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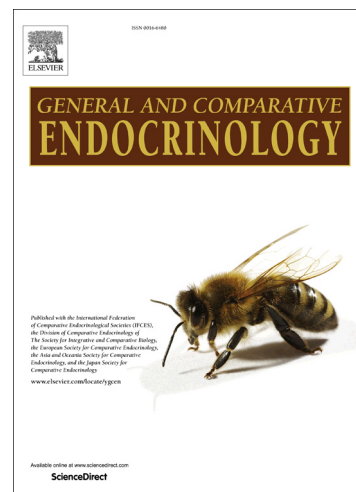
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RUNNINIG HEAD: **KETONE BODIES PRODUCTION IN RAT SERTOLI CELLS**

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ABSTRACT

Paracrine regulation of Sertoli cell function by germ cells is an outstanding characteristic of testicular physiology. It has been demonstrated that Sertoli cells produce ketone bodies and that germ cells may use them as energy source. The aim of the study was to analyze a possible regulation by germ cells of ketogenesis in Sertoli cells. Cultures of Sertoli cells (SC) obtained from 31-day-old rats were co-cultured with germ cells (GC). The results presented herein show that the presence of GC stimulated 3-hydroxybutyrate production and increased mRNA levels of two enzymes involved in ketogenesis —carnitine palmitoyltransferase 1a (CPT1a) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMGCoA) synthase— in SC. Additionally, GC increased monocarboxylate transporter 4 (*Mct4*) expression in SC, a transporter involved in ketone bodies exit. To evaluate if the observed effects might be mediated by soluble factors, SC cultures were incubated with germinal cell-conditioned medium (GCCM) or with two growth factors, bFGF and IGF1, which are known to be secreted by GC. We observed that GCCM and bFGF stimulated ketone bodies production but that IGF1 did not modify it. Also, we observed that GCCM and bFGF increased *Cpt1a* and *Mct4* mRNA levels. In summary, results presented herein demonstrate that Sertoli cells are able to produce ketone bodies and that its production is regulated in a paracrine way by germ cells. This study adds new information about communication between Sertoli cells and developing germ cells.

KEYWORDS

SERTOLI CELL, GERM CELL, KETONE BODIES, CPT1a, mHMGCoA SYNTHASE, MCT4

INTRODUCTION

Spermatogenesis is an intricate process highly dependent on Sertoli cell function, which is under endocrine as well as under the autocrine and paracrine control. These controls result from multiple and complex interactions between the different testicular cells (Parvinen 1982; Griswold 1995; Gnessi *et al.* 1997). To this respect, many studies have indicated that Sertoli cell functions are modulated according to the associated stages of spermatogenesis suggesting that germ cells can influence Sertoli cell function (Parvinen 1982; Tähkä 1989). More precisely, studies in vitro have shown that co-culture of germ cells with Sertoli cells modulates the secretion of androgen binding protein, transferrin, inhibin, among other Sertoli cell products (Le Magueresse *et al.* 1986; Castellon *et al.* 1989; Pineau *et al.* 1990). It has been postulated that the regulation exerted by germ cells on Sertoli cell function is the result of a cell-cell contact and of secreted soluble factors (Tähkä 1989; Skinner 1991).

Among Sertoli cell functions that might be of interest to germ cell development is the provision of adequate levels of energy substrates. Studies on the metabolism of glucose have shown that Sertoli cells actively metabolize glucose but the majority of it is converted to lactate and not oxidized via the citric acid cycle (Robinson and Fritz 1981; Grootegoed *et al*. 1986). On the other hand, post-meiotic germ cells are unable to use glucose for their energetic metabolism and they utilize lactate provided by Sertoli cells as an energy source (Jutte *et al.* 1981; Mita and Hall 1982). Although hormonal and paracrine regulation of lactate production by Sertoli cells has received considerable attention (Boussouar and Benahmed 2004; Galardo *et al*. 2017; Maldonado *et al.* 2016; Rato *et al.* 2015; 2016), fewer studies have focused on the analysis of other Sertoli cell products that may potentially be used as energy substrates by germ cells (Jutte *et al*. 1985; Alves *et al.* 2012). To this respect, Jutte *et al.* (1985) have demonstrated that Sertoli cells can oxidize palmitate to CO_2 and also, that this fatty acid is converted to ketone bodies, 3-hydroxybutyrate and acetoacetate. After

this precursor report on ketone bodies production, no further studies were undertaken to evaluate a possible physiological relevance of these observations. Fifteen years ago, Koga *et al.* (2000) showed that post-meiotic germ cells express a specific testicular isoform of succinyl-CoA transferase (SCOT-t). SCOT catalyzes the conversion of acetoacetate to acetoacetyl-CoA, which is a key step in ketone bodies catabolism and for this reason the authors postulated that ketone bodies may constitute energy substrates used by germ cells. A few years later, Tanaka *et al.* (2004) demonstrated that spermatozoa use ketone bodies as an energy source.

Ketogenesis occurs when acetyl-CoA levels obtained from fatty acid oxidation exceeds the capacity of mitochondria to oxidize this molecule. The process of ketogenesis comprises several biochemical steps that result in the conversion of acetyl-CoA to acetoacetate and 3-hydroxybutyrate. It has been shown that the transport of long chain-fatty acids into the mitochondria mediated by carnitine palmitoyltransferase 1 (CPT1) and the production of 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) catalyzed by mitochondrial HMGCoA synthase (mHMGCoA synthase) are important control sites of ketogenesis (Casals *et al.* 1992; Blázquez *et al.* 1998; Hegartdt 1999).

Three isoforms of CPT1 have been described so far: 1a (liver isoform), 1b (muscle isoform) and 1c (brain isoform). It has been observed that all isoforms of CPT1 are present in the testis (Adams *et al.* 1998; Price *et al.* 2002). Particularly, Adams *et al.* (1998) have observed that CPT1b is expressed in meiotic and post-meiotic germ cells but not in Sertoli cells. Recently, Regueira *et al.* (2014) demonstrated that *CPT1a* is expressed in rat Sertoli cells. CPT1c is localized in the endoplasmic reticulum rather than mitochondria and it does not participate in mitochondrial fatty acid oxidation (Sierra *et al.* 2008). So far, no data are available on CPT1c cellular localization in the testis.

The liver has been considered the organ with the highest capacity to produce ketone bodies. However, similar levels of expression of mHMGCoA synthase to those observed in liver have been demonstrated in the testis. The expression of this enzyme was initially localized in Leydig cells (Royo *et al.* 1993) and in a recent report also in Sertoli cells (Zimmermann *et al.* 2015).

Another molecular mechanism that may participate in the secretion of ketone bodies is their exit from the cell. Ketone bodies are charged molecules that cannot diffuse freely through cell membranes. It has been demonstrated that the latter compounds traverse membranes by means of monocarboxylate transporters (MCTs) and, specifically, that Sertoli cells express *Mct1* and *Mct4* (Galardo *et al.* 2007). It has also been proposed that MCT4 participates in monocarboxylates exit from the cell (Halestrap and Wilson 2012).

Considering that it has been shown that ketone bodies are used as energy substrates by germ cells, a possible regulation of ketone bodies production in Sertoli cells by germ cells is postulated. The aim of the present study was to analyze if germ cells regulate ketone bodies production and simultaneously the molecular mechanisms that participate in their production in Sertoli cells.

MATERIALS AND METHODS

Materials

Thirty one-day-old Sprague–Dawley rats were obtained from an animal care unit (Animal Care Laboratory, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina). Animals were killed by CO_2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of

Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Ethical Commitee from the Instituto de Biología y Medicina Experimental (Ref.: CE 011/2015, IByME). Human recombinant bFGF was purchased from Invitrogen (Carslab, CA, USA). Tissue culture media, human recombinant IGF1 and all other drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Sertoli cell and germ cell isolation and culture

Thirty one-day-old Sprague-Dawley rats were used to prepare Sertoli cells and germ cells. Testes were removed and decapsulated and Sertoli cell and germ cells isolation was performed as described by Schteingart *et al.* (1989) with modifications. Briefly, decapsulated testes were digested with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature. Seminiferous tubules pellet was washed and digested again with collagenase for 10 min at room temperature. The seminiferous tubules and the germ cells obtained after the digestion procedure were washed and recovered by centrifugation at 200xg for 3 min. The pellet was resuspended in HBSS and the small fragments of seminiferous tubules (SST) were collected by gravity sedimentation and saved for Sertoli cell isolation procedure. Germ cells present in the supernatant were collected by centrifugation at 200xg for 3 min. Germ cells were resuspended in HBSS containing 0.2% w/v BSA and contaminant Sertoli cells aggregates were sedimented by gravity for 10 min. Germ cells recovered from the supernatant were centrifuged at 200xg for 3 min and were purified by use of a discontinuous four-layer Percoll density gradient (20%-25%-32%-37%). The gradient was centrifuged at 800xg for 30 min at 4°C. The interface between 25% and 32% was collected, washed with culture medium, which consists of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 20 mM HEPES, 100 IU/ml penicillin, 2.5 µg/ml

amphotericin B, 1.2 mg/ml sodium bicarbonate (DMEM:HAM F12) to remove Percoll. Purified

germ cells collected by centrifugation at 200xg for 5 min were resuspended in DMEM:HAM F12

supplemented with 10 μg/ml transferrin, 5 μg/ml vitamin E, and 4 ng/ml hydrocortisone

(culture medium). Germ cells were cultured in tissue culture flasks at a density of $5x10^5$ cells/

cm^2 and at 10 $x10^5$cells/ cm^2 at 34°C in a mixture of 5% CO_2-95% air for 24 h. After this

incubation period tissue culture media containing germ cells were gently removed from tissue

culture flasks and centrifuged at 200xg for 5 min. Supernatant was used as germinal cell-

conditioned medium (GCCM) and the pellet contained purified germ cells were resuspended in

culture medium. In order to characterize the germ cell types present in the suspension, the

preparation was evaluated by flow cytometry to measure the DNA content as previously

described (Galardo *et al.* 2014). Briefly, cells were resuspended in DMEM-F12 supplemented

with 50% fetal bovine serum and fixed in ice-cold 70% ethanol. Propidium iodide was added to

fixed cells to a final concentration of 50 mg/ml. Flow cytometry was performed using a FACS

Caliber (Becton Dickinson). The preparation contained 27% tetraploid cells (spermatocytes) and

63% haploid cells (spermatids). Furthermore, the preparation contained small proportions of

diploid cells (10%) that may represent contamination with somatic cells.

The saved SST were submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove

peritubular cells, washed and then digested again with 0.05% w/v collagenase and 0.0003% w/v

soybean trypsin inhibitor in HBSS for 5 min at room temperature. The Sertoli cell suspension,

collected by sedimentation, was washed with HBSS containing 0.2% w/v BSA in order to

remove contaminant germ cells. Sertoli cells collected by gravity sedimentation was

resuspended in DMEM:HAM F12, filtered by Nitex and centrifuged at 200xg for 3 min. The

Sertoli cells pellet was resuspended in culture medium with 5 μg/ml insulin. Sertoli cells were

cultured in 6- or 24-multiwell plates (5 μg DNA/cm^2) for 24 h at 34°C in a mixture of 5% CO_2-

95% air. Purity of Sertoli cells reached 95% after 5 days in culture as seen by phase contrast microscopy. No myoid cell contamination was revealed when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to alpha-smooth muscle actin. Remaining cell contaminants were of germ cell origin.

Culture conditions

Sertoli cells – germ cells co-cultures: Sertoli cells were allowed to attach for 24 h in the presence of insulin, and medium was replaced at this time with fresh culture medium without insulin. Purified germ cells (2.5 or 5.0 x10^5 cel/cm^2) were seeded on top of Sertoli cell monolayer and incubated for a variable period of time. After 48- or 72-h of co-culture period, germ cells were removed by gentle aspiration and were used to perform RT-PCR analysis or to evaluate cell viability by trypan blue exclusion. In order to eliminate the germ cells attached to Sertoli cell monolayer, cultures were treated with hypotonic solution (20 mM Tris/HCl, pH 7·4) for 2 min (Riera *et al.* 2002). Sertoli cells harvested after 12-, 24- or 48-h incubation periods with GC were utilized for RT-PCR and RT-qPCR analyzes. Conditioned media obtained after 48- or 72-h of co-culture periods with GC were utilized to evaluate 3-hydroxybutyrate production.

Sertoli cells – GCCM cultures: Sertoli cells were allowed to attach for 24 h in the presence of insulin and medium was replaced at this time. Germ cell-conditioned media from 5.0x10^5 GC/cm^2 and from 10.0 x10^5 GC/cm^2 cultures were diluted 1:1 with fresh culture medium without insulin and supplemented with 100μM palmitate to obtain GCCM 2.5 or GCCM 5.0 respectively. Sertoli cells were incubated with the above mentioned GCCM for a variable period of time. Sertoli cells harvested after 12-, 24- or 48-h incubation periods with GCCM were utilized for RT-qPCR analyzes. On the other hand, conditioned media obtained

after a 72-h incubation period with GCCM were utilized to evaluate 3-hydroxybutyrate production.

Sertoli cells-paracrine factors cultures: Sertoli cells were allowed to attach for 24 h in the presence of insulin, and medium was replaced at this time with fresh medium without insulin and supplemented with 100μM palmitate. Cells were maintained under basal conditions or stimulated with variable doses of bFGF (5-30 ng/ml) or IGF1 (5-50 ng/ml) for a variable period of time. The rationale for the election of these doses is based on previous observations that 30 ng/ml bFGF and 50 ng/ml IGF1 elicit a maximal biological response in rat Sertoli cells (Riera *et al.* 2002; Meroni *et al.* 2004). Sertoli cells harvested after 12-, 24- or 48-h incubation periods with bFGF or IGF1 were utilized for RT-qPCR analyzes. On the other hand, conditioned media obtained after a 72-h incubation period with the peptides were utilized to evaluate 3-hydroxybutyrate production.

3-hydroxybutyrate determination

3-hydroxybutyrate was measured by a standard method involving conversion of NAD^+ to NADH determined as the rate of increase of absorbance at 340 nm. A commercial kit RANBUT (Randox Laboratories Limited, Crumlin, UK) was used. Conditioned media (900 μl) were lyophilized and resuspended in 100 μl of ultrapure water. A 50 μl aliquot was used to quantify 3-hydroxybutyrate levels. Cells were disrupted by ultrasonic irradiation in 500 μl of EDTA (2mM) and a 50 μl aliquot was saved for DNA determination. Results were expressed as nmol 3-hydroxybutyrate/μg DNA.

RT-PCR

Whole testis, purified Sertoli cells and germ cells were utilized to isolate total RNA using TRI Reagent (Sigma–Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260 nm. Reverse transcription was performed on 2 µg RNA at 42 °C for 50 min with a mixture containing 200 U MMLV reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix (Invitrogen). The cDNAs encoding *Cpt1a*, *mHmgCoA synthase* and *Mct4* were amplified from 1 µl of the cDNA reaction mixture using specific gene primers. The specific primers were: 5'-GGAACTCAAACCCATTCGTC-3' and 5'-GTTGGATGGTGTCTGTCTCT-3' for *Cpt1a* (Accession # NM_031559.2), 5'-GATGCACCGCAGAAAATATG-3' and 5'-GGAAGCCGTTTGG-3' for *mHmgCoA synthase* (Accession # NM_173094) and 5'- TGTGTGTGAACCGCTTTGG-3' and 5'-CAGACCCAAGCCAGTGATG-3' for *Mct4* (Accession# NM_030834.1). PCR was performed with GoTaq DNA polymerase (Promega Corporation) under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s; 60 °C for 30 s and extension at 72 °C for 50 s followed by 10 min at 72 °C. The PCR products were resolved by 2% w/v agarose gel and stained with ethidium bromide.

Real-time PCR (RT-qPCR)

Total RNA was isolated from Sertoli cells cultures using the TRI Reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260 nm. Reverse transcription was performed as previously mentioned in RT-PCR. Real-time PCR was performed using Step One Plus Real Time PCR System (Applied Biosystems, Warrington, UK). The specific primers for *Cpt1a*, *mHmgCoA synthase* and *Mct4* were the same used to RT-PCR and for *Hprt1* were 5´-AGTTCTTTGCTGACCTGCTG-3´ and 5´-TTTATGTCCCCCGTTGACTG-3´ (Accession # NM_012583.2). Amplification was carried out as recommended by the manufacturer: 25 µl reaction mixture contained 12.5 µl of SYBR Green

PCR Master mix (Applied Biosystems), the appropriate primer concentration and 2 μl of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. *Hprt1* was used as reference gene. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

Cell viability

Germ cell suspension was washed with PBS and then centrifuged for 5 min at 400xg, and the cell pellet was recovered. Cells were resuspended in fresh medium, and 0.4% trypan Blue was added. Cells that excluded the colorant (viable) and those that were stained (nonviable) were counted in a Neubauer chamber. Results are expressed as % of viable cells.

DNA quantitation

DNA quantitation was determined by the method of Labarca and Paigen (1980). Briefly, Sertoli cell monolayers were disrupted by ultrasonic irradiation in 500 μl of EDTA (2mM) and a 50 μl aliquot was used for DNA quantification. This aliquot was mixed with 450 μl 2mM EDTA and 1 ml of 0.3μg/ml Hoechst 33258 solution in buffer HSB (2M NaCl, 50mM NaH_2PO_4, pH 7.4). Fluorescence was evaluated in a fluorometer (Hoefer DNA fuorometer TKO100) at 460 nm. Values were compared against a standard curve prepared with increasing concentrations of DNA from calf thymus (Sigma).

Statistical analysis

All experiments were run in triplicates and repeated 3–4 times. One way ANOVA and post hoc analysis using Tukey–Krämer's multiple comparisons test were performed using GraphPad InSat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). P values <0.05 were considered statistically significant.

RESULTS

1. Effect of germ cells on ketone bodies production in rat Sertoli cells.

To analyze if germ cells (GC) were able to modulate ketone bodies production, Sertoli cell cultures were co-cultured with 2.5 and 5.0 x 10^5 GC/cm^2 during 48 or 72 h. Figure 1A shows that in co-cultures with 2.5 x10^5 and 5 x10^5 GC/cm^2, a significant increase in 3-hydroxybutyrate production in 72-h incubations was observed. The increase in 3-hydroxybutyrate production in 48-h incubations was only observed in co-cultures with the higher number of GC (5.0 x 10^5 GC/cm^2). The viability of germ cells at the end of incubation periods was also evaluated. Figure 1B shows that germ cell viability decreased if they were cultured in the absence of Sertoli cells. However, germ cells co-cultured with Sertoli cells maintained their viability at both periods of times analyzed.

2. Effect of germ cells on *Cpt1a*, *mHmgCoA synthase* and *Mct4* mRNA levels in Sertoli cells.

As mentioned in the introduction, the process of ketogenesis comprises several biochemical steps that result in the conversion of acetyl-CoA to acetoacetate and 3-

hydroxybutyrate. The transport of long chain-fatty acids into the mitochondria mediated by CPT1, the production of HMGCoA catalyzed by mHMGCoA synthase and the exit of ketone bodies from the cell through MCT4 may be important control sites that ensure ketone bodies secretion. Figure 2A shows that *Cpt1a* and *mHmgCoA synthase* mRNAs were detected in Sertoli cells but not in GC. This figure also shows that *Mct4* mRNA was detected in both cell types. The effect of co-culturing Sertoli cells with GC on *Cpt1a*, *mHmgCoA synthase* and *Mct4* expression in Sertoli cells was next examined. Figures 2B to 2D (left panels) show the results obtained in Sertoli cell cultures co-cultured with 5.0 x10^5 GC/cm^2 for variable periods of time. The right panels of the same figures show the results obtained in Sertoli cells co-cultured with variable doses of GC/cm^2 at the time of maximal regulation of *Cpt1a*, *mHmgCoA synthase* and *Mct4* expression. Figure 2B (left) shows that co-culture of Sertoli cells for 24 h with 5.0 x10^5 GC/cm^2 increased *Cpt1a* mRNA levels. Figure 2B (right) shows that in 24-h incubations 2.5 x10^5 GC/cm^2 also promoted a significant increase in *Cpt1a* mRNA levels. Figure 2C shows that only 48 h co-cultures with 5.0 x 10^5 GC/cm^2 caused an increase in *mHmgGCoA synthase* mRNA levels. Figure 2D (left) shows that co-culture of Sertoli cells for 24 h or 48 h with 5.0 x10^5 GC/cm^2 provoked a significant increase in *Mct4* mRNA levels. Figure 2D (right) shows that in 24-h incubations, 2.5 x 10^5 GC/cm^2 also stimulated *Mct4* mRNA levels.

3. Effect of GCCM, bFGF and IGF1 on ketone bodies production in Sertoli cells.

As mentioned in the introduction, the regulation exerted by GC on Sertoli cell function may result from cell-cell contact and from secreted soluble factors. The next set of experiments was designed to analyze if GC-secreted soluble factors were involved in the observed regulation of ketone bodies production. To achieve this goal, Sertoli cell monolayers were incubated with GCCM obtained from GC cultured at different densities and with different doses of bFGF or IGF1 for 72 h. Figure 3A shows that GCCM 2.5 and GCCM 5.0 increased 3-

hydroxybutyrate levels in Sertoli cells. Figure 3B shows that 30 ng/ml bFGF increased 3-hydroxybutyrate production and Figure 3C shows that IGF1 did not modify 3-hydroxybutyrate production at any dose tested.

4. Effect of GCCM, bFGF and IGF1 on *Cpt1a*, *mHmgCoA synthase* and *Mct4* mRNA levels.

In the next set of experiments, we investigated to what extent the previously observed results on ketone bodies production in response to GCCM, bFGF and IGF1 could be accounted for by effects on the expression of genes involved in ketone bodies secretion. Left panels of figures 4A to 4C show the results obtained in incubations of Sertoli cells cultures with GCCM 5.0 for variable periods of time. The right panels of the same figures show the results obtained in Sertoli cells incubated with GCCM 2.5 and 5.0 at the time of maximal regulation of gene expression. Figure 4A (left) shows that GCCM 5.0 promoted an increase in *Cpt1a* mRNA levels in 12-h incubations. The same figure (right panel) shows that not only GCCM 5.0 but also GCCM 2.5 significantly increased *Cpt1a* mRNA levels in 12-h incubations. Figure 4B (left) shows that GCCM 5.0 decreased *mHmgCoA synthase* expression in 48-h incubations. Figure 4B (right) shows that GCCM 2.5 also decreased the expression of this gene. Figure 4C (left) shows that GCCM 5.0 increased *Mct4* mRNA levels in 24-h and 48-h incubations. The right panel shows that both 2.5 and 5.0 GCCM significantly increased *Mct4* expression in 24-h incubations.

As for the effect of growth factors, Figures 5 and 6 show respectively the results obtained in incubations with bFGF or IGF1, for different periods of time (left) and with variable doses (right). Figure 5A shows that *Cpt1a* mRNA levels increased only in incubations with 30 ng/ml bFGF for 24 h. Figure 5B shows that treatment with 30 ng/ml bFGF for 48 h decreased *mHmgCoA synthase* expression. Figure 5C shows that bFGF increased *Mct4* expression in all experimental conditions tested.

Figure 6 shows the results obtained for the analysis of possible effects of IGF1. Figures 6A and 6B show that IGF1 did not modify the expression either of *Cpt1a* or *mHmgCoA synthase*. Finally, Figure 6C shows that the increase in *Mct4* mRNA levels promoted by IGF1 was only observed in 12-h incubations with a 50 ng/ml dose.

DISCUSSION

It is generally accepted that Sertoli cells ensure an optimal microenvironment for spermatogenesis. The establishment of the blood-testis barrier, at the time of meiosis initiation, physically divides the seminiferous epithelium into a basal and an adluminal compartment. For this reason, those germ cells that reside in the adluminal compartment are isolated from circulation and rely on Sertoli cell factors to fuel their metabolism.

As mentioned in the introduction, Sertoli cells are able to produce ketone bodies from fatty acids. Additionally, meiotic germ cells express a specific isoform of SCOT, which suggests that these cells metabolize ketone bodies and use them as an energy source. The studies from Tanaka *et al.* (2004), which show that ketone bodies maintain the motility of sperm cells, support the latter suggestion. We hypothesized that if ketone bodies were important to germ cell development, paracrine regulatory mechanisms would exist. The present study intends to define to what extent and by what means germ cells are able to regulate ketogenesis in Sertoli cells.

Classically, the study of ketogenesis has been limited to the liver. Hepatic cells produce ketone bodies in response to fasting and in this way supplies this energetic metabolite to extrahepatic tissues. However, hepatic cells do not seem to be the only ones, since other cells have been shown to be ketogenic. In brain and retina, astrocytes and retinal pigment epithelial cells produce ketone bodies that are delivered to and used by neurons and retinal cells as energy substrates, demonstrating a direct metabolic cooperation in these tissues (Guzmán and Blázquez 2001; Adijanto *et al.* 2014). The pair of Sertoli and germ cells is considered a notable example of metabolic cooperation where ketone bodies may be one of the substrates involved in this

cooperation. As mentioned before, paracrine regulation of Sertoli cell function by germ cells is an outstanding characteristic of testicular physiology. To this respect, we observed that germ cells stimulated the secretion of ketone bodies in Sertoli cells. In order to analyze to what extent soluble secreted factors were involved in the above-mentioned regulation, the effect of germinal cell conditioned media (GCCM) on ketone bodies production was studied. We observed that, although to a lesser extent, GCCM increased ketone bodies secretion. The latter results indicated that the stimulatory effect of germ cells on ketogenesis is at least partially mediated by soluble factors of germ cell origin.

Growth factors, such as bFGF and IGF1, belong to the set of intratesticular regulators that are produced by germ cells (Han *et al.* 1993 Hansson *et al.* 1989). Several biological responses in Sertoli cells are modulated by these peptides, highlighting an important role of bFGF and IGF1 in paracrine regulation (Gnessi *et al.* 1997; Riera *et al.* 2002; 2003; Galardo *et al.* 2008; 2013; Pitetti *et al.* 2013; Meroni *et al.* 2004; Regueira *et al.* 2015). Related to this, we demonstrated that bFGF but not IGF1 increases the production of ketone bodies, which suggests that bFGF is, among others, one of the factors responsible for the regulation of ketogenesis by germ cells.

As mentioned in the introduction, the route of ketogenesis occurs in the mitochondria when acetyl-CoA generated from fatty acid β-oxidation is shunted to the HMGCoA pathway. The process comprises several biochemical steps and it has been observed that CPT1a activity is a master key for its regulation. A rise in CPT1a activity is accompanied by increased levels of ketone bodies in hepatocytes and astrocytes (McGarry and Brown 1997; Blázquez *et al.* 1998). In view of these data from the literature, a regulation by germ cells of *Cpt1a* expression associated with the increase in ketogenesis in Sertoli cells may be expected.

In the present study, we confirmed previous results from Regueira *et al.* (2014) that show *Cpt1a* expression in Sertoli cells. Additionally, we show that *Cpt1a* is not expressed in germ cells. The presence of *Cpt1a* in Sertoli cells but not in germ cells may explain why higher levels

of *Cpt1a* mRNA had been observed in immature rat testis as compared to mature rat testis where the ratio between Sertoli and germ cell number is much lower (Adams *et al.* 1998). A few studies have analyzed the ability of hormones to regulate *Cpt1a* expression. Particularly, it has been observed that glucagon and its second messenger cyclic AMP, which increase ketone bodies production, stimulate transcription of *Cpt1a* in hepatocytes (Chatelain *et al.* 1996; Louet *et al.* 2001). In contraposition, it has been observed that insulin produces a diminution in ketone bodies production accompanied by a decrease in the expression of *Cpt1a* in hepatocytes (Beynen *et al.* 1980; Park *et al.* 1995). The results presented herein show that germ cells, GCCM and bFGF increase *Cpt1a* expression in Sertoli cells, suggesting that regulation by germ cells is produced, at least in part, by soluble factors. On the other hand, IGF1, which does not alter ketone bodies production, does not modify *Cpt1a* mRNA levels and this reinforces the idea that this peptide is not among the germ cell soluble factors responsible for regulation of ketogenesis in Sertoli cells.

Another control point of ketogenesis is the one regulated by mHMGCoA synthase. Previous studies from Royo *et al.* (1993) in the testis had described the expression of mHMGCoA synthase in Leydig cells but not in Sertoli cells. However, the expression in Sertoli cells of this enzyme, strictly associated with ketogenic tissues, has been recently demonstrated (Zimmermann *et al.* 2015). In the present study, we show that *mHmgCoA synthase* is expressed in Sertoli cells but not in germ cells.

The relationship between physiological situations that alter ketogenesis and *mHmgCoA synthase* expression has been extensively explored in the liver. To this respect, those situations leading to increase in plasma ketone bodies, such as starvation or diabetes, show increased hepatic *mHmgCoA synthase* mRNA levels (Serra *et al.* 1993; Hegardt 1999). Furthermore, refeeding of starved rats or insulin-treated rats with the consequent decrease in plasma ketone bodies show decreased expression of *mHmgCoA synthase* in liver (Casals *et al.* 1992; Serra *et al.* 1993). Additional evidence for the relationship between ketogenesis and *mHmgCoA synthase* expression arose from studies in hepatic cell lines. For instance, in the hepatoma cell line FAO,

which produces extremely low levels of ketone bodies, a very low expression of *mHmgCoA synthase* is observed (Prip-Buus *et al.* 1992); while in HepG2 cells over-expression of *mHmgCoA synthase* synthase is sufficient to induce ketone bodies production (Vilá-Brau *et al.* 2011). In the present study, we observed that germ cells increase *mHmgCoA synthase* expression in Sertoli cells. This regulation is apparently mediated by mechanisms that involve cell-cell contact. This assumption is based on the observation that GCCM, IGF1 or bFGF did not exert effects or in fact, under certain experimental conditions they reduced expression of the enzyme.

It has been shown that the activation of peroxisome proliferator-activated receptors (PPARs) is involved in the regulation of gene expression in Sertoli cells (Regueira *et al.* 2014). Fatty acids and a wide range of lipid derived molecules may act as physiological ligands of PPARs (Bensinger and Tontonoz 2008). Noticeably, it has been shown that *mHmgCoA synthase* gene promoter contains a functional PPAR response element (PPRE) (Rodriguez *et al.* 1994) and that fatty acids regulate *mHMGCoA synthase* gene expression by means of PPAR activation in HepG2 cells (Rodriguez *et al.* 1994). Sertoli cells phagocyte and degrade apoptotic GC and residual bodies during spermatogenesis. Additionally, a temporal relationship between phagocytosis of residual bodies and increased lipid droplets has been observed (Kerr *et al.* 1984; Ueno and Mori 1990; Sasso-Cerri *et al.* 2001) and it has been postulated that these lipid droplets constitute the source of fatty acids that are used as energy substrates for Sertoli cells (Xiong et al. 2009). Therefore, it is feasible that these fatty acids may also act as endogenous ligands for PPARs leading to the regulation of *mHmgCoA synthase* expression by GC in Sertoli cells. Further studies will be necessary to corroborate if this mechanism is really involved in the regulation of *mHMGCoA synthase* expression by germ cells.

Once ketone bodies are synthetized, the regulation of the expression of those transporters that allow their exit from Sertoli cells may also constitute a mechanism contributing to the supply of ketone bodies to germ cells. Ketone bodies traverse the plasma membrane through a family of proton-linked monocarboxylate transporters -MCTs. MCTs 1–4 mediate lactate transport, but

they are just as capable of transporting other substrates. MCTs have a kinetic behavior for ketone bodies transport that is fairly similar to that for lactate. MCT4, which has a much lower affinity for monocarboxylates than MCT1 and MCT2, may be responsible for the exit of ketone bodies from the cells (Halestrap 2012). It is worth mentioning that germ cells, especially round spermatids, express the high affinity monocarboxylate transporter isoform MCT2, which might mediate the incorporation of ketone bodies into these cells (Brauchi *et al.* 2005). We have previously observed that Sertoli cells express *Mct4* (Galardo *et al.* 2007) and in the present study we show that this transporter is also expressed in germ cells. As for *Mct4* regulation in Sertoli cells, we observed that germ cells, GCCM and bFGF increase its expression. Considering that a relationship between expression of *Mct4* and production of ketone bodies in response to identical stimuli was demonstrated, it is tempting to speculate that regulation of *Mct4* expression might constitute an additional mechanism participating in the energetic support of germ cells.

In summary, results presented herein demonstrate that Sertoli cells are able to produce ketone bodies and that its production is regulated in a paracrine way by germ cells. In addition, results demonstrate that the latter effect of germ cells is accompanied by increased expression of *Cpt1a* and *mHMGCoA synthase,* genes whose regulation has been tightly associated to ketogenesis. Concomitantly, increased expression of the monocarboxylate transporter *Mct4* was observed and this fact is interpreted as a way to ensure the exit of ketone bodies from Sertoli cells.

It is widely accepted that the regulation of Sertoli cell functions by germ cells constitutes an essential condition to adapt somatic cell metabolism to constant demands of the rapidly changing germ cell population. The secretion of ketone bodies is presented as an additional component to the existent list of Sertoli cell functions that are regulated not only by hormones but also by haploid cells.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1. Effect of germ cells on 3-hydroxybutyrate production in rat Sertoli cells. A) Sertoli cells monolayers were maintained in basal conditions or co-cultured with germ cells (GC; 2.5 or 5.0 x10^5 cells/ cm^2) for 48- or 72 h. 3-hydroxybutyrate production was determined in the 48- or 72-h conditioned media. Results are expressed as mean ± S.D. of triplicate incubations in one representative experiment out of three. * p< 0.05; ***p< 0.001 vs Basal. B) Germ cells (5.0 x10^5 cell/ cm^2) were cultured in the absence (GC) or presence of Sertoli cells (GC/SC) for 48- or 72 h. Cells that excluded the colorant (viable) and those that were stained (nonviable) were counted in a Neubauer chamber. Results are expressed as % of viable cells, means ± S.D. of three independent experiments. ***p< 0.001 vs GC.

Figure 2. Effect of germ cells on *Cpt1a*, *mHmgCoA synthase* and *Mct4* mRNA levels in rat Sertoli cells. A) Characterization of *Cpt1a*, *mHmgCoA synthase* and *Mct4* expression in Sertoli cells and germ cells was performed. Total RNA of rat liver (L), testis (T), Sertoli cells (SC) or germ cells (GC) were extracted, analyzed by RT-PCR and visualized by ethidium bromide staining. Liver and testis were used as positive controls. NT indicates no template control. B-D) Sertoli cells monolayers were maintained in basal conditions or co-cultured with GC (5.0 x10^5 cells/ cm^2) for variable periods of time (B-D left panel). Additionally, Sertoli cells monolayers were co-cultured with 2.5 or 5.0 x10^5 cells/ cm^2 GC during the incubation periods indicated in the figure (B-D right panel). Total cellular RNA was then extracted. RT-qPCRs for *Cpt1a*, *mHmgCoA synthase* or *Mct4* were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. of four independent experiments. *p< 0.05; **p< 0.01, ***p< 0.001 vs Basal.

Figure 3. Effect of GCCM, bFGF and IGF1 on ketone bodies production in rat Sertoli cells. Sertoli cells monolayers were maintained in basal conditions or incubated for 72 h with: (A) GCCM (2.5 or 5.0) obtained as described in Materials and Methods, (B) variable doses of bFGF (5, 15 or 30 ng/ml) or (C) variable doses of IGF1 (5, 25 or 50 ng/ml). 3-hydroxybutyrate production was determined in conditioned media. Results are expressed as mean ± S.D. of triplicate incubations in one representative experiment out of three (** $p < 0.01$; ***$p < 0.001$ vs Basal).

Figure 4. Effect of GCCM on *Cpt1a, mHmgCoA synthase* and *Mct4* mRNA levels in rat Sertoli cells. Sertoli cells monolayers were maintained in basal conditions or incubated for variable periods of time with 5.0 GCCM (A-C left panel). Additionally, Sertoli cells were incubated with 2.5 and 5.0 GCCM during the incubation periods indicated in the figure (A-C right panel). Total cellular RNA was then extracted. RT-qPCRs for *Cpt1a, mHmgCoA synthase* or *Mct4* were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. of four independent experiments. *$p < 0.05$; **$p < 0.01$ vs Basal.

Figure 5. Effect of bFGF on *Cpt1a, mHmgCoA synthase* and *Mct4* mRNA levels in rat Sertoli cells. Sertoli cells monolayers were maintained in basal conditions or incubated for variable periods of time with 30 ng/ml bFGF (A-C left panel). Additionally, Sertoli cells were incubated with 15 and 30 ng/ml bFGF during the incubation periods indicated in the figure (A-C right panel). Total cellular RNA was then extracted. RT-qPCRs for *Cpt1a*, *mHmgCoA synthase* or *Mct4* were performed. The comparative ΔΔCt method was used to calculate relative gene

expression. Results are expressed as means ± S.D. of four independent experiments. *p< 0.05; **p< 0.01 ; ***p<0.001 vs Basal.

Figure 6. Effect of IGF1 on *Cpt1a*, *mHmgCoA synthase* and *Mct4* mRNA levels in rat Sertoli cells. Sertoli cells monolayers were maintained in basal conditions or incubated for variable periods of time with 50 ng/ml IGF1 (A-C left panel). Additionally, Sertoli cells were incubated with 25 and 50 ng/ml IGF1 for 12 h (A-C right panel). Total cellular RNA was then extracted. RT-qPCRs for *Cpt1a, mHmgCoA synthase* or *Mct4* were performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. of four independent experiments. *p< 0.05 vs Basal.

Highlights

- *Spermatocytes and spermatids increase 3-hydroxybutyrate production in Sertoli cells*

- *Germ cells regulate mRNA levels of CPT1a, mHMGCoA synthase and MCT4*

- *Germinal cell-conditioned medium and bFGF increase 3-hydroxybutyrate production*