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Hepatic Mitochondria in Diet-induced Obese Mice (Stereology and Molecular Analysis)

Mitocondrias Hepáticas en Ratones Obesos Inducidos por la Dieta (Estereología y Análisis Molecular)

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SUMMARY: The world population is going through an obesity epidemic that has severe consequences for the health system. This study focused on studying hepatic mitochondria in obese animals induced by a high-fat (HF) diet and used the model-based stereology in electron micrographs for the quantitative study. Besides, the gene expressions of molecular markers of mitochondrial biogenesis carnitine palmitoyltransferase 1a (*Cpt 1α*), mitochondrial transcription factor a (*Tfam*), uncoupling protein 3 (*Ucp 3*), and nuclear respiratory factor 1 (*Nrf 1*) were analyzed. The HF diet caused a weight gain of +1820 % comparing the control group (C) with the HF group (from 0.32±0.31 g to 5.5±0.39 g, $P<0.001$). The HF group showed fat droplets in the hepatocyte cytoplasm (steatosis) and less dense and large mitochondria in transmission electron microscopy. The mitochondria size (cross-section) did not show a significant difference between the groups C and HF. However, the mitochondria numerical density per area was 30 % less, the mitochondrial surface density (outer membrane) was 20 % less, and the mitochondrial volume density was 22 % less in the HF group than the C group. The gene expressions of molecular markers of mitochondrial biogenesis *Cpt 1α, Tfam, Ucp 3*, and *Nrf 1* decreased in the HF group compared to the C group. The quantitative results match perfectly with the molecular ones of mitochondrial biogenesis markers. In the future, it will be crucial to verify if and how these data recover with the reduction of obesity, which would be of significant interest given the current obesity epidemic that affects the world population.

KEY WORDS: Mitochondria; Mitochondrial biogenesis; Diet-induced obesity; Cell biology; Molecular biology.

INTRODUCTION

Mitochondria are organelles involved in intracellular lipid trafficking, exchanging lipids with the rest of the cell at membrane contact sites (endoplasmic reticulum-mitochondria encounter structure or ERMES) (Kornmann, 2020). Mitochondria regulate ATP generation, cell proliferation, cell death, and metabolism, and the management of innate immunity and the inflammatory response (Missiroli *et al*., 2020). Dysfunctional mitochondria are implicated in several pathophysiological conditions such as cancer, neurodegeneration, and aging (Kumar *et al*., 2020) and target cell death regulation (Bock & Tait, 2020).

Moreover, toxic agents might lead to mitochondrial membrane damage and internal membrane swelling, especially of hepatocytes (Dhanraj *et al*., 2020). Mitochondria can be studied with quantitative morphological tools such as model-based stereology (Mandarim-de-Lacerda, 2011). Sometimes, although no qualitative differences might be observed, a quantitative study allows the determination of a higher mean volume, number, and surface density of mitochondria (Black & Cornacchia, 1986). The significance of quantitative studies with mitochondria is because mitochondrial biogenesis includes mitochondrial proliferation and differentiation and is essential for tissue health through the balance of mitochondrial turnover (Whitaker *et al*., 2016).

The high-fat (HF) diet induces hepatic steatosis through multiple pathways: increasing hepatic inflow of fatty acids and hepatic fatty acid synthesis and decreasing fatty

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acid oxidation in the liver, overloading the mitochondrial capacity (Shin *et al*., 2019). Consequently, there is increased mitochondrial biogenesis and hepatocytes' interaction (Song *et al*., 2020).

This study aimed to observe the liver mitochondria's quantitative data and molecular markers of mitochondrial biogenesis related to diet-induced obesity in mice.

MATERIAL AND METHOD

Experimental design: animals and diet

All procedures are in accordance and approved by the Animal Ethics Committee of the University of State of Rio de Janeiro (Protocol number 047/2018).

Twenty 3-month-old C57BL/6 male mice were kept individually in a ventilated system under controlled conditions of light (12/12 h dark/light cycle), temperature (21±2 °C), and humidity (60±10 %) (Nexgen, Allentown Inc., PA, USA), with free access to food and water during 15 weeks.

The animals were randomly assigned into two groups (n=10/group): control (C, fed a control diet with 19 % of energy as protein; 17 % as fat, and 64 % as carbohydrates; total energy 15 kJ/g), and high-fat (HF, fed a high-fat diet with 19 % of energy as protein; 49 % as fat and 32% as carbohydrates; total energy 21 kJ/g). Diets were manufactured by PragSolucoes (Jau, SP, Brazil) following the recommendations for mature rodents (Aguila *et al*., 2021). The bodyweight of the animals was measured weekly.

Sacrifice and tissue extraction

The animals were food-deprived for six hours and killed under anesthesia (ketamine 60 mg/kg and xylazine 10 mg/kg, intraperitoneal). The livers were dissected, and several 1 mm^3 fragments from all lobes were fixed for 48 h in 2.5 % glutaraldehyde (Riedel-de-Haen, Germany) in 0.1 M cacodylate buffer (pH 7.2) and post-fixed for 3 h in 1 % osmium tetroxide (Sigma-Aldrich Louis, USA) or frozen at -80 ºC until analyses.

Fixed fragments were rinsed in PBS, dehydrated (graded series of acetone), and embedded in epoxy resin (Embed-812, EMS, Hatfield, PA, USA). Semithin sections (1 µm) and ultrathin sections (70 nm) were obtained with a Leica Ultracut-UCT (Leica Mikrosysteme GmbH, Austria). Semithin sections were stained with toluidine blue and studied in a Nikon microscope model 80i and DS-Ri1 digital camera (Nikon Instruments, Inc., New York, USA). Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed at 80 kV transmission electron microscopy (JEOL, Akishima, Tokyo, Japan).

Mitochondrial stereology

The model-based stereology was assessed on electron micrographs superimposing a test-system composed of test-lines (L_T) and test-points (P_T) in a frame with a known area (A_T) (Mandarim-de-Lacerda & del Sol, 2017). Briefly, in a random set of liver micrographs per group, mitochondrial data might be estimated by counting the mitochondrial profiles in A_T (N_A), the mitochondrial surface intersections with the test line (I), and the points hitting the mitochondria (P_p) as described previously (Reis-Barbosa *et al*., 2020). Therefore, we estimated the following data: a) N_A [mito, liver] := N [mito, liver]/A_T; b) volume density, Vv [mito, liver] := P_p [mito, liver]/P_T; c) surface density (outer membrane), Sv [mito, liver] := 2*I [mito, liver]/L_T; d) cross-sectional mitochondrial area, A [mito, liver] := Vv [mito/liver]/2N_A [mito, liver].

Real-time polymerase chain reaction (RT qPCR)

Total RNA of the liver (30 mg) was extracted (Trizol, Invitrogen, CA, USA), and quantified (spectroscopy, Nanovue, GE Life Sciences), then one microgram was treated with DNAse I. Then, oligo (dT) primers for mRNA and Superscript III reverse-transcriptase were applied to the synthesis of the first-strand cDNA (thermocycler CFX96 (Bio-Rad, Hercules, CA, USA, and SYBR Green mix). The endogenous beta-actin was used to normalize the expression of the selected genes. After the pre-denaturation and polymerase-activation program (4 min at 95 °C), 44 cycles of 95 °C for 10 s and 60 °C for 15 s the material followed a melting curve program (60-95 °C, the heating rate of 0.1 °C/s). RT qPCR analyzed the expression intensities of the genes, and the ratio of relative mRNA expression was calculated using $2^{-\Delta\Delta CT - \Delta CT}$ as the difference between the number of cycles (CT).

The primers were designed using Prime3 software (http://bioinfo.ut.ee/primer3-0.4.0/). We studied the mitochondrial biogenesis markers: carnitine palmitoyltransferase 1a (*Cpt 1a*) (TGTCAAAGATACCGTGAGCAG, GCCCACCAGGATTTTAGCTT); mitochondrial transcription factor a (*Tfam*) (AAGGGAATGGGAAAGGTAGA, AACAGGACATGGAAAGCAGAT); mitochondrial uncoupling protein 3 (*Ucp 3*) (CAGATCCTGCTGCTACCTAAT, GCATCCATAGTCCCTCTGTAT); nuclear respiratory factor 1 (*Nrf 1*) (TGAGCTGCTGTCCAAATACC, ACCTTCTGCTTCATCTGTCG).

Data analysis

The data are shown as the means and standard deviations, analyzed by the unpaired t-test and Welch correction (Prism version 9.01 for Windows, GraphPad Software, La Jolla, CA, USA).

RESULTS

Weight gain

The HF diet caused a weight gain of +1820 % comparing the C group with the HF group (from 0.32±0.31 g to 5.5±0.39 g, P<0.001).

Liver mitochondria

a. Stereology

The effect of chronic HF dietary intake appears in the hepatocytes' ultrastructural study. Compared with the control group fed a balanced diet, the HF group showed fat droplets in the hepatocyte cytoplasm (steatosis) and less dense and seemingly large mitochondria (Fig. 1).

The mitochondria size (cross-section) did not show a significant difference between the groups C and HF. However, the mitochondria numerical density per area was 30 % less, the mitochondrial surface density (outer membrane) was 20 % less, and the mitochondrial volume density was 22 % less in the HF group than the C group (Table 1).

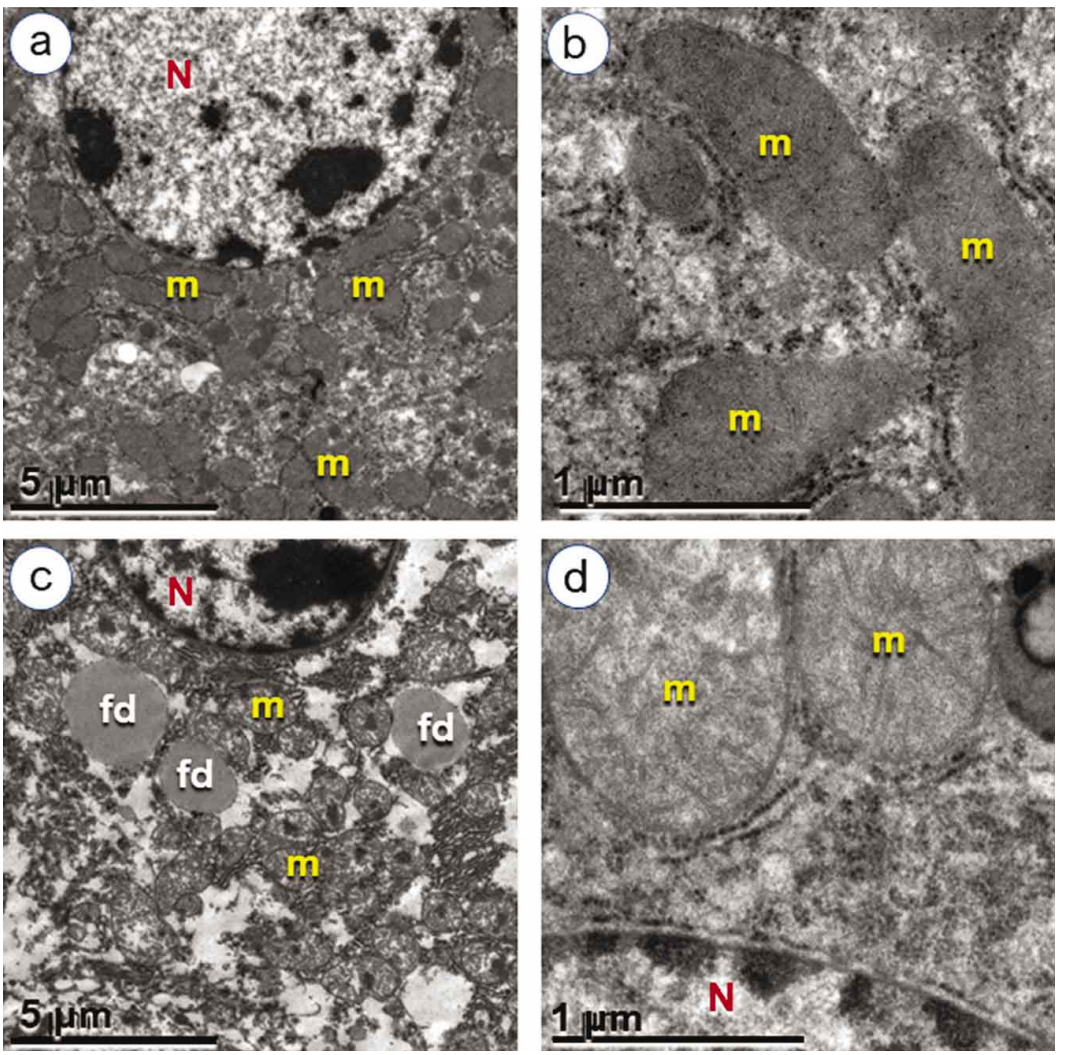

Fig. 1. Electron micrographs of hepatocytes (different magnifications) in the control (C) group (a and b) and the high-fat (HF) group (c and d). Mitochondria (m) are more electron-dense and appear grouped closer to the nucleus (N) in the C group than the HF group, probably because the cytoplasm contains numerous fat droplets (fd) in the HF group, which interpose between the mitochondria.

Table I. We compared the liver mitochondria in the groups C and HF with model-based stereology. Data are mean ± standard deviation (unpaired t-test and Welch correction, Prism v. 9.01 for Windows, GraphPad Software, La Jolla, CA, USA) in a sample of five animals per group.

Data	C	HF	*P*
A [mito, liver] (μm^2)	0.30±0.04	0.34±0.05	ns
N_A [mito, liver] ($10^3/mm^2$)	459±0.2	315±0.3	<0.001
S_V [mito, liver] (mm^2/mm^3)	1223±122.3	976±120.6	0.02
V_V [mito, liver] (%)	27±1.2	21±3.1	0.01

Abbreviations: A, cross-sectional area; C, control group; HF, high-fat group; mito, mitochondria; ns, not significant; P, probability; N_A, numerical density per area; S_V, surface density; V_V, volume density.

b. Molecular biogenesis markers

The molecular markers of mitochondrial biogenesis decreased in the HF group compared to the C group (Fig. 2).

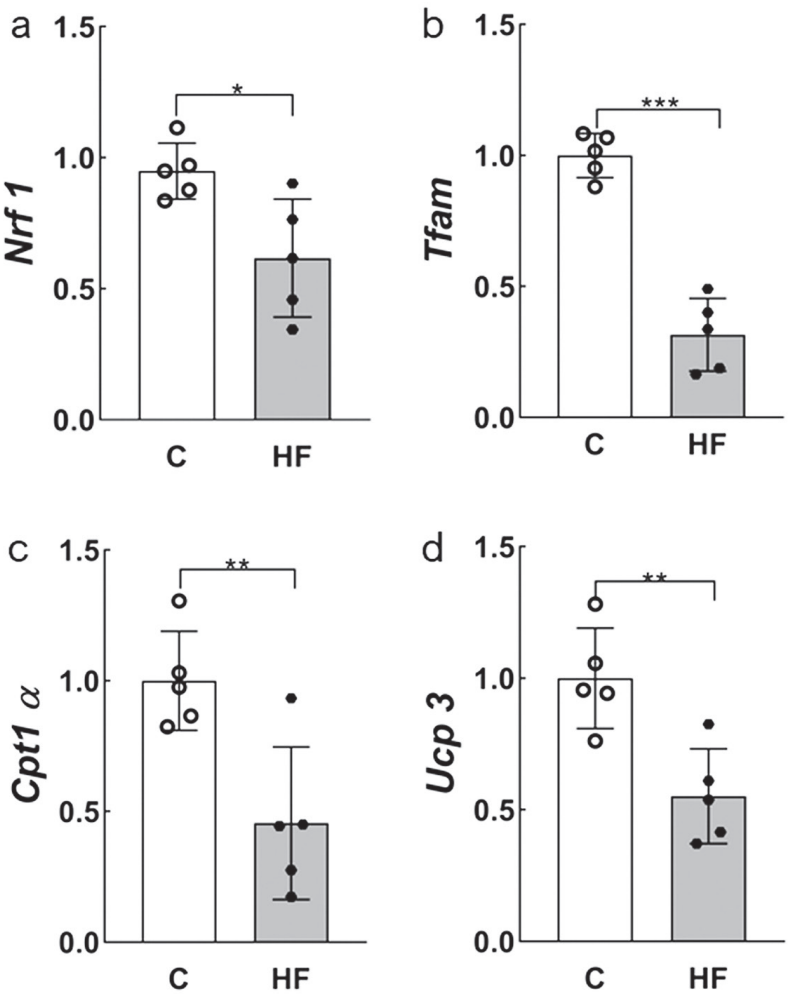

Fig. 2. Gene expressions (relative mRNA expressions) of the mitochondrial biogenesis markers: (a) nuclear respiratory factor 1 (*Nrf 1*); (b) mitochondrial transcription factor a (*Tfam*); (c) carnitine palmitoyltransferase 1a (*Cpt 1α*); (d) mitochondrial uncoupling protein 3 (*Ucp 3*). Data are expressed as the mean ± standard deviation (n=5/group). Unpaired t-test and Welch correction, *P < 0.05, **P < 0.01 and ***P < 0.001. Groups: control, C; High-fat, HF.

DISCUSSION

An intervention in the consumed diet can affect various metabolic pathways in different organs and tissues (Bargut *et al*., 2017; Cioffi *et al*., 2017). In the current study with diet-induced obesity, there were changes in mitochondrial biogenesis in hepatocytes. We demonstrated overwhelming changes in the hepatocyte due to diet-induced obesity in mice. These changes were observed at the ultrastructural and molecular levels. The quantitative mitochondrial data and the molecular markers of mitochondrial biogenesis were significantly reduced because of diet-induced obesity.

The HF diet was responsible, as expected, for an impressive weight gain in the animals saturating the white adipose tissue's packaging capacity. Then, the excess of fat is accumulated in non-adipose tissues, like the liver (Barbosa-da-Silva *et al*., 2013), with consequent effect on the mitochondrial oxygen consumption, mitochondrial volume, and inner membrane surface (Schwerzmann *et al*., 1986). All conditions triggered by the HF diet can significantly worsen hepatocytes, thus establishing a close relationship between mitochondrial biogenesis and respiration, and caused hepatocyte nuclear and mitochondrial cristae modifications with low capacity beta-oxidation (Lopez-Lluch, 2017).

The mitochondrial biogenesis related genes, including N*rf1, Tfam, Cpt-1α,* and *Ucp3*, were downregulated because of the diet-induced obesity. The transcriptions of *Nrf1* and *Tfam* are master regulators of mitochondrial biogenesis linked to obesity in mice (Veiga *et al*., 2017; Kozhukhar & Alexeyev, 2019). Thus, diet-induced obesity results in a distribution of *Tfam* gene expression in a minor abundant mitochondrial population, indicating that the HF diet decreased mitochondrial differentiation (Song *et al*., 2020).

The alteration of mitochondria by lipid intake is mediated by the activation of mitochondrial biogenesis generated by *Tfam, Pgc-1,* and *Nrf-1* (Davari *et al*., 2020). In general, Tfam is regulated by the *Pgc-1* coactivator, and *Nrf-1* binds together and forms a heterodimer for regulating mitochondrial function (Lu *et al*., 2020). The binding of *Nrf-1* and Pgc-1 coactivator improves the transcription of *Nrf-1* (Davari *et al*., 2020). Here, it is noteworthy to note that these pathways were lessened, corroborating mitochondrial bioenergetics' impairment linked with diabetes and fatty liver (Aguila *et al*., 2010).

In conclusion, the present study provides essential preliminary information about how the liver mitochondria of obese animals are depressed, which is relevant because of this organelle's bioenergetic role. The quantitative findings match perfectly with the molecular ones of mitochondrial biogenesis markers. Therefore, it will be crucial for future verification if and how these data recover with the reduction of obesity, which would be of significant interest given the current obesity epidemic that affects the world population.

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RESUMEN: La población mundial atraviesa una epidemia de obesidad que tiene graves consecuencias para el sistema de salud. Este estudio se centró en el análisis de las mitocondrias hepáticas en animales obesos inducidos por una dieta alta en grasas (HF) y utilizó la estereología basada en modelos en micrografías electrónicas para el estudio cuantitativo. Además, se analizaron las expresiones génicas de los marcadores moleculares de la biogénesis mitocondrial carnitina palmitoiltransferasa 1a (*Cpt 1α*), factor de transcripción mitocondrial a (*Tfam*), proteína desacoplante 3 (*Ucp 3*) y factor respiratorio nuclear 1 (*Nrf 1*). La dieta HF provocó un aumento de peso de +1820 % comparando el grupo de control (C) con el grupo HF (de 0,32 ± 0,31 g a 5,5 ± 0,39 g, *P* <0,001). El grupo HF mostró gotas de grasa en el citoplasma de los hepatocitos (esteatosis) y mitocondrias menos densas y grandes en la microscopía electrónica de transmisión. El tamaño de las mitocondrias (sección transversal) no mostró una diferencia significativa entre los grupos C y HF. Sin embargo, la densidad numérica de mitocondrias por área fue 30% menor, la densidad de superficie mitocondrial (membrana externa) fue 20 % menor y la densidad de volumen mitocondrial fue 22 % menor en el grupo HF que en el grupo C. Las expresiones génicas de los marcadores moleculares de la biogénesis mitocondrial *Cpt 1α, Tfam, Ucp 3* y *Nrf 1* disminuyeron en el grupo HF en comparación con el grupo C. Los resultados cuantitativos coinciden perfectamente con los moleculares de los marcadores de biogénesis mitocondrial. En el futuro, será crucial verificar si estos datos se recuperan y cómo se recuperan con la reducción de la obesidad, lo que sería de gran interés dada la actual epidemia de obesidad que afecta a la población mundial.

PALABRAS CLAVE: Mitocondrias; Biogénesis Mitocondrial; Obesidad inducida por la dieta; Biología celular; Biología molecular.

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