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Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility?

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ABSTRACT

Human feeding behavior and lifestyle are gradually being altered, favoring the development of metabolic diseases, particularly type 2 diabetes and obesity. Leptin is produced by the adipose tissue acting as a satiety signal. Its levels have been positively correlated with fat mass and hyperleptinemia has been proposed to negatively affect male reproductive function. Nevertheless, the molecular mechanisms by which this hormone affects male fertility remain unknown. Herein, we hypothesize that leptin acts on human Sertoli cells (hSCs), the “nurse cells” of spermatogenesis, altering their metabolism. To test our hypothesis, hSCs were cultured without or with leptin (5, 25 and 50 ng/mL). Leptin receptor was identified by qPCR and Western blot. Protein levels of glucose transporters (GLUT1, GLUT2 and GLUT3), phosphofructokinase, lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4) were determined by Western Blot. LDH activity was assessed and metabolite production/consumption determined by proton nuclear magnetic resonance. Oxidative damage was evaluated by assessing lipid peroxidation, protein carbonilation and nitration. Our data shows that leptin receptor is expressed in hSCs. The concentration of leptin found in lean, healthy patients, upregulated GLUT2 protein levels and concentrations of leptin found in lean and obese patients increased LDH activity. Of note, all leptin concentrations decreased hSCs acetate production illustrating a novel mechanism for this hormone action. Moreover, our data shows that leptin does not induce or protect hSCs from oxidative damage. We report that this hormone modulates the nutritional support of spermatogenesis, illustrating a novel mechanism that may be linked to obesity-induced male infertility.

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1. Introduction

Human eating habits and lifestyle have been dramatically changing. Increased consumption of energy together with a sedentary lifestyle has led to a positive energy balance. These changes contribute to an increased incidence of obesity and associated metabolic diseases. Until recently, these chronic metabolic diseases were only associated with aging. However, this paradigm is being shifted and a growing number of children, adolescents and young adults in reproductive age are affected by these pathological conditions [1].

Leptin, is a peptide hormone mainly produced in adipose tissue stores [2]. It was initially called “satiety hormone”, since it was thought to be solely produced by adipocytes of white adipose tissue, to control energy homeostasis and decrease food intake [3]. More recently, leptin has been reported to be also produced in other tissues [4]. The plasma concentration of leptin tends to be increased in most obese individuals and positively correlated with total body fat [5,6], with the exception of the rare individuals with congenital leptin deficiency [7]. In fact, leptin possesses anti-obesity functions, based on its ability to suppress appetite and decrease body weight and adiposity [2]. Notably, leptin is now a FDA approved [8] therapeutic for several medical conditions.

Abbreviations: 4-HNE, 4-hydroxynonenal; BTB, blood–testis barrier; cDNA, complementary deoxyribonucleic acid; DNP, Dinitrophenyl; FBS, fetal bovine serum; GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GLUT3, glucose transporter 3; hL, human liver; hSC, human Sertoli cell; ITS, insulin–transferrin–sodium selenite; LDH, lactate dehydrogenase; MCT4, monocarboxylate transporter 4; NT, nitro-tyrosine; Ob-R, leptin (or obesity) receptor; OS, oxidative stress; PFK, phosphofructokinase; ROS, reactive oxygen species; SC, Sertoli cell; SRB, sulforodamine B

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The biological actions of leptin are carried out through interaction with the specific membrane-spanning leptin (or obesity) receptor (Ob-R) [9]. This hormone also mediates metabolic signals to the reproductive system, denoting when sufficient fat stores are available to meet the caloric demands of the reproductive events [10]. It has been suggested that the hypothalamus may be the primary target for most of leptin's actions on the reproductive axis [11]. However, based on the characterization of leptin receptor distribution and the effects of leptin on *in vitro* systems, direct action sites for this hormone have been suggested both in female [12] and male reproductive tissues [13–15].

Several studies using a leptin deficient rodent model emphasized the role of leptin in male reproductive function. *Ob*/*ob* mice present an autosomal-recessive mutation on chromosome 6, promoting a profound decrease in circulating leptin levels. Among other characteristics, these mice are obese and infertile [10,16]. Of note, a low-dosage leptin treatment restored fertility in *ob*/*ob* male mice [17]. In fact, those leptin-treated mice presented increased testicular and seminal vesicle weight [10,16,17] and elevated sperm counts [10]. In humans, congenital leptin deficiency is associated with hypogonadotropic hypogonadism, which may be reverted upon recombinant leptin treatment [18]. Yet, although it is known that leptin crosses the Sertoli–Sertoli cell barrier (BTB) [19] and is present in the seminal plasma [14], the molecular mechanisms by which it regulates male reproductive function remain unknown. The Sertoli cell (SC), which is the somatic component of BTB, plays an essential role in spermatogenesis. These cells are responsible for the physical and nutritional support of the developing germ cells [20]. The preferential substrate of developing germ cells is lactate, which is produced by the SC from several metabolic sources, particularly glucose [21]. The metabolic cooperation established between SCs and developing germ cells is essential for the occurrence of spermatogenesis [20,22]. The SC metabolism is sensitive to hormonal fluctuations and presents an enormous metabolic plasticity [23]. Therefore, we hypothesized that exposure to leptin can affect spermatogenesis by modulating SC metabolism. To test our hypothesis, we firstly evaluated the expression of the Ob-R on human SCs (hSCs). Then, hSCs were exposed to three distinct concentrations of leptin (the physiological concentration found in lean, healthy patients and in seminal plasma; a concentration usually detected in obese patients and a concentration found in morbidly obese individuals). The effects on metabolite production/consumption and protein levels and/or activity of key glucose and monocarboxylate transporters and metabolic enzymes were determined. Finally, since leptin has been suggested to alter oxidative equilibrium in cells, oxidative damage in exposed hSCs was evaluated by assessing lipid peroxidation, protein carbonilation and nitration.

2. Material and methods

2.1. Chemicals

NZY M-MuLV Reverse Transcriptase, random hexamer primers, dNTPs, NZTaq 2× Green Master Mix, agarose and DNA ladder were obtained from NZYTech (Lisboa, Portugal). Leptin was obtained from Bachem (Bubendorf, Switzerland). Primers were obtained from STABVIDA (Oeiras, Portugal). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless stated otherwise.

2.2. Sertoli cells primary culture

Clonetics™ human SCs (MM-HSE-2305) were purchased from Lonza (Walkersville, USA). The hSCs were thawed following the manufacturer protocol optimized by our group. In brief, the vial with frozen cells was thawed at 33 °C and cells were placed in culture flask with Sertoli culture medium (1:1 mixture of DMEM-Ham's F12, pH 7.4) supplemented with 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 μg/mL gentamicin and 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 33 °C in an atmosphere of 6% CO_2. Sertoli cells cultured in the presence of 10% FBS in F12:DMEM remain mitotically active as described [24]. The cells used for all experiments were obtained between the third and eighth passage to ensure reproducibility. Each "n" corresponds to a cell passage and all experiments were performed in triplicate. After 96 h, cultures were examined by phase contrast microscopy and hSCs culture purity was determined as described [25].

2.3. Experimental groups

Cells were allowed to grow until reach 80–85% of confluence and serum-starved before treatment. The culture medium was then replaced by serum-free medium (DMEM: F12 1:1, pH 7.4) supplemented with insulin–transferrin–sodium selenite (ITS medium; final concentration of 10 mg/L; 5.5 mg/L; 6.7 μg/L, respectively). To evaluate the effect of leptin on the glycolytic profile of hSCs we defined a control group with ITS medium without leptin and three groups supplemented leptin (5 ng/mL, 25 ng/mL and 50 ng/mL). The concentration of 5 ng/mL was chosen agreeing with the physiological levels found in lean, healthy patients [26] and the concentration found in seminal plasma [14]. The concentration of 25 ng/mL was chosen based on the levels reported in the literature for obese patients [26]. We also found relevant to evaluate the effects of a concentration reported in morbidly obese men (50 ng/mL) [27]. After 24 h of treatment, culture medium was collected. Cells were detached, counted with a Neubauer chamber and collected. Viability was evaluated by the Trypan Blue Exclusion test.

2.4. Cytotoxicity assay

The cytotoxicity of hSCs to leptin was determined by the colorimetric sulforodamine B (SRB) assay [28]. In brief, cells were seeded and treated with selected concentrations of leptin. After treatment, cells were washed twice in phosphate buffered saline solution and fixed overnight in 1% acetic acid in methanol. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1 h at 37 °C. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cell proteins was extracted with 10 mM Tris solution (pH 10) and the optical densities of the resulting media were determined at 540 nm. No cytotoxicity was observed for the doses of leptin used in this work (data not shown).

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

The extraction of total RNA (tRNA) from hSCs was performed using the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, USA) as indicated by the manufacturer. tRNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, München, Germany). Human liver (hL) tRNA was purchased from AMS Biotechnology (Abingdon, UK). tRNA from hSCs and hL was reversely transcribed as described [29]. The resulting complementary deoxyribonucleic acid (cDNA) was used with exon-exon spanning primers set designed to amplify Ob-R (Forward primer: TCTGGACTGCTCACGGTCAT; Reverse primer: ACCCAGCATTTTCACGGTTTG), Sox-9 (Forward primer: AGGAAGTCGGTGAAGAACGG; Reverse primer: AAGTCGATAGGGGGCTGTCT) and GATA-4 (Forward primer: CTAGCAGCTTCTGCGCCTGT; Reverse primer: GTGGTTCCGGAAGCTGATGTA). PCR were carried out as described [30]. Primers' optimal annealing temperature was set to 62 °C to Ob-R, 56 °C to Sox-9 and 58 °C to GATA-4. 35 cycles were required for the exponential amplification phase of fragments (180 bp to Ob-R and GATA-4 and 275 bp to Sox-9). hL was used as positive control for Ob-R experiments and cDNA-free sample was used as negative control. Samples were run in 1.5% agarose gel electrophoresis (120 mV, 40 min) and visualized using software Molecular Imager FX Pro Plus MultiImager (BioRad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the expected products was compared to a DNA ladder.

2.6. Western blot

Western Blot was performed as described [20]. In brief, protein samples (50 μg) were fractionated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 containing 5% skimmed dried milk, incubated overnight at 4 °C with primary antibodies and the conditions presented in Table 1. Mouse anti-β-actin was used as protein loading control. The immune-reactive proteins were detected separately with secondary antibodies and the conditions presented in Table 1. Membranes were reacted with ECF detection system (GE, Healthcare, Weßling, Germany) and imaged with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), according to standard methods.

2.7. Determination of lactate dehydrogenase (LDH) activity

LDH activity was determined using a commercial assay kit (Promega, Madison, USA), following the manufacturer's instructions as described [30]. The activities measured were calculated using the molar absorptivity of formazan and expressed in nmol/min/mg of protein.

2.8. Measurement of oxidative damages

Protein carbonyl, nitro-tyrosine (NT) and 4-hydroxynonenal (4-HNE) group levels were determined by Slotblot. Protein carbonyl groups were determined as described [31]. To determine NT and 4-HNE protein levels, 2.5 μg of protein was diluted in phosphate buffer saline to a final volume of 100 μL and transferred to PVDF membranes. Membranes were then incubated with primary antibodies and the conditions presented in Table 1. The immune-reactive proteins were detected separately with the respective secondary antibodies and the conditions presented in Table 1. Membranes were reacted with ECF™ substrate (GE Healthcare, Buckinghamshire, UK). Densities from each band were quantified using Quantity One software (BioRad, Vilber Lourmat, France).

2.9. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

^{1}H NMR spectroscopy was used to determine metabolite concentrations in the extracellular media of hSCs. Fully relaxed ^{1}H NMR spectra of extracellular media were obtained at 14.1 T, 25 °C, using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods [32]. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); H1-α glucose (doublet, 5.22); acetate (singlet, 1.9); pyruvate (singlet, 1.35). The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn, Livermore, USA). The results are expressed as consumption or production of each metabolite in absolute values of pmol/cell.

2.10. Statistical analysis

Statistical differences between experimental groups were assessed by two-way ANOVA, followed by Bonferroni's post-test. All data are shown as mean ± SEM (n = 6 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant.

3. Results

3.1. Leptin receptor (Ob-R) is expressed in human Sertoli cells

Though the seller made available a specific characterization of the cells, we further confirmed that the purchased hSCs expressed specific SCs markers (GATA-4 and Sox-9) (data not shown). The Ob-R was previously identified in rodent testis by RT-PCR and immunohistochemistry [33]. In human testis this receptor was only identified by immunohistochemistry [15,34]. For SCs, the Ob-R was only identified in rodent cells by hybridization *in situ* [35]. Therefore, before analyzing the effect of leptin on hSCs, we investigated the presence of Ob-R in these cells. Using RT-PCR, we were able to detect a 180 bp amplicon in cultured hSCs, corresponding to the presence of Ob-R mRNA (Fig. 1, Panel A). In addition, using a specific Ob-R antibody, we were able to detect a double staining of approximately 100 kDa and 125 kDa (Fig. 1, Panel B) corresponding to the presence of the short and long forms of Ob-R.

3.2. GLUT2 is present in human Sertoli cells and its protein levels are increased after exposure to a concentration of leptin found in lean men

When in culture, hSCs metabolize glucose to lactate, which is critical for the progression of spermatogenesis. Exposure to leptin is not able to significantly alter the uptake of glucose by hSCs (Fig. 2, Panel D). The glucose enters in cells through glucose membrane transporters (GLUTs). In hSCs, two GLUTs isoforms that participate in extracellular glucose uptake have already been identified: GLUT1 and GLUT3 (for review [20]). Exposure to leptin did not alter GLUT1 or GLUT3 protein levels (Fig. 2, Panel A and C, respectively). Of note, we were able to identify GLUT2 protein levels for the first time in hSCs. Using a specific

Table 1
List of antibodies used in this study.

Antibody	Source	Molecular weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	55	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT3	Goat	48	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
PFK	Rabbit	85	1:400	Santa Cruz Biotechnology, Heidelberg, Germany	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
LDH	Rabbit	37	1:10,000	Abcam, Cambridge, MA, USA	ab52488
GLUT2	Rabbit	61	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
β-Actin	Mouse	42	1:5000	Sigma-Aldrich, Roedermark, Germany	A5441
DNP	Rabbit	–	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	–	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Nitro-Tyrosine	Rabbit	–	1:2500	Cell Signaling Technology, Danvers, MA, USA	9691S
Mouse	Goat	–	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	–	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Rabbit	–	1:5000	Sigma-Aldrich, Roedermark, Germany	A4187

GLUT1 — glucose transporter 1; GLUT3 — glucose transporter 3; PFK-1 — phosphofructokinase 1; MCT4 — monocarboxylate transporter 4; LDH — lactate dehydrogenase; GLUT2 — glucose transporter 2; DNP — Dinitrophenyl; 4-HNE — 4-hydroxynonenal.

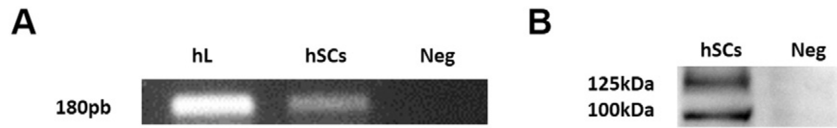

Fig. 1. Expression of leptin receptor (Ob-R) in cultured human Sertoli cells (hSCs). Identification of Ob-R by reverse transcriptase polymerase chain reaction (Panel A) and Western Blot (Panel B). hL: human liver; hSCs: human Sertoli cells; and Neg: Negative control.

antibody for GLUT2 we detected the specific staining for this protein at 61 kDa (Fig. 2, Panel B). In addition, the exposure of hSCs to the physiological concentration of leptin (5 ng/mL) increased GLUT2 protein levels to 1.13 ± 0.08 fold variation to control (Fig. 2, Panel B).

3.3. Levels of leptin reported in morbidly obese men decreased pyruvate consumption by human Sertoli cells

Glucose is take up by hSCs through GLUTs and then metabolized. The first rate-limiting step in glucose metabolism is the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase (PFK). Thus, we analyzed the protein levels of this enzyme. Exposure to all studied leptin concentrations was not able to alter PFK protein levels (Fig. 3, Panel A). Pyruvate consumption by hSCs treated with leptin was found to be decreased only when cells were exposed to a concentration reported in morbidly obese men (Fig. 3, Panel B). One of the metabolic pathways involved in the consumption of pyruvate, known to occur in hSCs, is its conversion to alanine by a reversible reaction catalyzed by alanine aminotransferase. The production of alanine was not altered when these cells were exposed to the different concentrations of leptin used (Fig. 3, Panel C) and closely resembled the patterns of pyruvate consumption.

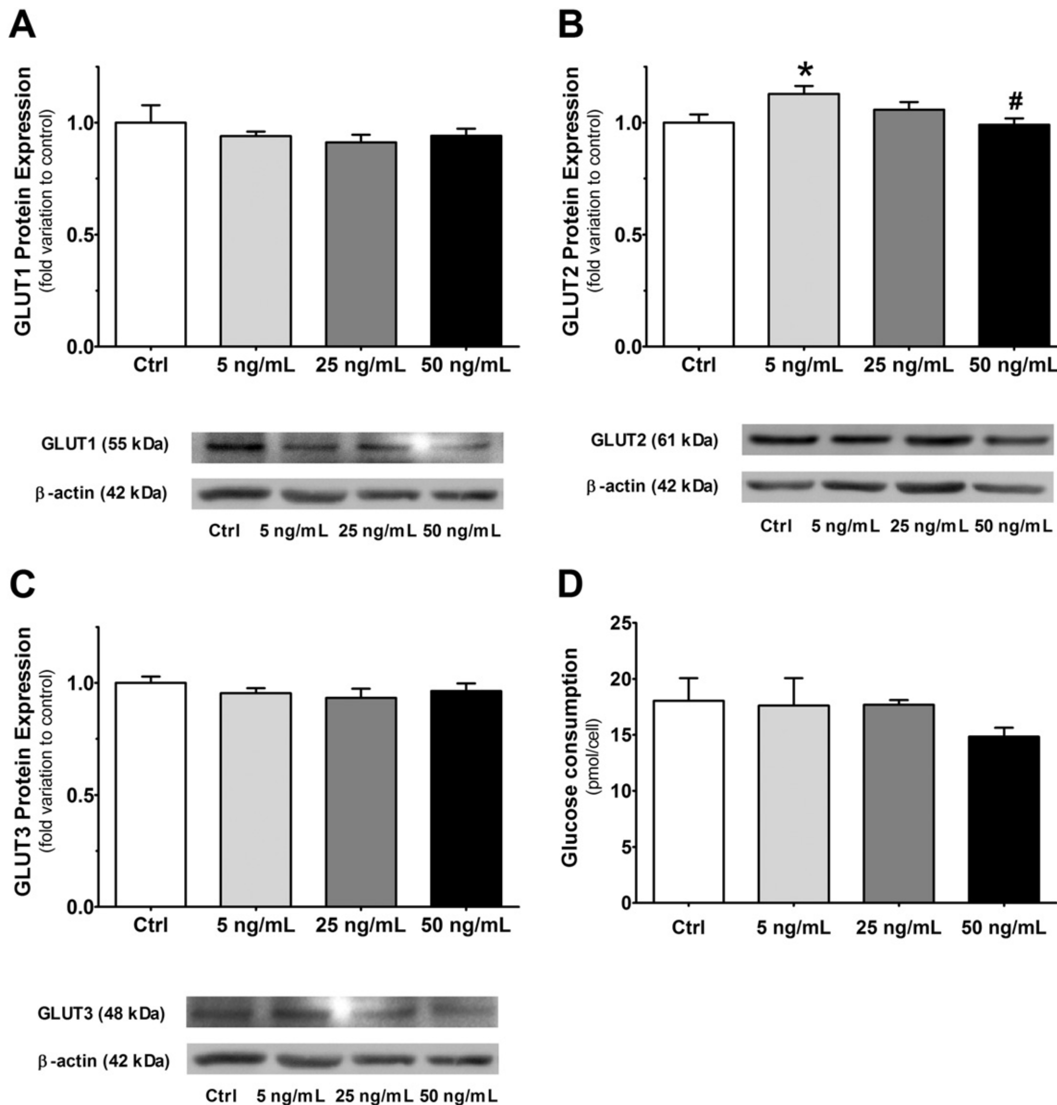

Fig. 2. Effect of leptin in glucose transporters and glucose consumption in human Sertoli cells (hSCs). Protein levels of glucose transporter 1 (GLUT1) (Panel A) and glucose transporter 2 (GLUT2) (Panel B) and glucose transporter 3 (GLUT3) (Panel C), as well as glucose consumption (Panel D) by hSCs after exposure to leptin. Panels A–C also show representative Western Blot experiments. Panels A–C show pooled data of independent experiments, indicating the protein levels of GLUT1, GLUT2 and GLUT3, respectively. Panel D shows pooled data of independent experiments, indicating the consumption of glucose in pmol/cell. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control and # relative to 5 ng/mL.

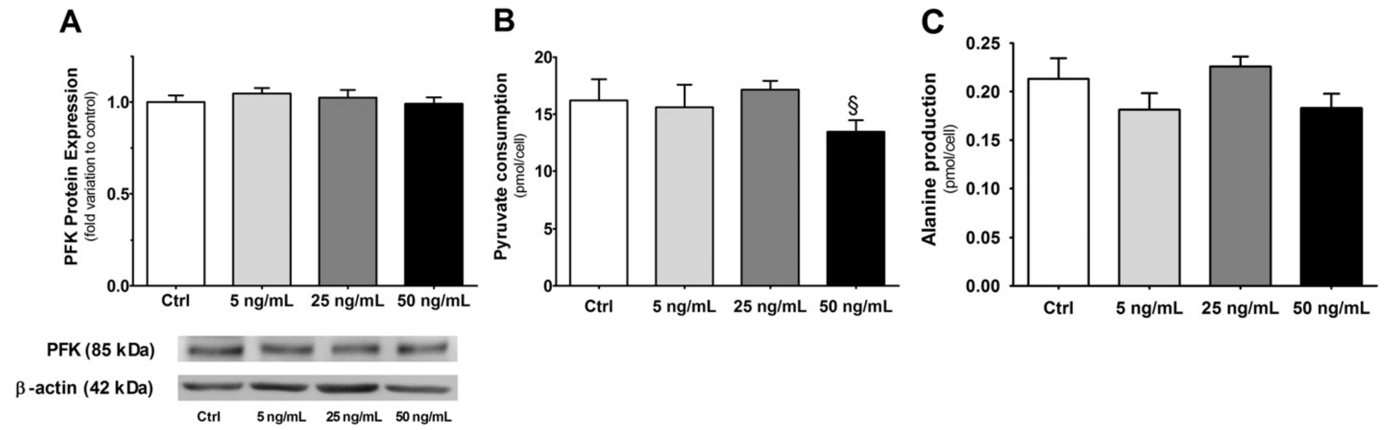

Fig. 3. Effect of leptin in glycolysis of human Sertoli cells (hSCs). Protein levels of phosphofructokinase (PFK) (Panel A), as well as pyruvate consumption (Panel B) and alanine production (Panel C) by hSCs after exposure to leptin. Panel A also show representative Western Blot experiments. Panel A shows pooled data of independent experiments, indicating the protein levels of PFK. Panels B–C show pooled data of independent experiments, indicating the consumption of pyruvate and production of alanine in pmol/cell. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: § relative to 25 ng/mL.

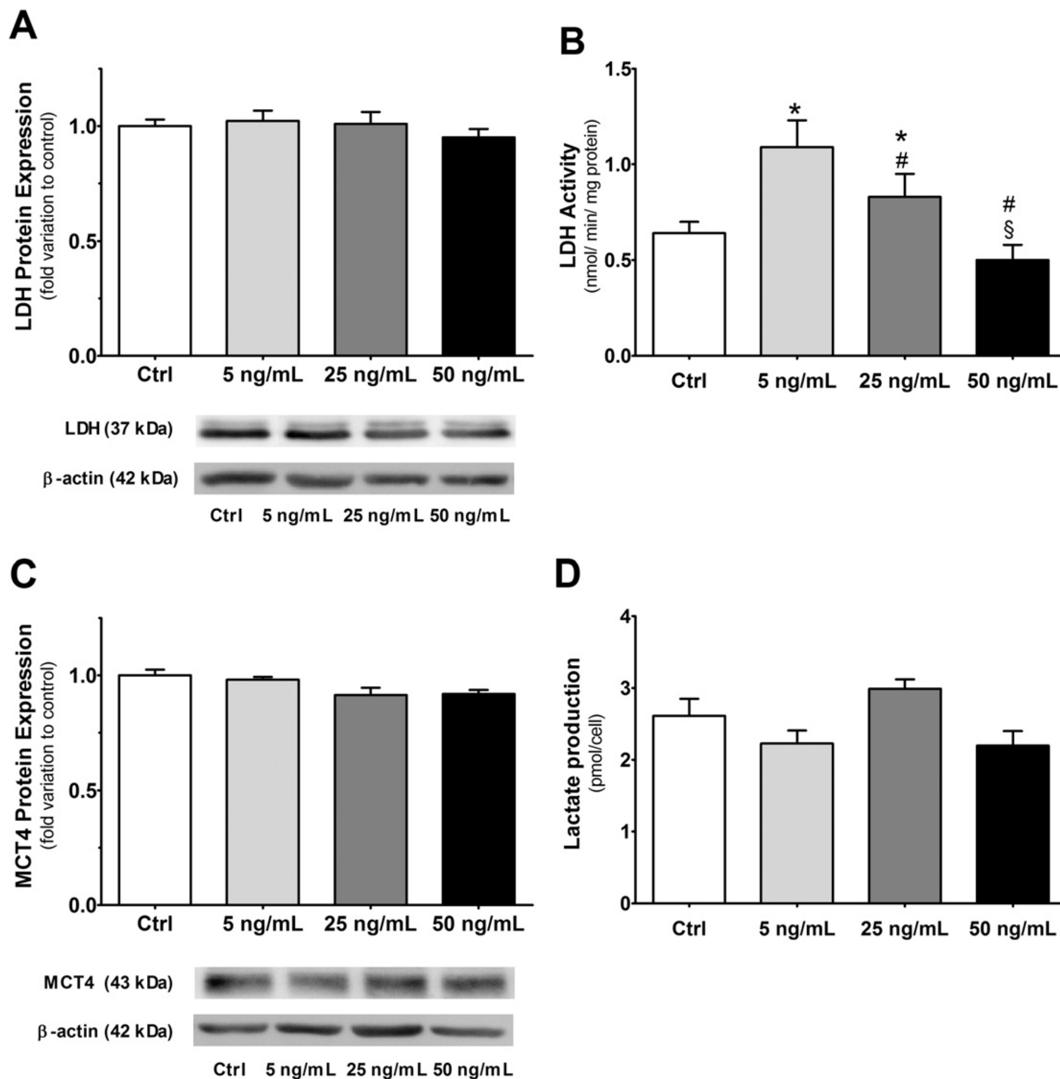

Fig. 4. Effect of leptin in lactate metabolism of human Sertoli cells (hSCs). Lactate dehydrogenase (LDH) protein levels (Panel A) and activity (Panel B), as well as, protein levels of monocarboxylate 4 (MCT4) (Panel C) and lactate production (Panel D) by hSCs after exposure to leptin. Panels A and C show pooled data of independent experiments, indicating the protein levels of LDH and MCT4, respectively. Panels B and D show pooled data of independent experiments, indicating the activity of LDH or production of lactate in pmol/cell. Panels A and C also show representative Western Blot experiments. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control; # relative to 5 ng/mL, and § relative to 25 ng/mL.

3.4. Leptin concentrations found in lean and obese men modulate LDH activity

LDH is crucial for the production of lactate by SCs. LDH protein levels were not altered in hSCs exposed to increasing doses of leptin (Fig. 4, panel A). However, LDH activity was increased when hSCs were exposed to leptin concentrations usually found in lean and obese men as compared with cells in control conditions and cells exposed to a concentration of leptin found in morbidly obese patients. The LDH activity increased from 0.64 ± 0.06 nmol/min/mg of protein in control conditions to 1.09 ± 0.14 and 0.83 ± 0.12 nmol/min/mg of protein when cells were exposed to 5 ng/mL and 25 ng/mL, respectively (Fig. 4, Panel B). Furthermore, LDH activity in hSCs exposed to 5 ng/mL (1.09 ± 0.14 nmol/min/mg) was significantly higher than that of cells exposed to 25 ng/mL (0.83 ± 0.12 nmol/min/mg). Of note, a concentration of leptin reported in morbidly obese individuals decreased LDH activity to 0.50 ± 0.08 nmol/min/mg of protein (Fig. 4, Panel B), when compared with cells from all other experimental conditions. Yet, although alterations in LDH activity were observed among hSCs of the different experimental groups, no differences were detected in the export/production of lactate by hSCs exposed to the different concentrations of leptin (Fig. 4, Panel C and Panel D, respectively).

3.5. Acetate production by human Sertoli cells is decreased after exposure to leptin and no oxidative damages were detected in the cells after treatment with leptin

SCs are known as lactate producers, however it was recently reported that they also produce high amounts of acetate [22]. The production of acetate in hSCs treated with leptin presented a decrease when compared to control condition. The acetate production by hSCs in control condition was 1.31 ± 054 pmol/cell and 0.46 ± 0.15 pmol/cell, 0.62 ± 0.10 pmol/cell and 0.52 ± 0.15 pmol/cell in cells treated with 5 ng/mL, 25 ng/mL and 50 ng/mL, respectively (Fig. 5, Panel A). Pyruvate metabolism, particularly the lactate/alanine ratio, is linked to intracellular redox homeostasis since it reflects the $NADH/NAD^+$ equilibrium [36]. Exposure to leptin, in any of the concentrations used, does not alter the intracellular redox state of hSCs (Fig. 5, Panel B). The high glycolytic rates detected in hSCs can lead to increased levels of oxidative stress (OS). Few studies have reported that different leptin levels are associated with variations in OS biomarkers levels [37,38]. Protein carbonylation/nitration and lipid peroxidation are excellent biomarkers of OS. DNP, 4-HNE and NT are products formed from the attack of free radicals to proteins and membranes. Our data showed that exposure of hSCs to concentrations of leptin found in lean, obese and morbidly obese men, did not alter carbonyl group (Fig. 6, Panel A), NT group (Fig. 6, Panel B) nor 4-HNE group (Fig. 6, Panel C) levels.

4. Discussion

Leptin is crucial in the regulation of body glucose metabolism. Most obese patients present a high level of leptin, which is correlated with their adiposity [39]. Obesity has reached pandemic proportions and the number of obese individuals is expected to dramatically increase in the next decades. One of the negative health consequences associated with obesity in men is reduced fertility [40]. Subfertility/infertility in overweight and obese men has been proposed to be due to suppression and/or alteration of SC function [41]. One of SCs main functions is the metabolic support of spermatogenesis (for review [20]). Since leptin is reported to be involved in the metabolic control of reproduction (for review [42]) and SCs produce the lactate needed for developing germ cells, we hypothesized that leptin can modulate SCs metabolism, affecting the normal occurrence of spermatogenesis and altering the fertility potential of males. The action of leptin [43] in male reproductive function has been reported [10,17] and Ob-R was identified in rodent and porcine testicular and epididymal tissue [13,44] and Leydig cells [35], while there is no consensus on its presence in SC [35,44]. In humans, the presence of the Ob-R was reported in testicular tissue [15,34] though the authors reported lack of immunoreactivity inside the tubules and suggested that hSCs did not express this receptor. Yet, our results attained by PCR and confirmed by Western blot, are the first to clearly demonstrate that Ob-R is expressed in isolated hSCs.

The SCs take glucose from circulation and produce metabolites essential to sustain the development of germ cells. Although the presence of GLUT1 has not been observed yet in human testis by immunohistochemistry [45], recent studies using molecular biology techniques [20, 46,47] reported that extracellular glucose uptake by SCs is mainly achieved by GLUT1 and GLUT3. This GLUT1/GLUT3 system is very sensitive to hormonal exposure, particularly insulin [22]. GLUT2 expression as already been identified in 42GPA9 cell line immortalized from mouse SCs by PCR [43] and though expression of GLUT2 was suggested to be non-detectable in human testis by immunohistochemistry [45], we report, for the first time, that cultured hSCs abundantly express GLUT2, illustrating that this transporter can play a crucial role in glucose transport. Leptin interferes with cellular glucose transport. For instance, it is reported to decrease GLUT3 expression in the hypothalamic neuronal cell line RCA6 [48]. However, in cells with high glycolytic flux, such as breast tumor cells, leptin does not alter GLUT1 expression [49]. In hSCs, leptin does not alter GLUT1 and GLUT3 protein levels. Nevertheless, GLUT2 protein levels are increased after exposure to a concentration of leptin reported in the plasma of lean men [26]. However, when hSCs were exposed to the concentration found in the plasma of obese and morbidly obese men, no alteration was observed in GLUT2 protein levels, relatively to control. Evidence shows that this hexose transporter is involved in the control of food intake [50] and, in liver, leptin also upregulates GLUT2 levels [51]. Although the exact contribution of GLUT2

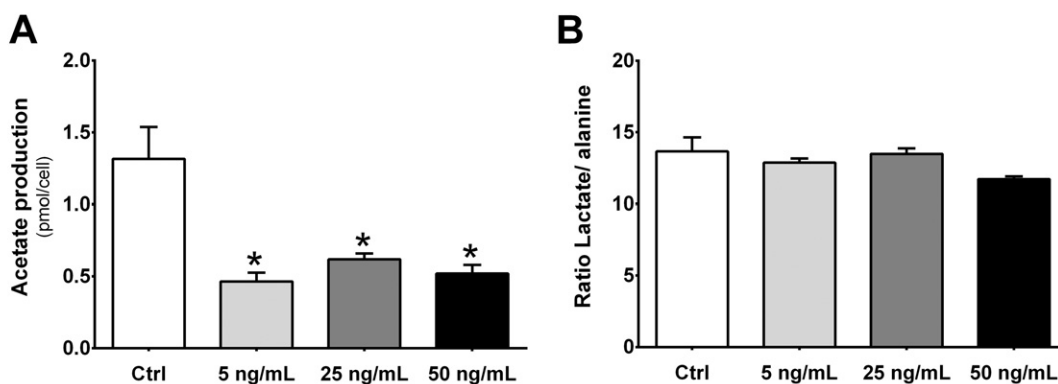

Fig. 5. Effect of leptin in acetate production by human Sertoli cells (hSCs) and intracellular redox state. Acetate production (Panel A) by hSCs and lactate/alanine ratio (Panel B) after exposure to leptin. Panel A shows pooled data of independent experiments, indicating the production of acetate in pmol/cell and Panel B shows pooled data of independent experiments, indicating the lactate/alanine ratio. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control.

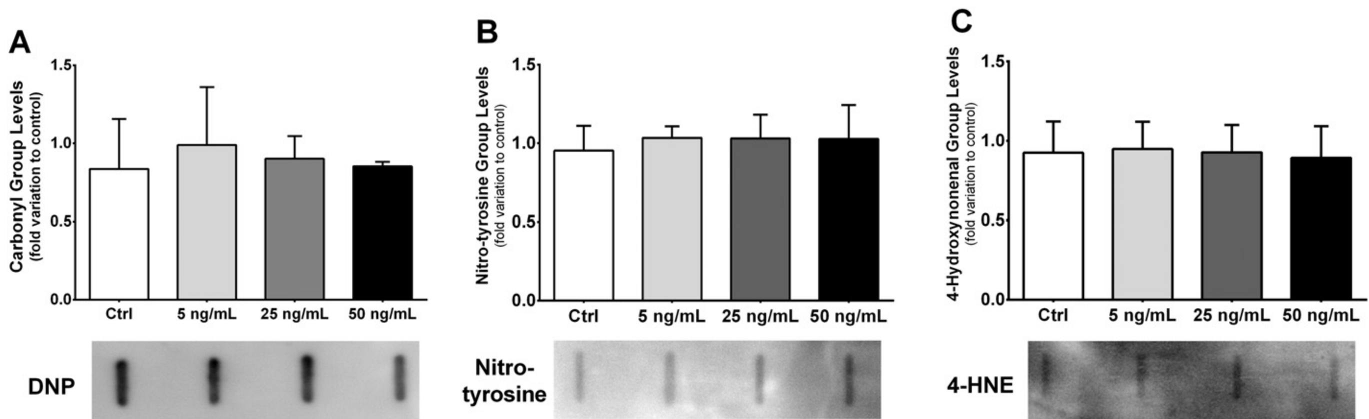

Fig. 6. Induction of oxidative damages by leptin in human Sertoli cells (hSCs). Carbonyl (Panel A), nitro-tyrosine (NT) (Panel B) and 4-hydroxynonenal (4-HNE) (Panel C) group levels measured in hSCs after exposure to leptin. Panels A, B and C show pooled data of independent experiments, indicating the expression levels of carbonyl, NT and 4-HNE, respectively. Panels A, B and C also show representative Slotblot experiments. Results are expressed as mean ± SEM (n = 6 for each condition).

to the pool of glucose taken by hSCs from the interstitial fluid remains to be unveiled, the stimulation of GLUT2 protein levels by the concentration of leptin found in lean men, illustrates a crucial mechanism dependent of this hormone. However, glucose consumption by hSCs was not altered by exposure to leptin. We have previously discussed (for review [23]) that SCs present a high metabolic plasticity. For instance, when cultured in insulin deprivation conditions [22], or after exposure to endocrine disruptors [52], these cells change the expression of glucose transporters to sustain glucose uptake. Leptin modulates GLUT2 protein levels, illustrating an effect also reported in other highly metabolic systems such as liver [53]. Notably, the concentration of leptin found in morbidly obese men induced a decrease in GLUT2 protein levels when compared with the concentration detected in lean men providing evidence that this mechanism may be relevant to obesity-related male subfertility/infertility. Nevertheless, further studies will be needed to test this hypothesis and disclose the relevance of this difference in GLUT2 expression.

In SCs, the vast majority of glucose is metabolized to pyruvate which is then converted to lactate. Although no changes were detected on the protein levels of PFK or LDH, LDH activity was stimulated by the concentration of leptin detected in lean men. Notably, LDH activity decreased by exposure to leptin in a dose-dependent manner, being progressively smaller when hSCs were exposed to doses observed in obese and morbidly obese men. This suggests that LDH activity may be a key control point of leptin action in spermatogenesis. However, the observed changes in GLUT2 protein levels and LDH activity did not result in alteration on the production of lactate by hSCs. This illustrates that although leptin modulates mechanisms associated to lactate production, these cells present a metabolic plasticity that under our experimental conditions allows them to sustain the production of lactate.

Although the lactate produced by SCs is referred to as the central metabolite for spermatogenesis, these cells synthesize other metabolites essential for the development of germ cells. Germ cells are constantly being duplicated and their proliferation rate is quite high. hSCs produce and export high amounts of acetate [22] that is proposed to be essential to maintain the high rate of lipid synthesis in developing germ cells [22], an hypothesis that was not yet tested but likely to occur. Leptin affects lipid catabolism in human placenta [54] and promotes the synthesis of acyl-coenzyme A, a pivotal intermediate in fatty acids metabolism, during monocyte-macrophage differentiation [55]. Moreover, it is reported that acetate may have an inhibitory effect on leptin secretion in epididymal adipocytes [56]. As referred, during spermatogenesis, there is a high rate of lipid synthesis and remodeling [57] and acetate is known to be the most reliable carbon source for lipid synthesis [58]. Acetate production by SCs is very sensitive to hormonal treatment, particularly insulin [22] and melatonin [59]. Our results show that acetate production by hSCs is severely decreased after exposure to all concentrations of leptin. This is consistent with reports showing that leptin decreases acetate degradation in isolated adipocytes and its incorporation into lipids [60]. In fact, leptin directly suppresses some biochemical pathways in peripheral tissues and cells, particularly those involving acetyl-CoA carboxylase [61]. Since all leptin concentrations, including the concentration found in lean men, altered acetate production by hSCs, further studies are needed to explore the hypothesis that this is a relevant mechanism for obesity-related subfertility/infertility by having a synergistic action with other hormones and factors.

Though absolute differences in metabolites concentration detected are due to exposure to leptin and thus reveal the direct action of this hormone in hSCs, we must highlight that absolute quantifications differ from our previous reports [22,62,63]. This difference in absolute quantification is due to several factors, particularly those that might not allow a simple comparison: the ITS supplement is very different; the stoichiometric pressure over some pathways, instead of other is very distinct; and this study was done after 24 h of culture while the others were performed at least in 48 h. Moreover, the hSCs used were purchased and only then expanded while our previous studies were done using hSCs isolated from fresh biopsies. The results attained allowed us to assess the effect of leptin in hSCs.

Compelling evidence suggests that leptin's action induces oxidative stress (OS) and reactive oxygen species (ROS) may serve as second messengers in leptin-mediated signaling. Moreover, it has been reported that leptin induces OS in human endothelial cells (10 ng/ml) [64] and plasma of Wistar rats [65]. The cellular glucose sensing machinery and cell metabolism are closely related to OS, particularly the lactate/alanine ratio [29]. Indeed, this ratio is an index of cellular redox state [66]. The higher is the NADH level in the cytosol, the more extensive is the conversion of pyruvate to lactate by LDH, and such leads to a considerable reduction of the alanine pools through the action of alanine transaminase. Of note, when exposed to some compounds such as the antidiabetic metformin [30], SCs adapt their metabolic behavior to maintain the $NADH/NAD^+$ equilibrium. Interesting, in hSCs, the intracellular redox equilibrium remains unaltered after exposure to leptin. Moreover, the group levels of carbonyl, NT and 4-HNE were not altered after hSCs were exposed to leptin, illustrating that this hormone does not promote oxidative damage in these cells even at concentration found in morbidly obese men.

Obesity and its associated co-morbidities have reached pandemic proportions. Men suffering with those diseases have fertility problems that end-up in subfertility and infertility. Among the several hormonal and metabolic alterations caused by the expansion of fat mass, increased leptin levels is a well-known characteristic of these individuals. Leptin

acts in several tissues and cellular systems altering their metabolic behavior. For instance, leptin was reported to stimulate the metabolic activity of NK-92 cells, which belong to the innate immune system and mediate several anti-tumor responses [67]. Besides, hyperleptinemia is associated with carcinogenesis [68], which is known to be related with an increased glycolytic profile of cells [69]. Within the testis, the glycolytic activity of SCs is pivotal to the normal occurrence of spermatogenesis. Herein we report that Ob-R mRNA and protein are expressed in hSCs and that leptin directly acts on these cells modulating their metabolic behavior. Leptin stimulates GLUT2 protein levels and LDH activity, and severely decreases the production of acetate by hSCs. This is a first assessment of how leptin can interfere with the metabolic support of spermatogenesis by hSCs. Further studies will be needed to fully disclose the role of leptin in Sertoli-germ cells interaction and its relevance for male fertility.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

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