DR VANINA GABRIELA DA ROS (Orcid ID : 0000-0003-2367-0042)
DR MARIA FERNANDA RIERA (Orcid ID : 0000-0003-0762-1662)

Article type : Original Article

Postnatal metformin treatment alters rat Sertoli cell proliferation and daily sperm production

Rindone Gustavo Marcelo 1, Gorga Agostina 1, Pellizzari Eliana Herminia 1, Camberos María del Carmen 1, Galardo María Noel 1, Da Ros Vanina Gabriela 2, Buffone Mariano Gabriel 2, Meroni Silvina Beatriz 1, Riera María Fernanda 1.

1-Centro de Investigaciones Endocrinológicas "Dr. César Bergadá" (CEDIE) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – FEI – División de Endocrinología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina.
2-Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires, Argentina.

RUNNINIG HEAD: METFORMIN DECREASES SERTOLI CELL PROLIFERATION

KEYWORDS: METFORMIN, SERTOLI CELLS, PROLIFERATION, CELL CYCLE REGULATORS, AMPK, DAILY SPERM PRODUCTION.

CORRESPONDENCE TO:

Riera Maria Fernanda, PhD
Centro de Investigaciones Endocrinológicas "Dr. César Bergadá" (CEDIE) CONICET – FEI – División de Endocrinología, Hospital de Niños Ricardo Gutiérrez
Gallo 1330, (C1425EDF) Buenos Aires, Argentina
TEL: 5411-4963 5931; FAX: 5411-4963 5930
e-mail: friera@cedie.org.ar

ABSTRACT

Background: The direct correlation between Sertoli cell number and sperm production capacity highlights the importance of deciphering external factors that modify Sertoli cell proliferation. A growing body of evidence *in vitro* suggests that metformin, the main pharmacological agent for type 2 diabetes treatment in children, exerts anti-proliferative effects on Sertoli cells.

Objective: The aims of this study were to investigate the effect of metformin administration during postnatal period on Sertoli cell proliferation and on cell cycle regulators expression and to analyze the impact of this treatment on the sperm production capacity in adulthood.

Materials and methods: Sprague-Dawley rat pups were randomly divided into two groups: MET (receiving daily 200 mg/kg metformin, from Pnd3 to Pnd7 inclusive) and control (receiving vehicle). BrdU incorporation was measured to assess proliferation. Gene expression analyses were performed in Sertoli cells isolated from animals of both groups. Daily sperm production and sperm parameters were measured in adult male rats (Pnd90) that received neonatal treatment.

Results: MET group exhibited a significant decrease in BrdU incorporation in Sertoli cells. Concordantly, MET group showed a reduction in Cyclin D1 and E2 expression and an increase in p21 expression in Sertoli cells. In addition, metformin-treated animals displayed lower values of daily sperm production on Pnd90.

Discussion and conclusion: These results suggest that metformin treatment may lead to a decrease in Sertoli cell proliferation, a concomitant altered expression of cell cycle regulators and ultimately, a reduction in daily sperm production in adult animals.

1. INTRODUCTION

Sertoli cells play a central role in the development of a functional testis by providing structural and nutritional support for the germ cell epithelium. Each Sertoli cell can maintain a limited number of germ cells, so the quantity of Sertoli cells in the adult testis determines daily sperm production[1-3]. Besides its clear role towards germ cell population, recent findings have also shown a correlation between the amount of Sertoli cells and Leydig cell number in adulthood[4,5]. The Sertoli cell population arises from proliferative events in fetal, neonatal and prepubertal periods. In rats, Sertoli cells proliferate throughout fetal life and up to postnatal day 15. Then they stop dividing and begin a process of maturation that includes the formation of the blood-testis barrier and the cell differentiation that allows them to fully support spermatogenesis[6].As only immature Sertoli cells proliferate, the final pool of Sertoli cells will be established before adulthood[7-9].

Mounting evidence points to a continuing decline in human sperm counts over the past few decades[10]. The bases of these observations remain undetermined; however, epidemiological, clinical, and experimental studies suggest that there are multiple possible causes for the progressive impairment of male reproductive function. Among these, drugs used for the treatment of several pathologies in early stages of life should be considered. These treatments may alter Sertoli cell proliferation and maturation and might be somehow related to the aforementioned impairment of the testicular function.

The rise in the prevalence of childhood obesity has markedly increased type 2 diabetes (T2D) diagnosis in children and young people. Besides imperative lifestyle changes, drug therapy is often required for pediatric T2D management. Currently, metformin is the only oral agent approved by the US Food and Drug Administration for T2D treatment in 10-year-old children or older. Beneficial clinical outcomes have positioned metformin as a first-line strategy and have led to studies exploring new applications in pediatric obesity and prediabetes[11-14]. Given that metformin is being used in children whose Sertoli cells are going through the prepubertal stage of proliferation[15], it is relevant to study the effect of this drug on testicular function

In addition to its strong antidiabetic properties, numerous studies have shown the anti-proliferative effects of metformin in different cell types, including testicular cells[16-20]. In this context, some studies have investigated the effect of metformin on Sertoli cell proliferation. In mice, administration of metformin to pregnant animals reduces the number of Sertoli cells of male offspring[18]. Prepubertal chickens treated with metformin for 3 weeks showed decreased testis weight and seminiferous tubule diameter[19]. Moreover, *in vitro* studies have shown that metformin decreases postnatal proliferation of chicken and rat Sertoli cells[19,20]. This decrease in Sertoli cell proliferation elicited by metformin is accompanied by reduced expression of cyclins such as Cyclin D1 and increased expression of the cyclin-dependent kinase inhibitor 1A (*Cdkn1a*) commonly known as p21. Additionally, metformin counteracts the mitogenic effects of follicle stimulating hormone (FSH) on postnatal rat Sertoli cell cultures[20]. However, it is unknown whether postnatal treatment with metformin has any effect on the regulation of mammalian Sertoli cell proliferation and if so, which mechanisms may play a role.

The aim of the present study is to investigate the effect of metformin treatment during the neonatal period of the rat on Sertoli cell proliferation and to analyze a potential impact on the spermatogenic capacity in adult animals.

2. MATERIALS AND METHODS

1. *Materials*

Metformin (1,1-Dimethylbiguanide hydrochloride), tissue culture media and all other drugs were purchased from Sigma-Aldrich (St Louis, MO, USA).

2. *Animals*

Pregnant Sprague-Dawley rats were purchased from the Central Animal Facility of the University of Veterinary (Universidad de Buenos Aires, Argentina). Rats were housed under controlled conditions of temperature and humidity, 12-hour light/dark cycles and had free access to water and commercial pellet laboratory chow (Rat-Mouse Diet, Asociación de Cooperativas Argentina, Buenos Aires, Argentina). Pregnant rats were kept in individual cages. The day the offspring was born was considered as Postnatal day 0 (Pnd0). Experimental design was carried out according to the guidelines recommended by the National Institute of Health and approved by the Local Institutional Committee for the Care and use of Laboratory Animals (CICUAL, Resolution Nº 2017/007).

3. *Experimental design*

At Pnd3, pups were sexed according to the anogenital distance and litters were adjusted to ten pups per mother. In each litter, animals were weighted and male pups were randomized into two groups: control and metformin. The control group received vehicle (sterile saline solution) and metformin group (MET) received a dose of 200 mg/kg metformin (Sigma-Aldrich). The selected dose was chosen considering different criteria: the formula for dose translation between species based on the Body Surface Area calculation method[21] and previous studies that have determined that administration of a dose of 200 mg/kg metformin in rodents is necessary to achieve similar plasma concentrations to those observed in patients treated with metformin[22,23]. Administration was performed daily by intraperitoneal injection from Pnd3 to Pnd7, during this postnatal period Sertoli cells are actively proliferating[7].

At Pnd8 a set of animals of each group were decapitated; testes were removed and used for Sertoli cells isolation or histological analysis. Blood collected by exsanguination after decapitation was

used for serum glucose levels determination using a standard enzymatic method (Wiener Laboratories, Rosario, Argentina).

Another set of pups (control and MET) were weaned on Pnd21 and housed without further treatment. At Pnd90, animals were weighed and anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg). Testis and epididymis were removed and then the animals were euthanized by cervical dislocation. Sperm motility analysis and spermatid head counts were performed on epididymis and testicular samples, respectively.

4. Histological analysis

Testes were fixed in Bouin solution and embedded in paraffin. Sections (3-5 μm) obtained from the poles and equatorial areas were stained with haematoxylin and eosin and the testis morphology was examined by light microscopy. Tubule diameter was assessed in round transverse sections using Image J software (National Institutes of Health, USA).

5. Quantification of cell proliferation by BrdU incorporation

At Pnd8, animals from control (n=7) and MET (n=7) groups were injected with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich) 90 min before euthanasia, allowing BrdU to be incorporated in proliferating cells. Then, the testes were removed, fixed in Formalin 10% and embedded in paraffin. For each testis, 3 sections (3-5 μm) distant from one another by at least 50 μm were selected and subjected to antigen retrieval, which consisted in the immersion of sections in citrate buffer (pH 6.0) and heating for five cycles of 90 seconds at maximum power in a microwave. Sections were cooled at room temperature for 20 min. H_2O_2 was used to quench endogenous peroxidase activity. Then, a blocking step was implemented with a horse serum dilution. BrdU immunodetection was performed using mouse monoclonal BrdU antibody (1/200; Dako, Glostrup, Denmark), and was labelled using the Vectastain Universal ABC detection system (Vector Laboratories, CA, USA) and 3 3′diaminobenzidine chromogen (DAB) (Dako). Negative controls were processed without the BrdU antibody to test non-specific secondary antibody binding. The sections were counterstained with haematoxylin. For image acquisition, an Eclipse 50i microscope with a DS Fi1 digital camera and NIS Elements version 3.0 BR imaging software (Nikon Instruments, Melville, N.Y., USA) were used. Cell type identification was performed observing the distinctive nuclear and cytoplasmic morphological features. A total of 500 Sertoli cells or 100 germ cells, proliferating (BrdU stained) and quiescent, were blindly

counted per testis section using Image J software (National Institutes of Health, USA). Values are expressed as the percentage of BrdU positive cells over total.

6. Quantification of cell apoptosis by TUNEL

Testes from control (n=6) and MET (n=6) groups removed on Pnd8 were fixed in Formalin 10% and embedded in paraffin. For each testis, 2 sections (3-5 μm) distant from one another by at least 50 μm were selected and blocked with a horse serum dilution. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction using an In Situ Cell Death Kit (Roche Applied Science, Indiana, USA) was performed according to the manufacturer's recommendations. Briefly, testis sections were incubated with TUNEL reaction mixture for 60 min at 37 ^{0}C in a wet chamber. Then, testis sections were washed and incubated with DAPI solution and coverslips were mounted using Vectashield mounting medium (Vector Laboratories, CA, USA). Epifluorescence microscopy was performed using an Axio scope A1 microscope (Zeiss, Germany) coupled with an Axiocam 503 color camera (Zeiss). Assay specificity was assessed using the TUNEL reaction mixture without terminal transferase (negative controls). The number of TUNEL-positive cells was quantified in each tissue section by counting TUNEL-positive cells per round seminiferous tubule using Image J software (National Institutes of Health, USA). Two testicular histological sections were counted per rat, with a minimum of 100 randomly selected tubules in each tissue section. The data are presented as the mean ± S.D. (n=6 per group).

7. Determination of serum hormone levels

Blood collected by exsanguination after decapitation was used for serum hormone levels determination. Thyroxine, triiodothyronine, testosterone and estradiol concentration were directly measured using electrochemiluminescent immunoassay (ECLIA, Roche Diagnostics). The limits of quantification were 10 ng/dL for testosterone and 10 pg/mL for estradiol.

8. Sertoli cell isolation

Sertoli cells were isolated from testes of control (n=3) and MET (n=3) animals on Pnd8. Testes were processed in parallel following the experimental procedure described by Riera *et al*[24]. Briefly, testes were incubated in culture medium containing 0.03% w/v collagenase and 0.003% w/v soybean trypsin inhibitor, for 5 min at room temperature. Culture medium consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 0.1 % w/v

BSA, 100 IU/ml penicillin, 2.5 µg/ml amphotericin B and 1.2 mg/ml sodium bicarbonate. After the initial dispersion, seminiferous tubules were collected by sedimentation and supernatants were discarded to remove interstitial cells. Seminiferous tubules were treated with 1 M glycine-2 mM EDTA (pH 7.4) to remove peritubular cells. Tubules washed with culture media and recovered by sedimentation were treated with collagenase for a second time. Tubules were incubated for 10 min at room temperature with a solution of 0.03% w/v collagenase and 0.003% w/v soybean trypsin inhibitor and 0.03% w/v DNase. The resultant Sertoli cell suspension collected by centrifugation at 200 g for 3 minutes was resuspended in the culture medium described above without BSA. A volume of cell suspension corresponding to 100 µg of DNA was centrifuged and the supernatant was discarded. Total RNA was isolated from cell pellets. For each group, six independent Sertoli cell isolations were performed.

9. RNA isolation and quantitative real-time PCR (RT-qPCR) analysis

Total RNA was isolated from Sertoli cell pellets with 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 described.[20] Real-time PCR was performed using the Step One Real Time PCR System (Applied Biosystems, Warrington, UK). Table 1 shows the specific primers used to analyze the expression of cell cycle regulators, maturation markers of Sertoli cells, Actin and beta-2 microglobulin. Amplification was carried out as recommended by the manufacturer: 25 µl reaction mixtures contained 12.5 µl of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µl of cDNA. Relative cDNA concentrations were established by the standard curve method, using sequential dilutions of a cDNA sample. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec 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. Relative gene expression was calculated using the relative standard curve method. The data were normalized to Actin (*Actb*) and beta-2 microglobulin (*B2m*).

Western blot analysis

Testes from control (n=6) and MET (n=6) groups were decapsulated, immersed in 300 µl lysis buffer [Tris 10 mM pH 7.5, 150 mM, NaCl, 1 mM EDTA, 1 mM EGTA, 1% w/v Triton X-100, 100 mM NaF, 10 mM Na4P2O7, 10 mM Na3VO4 and protease inhibitor cocktail (Sigma-Aldrich,

P-8340)] and placed on ice. Then, testes were carefully minced with a razor blade several times, incubated in lysis buffer for 30 min and centrifuged at 16000 g for 30 min at 4°C. Western blot analysis was performed as previously described[20]. Membranes were probed with antibodies that specifically recognize phosphorylated Acetyl-CoA carboxylase and AKT serine/threonine kinase (P-ACC and P-AKT) (Cell Signaling Technology, Inc., Danvers, MA, USA) and with an antibody against the non-phosphorylated form of ACC or AKT (Cell Signaling Technology). 1:1000 dilutions of primary antibodies were used. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD, USA).

10. ***Daily sperm production (DSP)***

Testes from control (n=5) and MET (n=5) groups were collected and weighed at Pnd90. Testes were processed following the experimental procedure described by Fernandes *et al*[25]. The tunica albuginea was removed, and the parenchyma was homogenized in 0.9% w/v NaCl containing 0.5% w/v Triton X-100. Then, the homogenates were sonicated for 30 sec. Samples were diluted at 1:10 and transferred to a Neubauer chamber where counting was performed in quadruplicate. Elongated spermatid nuclei with the characteristic shape of step 19 spermatids and resistant to homogenization were counted to determine the number of spermatid nuclei. To calculate the DSP, the number of spermatids was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium[26].

11. ***Sperm morphology and motility***

Spermatozoa were recovered from the cauda epididymis by incising the large diameter tubules in 1 ml of capacitation medium described by Kaplan and Kraicer[27] and placing them in conical tubes at 37°C in an atmosphere containing 5% CO_2. After sperm dispersion, an aliquot of the upper sperm suspension (500 µl) was diluted. Then, 10 µl of the sperm dilution was placed on pre-warmed slides to determine the percentage of motile sperm by phase-contrast microscopy. To evaluate morphology, sperm samples were stained with haematoxylin and eosin after air-drying at room temperature. Examination was performed by light microscopy.

12. Statistical analysis

Normal distribution and variance homogeneity were assessed in all data sets. Most of the data were analyzed using one-way ANOVA. Gene data analyses were carried out using two-way ANOVA, considering the experiment variable as a random factor. Tukey's test was used as a post-hoc analysis. Western blot data analysis was performed using a one-tailed student's t test. Statistical evaluation was performed using InfoStat 2020e version (Grupo InfoStat, FCA, UNC, Argentina) and differences between groups means were considered statistically different at a P value<0.05.

2. RESULTS

2.1. *Effect of metformin treatment on body weight and glucose serum levels*

We observed no differences in maternal care and nursing between experimental groups. No signs of toxicity in the litters or alterations in weight gain were observed between treated and control pups during the course of the experiment (data not shown). Table 2 shows that no differences were found in body weight and serum glucose levels between control and metformin (MET) groups at Pnd8.

2.2. *Effect of metformin treatment on Sertoli and germ cell proliferation and on testicular cell apoptosis*

To evaluate proliferation, MET was administered from Pnd3 to Pnd7 and BrdU incorporation was assessed on Pnd8. Figure 1A shows that MET treatment decreased the percentage of Sertoli cells incorporating BrdU and did not affect the percentage of BrdU positive germ cells. Figure 1B shows that there were no differences in testis histological characteristics between control and MET group. In addition, testis weight and tubule diameter were not affected in treated animals (Table 3). To analyze if the decrease in BrdU-positive Sertoli cells may be associated with increased cell apoptosis, a TUNEL assay was performed. Figure 1C shows that the number of TUNEL-positive seminiferous tubule cells was similar in control and MET-treated animals.

2.3. *Effect of metformin treatment on the expression of cyclins and cell cycle inhibitors in Sertoli cells*

Having demonstrated that neonatal MET treatment decreases Sertoli cell proliferation, we evaluated if this effect was associated with the expression of cell cycle regulators, cyclins and cell cycle inhibitors. To this end, Sertoli cells were isolated from control and MET groups on Pnd8 and mRNA levels of Cyclin D1-D3 (*Ccnd1-Ccnd3)* Cyclin E1-E2 (*Ccne1-Ccne2)*, p21 (*Cdkn1a*) and p27 (*Cdkn1b*) were determined. Figure 2A shows that *Ccnd1* and *Ccne2* mRNA levels were significantly lower in Sertoli cells of the MET group than in controls, whereas *Ccnd2, Ccnd3 and Ccne1* mRNA levels showed no differences. Figure 2B shows that *Cdkn1a* mRNA levels were higher and *Cdkn1b* mRNA levels were lower in Sertoli cells of MET group than in control ones.

2.4. Effect of metformin on phosphorylated ACC and AKT levels.

In our previous report, we showed that in vitro metformin exerts its anti-proliferative action by activating AMP- activated protein kinase (AMPK)[20], so we decided to explore whether this activation also occurs in vivo. To this end, 2 h after MET administration, we evaluated phosphorylated ACC (P-ACC) levels —reflecting AMPK activation— in the testis. Figure 3 (left panel) shows that P-ACC levels were increased in the testis of MET group compared with controls. In addition, we analyzed phosphorylated AKT (P-AKT) levels, a signaling kinase that participates in the stimulation of Sertoli cell proliferation[24]. MET-treated rats showed decreased P-AKT levels in the testis (Figure 3, right panel).

2.5. *Effect of metformin treatment on the expression of markers of Sertoli cell maturation*

Given that MET exposure decreased Sertoli cell proliferation, we decided to evaluate if this effect led to an induction of cell maturation. To this end, Sertoli cells were isolated on Pnd8 and we evaluated the expression of an immature Sertoli cell marker, the anti-Mullerian hormone (*Amh*), and of mature Sertoli cell markers, such as androgen receptor (*Ar*), claudin 11(*Cldn11*) and connexin 43 (*Gja1*). In addition, FSH receptor (*Fshr*) mRNA levels were analyzed. Figure 4 shows that *Ar* mRNA levels were lower in Sertoli cells isolated from the MET group than control. No differences between control and MET groups regarding mRNA levels of *Amh*, *Cldn11, Gja1* and *Fshr* were observed.

Coexisting hormonal stimuli regulate Sertoli cell proliferation and maturation. Among them, testosterone and estradiol stimulate proliferation and also the maturation of Sertoli cells.

Besides, thyroid hormones inhibit cell proliferation and stimulate Sertoli cell functional maturation[9,28]. Thus, we evaluated if MET treatment modifies serum testosterone, estradiol and thyroid hormones levels on Pnd8. Thyroxine and triiodothyronine levels were similar in control and MET-treated animals, whereas serum testosterone and estradiol levels were not detectable in either of the groups at this age (Supplementary Table 1).

2.6. *Effect of neonatal metformin treatment on sperm parameters in adult animals*

Taking into account that neonatal treatment with MET decreased Sertoli cell proliferation and that each Sertoli cell supports a limited number of germ cells, we assessed whether neonatal exposure to MET affects daily sperm production in the adult animal. To achieve this goal, a set of animals was treated with MET from Pnd3 to 7 and then allowed to grow until Pnd90. Then, the number of step 19 spermatids and daily sperm production (DSP) were analyzed. Figure 5A shows that there was a significant decrease in the number of spermatids per testis and DSP in animals exposed to MET compared with controls. This figure also shows that adult testis weight was not modified by MET treatment. Figure 5B shows that seminiferous tubules had normal architecture and complete spermatogenesis in control and MET-treated animals. Additionally, morphology and motility of sperm were evaluated. Figure 5C shows that there were no differences in morphology and motility of epididymal spermatozoa between control and MET groups.

3. DISCUSSION

Sertoli cells proliferate during specific periods in life, including fetal, neonatal and the beginning of puberty in most species. In the rat, there is not a clear distinction between neonatal and pubertal periods of Sertoli cell proliferation because there is not a time gap between them[6]. After these proliferative periods, Sertoli cells remain numerically stable throughout life. Given that the number of Sertoli cells determines sperm production capacity, deciphering the external factors that may alter Sertoli cell proliferation is relevant to understand its contribution to the underlying causes responsible for the progressive decrease in human sperm production observed during the last decades.

Metformin is one of the most widely prescribed anti-hyperglycemic agents for the treatment of T2D in adults. Recently, the effect of metformin on cell proliferation has garnered much attention. Numerous *in vitro* studies have demonstrated the anti-proliferative activity of metformin in cancer cell lines and in fetal and postnatal Sertoli cells[18,20,29,30]. As for in vivo

studies, even though Tartarin et al[18] have not analyzed cell proliferation, they observed a decrease of about ten percent in the number of GATA4-positive Sertoli cells in mice exposed in utero to metformin. Considering that they did not observe an increase in apoptosis, they concluded that metformin may alter Sertoli cell proliferation during fetal life. In the present study, we observed that the treatment of neonatal rats with metformin decreases Sertoli cell proliferation by sixteen percent, if we express our results as percent of control, without changes in seminiferous tubule cell apoptosis. However, we found no statistical differences in tubule diameter or testis weight between groups. Despite the latter, these results indicate that metformin decreases Sertoli cell proliferation during both fetal and postnatal proliferative periods in mammals.

As for germ cells, Tartarin *et al*[18] observed that there was no modification in germ cell number of mice exposed *in utero* to metformin. Faure *et al*[19] obtained rather different results in an avian model. The authors observed that the *in vitro* treatment with metformin decreases BrdU incorporation in germ cells. In the same study, this result was not confirmed *in vivo* as they observed that metformin treatment in prepubertal chicken does not modify the number of spermatogonia. In agreement with Tartarin *et al*[18], our study shows that germ cell proliferation is not affected in 8-day-old animals previously treated with metformin, suggesting that in mammals, metformin treatment during the fetal or neonatal period does not affect germ cell proliferation.

The signaling pathways by which metformin exerts its actions have not yet been fully clarified. Reports have shown that it acts through AMPK-independent and dependent mechanisms. AMPK is a serine/threonine protein kinase that maintains cellular energy homeostasis and is activated in response to ATP depletion leading to an increase in the intracellular AMP:ATP ratio[31]. In this respect, it has been reported that metformin inhibits the mitochondrial respiratory chain complex 1 that induces a drop in ATP concentration, activating AMPK[32]. Several studies have shown that metformin exerts its anti-proliferative action in different cell types partly by activating AMPK[33-35]. In the present study, we found that metformin activates AMPK in the testis, as evidenced by increased phosphorylation of ACC, a known AMPK target. This finding is in agreement with previous in vitro studies that reported that metformin activates AMPK and inhibits proliferation in Sertoli cell cultures[19,20]. In addition, activation of AMPK induced by A-76966, a specific activator of this kinase, reduces Sertoli cell proliferation[24]. Therefore, it is tempting to speculate that AMPK activation may participate in the observed inhibitory effect of metformin on Sertoli cell proliferation. On the other hand, it is known that PI3K/AKT pathway participates in the hormonal regulation of Sertoli cell proliferation[24]. It has been reported that metformin inhibits

this pathway as part of its mechanism to decrease proliferation in several cell types[36-38]. Our results show that metformin treatment decreases phosphorylated AKT levels, suggesting an inhibitory effect in the PI3K/AKT pathway of this drug. Altogether, we postulate that metformin may inhibit Sertoli cell proliferation by modulating the AMPK and PI3K/AKT signaling pathways.

In the present study, we also tried to uncover some of the molecular mechanisms by which metformin affects Sertoli cell proliferation. Cyclins and cell cycle inhibitors tightly control cell cycle progression. Cyclin D1 is important for the G1-S transition in Sertoli cells and FSH —the main mitogen for these cells— increases Cyclin D1 expression as a part of its mechanism of action to regulate cell proliferation[39]. On the other hand, the cell cycle inhibitor p21 is involved in cell cycle arrest and in the maturation of Sertoli cells. Retinoic acid, the thyroid hormones and signaling proteins such as AMPK and Sirtuin 1 increase p21 expression as part of their anti-proliferative action[24,40,41]. In the present study, we show that Sertoli cells isolated from metformin treated animals present reduced *Ccnd1* and *Ccne2* mRNA levels and increased *Cdkn1a* mRNA levels. These results agree with our *in vitro* observations, that indicate that metformin decreases basal and FSH-stimulated *Ccnd1* and *Ccne2* and increases *Cdkn1a* mRNA levels in immature Sertoli cell cultures[20]. Altogether, these data suggest that *in vivo* metformin exerts its anti-proliferative actions on Sertoli cells through changes in the expression of cyclins and/or cell cycle inhibitors.

The levels of p27 in Sertoli cells are inversely correlated with their proliferative activity and reach the highest level in mature cells. In line with this, inhibition of Sertoli cell proliferation elicited by thyroid hormone and retinoic acid is accompanied by an increase in the expression p27 [40]. Surprisingly, we observed that the treatment of neonatal rats with metformin decreases *Cdkn1b* expression in Sertoli cells, even though their proliferative activity is reduced. Studies in knockout animals for this cell cycle inhibitor demonstrated that in addition to its role in cessation of proliferation, loss of p27 impairs differentiation of Sertoli cells[42]. Moreover, immunoexpression of p27 coincides with maturation of Sertoli cells in mice, rats and humans[43,44]. Altogether, these data led to consider p27 as a maturation marker of Sertoli cells[6], and it is thus possible to speculate that reduced p27 mRNA levels observed in metformin treated animals may be related to an alteration in the maturation process of Sertoli cells.

In most species, cessation of proliferation of Sertoli cells triggers a maturation process that consists in the establishment of the BTB and the acquisition of full capacity to sustain germ cells.

The latter process involves marked changes in gene expression. For example, AMH is widely accepted as an immature Sertoli cell marker because its expression decreases during maturation. On the other hand, among the genes that have been considered as maturation markers, besides the above mentioned p27, are connexin 43 and claudin 11 —both proteins involved in BTB formation— and androgen receptor[6,45,46]. We observed that neonatal metformin treatment does not modify neither *Amh* expression in Sertoli cells nor that of proteins involved in the establishment of BTB such as *Gja1* and *Cldn11*. Metformin slightly decreased *Ar* mRNA levels, a classical marker of Sertoli cell maturation, and did not modify *Fshr* expression. In addition, considering that thyroid hormones promote cessation of Sertoli cell proliferation, we determined their levels. Our results show that metformin treatment did not modify serum thyroxine and triiodothyronine levels. Altogether, these results suggest that the *in vivo* metformin effects are mostly related to an anti-proliferative action rather than an accelerated maturation process of Sertoli cells.

As the number of germ cells that each mature Sertoli cell supports is limited, Sertoli cell number is one of the main determinants of spermatogenic efficiency and therefore of sperm count in adulthood[2,3,47]. The results presented herein show that neonatal exposure to metformin decreased the number of spermatids and daily sperm production in adult animals but did not modify testis weight. In addition, metformin exposure did not alter epididymal sperm morphology or motility. In this context, Forcato *et al*[48] have recently shown that male offspring of pregnant rats that had been treated with metformin during gestation show no modification of sperm count parameters; however, there is a decrease in daily sperm production in the progeny of pregnant rats that had been exposed to metformin not only during gestation but also in lactation periods. The latter observations and our results led us to speculate that metformin treatment during the postnatal Sertoli cell proliferation period may have an impact on sperm production capacity in adulthood.

Finally, fertility evaluation in several animal models has shown that rodents produce and ejaculate a vast excess of sperm and that fertility in rats is only compromised after a severe reduction in sperm counts[49,50]. Hence, we consider that the observed modest decrease in DSP without further changes in sperm morphology and motility is unlikely to compromise fertility in metformin-treated rats. However, interspecies comparisons leave humans in a disadvantageous position; men have a lower sperm production rate efficiency[50] and consequently, the quantity of sperm per ejaculate is only a few-fold higher than the number at which fertility is impaired[51]. Considering the above-mentioned differences between rats and humans, we suggest that it would

be helpful to study whether sperm production and/or fertility are reduced in adult patients that had been treated with metformin during childhood.

Proliferation of human immature Sertoli cells occurs at the onset of puberty, when the increase in FSH levels induces mitosis in this cell type. Importantly, the incidence of both T2D and obesity in children has risen at staggering rates leading to the use of metformin in the pediatric population, during a period that coincides with the last stage of Sertoli cell proliferation[12-14]. Considering that the *in vitro* and *in vivo* findings suggest that metformin decreases postnatal Sertoli cell proliferation and affects sperm production capacity, attention must be paid to possible alterations in pediatric patients treated with this anti-hyperglycemic agent. In conclusion, and from a broader perspective, the results presented herein reinforce the notion of the potential effects of metformin on Sertoli cell proliferation and support the hypothesis that metformin treatment during the onset of puberty might have a long-term impact on male reproductive function.

4. 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.

5. FUNDING

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2014/945, PICT 2015/0228 and PICT 2015/2294) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 2015/127).

6. AUTHOR CONTRIBUTION

GMR, AG, EHP, CMC, VGDR and MGB performed the experiments. GMR, GA, EHP, VGDR, MGB and MNG collected and analyzed data. GMR and AG designed the figures. SBM and MFR designed and directed the study. GMR, SBM and MFR wrote the paper. All authors reviewed and approved the final manuscript.

7. ACKNOWLEDGEMENTS

The technical help of Mercedes Astarloa, Evelin Barrios, Mariana Cruz and Marcela Venara is gratefully acknowledged. We express gratitude to Dr Gabriela Ballerini for helping us with hormone levels determinations.

REFERENCES

1. Wing TY, Christensen AK. Morphometric studies on rat seminiferous tubules. Am J Anat 1982;165:13-25.

2. Johnson L, Zane RS, Petty CS, Neaves WB. Quantification of the human Sertoli cell population: its distribution, relation to germ cell numbers, and age-related decline. Biol Reprod 1984;31:785-795.

3. Berndtson WE, Thompson TL. Changing relationships between testis size, Sertoli cell number and spermatogenesis in Sprague-Dawley rats. J Androl 1990;11:429-435.

4. Rebourcet D, O'Shaughnessy PJ, Monteiro A, Milne L, Cruickshanks L, Jeffrey N, Guillou F, Freeman TC, Mitchell RT, Smith LB. Sertoli cells maintain Leydig cell number and peritubular myoid cell activity in the adult mouse testis. PLoS One 2014;9:e105687.

5. Rebourcet D, Darbey A, Monteiro A, Soffientini U, Tsai YT, Handel I, Pitetti JL, Nef S, Smith LB, O'Shaughnessy PJ. Sertoli Cell Number Defines and Predicts Germ and Leydig Cell Population Sizes in the Adult Mouse Testis. Endocrinology 2017;158:2955-2969.

6. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction. 2003;125(6):769-784.

7. Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. Biol Reprod. 1971;4(1):84-87.

8. Orth JM. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anat Rec. 1982;203(4):485-492.

9. Meroni SB, Galardo MN, Rindone G, Gorga A, Riera MF, Cigorraga SB. Molecular Mechanisms and Signaling Pathways Involved in Sertoli Cell Proliferation. Front Endocrinol (Lausanne).2019;10:224.

10. Levine H, Jorgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, Pinotti R, Swan SH. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update. 2017;23(6):646-659.

11. Khokhar A, Umpaichitra V, Chin VL, Perez-Colon S. Metformin Use in Children and Adolescents with Prediabetes. Pediatr Clin North Am Pediatric Clinics of North America. 2017;64(6):1341-1353.

12. Brufani C, Crino A, Fintini D, Patera PI, Cappa M, Manco M. Systematic review of metformin use in obese nondiabetic children and adolescents. Horm Res Paediatr. 2013;80(2):78-85.

13. Kendall D, Vail A, Amin R, Barrett T, Dimitri P, Ivison F, Kibirige M, Mathew V, Matyka K, McGovern A, Stirling H, Tetlow L, Wales J, Wright N, Clayton P, Hall C. Metformin in obese children and adolescents: the MOCA trial. J Clin Endocrinol Metab. 2013;98(1):322-329.

14. Adeyemo MA, McDuffie JR, Kozlosky M, Krakoff J, Calis KA, Brady SM, Yanovski JA. Effects of metformin on energy intake and satiety in obese children. Diabetes Obes Metab. 2015;17(4):363-370

15. Rey RA, Campo SM, Ropelato MG, Bergadá I. Hormonal Changes in Childhood and Puberty. In Puberty, Kumanov P & Agarwal A. Springer International Publishing: Switzerland; 2016; p 23–37.

16. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, Micic D, Trajkovic V. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. Cell Mol Life Sci. 2007;64(10):1290-1302.

17. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y, Bost F. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene. 2008;27(25):3576-3586.

18. Tartarin P, Moison D, Guibert E, Dupont J, Habert R, Rouiller-Fabre V, Frydman N, Pozzi S, Frydman R, Lecureuil C, Froment P. Metformin exposure affects human and mouse fetal testicular cells. Hum Reprod. 2012;27(11):3304-3314

19. Faure M, Guibert E, Alves S, Pain B, Rame C, Dupont J, Brillard JP, Froment P. The insulin sensitiser metformin regulates chicken Sertoli and germ cell populations. Reproduction. 2016;151(5):527-538.

20. Rindone GM, Gorga A, Regueira M, Pellizzari EH, Cigorraga SB, Galardo MN, Meroni SB, Riera MF. Metformin counteracts the effects of FSH on rat Sertoli cell proliferation. Reproduction. 2018;156(2):93-101.

21. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2008;22(3):659-661.

22. Barbieri F, Thellung S, Ratto A, Carra E, Marini V, Fucile C, Bajetto A, Pattarozzi A, Wurth R, Gatti M, Campanella C, Vito G, Mattioli F, Pagano A, Daga A, Ferrari A, Florio T. In vitro and in vivo antiproliferative activity of metformin on stem-like cells isolated from spontaneous canine mammary carcinomas: translational implications for human tumors. BMC Cancer. 2015;15:228.

23. Chandel NS, Avizonis D, Reczek CR, Weinberg SE, Menz S, Neuhaus R, Christian S, Haegebarth A, Algire C, Pollak M. Are Metformin Doses Used in Murine Cancer Models Clinically Relevant? Cell Metab. 2016;23(4):569-570.

24. Riera MF, Regueira M, Galardo MN, Pellizzari EH, Meroni SB, Cigorraga SB. Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation. Am J Physiol Endocrinol Metab. 2012; 302(8):E914-923

25. Fernandes GS, Arena AC, Fernandez CD, Mercadante A, Barbisan LF, Kempinas WG. Reproductive effects in male rats exposed to diuron. Reprod Toxicol. 2007;23(1):106-112.

26. Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil. 1978;54(1):103-107.

27. Kaplan R, Kraicer PF. Effect of elevated calcium concentration on fertilization of rat oocytes in vitro. Gamete Res Gamete Research. 1978;1(3-4):281-285.

28. Escott GM, da Rosa LA, Loss Eda S. Mechanisms of hormonal regulation of sertoli cell development and proliferation: a key process for spermatogenesis. Curr Mol Pharmacol 2014;7:96-108.

29. Zhuang Y, Miskimins WK. Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27Kip1 or p21Cip1. J Mol Signal. 2008;3:18.

30. Sikka A, Kaur M, Agarwal C, Deep G, Agarwal R. Metformin suppresses growth of human head and neck squamous cell carcinoma via global inhibition of protein translation. Cell Cycle. 2012;11(7):1374-1382.

31. Gowans GJ, Hardie DG. AMPK: a cellular energy sensor primarily regulated by AMP. Biochem Soc Trans 2014;42:71-75.

32. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia 2017;60:1577-1585.

33. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 2006;66:10269-10273.

34. Qu Z, Zhang Y, Liao M, Chen Y, Zhao J, Pan Y. In vitro and in vivo antitumoral action of metformin on hepatocellular carcinoma. Hepatol Res 2012;42:922-933.

35. White-Al Habeeb NM, Garcia J, Fleshner N, Bapat B. Metformin Elicits Antitumor Effects and Downregulates the Histone Methyltransferase Multiple Myeloma SET Domain (MMSET) in Prostate Cancer Cells. Prostate 2016;76:1507-1518.

36. Liu Y, Zhang Y, Jia K, Dong Y, Ma W. Metformin inhibits the proliferation of A431 cells by modulating the PI3K/Akt signaling pathway. Exp Ther Med 2015;9:1401-1406.

37. He Y, Tan X, Hu H, Wang Q, Hu X, Cai X, Guan Y, Chen B, Jing X. Metformin inhibits the migration and invasion of esophageal squamous cell carcinoma cells by downregulating the protein kinase B signaling pathway. Oncol Lett 2018;15:2939-2945.

38. Li Z, Wang L, Luo N, Zhao Y, Li J, Chen Q, Tian Y. Metformin inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the phosphorylation of Akt. Oncol Lett 2018;15:7948-7954.

39. Crepieux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, Guillou F, Reiter E. The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene. 2001;20(34):4696-4709.

40. Buzzard JJ, Wreford NG, Morrison JR. Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat sertoli cells. Endocrinology. 2003;144(9):3722-3731.

41. Gorga A, Rindone GM, Regueira M, Pellizzari EH, Camberos MC, Cigorraga SB, Riera MF, Galardo MN, Meroni SB. Effect of resveratrol on Sertoli cell proliferation. J Cell Biochem. 2018;119(12):10131-10142.

42. Holsberger DR, Buchold GM, Leal MC, Kiesewetter SE, O'Brien DA, Hess RA, Franca LR, Kiyokawa H, Cooke PS. Cell-cycle inhibitors p27Kip1 and p21Cip1 regulate murine Sertoli cell proliferation. Biol Reprod. 2005;72(6):1429-1436.

43. Beumer TL, Kiyokawa H, Roepers-Gajadien HL, van den Bos LA, Lock TM, Gademan IS, Rutgers DH, Koff A, de Rooij DG. Regulatory role of p27kip1 in the mouse and human testis. Endocrinology. 1999;140(4):1834-1840.

44. Cipriano SC, Chen L, Burns KH, Koff A, Matzuk MM. Inhibin and p27 interact to regulate gonadal tumorigenesis. Mol Endocrinol. 2001;15(6):985-996.

45. Sridharan S, Brehm R, Bergmann M, Cooke PS. Role of connexin 43 in Sertoli cells of testis. Ann N Y Acad Sci. 2007;1120:131-143.

46. Mazaud-Guittot S, Meugnier E, Pesenti S, Wu X, Vidal H, Gow A, Le Magueresse-Battistoni B. Claudin 11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. Biol Reprod. 2010;82(1):202-213.

47. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology. 1988;122(3):787-794.

48. Forcato S, Novi D, Costa NO, Borges LI, Goes MLM, Ceravolo GS, Gerardin DCC. In utero and lactational exposure to metformin induces reproductive alterations in male rat offspring. Reprod Toxicol. 2017;74:48-58.

49. Aafjes JH, Vels JM, Schenck E. Fertility of rats with artificial oligozoospermia. J Reprod Fertil 1980;58:345-351.

50. Amann RP. Detection of alterations in testicular and epididymal function in laboratory animals. Environ Health Perspect 1986;70:149-158.

51. Working PK. Male reproductive toxicology: comparison of the human to animal models. Environ Health Perspect 1988;77:37-44.

FIGURE LEGENDS

Fig.1. Effect of metformin treatment on cell proliferation and apoptosis

A) BrdU incorporation was evaluated in testicular sections from control and MET groups. Representative images of seminiferous tubules showing immunoreactivity for BrdU (brown) are shown. Scale bars represent 25 μm. In the graphs, each bar represents % BrdU-positive Sertoli cells (SC, left panel) or % BrdU-positive germ cells (GC, right panel). Values represent mean±S.D. obtained from 7 animals in each group. *P<0.05 vs control. B) H&E staining of testis from 8-day-old control and MET rats. Scale bars, 50 μm. C) Testicular representative photomicrographs of TUNEL assay. Arrowheads indicate TUNEL-positive cells. Scale bars represent 50 μm. In the graphs, each bar represents the number of TUNEL-positive cells per tubule. Values represent the mean±S.D. obtained from 6 animals in each experimental group.

Fig.2. Effect of metformin treatment on Cyclin D1-D3, Cyclin E1-E2, p21 and p27 mRNA levels in Sertoli cells

Sertoli cells were isolated from control and MET groups on Pnd8. Then total RNA was extracted and RT-qPCR was performed. *Ccnd1-Ccnd3* and *Ccne1-Ccne2* (panel A) and *Cdkn1a-1b* (panel B) mRNA levels were evaluated. Graphics show pooled data from six independent experiments. Results are expressed as the mean±S.E.M of the ratio between relative quantities of target genes and *Actb*. *P<0.05 vs control.

Fig.3. Effect of metformin treatment on P-ACC and P-AKT levels in testis

Testes were homogenized and Western blot analysis was performed using specific antibodies for phosphorylated ACC (P-ACC), total ACC (T-ACC), P-AKT or T-AKT. The upper panels show three representative comparisons between control (C) and MET animals out of six. The lower panels show pooled data of six animals from each group indicating the fold variation in phosphorylation (ratio of P-ACC to T-ACC and of P-AKT to T-AKT in each sample) relative to control. Results are expressed as mean±S.D. *P<0.05; **P<0.01 vs control.

Fig.4. Effect of metformin treatment on *Amh, Ar, Fshr, Gja1 and Cldn11* mRNA levels in Sertoli cells

Sertoli cells were isolated from control and MET groups on Pnd8. Then total RNA was extracted and RT-qPCR was performed. *Amh, Ar, Fshr*, *Gja1* and *Cldn11* mRNA levels were evaluated.

Graphics show pooled data from six independent experiments. Results are expressed as the mean±S.E.M of the ratio between relative quantities of target genes and *Actb*. *P< 0.05 vs control.

Fig.5. Effect of neonatal metformin treatment on sperm parameters in adult animals

Testicular and sperm parameters were assessed in animals from control and MET groups on Pnd90. A) The number of spermatids/testis, DSP/testis and testis weight were measured in samples collected from control and MET groups. Results are expressed as the mean±S.E.M. obtained from 5 animals in each group. *P< 0.05 vs control. B) H&E staining of testis from control and MET rats. Scale bars represent 100 μm. C) Cauda epididymis was collected to assess sperm morphology and motility. H&E staining of epididymal spermatozoa from control and MET groups (left panel). Scale bars represent 50 μm. Results of sperm motility are expressed as the mean±S.E.M. obtained from 5 animals in each group (right panel).

Table 1. Rat-specific primer sets for analysis by RT-qPCR.

Gene	Accession number	Primer sequence	Product length (bp)
Cyclin D1 (*Ccnd1*)	NM_171992.4	5'-CTACCGCACAACGCACTTTC-3' 5'-AAGGGCTTCAATCTGTTCCTG-3'	85
Cyclin D2 (*Ccnd2*)	NM_022267.1	5'-CTGACCAAGATCACCCACAC-3' 5'-CTCTTAGACGGAACTGCTGAAG-3'	99
Cyclin D3 (*Ccnd3*)	NM_012766.1	5'-GAAACCACACCCCTGACTATTG-3' 5'-AGGTCCCACTTGAGCTTCC-3'	113
Cyclin E1 (*Ccne1*)	NM_001100821.1	5'-ACAGCTTATTGGGATTTCAGC-3' 5'-GGAGCAAGCACCATCAGTAAC-3'	103
Cyclin E2 (*Ccne2*)	NM_001108656.1	5'-AGCCAGACTCTCCACAAGAAG-3' 5'-ATTCCTCCAGACAGTACAGGTG-3'	145
p21 (*Cdkn1a*)	NM_080782.3	5'-GTCTTGCACTCTGGTGTCTCA-3' 5'-GCACTTCAGGGCTTTCTCTT-3'	156
p27 (*Cdkn1b*)	NM_031762.3	5'-TTCGACGCCAGACGTAAAC-3' 5'-TTCAATGGAGTCAGCGATATG-3'	126
Actin (*Actb*)	NM_031144.3	5´-TGGCACCACACTTTCTACAAT-3´ 5´-GGTACGACCAGAGGCATACA-3´	189
beta-2 microglobulin (*B2m*)	NM_012512.2	5´-CGTGATCTTTCTGGTGCTTG-3´ 5´-GAGGTGGGTGGAACTGAGAC-3´	143
Anti-Mullerian hormone *(Amh*)	NM_012902.1	5´-TCAAGACCTAGCCACCTTCG-3´ 5´-AAGAGCTGAGGCTCCCATATC-3´	150
Androgen receptor (*Ar*)	NM_012502.1	5´-GTGAAATGGGACCTTGGATG-3´ 5´-GGTGGGAAGTAATAGTCGATGG-3´	103
FSH receptor (*Fshr*)	NM_199237.1	5´-CAGCAAGGTGACAGAGATTCC-3´ 5´-ATGATCCTTTCGGGATGACTC-3´	95
Claudin 11 *(Cldn11*)	NM_053457.2	5´-TGGTCTCTACCACTGCAAGC-3´ 5´-CCAGAACTGAGGCAGCAATC-3´	95
Connexin 43 (*Gja1*)	NM_012567.2	5´-ACTTCAGCCTCCAAGGAGTTC-3´ 5´-ATGTCTGGGCACCTCTCTTTC-3´	79

Table 2. Body weight and blood glucose levels of animals from Control and MET groups.

	Control	MET
Body weight (g)		
On Pnd3	8.7±1.2	8.8±0.9
On Pnd8	15.3±1.8	15.1±0.7
Blood glucose (mg/dL)	108±11	117±12

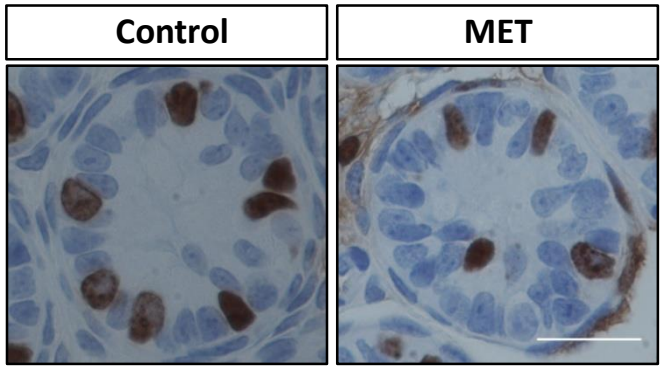
Values represent mean±S.D. obtained from 12 rats in each experimental group.

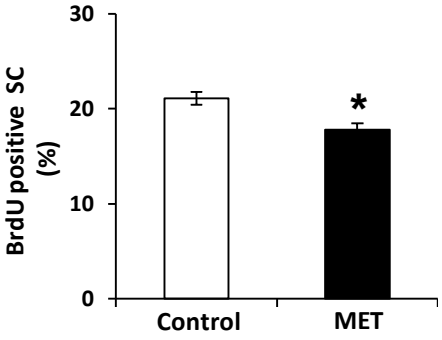
Table 3. Morphometrics parameters in Control and MET groups on Pnd8.

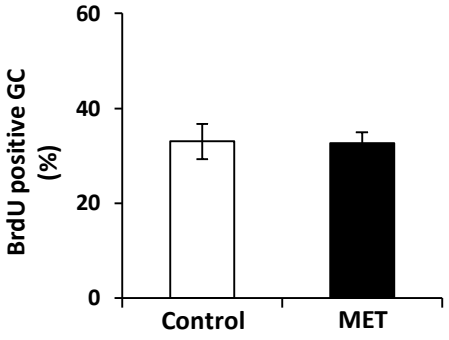
	Control	MET
Testis weight (mg)	13.4±2.0	14.2±3.5
Tubule diameter (μm)	49.7±2.6	50.3±2.7

Values represent mean ± S.D. obtained from 7 rats in each experimental group.

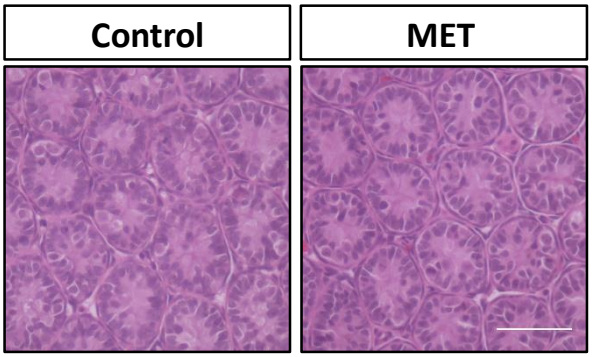
A


Control
MET

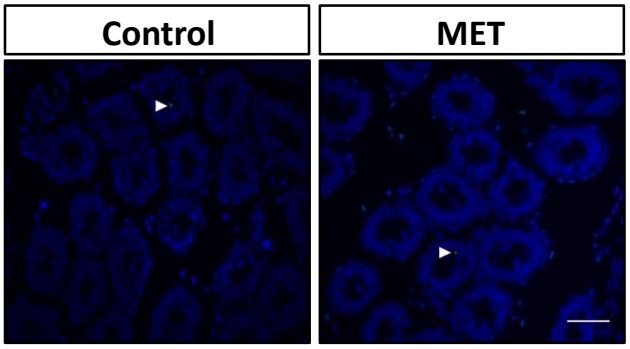

BrdU positive SC (%)
30
20
10
0
*
Control
MET

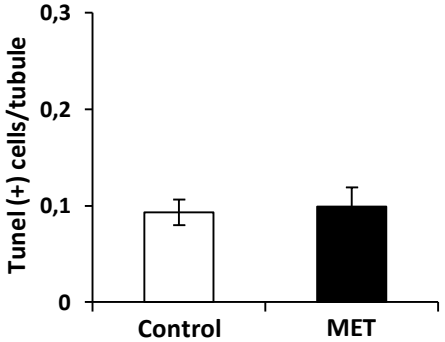

BrdU positive GC (%)
60
40
20
0
Control
MET

B


Control
MET

C


Control
MET


Tunel (+) cells/tubule
0,3
0,2
0,1
0
Control
MET