 2 (Nrf-2), nuclear factor kapa-b (NF-κB), and mitogen activated protein (MAP) kinase [72].

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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Cell Physiol Biochem Press GmbH&Co. KG

630

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

Conclusion

High-fat and combined high-fat with sucrose diets promoted cardiac oxidative stress, in the presence and absence of obesity, respectively. However, this process was not mediated by the AT1 and Nox2 pro-oxidant pathways or by the quantitative reduction of antioxidant enzymes.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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631

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:618-634

DOI: 10.33594/000000441
Published online: 27 October 2021

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Cell Physiol Biochem Press GmbH&Co. KG

634

Gasparini et al.: Hypercaloric Diets and Cardiac Oxidative Stress

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DOI: 10.33594/000000441
Published online: 27 October 2021

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