

# Effect of Leptin in Human Sertoli Cells Mitochondrial Physiology

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## Abstract

Leptin is an adipose tissue hormone that acts as energy sensor and reproductive function regulator. Recent reports suggest that leptin is involved in mitochondrial biogenesis in different tissue cells. Herein, we hypothesized that leptin could also affect Sertoli cells mitochondrial dynamics and biogenesis. Human Sertoli cells (hSCs) were cultured in the presence of different leptin concentrations (5, 25 and 50 ng/mL) or vehicle for 24 h. The three different leptin concentrations were selected to mimic the circulating levels found either in normal weight, obese, and morbidly obese individuals, respectively. Leptin receptor (LEPR) expression was evaluated as well as mitochondrial membrane potential, complexes levels, complex II activity and basal respiration. Moreover, mitochondrial DNA copy number and expression of mitochondrial biogenesis markers were assessed. In hSCs, leptin concentrations similar to those found both in lean men decreased mitochondrial complex II protein levels, but no changes in its activity were observed. This is in agreement with basal respiration and mitochondrial membrane potential assessments, which indicate no alterations in mitochondrial fitness. Furthermore, no changes in mitochondrial biogenesis markers were observed upon leptin exposure, although SIRT1/3 levels were increased after exposure to the highest leptin concentration. Overall, the increase in SIRT1/3 levels suggests a role for leptin in glycolysis, which given the relevance of SCs glycolytic flux for germ cells nutritional support further reinforces that this mechanism can be linked to obesity-related subfertility/infertility.

**Keywords** Leptin · Male fertility · Mitochondria · Sertoli cells · Spermatogenesis

## Introduction

Leptin is an adipose tissue hormone with a paramount role in regulating energy homeostasis by influencing several pathways in the central nervous system and peripheral tissues. Under physiological conditions, leptin is secreted by the adipocytes into the bloodstream in proportion to body fat mass. Thus, circulating leptin levels are usually proportional to each individual energy reserves [1]. Leptin actions are mediated through the leptin receptor (LEPR), which besides regulating whole body energy homeostasis also influences the reproductive potential, denoting that enough energy is available to meet the caloric demands of this biological process [2]. Leptin was first described as an adipocyte secreted hormone that acts on the basal hypothalamus to decrease food intake and increase energy expenditure, although later leptin receptors were also identified in tissues other than the central nervous system, being responsible for conveying additional peripheral actions, including a role in glucose metabolism regulation that is crucial for whole body energy homeostasis (for review [3]).

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The first clear evidence of leptin's involvement in the reproductive function was derived from the observation that leptin deficient obese *ob/ob* mice infertility could be surpassed with leptin replacement [4]. It was later further shown that leptin could also cross the blood-testis barrier (BTB) and be found in the seminal plasma [5]. Adjacent Sertoli cells (SCs) form the BTB thus being key players in spermatogenesis, by providing physical and nutritional support to germ cells. This metabolic cooperation is susceptible to the influence of circulating hormones, which can modulate the metabolic support for spermatogenesis [6]. Our group has identified the presence of LEPR in human SCs (hSCs) in addition to some molecular mechanisms elicited by leptin action in these cells' metabolism [7]. Furthermore, leptin has shown to modulate mitochondrial dynamics and biogenesis in other systems through regulation of genes involved in mitochondrial biogenesis [8, 9]. Interestingly, mitochondria are intimately related with male reproduction with several reported functions in testicular cells, namely, in the apoptosis and metabolism of those cells [10, 11]. These novel data supported the hypothesis that leptin could have a direct effect on SCs' mitochondrial function. Herein, we tested whether leptin exposure could affect mitochondrial dynamics and biogenesis in human SCs with potential consequences in their ability to support spermatogenesis, or if leptin just has a role on SCs' glycolysis efficiency control as proclaimed in a previous work from our group [7].

## Materials and Methods

### Chemicals

Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Leptin was obtained from Bachem (Bubendorf, Switzerland). Insulin–transferrin–sodium selenite (ITS) supplement and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye were purchased from Life Technologies (Gaithersburg, MD, USA). Mammalian Protein Extraction Reagent (M-PER) and BCA Protein Assay Kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dried milk was obtained from Regilait (Saint-Martin-Belle-Roche, France). WesternBright™ ECL substrate was purchased from Advansta (Menlo Park, CA, USA). NZY M-MuLV Reverse Transcriptase (M-MLV RT), random hexamer primers, dNTPs, NZYtaq Green Master Mix, Greensafe, NZY qPCR Green Master Mix, and NZYDNA Ladder VI were obtained from NZYTech (Lisbon, Portugal). Primers were obtained from STABVIDA (Oeiras, Portugal). Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Tetra Acetic acid (EDTA), Bovine Serum Albumin (BSA), trypsin–EDTA, and all other

chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

### Human Sertoli Cells Culture and Treatments

Clonetics™ human Sertoli cells (hSCs) (MM-HSE-2305) were purchased from Lonza (Walkersville, MD, USA). hSCs were thawed following manufacturer's protocol. In brief, the vial containing frozen cells was thawed at 33 °C, and cells were placed in 75-cm<sup>2</sup> flasks (VWR collection, Amadora, Portugal) at 37 °C, 5% CO<sub>2</sub> maintained in Sertoli culture medium (mixture of DMEM:Ham's F12 1:1, supplemented with 10% heat inactivated FBS, 25 mg/mL gentamicin, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 2.5 µg/mL fungizone, and 15 mM HEPES at pH 7.4).

Cells were allowed to grow until reaching 80–85% confluence and then washed thoroughly. The medium was replaced by serum-free media (DMEM:F12, 1:1, with ITS supplement: insulin 10 mg/L, transferrin 5.5 mg/L, and sodium selenite 6.7 µg/L, pH 7.4). To evaluate leptin effects on hSCs, culture media was supplemented with either one of three different leptin concentrations (5, 25, and 50 ng/mL) and compared with a leptin-free control group. The different leptin concentrations were selected to mimic plasma circulating levels found in lean healthy males [12], in patients with obesity [12] or morbid obesity [13], respectively. After a 24-h exposure to leptin supplemented or control medium in an atmosphere of 5% CO<sub>2</sub> at 37 °C, cells were detached with a trypsin–EDTA solution and collected according to standard cell culture procedure [14, 15]. Total cell number was determined using a Neubauer chamber, and viability was evaluated by the Trypan Blue Exclusion test. Then, cells were collected for protein and RNA/DNA extractions. The toxicity profile of leptins' concentrations used in the current cell culture studies was determined by the sulforhodamine B (SRB) assay as previously reported by our group [7]. No cytotoxicity was observed for the leptin doses used in this work (data not shown).

### Mitochondrial Membrane Potential ( $\Delta\Psi_m$ ) Assay

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) was determined by measuring the fluorescence of JC-1 dye (1,1',3,3'-Tetraethyl-5,5',6,6'-tetrachloro-imidacarbocyanine iodide). Briefly, cells were seeded in a 96-well black microplate (fluorescence quantitation—microplate reader) as wells as on sterile glass coverslips (in 12-well plates—fluorescence microscopy). Cells with 70% (for microplate reader) or 50% (microscopy) confluence were treated with different concentrations of leptin during 24 h. Cells treated with 10% DMSO before reading were considered as a depolarization positive control (microplate reader). At the end of treatment, cells were incubated with JC-1 for 30 min at 37 °C, 5% CO<sub>2</sub>. Afterwards, cells were washed with Hank's Balanced Salt Solution

(HBSS) supplemented with 17.5-mM glucose and 10-mM HEPES, as well as with calcium and magnesium salts. Fluorescence was determined at 485/528 nm and 530/590 nm using a Synergy™ HTX Multi-Mode Microplate Reader, BioTek (Winooski, VT, USA). Fluorescence ratio was measured considering JC-1 aggregates and monomers.

Additionally, SCs cultured on coverslips were mounted on slides to be observed under a Nikon fluorescence microscope (Tokyo, Japan). Processing was made using NIS-Elements Research Software. The accumulation of JC-1 dye in mitochondria depends on the  $\Delta\Psi_m$  of the cells. In healthy cells with high mitochondrial membrane potential, JC-1 forms aggregates that emit an intense red fluorescence, while in cells with dysfunctional mitochondria, JC-1 does not migrate to mitochondria, remaining in the monomeric form (green fluorescence). Alterations in the aggregates/monomers ratio indicates changes in  $\Delta\Psi_m$  which can represent mitochondrial depolarization or hyperpolarization. Images are presented as JC-1 aggregates and monomers fluorescence and later by performing a merging step to address mitochondrial shape (yellow color; red-green overlay).

### Basal Oxygen Consumption Rate Measurements in Intact Human Sertoli Cells

Cells were cultivated in 100 mm diameter cell culture plates reaching around 70–80% confluence. Prior to oxygen measurements, cells were trypsinized, resuspended in 2 mL respiration medium (specific for Sertoli Cells), and counted in a Neubauer chamber to address cell density. Respiration medium was based on cell culture medium, with a total of 17.5-mM glucose and 4-mM L-glutamine, without serum, and supplemented with 1% ITS (0.01 mg/ml recombinant human insulin, 0.0055 mg/ml human transferrin substantially iron-free-, and 0.005 µg/ml sodium selenite). To assess the oxygen consumption rates, we did closed-chamber oxygen measurement in a thermostated (by a water jacket, at 37 °C) 1 mL volume chamber, using a Clark-type electrode from Hansatech Oxytherm System, connected to a computer with Oxytrace Plus data acquisition software [16]. Each assay starts with a 30-min initial respiration, and oxygen consumption rate (OCR) was measured in the last 5 min. OCR is represented by the modulus of oxygen concentration (pM)/min and normalized to cell content ( $10^6$  cells).

### Isolation of Human Sertoli Cells Mitochondria

hSCs were cultivated (100 mm diameter plates) and treated during 24 h with leptin as described previously. Mitochondria-rich fractions were obtained from human hSCs by differential centrifugations after cell homogenization with Glass-Teflon Potter Elvehjem, using the traditional procedures with minor modifications [16]. After isolation

mitochondrial protein concentration was determined (optical density measured at 595 nm) using the Pierce™ BCA Assay kit according to manufacturer's instructions.

### Mitochondrial Complex II Activity

Isolated mitochondria were submitted to three cycles of freezing/thawing to give substrate access to the mitochondrial matrix. Assay was conducted at 37 °C using up to 10 µg of mitochondrial protein. Complex II (Succinate-Coenzyme Q reductase) activity was evaluated spectrophotometrically following the reduction of 2,6-dichloroindophenol (DCPIP – color change, from blue to colorless), which is used as an exogenous final acceptor of the electrons resulting from the succinate oxidation promoted by enzyme. Thus, the time-dependent decrease of absorbance intensity at 600 nm (extinction coefficient  $20.7 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ ) promoted by mitochondria-rich fraction was recorded in separate wells of one 96-well microplate, using a microplate spectrofluorometer (Synergy H1, BioTek, Winooski, Vermont, USA), similarly to the protocol described in literature with minor modifications [17]. Enzyme activity was expressed as nM DCPIP reduced/min/mg of protein.

### Total Protein Extraction of Human Sertoli Cells

hSCs proteins were extracted using the M-PER buffer, supplemented with 1% protease inhibitor cocktail and 100-mM sodium orthovanadate, following the manufacturer's instructions. Total protein concentration was determined using the Pierce™ BCA Protein Assay Kit according to manufacturer's instructions. Optical densities of samples were determined at 595 nm.

### Western Blot of Human Sertoli Cells

Western blot was performed as previously described [18] to analyze individual protein levels of oxidative phosphorylation (OXPHOS) complexes. For that, protein samples (100 µg) were mixed with sample buffer (60 mM Tris.HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and denatured for 15 min at 37 °C. Proteins were fractionated in 15% polyacrylamide gels, and electrophoresis was carried out for 90 min. Afterwards, proteins were transferred from gels to previously activated polyvinylidene difluoride membranes (Merck Milipore, Darmstadt, Germany) in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) and then blocked for 3 h in a 5% non-fat milk solution at room temperature. The membranes were incubated overnight at 4 °C with mouse anti-OXPHOS (1:1000, ab110413,

Abcam, UK). The antibody cocktail used represents a mixture of primary antibodies against 5 proteins: NDUFB8 (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8), representing the complex I; SDH8 (Succinate dehydrogenase assembly factor 4), complex II; UQCRC2 (Cytochrome b-c1 complex subunit 2), complex III; MTCO-1 (mitochondrially encoded cytochrome c oxidase I), complex IV; and ATP5A (ATP synthase F1 subunit alpha), complex V. As a protein-loading control, mouse anti- $\beta$ -actin was used (1:5000, MA5–15739, Thermo Fisher, USA). The immune-reactive proteins were detected with goat anti-mouse (1:5000, A3562, Sigma-Aldrich, Germany). Membranes were reacted with WesternBright™ ECL and visualized with the Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained using the Image Lab Software (Bio-Rad, Hemel Hempstead, UK) considering standard methods.

### RNA and DNA Extraction of Human Sertoli Cells

Extraction of total RNA (tRNA) from cells was performed using the GRS Total RNA Kit (GRiSP, Porto, Portugal), as indicated by the manufacturer. DNA from hSCs was extracted using the E.Z.N.A. Tissue DNA Kit (Omega bio-tek, Norcross, GA, USA), following manufacturer's instructions. RNA and DNA concentration and absorbance ratios used to measure sample purity were determined using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

### Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

tRNA obtained for each sample was reversely transcribed in a mixture containing 2.5  $\mu$ L of random hexamer primers (50 ng/ $\mu$ L), 1  $\mu$ L of dNTPs (10 mM), 1  $\mu$ g of tRNA, and sterile H<sub>2</sub>O up to a volume of 17  $\mu$ L. The mixture was then incubated during 5 min at 65 °C. Then, 1  $\mu$ L of M-MLV RT and 2  $\mu$ L of reaction buffer were added and incubated sequentially at 25 °C for 10 min, 37 °C for 50 min and 70 °C for 15 min. The resulting complementary DNA (cDNA) was used with exon-exon spanning primer sets designed to amplify the housekeeping gene 18S ribosomal RNA (*18S*) to evaluate sample integrity (Table 1). Each polymerase chain reaction (PCR) contained 1  $\mu$ L of cDNA in 12.5  $\mu$ L of final volume of a mixture containing 6.5  $\mu$ L of NZYTaq Green Master Mix, 0.1  $\mu$ L (50  $\mu$ M) of each primer and sterile H<sub>2</sub>O. Adipose tissue was used as positive control and cDNA-free sample was used as a negative control. At the end of the experiments, samples were run in 1% agarose gel electrophoresis with 2  $\mu$ L of Greensafe in 200 mL, during 30 min at 120 V, to confirm sample integrity. The agarose gel was visualized in Bio-Rad GelDoc XR (Bio-Rad, Hemel Hempstead, UK) using the

software Quantity One (Bio-Rad, Hemel Hempstead, UK). The size of the expected products was compared with a DNA ladder.

### Quantitative PCR (qPCR) of Human Sertoli Cells

mRNA expression levels of PPARG coactivator 1 alpha (*PPARGC1A*), nuclear respiratory factor 1 (*NR1F1*), Sirtuin 1 (*SIRT1*), Sirtuin 3 (*SIRT3*), and leptin receptor (*LEPR*) were evaluated by qPCR. Specific primers were designed for target amplification and housekeeping transcripts (Table 1). qPCR was carried out in a CFX Connect™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA). Amplification efficiency was determined for all primer sets using serial dilutions of cDNA (1:3, 1:15, and 1:75). qPCR conditions and reagent concentrations were previously optimized, and amplicons' specificity was determined by melting curves. qPCR amplifications used 1  $\mu$ L of diluted 1:15 cDNA in a 20- $\mu$ L reaction volume containing 10- $\mu$ L NZY qPCR Green Master Mix, 0.8  $\mu$ L (10 mM) of forward and reverse primers for each gene, and sterile H<sub>2</sub>O. Amplification conditions comprised an initial denaturation step of 5 min at 95 °C, followed by a specific number of runs for each set of primers of a 3 steps cycle: (1) a denaturation step of 30 s at 95 °C, (2) an annealing step of 30 s with a specific temperature for each set of primers, and (3) an extension step of 1 min at 72 °C. Target genes, sequences, and annealing temperatures are described in Table 1. Beta-2-microglobulin (*B2M*) transcript levels were used as a reference gene to normalize the mRNA expression levels of target genes. Fold variation of the expression levels was calculated following the mathematical model proposed by Pfaffl using the formula:  $2^{-\Delta\Delta C_t}$  [19].

### Mitochondrial DNA Copy Number of Human Sertoli Cells

Mitochondrial DNA (mtDNA) copy number in hSCs was assessed through qPCR analysis, as described before [20]. Briefly, the reaction mixture consisted in 10  $\mu$ L of NZY qPCR Green Master Mix, 0.8  $\mu$ L (10 mM) of forward and reverse primers, 20 ng of mtDNA and sterile H<sub>2</sub>O. qPCR was carried out in a CFX Connect™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA). Nuclear encoded beta-2-microglobulin (*B2M<sub>nc</sub>*) gene transcript levels were used as a reference gene to normalize the mtDNA expression levels of mitochondrially encoded NADH dehydrogenase 1 (*ND1*), following the mathematical model proposed by Pfaffl (Table 1) [19].

### Statistical Analysis

All experimental data are shown as mean  $\pm$  SEM ( $n = 3-6$  for each condition, done in triplicate). Statistical analysis was executed using a one-way ANOVA and Students' *t* test in

**Table 1** Genes, oligonucleotide sequence, and respective conditions for PCR amplification of human Sertoli cells

Gene	Sequence (5'-3')	AT (°C)	Amplicon size (bp)	Species of origin
<i>PPARGC1A</i>	Forward: TGGTTGCCTGCATGAGTGT Reverse: CACCACTTGAGTCCACCCAG	56	253	human
AN: NM_013261.3				
<i>NRF1</i>	Forward: CAGCCGCTCTGAGAACTTCA Reverse: GGCCGTTCCGTTTCTTTCC	58	300	human
AN: NM_005011.4				
<i>SIRT1</i>	Forward: ACAGGTTGCGGAATCCAAA Reverse: GTTCATCAGCTGGGCACCTA	60	155	human
AN: AF083106.2				
<i>SIRT3</i>	Forward: CCCAAGCCCTTTTCACTTT Reverse: CGACACTCTCTCAAGCCCA	56	148	human
AN: NM_001370314.1				
<i>LEPR</i>	Forward: TCAGTGGGGCTATTGGACTG Reverse: GGTGGCATGCAAGACAACCTTA	62	197	human
AN: NM_002303.5				
<i>B2M</i>	Forward: ATGAGTATGCCTGCCGTGTG Reverse: CAAACCTCCATGATGCTGCTTAC	60	93	human
AN: NM_004048.2				
<i>MT-ND1</i>	Forward: CGATCCGCTACGACCAACT Reverse: AGGTTTGAGGGGAATGCTG	58	121	human
AN: NC_012920.1				
<i>B2M<sub>nc</sub></i>	Forward: GAGGCTATCCAGCGTGAGTC Reverse: GACGCTTATCGACGCCCTAA	58	306	human
AN: NG_012920.1				
<i>18S</i>	Forward: AAGACGAACCAGAGCGAAAAG Reverse: GGCGGTTCATGGGAATAA	56	149	human
AN: NR_046237.1				

**Abbreviations:** *PPARGC1A* (PPARG coactivator 1 alpha); *NRF1* (Nuclear respiratory factor 1); *SIRT1* (Sirtuin 1); *SIRT3* (Sirtuin 3); *LEPR* (Leptin receptor); *B2M* (Beta-2-Microglobulin); *MT-ND1* (Mitochondrially encoded NADH dehydrogenase 1); *B2M<sub>nc</sub>* (Nuclear encoded beta-2-microglobulin); *18S* (18S ribosomal RNA); AT (Annealing temperature); BP (Base pairs); AN (Genbank Accession Number)

GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).  $P < 0.05$  was considered significantly different.

## Results

### Exposure of Human Sertoli Cells to Leptin Does Not Alter *LEPR* mRNA Levels

*LEPR* has been previously identified in human testis by several techniques [21–23]. Of note, our group has recently reported the presence of this receptor in hSCs [7]. To further characterize the role of leptin in hSCs, we analyzed mRNA levels of *LEPR* by qPCR after exposure to leptin. However, leptin exposure had no significant effect in mRNA levels of *LEPR* at any of the studied concentrations as compared with the leptin-free group (Fig. 1).

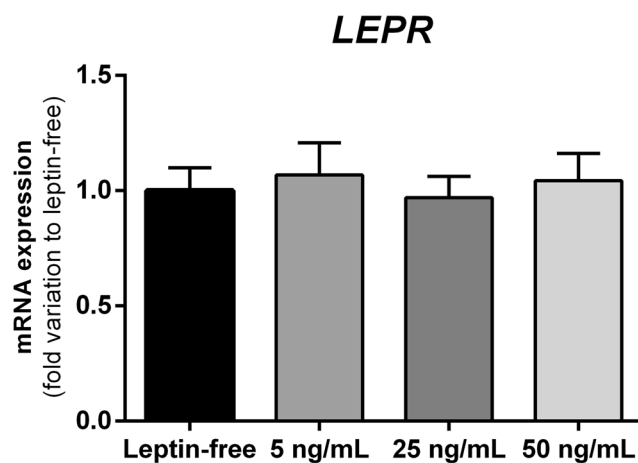
### Mitochondrial Membrane Potential ( $\Delta\Psi_m$ ) Is Not Altered after Exposure to Leptin in Human Sertoli Cells

Lately, several reports have shown leptin's involvement in mitochondrial dynamics. In order to disclose whether leptin modulates mitochondrial dynamics in hSCs, we analyzed the mitochondrial membrane potential in hSCs after exposure to leptin. Our results show no alterations in  $\Delta\Psi_m$  of hSCs treated with leptin when compared with the leptin-free group (Fig. 2a). These findings were further confirmed by the red/green fluorescence observed by JC-1 staining in leptin treated cells, which showed no significant difference in  $\Delta\Psi_m$  when

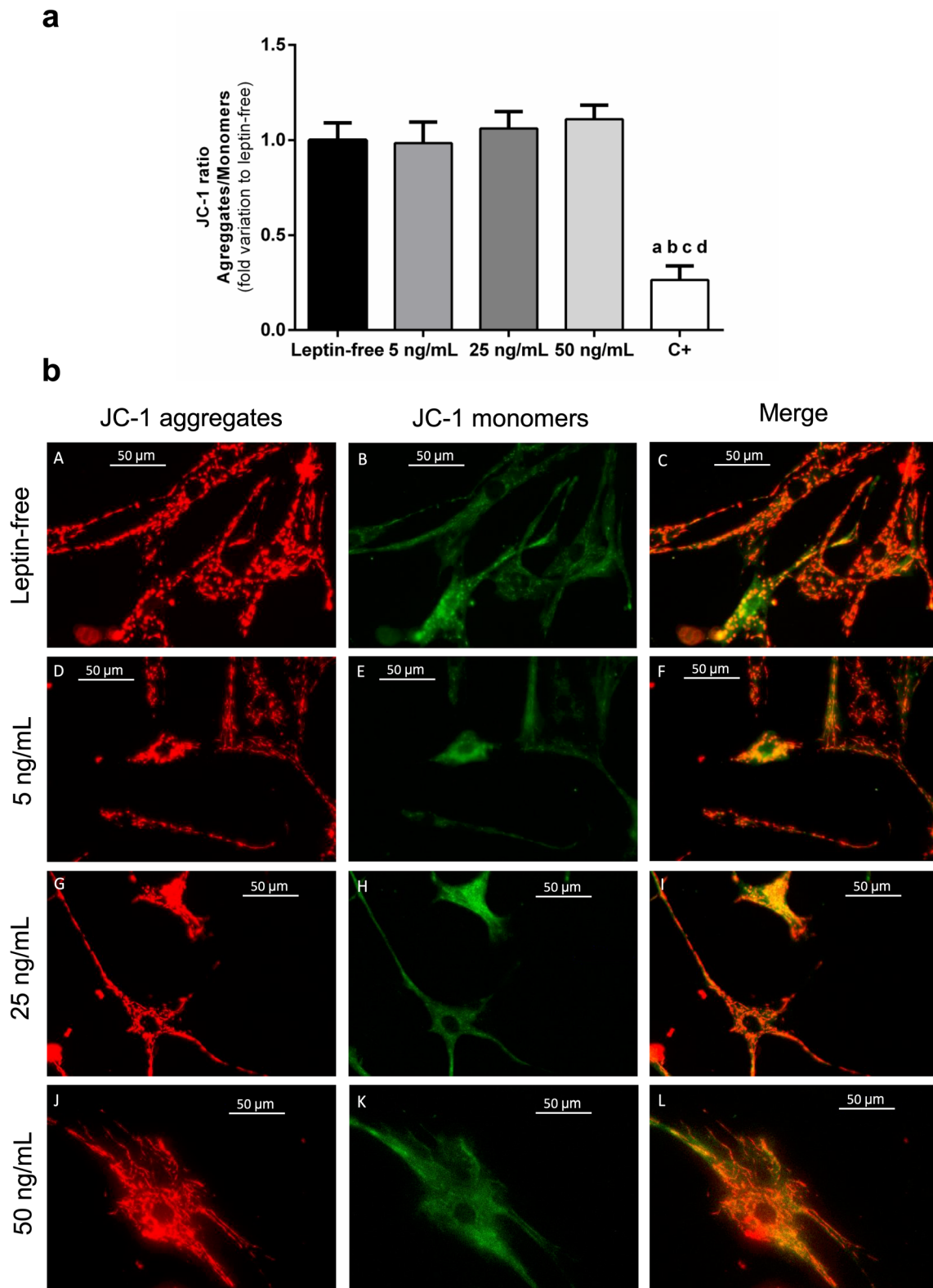
compared with the leptin-free group (Fig. 2b). Furthermore, no differences were observed in mitochondrial morphology/area between leptin-treated cells and non-treated cells (Fig. 2b).

### Exposure of Human Sertoli Cells to Leptin Decreases Succinate Dehydrogenase Subunit of Complex II with Slightly Impact in its Activity

To address mitochondrial dynamics, we analyzed the protein levels of mitochondrial complexes in hSCs after



**Fig. 1** Effect of exposure to leptin in levels of *LEPR* in human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating mRNA levels of *LEPR* in hSCs cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 6$  for each condition)



exposure to leptin. Our results show that complex II protein levels were decreased after cell exposure to 5 ng/mL ( $0.63 \pm 0.06$  – fold variation to leptin-free) relative to non-exposed cells (Fig. 3a). However, despite

lower protein levels in the cells exposed to 5 ng/mL of leptin, complex II activity was not significantly altered in the leptin treated groups relative to non-exposed cells (Fig. 3b).

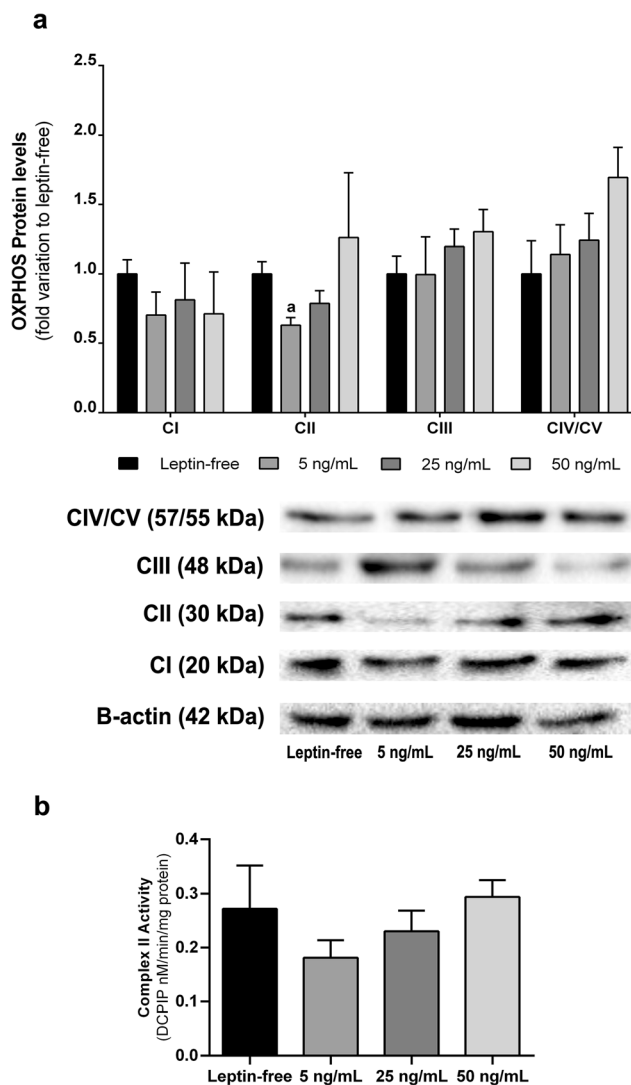
◀ **Fig. 2** Effect of exposure to leptin (5, 25, and 50 ng/mL) on mitochondrial membrane potential of human Sertoli cells (hSCs), using the JC-1 fluorescent probe. Panel a represents the fluorescence ratio of JC-1 measured at two wavelengths (Ex = 485 nm, Em = 530 nm; Ex = 535 nm, Em = 590 nm), calculated between red aggregates (representing hyperpolarized mitochondria) and green monomers (depolarized mitochondria). C+ represents a depolarizing control (DMSO 10%); Panel b comprises representative pictures of JC-1 fluorescence, captured in a Nikon Elipse400 microscope, at 400x magnification (red aggregates measured with a G-2A filter, EX510–560; DM575; and BA590—A, D, G, and J; green monomers measured with a FITC filter, EX480/40; DM510; and BA590—B, E, H, and K; and merged pictures—C, F, I and L. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 3$  for each condition)

### Leptin Does Not Alter Basal Respiration in Human Sertoli Cells

To access mitochondrial bioenergetic modulation by leptin, we evaluated its effects on cell respiration through intact cell respirometry. Despite the tendency of basal respiration depletion, leptin did not significantly disrupt the oxidative phosphorylation (Fig. 4), which is in agreement with our JC-1 fluorescence results described above.

### SIRT1 and SIRT3 Are Upregulated in Human Sertoli Cells Exposed to the Highest Concentration of Leptin that Mimics the One Present in Morbidly Obese Men

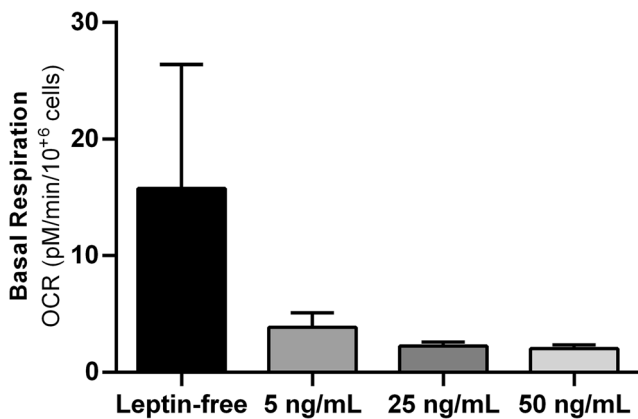
In parallel with JC-1 fluorescence imaging, we also assessed whether leptin could alter the number of mitochondrial copies and the transcript levels of *PPARGC1A*, *NRF1*, *SIRT1*, and *SIRT3* by qPCR, to address if leptin is involved in mitochondrial biogenesis in human Sertoli cells. The mRNA levels of *PPARGC1A* and *NRF1*, the master regulator of mitochondrial biogenesis, and a downstream effector of PGC-1 $\alpha$ , respectively, were not altered in leptin treated groups when compared with non-exposed cells (Fig. 5a and b). Concerning *SIRT1*, which also has been extensively implicated in mitochondrial biogenesis, mRNA levels were significantly increased in hSCs on the group treated with 50 ng/mL of leptin ( $1.50 \pm 0.09$ -fold variation to leptin-free) when compared with the leptin-free group and with hSCs exposed to 5 ng/mL and 25 ng/mL of leptin ( $0.92 \pm 0.18$  and  $0.88 \pm 0.10$ -fold variation to leptin-free, respectively) (Fig. 5c). *SIRT3*, the main mitochondrial deacetylase, which is also involved in mitochondrial biogenesis, was upregulated after exposure to 50 ng/mL of leptin. mRNA levels were significantly increased ( $2.10 \pm 0.15$ -fold variation to leptin-free) when compared with hSCs exposed to 5 ng/mL and 25 ng/mL of leptin ( $1.10 \pm 0.27$  and  $0.95 \pm 0.20$ -fold variation to leptin-free, respectively) (Fig. 5d). Furthermore, leptin exposure did not alter the mitochondrial copy number in hSCs (Fig. 5e).



**Fig. 3** Effect of exposure to leptin (5, 25, and 50 ng/mL) in human Sertoli cells (hSCs) mitochondrial complexes and complex II activity. Panel a shows pooled data of independent experiments, indicating mitochondrial complexes protein levels of hSCs. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 6$  for each condition). Panel b shows pooled data of independent experiments, indicating mitochondrial complex II activity of hSCs. Activity is expressed as nM of DCIPIP reduced/min/mg of mitochondrial protein. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 3$  for each condition). Significantly different results ( $P < 0.05$ ) are indicated as a relative to leptin-free group

### Discussion

Leptin is a crucial regulatory signal of whole-body homeostasis, acting as an energy sensor and a metabolic regulator of several processes, including reproductive function [24, 25]. Thus, leptin deficiency has been described as an impairment to reproductive function [26], while excessive leptin levels as frequently observed in overweight and obese individuals also seem to influence negatively male and female reproductive



**Fig. 4** Effect of exposure to leptin (5, 25, and 50 ng/mL) on intact and non-permeabilized human Sertoli cells (hSCs) basal oxygen consumption rates, measured using a Clark-type electrode. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 3$  for each condition)

function [27]. At a cellular level, leptin was recently shown to regulate mitochondrial function, particularly by modulating mitochondrial dynamics, biogenesis, and functioning [8]. In addition to the action on the central nervous system, leptin also acts in peripheral tissues, including the testes. In fact, leptin was reported to have several effects in male reproductive system [28]. Our group has recently identified the leptin receptor in hSCs, which are the somatic cells responsible for the physical and nutritional support of germ cells. In addition, we have shown that leptin alters the glycolytic and oxidative profiles in these same cells [7]. In this work our aim was to gain further insight whether leptin could influence reproductive function by modulating mitochondrial function and dynamics in human SCs or if leptin just has a role on SCs' glycolysis efficiency control as proclaimed in a previous work from our group, therefore expanding the knowledge on the molecular mechanisms by which this hormone could potentially interfere in spermatogenesis.

Although we recently identified that hSCs express *LEPR* [7], whether *LEPR* is responsive to different leptin stimuli or the molecular mechanisms by which leptin could act on hSCs are still undisclosed. Here, we show that *LEPR* expression in hSCs was not influenced by leptin exposure in any of the concentrations tested, suggesting that these cells do not respond to leptin stimuli in the tested timeframe and concentrations. Interestingly, an increase in *LEPR* expression was reported in several other systems [29, 30], including malignant mammary tissues, being generally considered a poor prognostic marker. However, our results elicit that leptin action when overproduced is not mediated by a response in *LEPR* expression in hSCs.

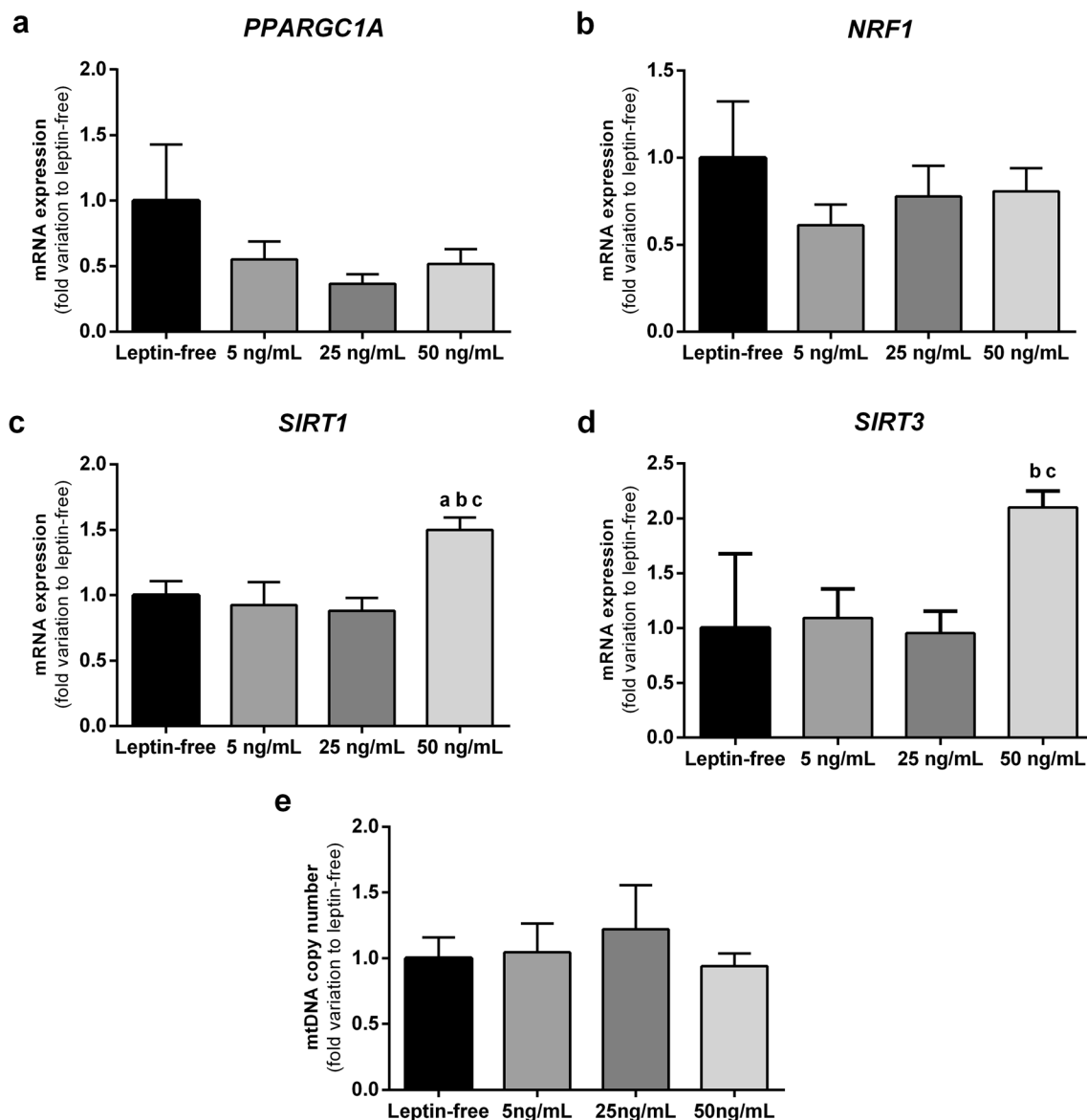
Some studies highlighted a possible role for leptin in modulating mitochondrial function and dynamics [9, 31]. To address mitochondrial morphology and mitochondrial membrane potential, we used JC-1 probe, considering aggregates/

monomers ratio as an indicator of these parameters. However, our results did not show differences in  $\Delta\Psi_m$  between leptin-treated groups and non-exposed cells, revealing that mitochondrial electron transport chain as a whole was not affected by increasing concentrations of leptin. Additionally, through JC-1 imaging, we observed a diffuse mitochondrial network distribution which was consistent between all groups indicating no alterations upon leptin treatment.

To date, although leptin has been recognized as a pivotal modulator of metabolism in several cells and tissues, the evidence linking leptin to SCs' metabolism is scarce. Mitochondrial electron transport chain components are relevant targets to explain any kind of modulation of OXPHOS and the way it integrates redox mediators (NADH/NAD<sup>+</sup>, FADH<sub>2</sub>/FAD, etc.) to the final phosphorylation of ADP to ATP. Despite no alterations in *LEPR* expression, exposure to leptin concentrations similar to those found in lean decreased mitochondrial complex II protein levels in hSCs. This result prompted us to evaluate complex II activity, which was not altered in the hSC of the various experimental groups, including the 5 ng/mL of leptin group. Interestingly, we can observe the presence of a slight "Hormesis effect". Nonetheless, in higher concentrations of leptin, the effect seems to be diluted, which can be explained due to activation of rescue mechanisms to re-establish the proper mitochondrial function (in this case, regularization of basal levels of CII expression). In parallel with mitochondrial potential assessment, mitochondrial basal respiration awareness can disclose the full picture of mitochondrial physiology. Our data, regarding basal respiration, reveals no significant alterations between leptin treated groups and non-exposed cells. Overall, considering bioenergetics parameters (addressing mitochondrial physiology) and mitochondrial distribution in cells (mitochondrial morphology), leptin did not alter oxidative phosphorylation as a whole. Furthermore, despite lower complex II expression in the group treated with 5 ng/mL, its enzymatic activity was not significantly changed. Considering mitochondrial area/shape and mitochondrial network in hSCs after treatment with leptin, we can observe that leptin treatment did not change mitochondrial area or shape between the different concentrations of leptin. Moreover, mitochondrial complex II has a small contribution to the overall electron transport chain process, corroborating that leptin has low impact on oxidative phosphorylation.

Previous studies also have shown that leptin stimulates fatty acid oxidation, reactive oxygen species production and glucose uptake in endothelial and muscle cells [32, 33]. In addition, leptin was shown to stimulate mitochondrial biogenesis in adipocytes and cardiomyocytes from ob/ob mice [31, 34]. To determine whether leptin could modulate mitochondrial biogenesis in hSCs, we measured the expression of key markers of mitochondrial biogenesis and function. The results showed that mitochondrial DNA copy number in hSCs





**Fig. 5** Effect of exposure to leptin in transcript levels of PPARG coactivator 1 alpha (PPARGC1A), nuclear respiratory factor 1 (NRF1), Sirtuin 1 (SIRT1), SIRT3, and in mitochondrial copy number in human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating mRNA levels of PPARGC1A, NRF1, SIRT1, SIRT3, and mitochondrial copy number in hSCs (panel a, b, c, d, and e,

respectively) cultured in the absence or presence (5, 25, and 50 ng/mL) of leptin. One-way ANOVA was used for statistical analysis. Results are expressed as mean  $\pm$  SEM ( $n = 6$ ) for PPARGC1A, NRF1, SIRT1, and mitochondrial copy number and  $n = 3$  for SIRT3. Significantly different results ( $P < 0.05$ ) are indicated as (a) relative to leptin-free group, (b) relative to 5 ng/mL group, and (c) relative to 25 ng/mL group

remained unchanged after exposure to leptin, while similar results were found for mitochondrial biogenesis markers, such as *PPARGC1A* levels, which is the master regulator of mitochondrial biogenesis [35]. Furthermore, the expression of the downstream *PPARGC1A* target, *NRF1*, a transcription factor involved in the expression of genes codified by the mitochondrial genome [36], was not altered in hSCs exposed to any of the chosen concentrations of leptin. Interestingly, *SIRT1* levels, which are involved in *PPARGC1A* deacetylation [37], were increased in the group treated with 50 ng/mL of leptin as compared with the other groups. *SIRT1*, besides

being involved in mitochondrial biogenesis, is heavily tied to cellular metabolism control by functioning as a metabolic sensor involved in lipid and glucose metabolism [38, 39]. A previous study has described a correlation between *SIRT1* abundance in seminal plasma and male fertility [40]. Although the presence of specific proteins in seminal fluid may come from different pools of cells from the reproductive tract, the results on this paper shed some light on the role of this deacetylase on spermatogenesis. Indeed, a work by Mu et al. [41] found that the activation of *SIRT1* was associated with the attenuation of endoplasmic reticulum (ER) stress in

the testes of obese mice. These results reflect in part the contribution of Sertoli cells to SIRT1 involvement in male fertility and on the event of spermatogenesis. Moreover, SIRT3, the primary mitochondrial deacetylase, has been described in several systems as a regulator of the activity of enzymes that coordinate global shifts in cellular metabolism. In its panoply of functions, SIRT3 promotes the tricarboxylic acid (TCA) cycle and electron transport chain function and reduces oxidative stress [42]. In the group treated with 50 ng/mL of leptin, SIRT1 and SIRT3 transcripts levels were significantly increased when compared with the other groups, but no direct correlation with the effects on complex II expression and activity (where leptin 5 ng/mL present a relevant alteration—decrease in expression and in accordance with activity slow-down) was observed. 50 ng/mL of leptin may evoke SIRT1- and SIRT3-mediated processes not directly connected to Krebs cycle and OXPHOS modulation, at least considering a 24-h treatment.

SCs uptake glucose to produce essential metabolites for germ cells' development. Our group has previously shown that leptin modulates glucose transporter protein levels, in particularly decreasing GLUT2, which may be linked with obesity-related male subfertility/infertility [7]. The results obtained in this study show that exposure to the leptin concentration reported in morbidly obese men increases *SIRT1* and *SIRT3* levels in hSCs. SIRT1 modulates the activity of PPARGC1A in glycolytic genes through deacetylation, which induces a strong glycolytic pathway dysregulation, compromising even further the nutritional support of spermatogenesis. Furthermore, SIRT1 modulation of PPARGC1A does not directly regulate the effects of PPARGC1A in mitochondrial genes, which could explain why no differences were observed in mitochondrial biogenesis markers [43]. Thus, our results support that the SIRT1 changes are not directly associated with mitochondrial function but rather with glycolysis control as our previous work demonstrated [7]. Moreover, the mitochondrial deacetylase SIRT3, was also upregulated at 50 ng/mL. Liu et al., reported that in renal cell carcinoma, *SIRT3* overexpression was associated with enhanced mitochondrial biogenesis [44]. However, in our work, no alterations in the selected mitochondrial biogenesis markers were observed. Interestingly, upregulation of *SIRT1* transcript levels is also associated with enhanced mitochondrial biogenesis [37], but our results, once again, did not revealed this. Also, mitochondrial physiology endpoints (mitochondrial potential and basal respiration) were not altered as we reported above, confirming no influence in mitochondrial biogenesis by SIRT1/3 leptin-mediated upregulation, in our cell model.

Mitochondrial assessment in hSCs is essential to evaluate the full picture of bioenergetics regulation. In regular conditions, we postulate that SCs present a sustained mitochondrial activity, coupled with anaplerotic reactions to refill Krebs

cycle intermediates and to coordinate with the essential biosynthetic processes. It seems that upon several hormonal/signaling challenges, particularly by leptin, hSCs mitochondria have a huge “buffer” capacity, and gathering the findings published by our group, other metabolic pathways, like glycolysis, swing around a pivotal point to achieve the right bioenergetics conditions, assuming a quantitatively more relevant metabolic role of glycolysis than OXPHOS per se.

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**Data Availability Statement** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with Ethical Standards

The work presented in this paper resulted from the use of Clonetics™ human Sertoli cells (hSCs) (MM-HSE-2305) purchased from Lonza (Walkersville, MD, USA). Thus, ethics approval is not required according to current law and the national ethical guidelines of our country or European Directives. Nevertheless, our laboratory is authorized to work with human material, particularly human Sertoli cells and sperm samples, according to the authorization from the Joint Ethics Committee CHUP/ICBAS with the approval number 2019/CE/P017(266/CETI/ICBAS).

**Conflict of Interest** The authors declare no conflict of interest.

## References

1. Cui H, López M, Rahmouni K. The cellular and molecular bases of leptin and ghrelin resistance in obesity. *Nat Rev Endocrinol*. 2017;13(6):338–51. <https://doi.org/10.1038/nrendo.2016.222>.
2. Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, et al. Leptin is a metabolic signal to the reproductive system. *Endocrinology*. 1996;137(7):3144–7. <https://doi.org/10.1210/endo.137.7.8770941>.
3. Moreira BP, Monteiro MP, Sousa M, Oliveira PF, Alves MG. Insights into leptin signaling and male reproductive health: the missing link between overweight and subfertility? *Biochem J*. 2018;475(22):3535–60. <https://doi.org/10.1042/bcj20180631>.
4. Cleary MP, Bergstrom HM, Dodge TL, Getzin SC, Jacobson MK, Phillips FC. Restoration of fertility in young obese (Lep(Ob) Lep(Ob)) male mice with low dose recombinant mouse leptin treatment. *Int J Obes Relat Metab Disord*. 2001;25(1):95–7.
5. Banks WA, McLay RN, Kastin AJ, Sarmiento U, Scully S. Passage of leptin across the blood-testis barrier. *Am J Phys*. 1999;276(6 Pt 1):E1099–104.
6. Oliveira PF, Martins AD, Moreira AC, Cheng CY, Alves MG. The Warburg effect revisited—lesson from the Sertoli cell. *Med Res Rev*. 2015;35(1):126–51. <https://doi.org/10.1002/med.21325>.
7. Martins AD, Moreira AC, Sa R, Monteiro MP, Sousa M, Carvalho RA, et al. Leptin modulates human Sertoli cells acetate production

- and glycolytic profile: a novel mechanism of obesity-induced male infertility? *Biochim Biophys Acta*. 2015;1852(9):1824–32. <https://doi.org/10.1016/j.bbadis.2015.06.005>.
8. Blanquer-Rosselló MM, Santandreu FM, Oliver J, Roca P, Valle A. Leptin modulates mitochondrial function, dynamics and biogenesis in MCF-7 cells. *J Cell Biochem*. 2015;116(9):2039–48. <https://doi.org/10.1002/jcb.25158>.
  9. Singh A, Wirtz M, Parker N, Hogan M, Strahler J, Michailidis G, et al. Leptin-mediated changes in hepatic mitochondrial metabolism, structure, and protein levels. *Proc Natl Acad Sci U S A*. 2009;106(31):13100–5. <https://doi.org/10.1073/pnas.0903723106>.
  10. Rajender S, Rahul P, Mahdi AA. Mitochondria, spermatogenesis and male infertility. *Mitochondrion*. 2010;10(5):419–28. <https://doi.org/10.1016/j.mito.2010.05.015>.
  11. Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. *Int J Androl*. 2012;35(2):109–24. <https://doi.org/10.1111/j.1365-2605.2011.01218.x>.
  12. Krempler F, Hell E, Winkler C, Breban D, Patsch W. Plasma leptin levels: interaction of obesity with a common variant of insulin receptor substrate-1. *Arterioscler Thromb Vasc Biol*. 1998;18(11):1686–90. <https://doi.org/10.1161/01.atv.18.11.1686>.
  13. Fathy SM, Morshed G. Peripheral blood lymphocyte subsets (CD4+, CD8+ T cells), leptin level and weight loss after laparoscopic greater curvature plication in morbidly obese patients. *Arch Med Sci*. 2014;10(5):886–90. <https://doi.org/10.5114/aoms.2014.46209>.
  14. Oliveira PF, Sousa M, Barros A, Moura T, Rebelo da Costa A. Intracellular pH regulation in human Sertoli cells: role of membrane transporters. *Reproduction*. 2009;137(2):353–9. <https://doi.org/10.1530/rep-08-0363>.
  15. Oliveira PF, Alves MG, Rato L, Silva J, Sa R, Barros A, et al. Influence of 5alpha-dihydrotestosterone and 17beta-estradiol on human Sertoli cells metabolism. *Int J Androl*. 2011;34(6 Pt 2):e612–20. <https://doi.org/10.1111/j.1365-2605.2011.01205.x>.
  16. Silva AM, Oliveira PJ. Evaluation of respiration with clark-type electrode in isolated mitochondria and permeabilized animal cells. *Methods Mol Biol*. 2018;1782:7–29. [https://doi.org/10.1007/978-1-4939-7831-1\\_2](https://doi.org/10.1007/978-1-4939-7831-1_2).
  17. Monteiro-Cardoso VF, Silva AM, Oliveira MM, Peixoto F, Videira RA. Membrane lipid profile alterations are associated with the metabolic adaptation of the Caco-2 cells to aglycemic nutritional condition. *J Bioenerg Biomembr*. 2014;46(1):45–57. <https://doi.org/10.1007/s10863-013-9531-y>.
  18. Martins AD, Sa R, Monteiro MP, Barros A, Sousa M, Carvalho RA, et al. Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics. *Mol Cell Endocrinol*. 2016;434:199–209. <https://doi.org/10.1016/j.mce.2016.07.008>.
  19. Pfäffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
  20. Santos RX, Correia SC, Alves MG, Oliveira PF, Cardoso S, Carvalho C, et al. Mitochondrial quality control systems sustain brain mitochondrial bioenergetics in early stages of type 2 diabetes. *Mol Cell Biochem*. 2014;394(1–2):13–22. <https://doi.org/10.1007/s11010-014-2076-5>.
  21. El-Hefnawy T, Ioffe S, Dym M. Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology*. 2000;141(7):2624–30. <https://doi.org/10.1210/endo.141.7.7542>.
  22. Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M. Expression of leptin and leptin receptor in the testis of fertile and infertile patients. *Andrologia*. 2007;39(1):22–7. <https://doi.org/10.1111/j.1439-0272.2006.00754.x>.
  23. Tena-Sempere M, Manna PR, Zhang FP, Pinilla L, González LC, Diéguez C, et al. Molecular mechanisms of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *J Endocrinol*. 2001;170(2):413–23. <https://doi.org/10.1677/joe.0.1700413>.
  24. Fernández-Formoso G, Pérez-Sieira S, González-Touceda D, Dieguez C, Tovar S. Leptin, 20 years of searching for glucose homeostasis. *Life Sci*. 2015;140:4–9. <https://doi.org/10.1016/j.lfs.2015.02.008>.
  25. Rosenbaum M, Leibel RL. 20 years of leptin: role of leptin in energy homeostasis in humans. *J Endocrinol*. 2014;223(1):T83–96. <https://doi.org/10.1530/joe-14-0358>.
  26. Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet*. 1996;12(3):318–20. <https://doi.org/10.1038/ng0396-318>.
  27. Malik IA, Durairajanayagam D, Singh HJ. Leptin and its actions on reproduction in males. *Asian J Androl*. 2019;21(3):296–9. [https://doi.org/10.4103/aja.aja\\_98\\_18](https://doi.org/10.4103/aja.aja_98_18).
  28. Landry D, Cloutier F, Martin LJ. Implications of leptin in neuroendocrine regulation of male reproduction. *Reprod Biol*. 2013;13(1):1–14. <https://doi.org/10.1016/j.repbio.2012.12.001>.
  29. Bruno A, Gerbino S, Ferraro M, Siena L, Bonura A, Colombo P, et al. Fluticasone furoate maintains epithelial homeostasis via leptin/leptin receptor pathway in nasal cells. *Mol Cell Biochem*. 2014;396(1–2):55–65. <https://doi.org/10.1007/s11010-014-2142-z>.
  30. Choi YS, Oh HK, Choi JH. Expression of adiponectin, leptin, and their receptors in ovarian endometrioma. *Fertil Steril*. 2013;100(1):135–41.e1–2. <https://doi.org/10.1016/j.fertnstert.2013.03.019>.
  31. Yan W, Zhang H, Liu P, Wang H, Liu J, Gao C, et al. Impaired mitochondrial biogenesis due to dysfunctional adiponectin-AMPK-PGC-1 $\alpha$  signaling contributing to increased vulnerability in diabetic heart. *Basic Res Cardiol*. 2013;108(3):329. <https://doi.org/10.1007/s00395-013-0329-1>.
  32. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzmán M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase  $\alpha$ . *J Biol Chem*. 2001;276(27):25096–100. <https://doi.org/10.1074/jbc.M007383200>.
  33. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, et al. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature*. 2002;415(6869):339–43. <https://doi.org/10.1038/415339a>.
  34. Luo GF, Yu TY, Wen XH, Li Y, Yang GS. Alteration of mitochondrial oxidative capacity during porcine preadipocyte differentiation and in response to leptin. *Mol Cell Biochem*. 2008;307(1–2):83–91. <https://doi.org/10.1007/s11010-007-9587-2>.
  35. Rohas LM, St-Pierre J, Uldry M, Jäger S, Handschin C, Spiegelman BM. A fundamental system of cellular energy homeostasis regulated by PGC-1 $\alpha$ . *Proc Natl Acad Sci U S A*. 2007;104(19):7933–8. <https://doi.org/10.1073/pnas.0702683104>.
  36. Jomayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. *Essays Biochem*. 2010;47:69–84. <https://doi.org/10.1042/bse0470069>.
  37. Tang BL. Sirt1 and the Mitochondria. *Mol Cell*. 2016;39(2):87–95. <https://doi.org/10.14348/molcells.2016.2318>.
  38. Li C, Liu C, Nissim I, Chen J, Chen P, Doliba N, et al. Regulation of glucagon secretion in normal and diabetic human islets by gamma-hydroxybutyrate and glycine. *J Biol Chem*. 2013;288(6):3938–51. <https://doi.org/10.1074/jbc.M112.385682>.
  39. Ye X, Li M, Hou T, Gao T, Zhu WG, Yang Y. Sirtuins in glucose and lipid metabolism. *Oncotarget*. 2017;8(1):1845–59. <https://doi.org/10.18632/oncotarget.12157>.
  40. Mostafa T, Nabil N, Rashed L, Makeen K, El-Kasas MA, Mohamaed HA. Seminal SIRT1 expression in infertile oligoastheno-teratozoospermic men with varicocele. *Andrology*. 2018;6(2):301–5. <https://doi.org/10.1111/andr.12462>.

41. Mu Y, Yin TL, Yin L, Hu X, Yang J. CTRP3 attenuates high-fat diet-induced male reproductive dysfunction in mice. *Clin Sci (Lond)*. 2018;132(8):883–99. <https://doi.org/10.1042/cs20180179>.
42. Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, et al. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Hum Reprod Update*. 2018;24(3):267–89. <https://doi.org/10.1093/humupd/dmy003>.
43. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature*. 2005;434(7029):113–8. <https://doi.org/10.1038/nature03354>.
44. Liu H, Li S, Liu X, Chen Y, Deng H. SIRT3 overexpression inhibits growth of kidney tumor cells and enhances mitochondrial biogenesis. *J Proteome Res*. 2018;17(9):3143–52. <https://doi.org/10.1021/acs.jproteome.8b00260>.

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