Metformin exposure affects human and mouse fetal testicular cells

P. Tartarin 1,†, D. Moison 2,3,4,†, E. Guibert 1, J. Dupont 1, R. Habert 2,3,4, V. Rouiller-fabre 2,3,4, N. Frydman 2,3,4,5, S. Pozzi 5, R. Frydman 5, C. Lectureuil 2,3,4,†, and P. Froment 1,†,*

1INRA UMR 85, Unité de Physiologie de la Reproduction et des Comportements, Nouzilly F-37380, France 2Univ Paris Diderot, Sorbonne Paris Cité, Laboratory of Development of the Gonads, Unit of Stem Cells and Radiation, BP 6, Fontenay-aux-Roses 92265, France 3CEA, DSV, ICRM, SCSCR, LDRG. Fontenay-aux-Roses 92265, France 4INSERM, Unité 967, Fontenay-aux-Roses 92265, France 5Service de Gynécologie-Obstétrique, Université Paris Sud, Hôpital Antoine Béclère, Clamart, France

*Correspondence address: Tel: +33-2-47-42-78-24; Fax: +33-2-47-42-77-78; E-mail: pfroment@tours.inra.fr

Submitted on November 16, 2011; resubmitted on May 29, 2012; accepted on June 14, 2012

BACKGROUND: Metformin is a drug used in the treatment of diabetes and of some disorders related to insulin resistance, such as polycystic ovary syndrome. Gestational diabetes can cause complications for both mother and child, and some studies have shown a beneficial effect of metformin during pregnancy without an increase in perinatal complications. However, the effects on the gonads have not been properly studied. Here we investigated the effect of metformin administered during pregnancy on the development and function of the fetal testis.

METHODS: A dual approach in vitro and in vivo using human and mouse models was chosen. Cultures of human and murine organotypic testes were made and in vivo embryonic testes were analysed after oral administration of metformin to pregnant mice.

RESULTS: In human and mouse organotypic cultures in vitro, metformin decreased testosterone secretion and mRNA expression of the main factors involved in steroid production. In vitro, the lowest observed effect concentration (LOEC) on testosterone secretion was 50 μM in human, whereas it was 500 μM in mouse testis. Lactate secretion was increased in both human and mouse organotypic cultures with the same LOEC at 500 μM as observed in other cell culture models after metformin stimulation. In vivo administration of metformin to pregnant mice reduced the testicular size of the fetal and neonatal testes exposed to metformin during intrauterine life. Although the number of germ cells was not affected by the metformin treatment, the number of Sertoli cells, the nurse cells of germ cells, was slightly yet significantly reduced in both periods (fetal period: P = 0.007; neonatal period: P = 0.03). The Leydig cell population, which produces androgens, and the testosterone content were diminished only in the fetal period at 16 days post-coitum.

CONCLUSIONS: This study showed a potentially harmful effect of metformin treatment on the development of the fetal testis and should encourage future human epidemiological studies.

Key words: metformin / fetal testis / steroidogenesis

Introduction

Metformin, a biguanide, is the oldest insulin sensitizer prescribed in the therapeutic management of type 2 diabetes mellitus and most used for reproductive abnormalities associated with insulin resistance (Diamanti-Kandarakis et al., 2010). Although the molecular mechanisms involved in metformin activity are not completely understood, it has been reported that metformin is transported into cells by organic cation transporters and could inhibit the respiratory complex I and/or activate the energy sensor AMP-activated protein kinase (AMPK) as described in hepatocytes and muscle (Zhou et al., 2001; Vander Heiden, 2011). Modifications to mitochondrial coupling and the cellular energy state (ATP/AMP ratio) induced by metformin could, at least in part, activate AMPK, switching off the anabolic process, and could be an explanation for its pleiotropic actions. However, different studies have demonstrated that metformin might act independently of AMPK, considering a direct regulation of the gluconeogenesis flux or a short-term inhibition of the mammalian TOR complex I by metformin (Foretz et al., 2010; Kalender et al., 2010).
Although the liver is the primary target organ, metformin can also act on other tissues including reproductive tissues. For 20 years, insulin-sensitizing drugs, such as metformin, have also been used for reproductive abnormalities associated with insulin resistance (Diamanti-Kandarakis et al., 2010) such as polycystic ovary syndrome (PCOS), a common endocrine disorder affecting women of reproductive age with a prevalence of up to 10%. In these patients, considering ovulation induction or IVF in women with PCOS, metformin co-administration improves insulin sensitivity, the ovulation rate, the pregnancy outcome and reduces the risk of ovarian hyperstimulation syndrome (Diamanti-Kandarakis et al., 2010). Although the available evidence is still insufficient to establish the benefit of metformin use during pregnancy (Legro, 2010), some studies propose that the adverse impact of insulin resistance during pregnancy (risk of early miscarriage, gestational diabetes) could be ameliorated by metformin therapy (Jakubowicz et al., 2002; Vanky et al., 2005; Thatcher and Jackson, 2006; Glueck et al., 2011). However, metformin could directly affect fetal physiology and embryonic development. Indeed, in ex vivo model systems, metformin was shown to cross the human placenta (Kovo et al., 2008) and it has been detected in the umbilical cord blood at the same concentrations as in the maternal venous blood (Charles et al., 2006). A meta-analysis made by Gutzin et al. (2003) concluded that metformin used during the first gestational trimester did not induce major neonatal malformations. Nevertheless, Carlsen and Vanky (2010) have reported an increase in sex hormone-binding globulin (SHBG) levels in newborn babies exposed to metformin during the first trimester of pregnancy. SHBG is a glycoprotein that binds androgens and estradiol and modulates their biological effects. The available evidence is still inadequate to establish the safety of fetuses upon metformin exposure. In particular, the effects of metformin exposure on the fetal testis, which can occur as early as the first trimester, a sensitive phase for male reproductive development, have never been studied.

Spermatogenesis is a continuous process in the adult testis and results from the interactions between germ cells, nurse Sertoli cells and steroid-secreting Leydig cells, beginning in the fetal period. The first morphological sign of testicular differentiation is the maturation of Sertoli cells, leading to the formation of testicular cords, 11.5 days post-coitum (dpc) in mouse and between 6 and 7 weeks post-fertilization in humans. Fetal Leydig cells then secrete testosterone from 12 dpc in mice (Tapanainen et al., 1981; Habert et al., 2001; Lambrot et al., 2006a). Inside the semiferiferous cords, germ cells called gonocytes proliferate and give rise to spermatogonial stem cells during post-natal life. The integrity of gonocytes is crucial for fertility in adulthood and for avoiding the transmission of genetic alterations. An altered fetal androgen production interferes with masculinization and can result in disorders of sexual differentiation or phenotypic sex reversal. These disorders are known to be risk factors for low sperm counts and development of testicular cancer in adulthood (Skakkebaek et al., 2001).

Metformin exposure might affect fetal testicular development, for example in the studies reported above and in recent work describing a decrease in steroid production after metformin administration in ovarian granulosa cell culture (Tosca et al., 2007). To test this hypothesis, two complementary approaches were used in this study: (i) using fetal human and mouse testis organ cultures tested for toxicological studies (Lambrot et al., 2006a) and (ii) using mice to evaluate the long-term consequences of fetal exposure in vivo; we administered metformin to pregnant mice and analysed the effect on the fetal or neonatal testes of male offspring.

**Materials and Methods**

**Chemicals and solutions**

The culture medium was phenol red-free Dulbecco’s Modified Eagle Medium/Ham F12 (1:1; Invitrogen, Carlsbad, CA, USA) supplemented with 80 μg/ml gentamicin (Invitrogen). Ovine LH (oLH; NIH-LH S19; 1.01 IU/mg) was a gift from Dr A.F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA) and used for mouse cultures, and LH from the human pituitary (100 ng/ml) was used in human cultures (≥5,000 IU/mg; Sigma). Metformin was purchased from Sigma Aldrich (St Louis, MO, USA).

**Animals**

Mice (NMRI, Charles Rivers, France) were housed under controlled photoperiod (lights on 08:00–20:00) with ad libitum access to tap water. All animal studies were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals. Males were caged with females and the morning of vaginal plug identification was designated as embryonic day 0.5 of pregnancy (E0.5).

**Organ culture**

Culture of human fetal gonads

Human fetal gonads were obtained from pregnant women referred to the Department of Obstetrics and Gynaecology at the Antoine Béclère Hospital, Clamart (France) for legally induced abortion in the first trimester of pregnancy, i.e. from the 7th until the 12th week post-conception, as previously described (Lambrot et al., 2006b). The women were informed in a leaflet which they signed that a scientific programme could be carried out on their fetus if they gave their consent. Ethical approval was obtained from the Antoine Béclère Hospital Ethics Committee and from the Agence de la biomédecine (French Bioethics Law of 6 August 2004).

We investigated whether metformin exposure in utero could affect the activity of the human fetal gonad. Thus, each human gonad was cut into small pieces: three pieces of human tissues were placed on 10-mm-diameter Millicell CM filters (Millipore, Billerica, MA) in the same conditions as described for the culture of mouse fetal testis. Testis fragments were precultured for 1 day in a control medium and then for 3 days in the absence or presence of metformin at different concentrations (50, 500 μM, 1 and 5 mM). The 50 μM metformin concentration is in the range of concentrations measured in human plasma during therapeutic treatment (Wilcock and Bailey, 1994; Robert et al., 2003).

**Culture of mouse fetal gonads**

Organ cultures were prepared as previously described (Lambrot et al., 2006b; Livera et al., 2006). Briefly, on Day 13.5 of pregnancy (E13.5), intact testes were recovered without their mesonephros. They were placed on 10-mm-diameter Millicell CM filters (Millipore, Billerica, MA, USA; pore size 0.45 μm) floating on 320 μl of culture medium in tissue culture dishes. Testes were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO₂. The medium was changed every 24 h and the culture was continued for 3 days (Supplementary data 1A). Testes were cultured in the presence of LH (100 ng/ml) and exposed or not (control) to different metformin doses between 50 μM (therapeutic dose) and 5 mM (high dose). At the end of the culture, testes were
fixed in formalin, embedded in paraffin and cut into 5-μm sections. For RNA analysis, a pool of at least three testes was immediately dry frozen with liquid nitrogen and stored at −80°C. The entire media samples were kept at −20°C until use for testosterone and lactate radioimmunoassays.

Mouse sertoli cell culture and cell proliferation
As previously described (Froment et al., 2007), Sertoli cells were prepared from live Swiss 1 dpp neonate male mice for each culture. Sertoli cells were isolated by two successive collagenase digestions (Sigma) and a 0.1% hyaluronidase treatment (Sigma). Cells were cultured in chamber slides (2 × 104 cells/chamber; Nunc, Naperville, IL, USA) in HEPES-buffered F12/Dulbecco’s modified Eagle’s medium (PAA, Austria) with 1% fetal calf serum at 33°C in a humidified atmosphere of 5% CO₂ in air. Sertoli cells were stimulated with increasing concentrations of metformin for 48 h and then labelled for 24 h with 10 μM BrdU (Sigma) and fixed for 10 min in 4% paraformaldehyde/phosphate-buffered saline. By indirect immunofluorescence, BrdU-positive cells were identified and counted with a minimum of 500 cells in each condition. Results correspond to the average of two independent experiments.

In vivo treatments
The first 13 days of gestation in mice is the period corresponding to the first trimester of pregnancy in women. This period is also a sensitive period for the testis development, due to the development of seminiferous cords, and proliferation and apoptosis of germ cells which occur until E15.5 in mice. For in vivo studies on neonatal testes, metformin or saline were orally administered by one daily oral gavage or saline (control group) was administered by one daily oral gavage to neonatal testes. Metformin (300 mg/kg/day) is needed to obtain a similar therapeutic effect in diabetic animals as in humans (50 μM; Foretz et al., 2010; Wilcock and Bailey, 1990).

For testicular morphometry, the ellipsoid volume: 0.71 × length² × width as described in Rivkees et al. (1987).

Histological analysis of testes
Sectioned testes were stained with haematoxylin and eosin and the diameters of round transverse sections of seminiferous tubules (at least 30 measurements per testis) were measured using an ocular measuring device. The size of the testis estimated was used for determining the ellipsoid volume: 0.71 × length² × width as described in Rivkees et al. (1987).

Testicular immunohistochemistry and cell counting
Serial sections were mounted on slides, deparaffinized and rehydrated. They were then immunostained by a standard procedure as previously described (Livera et al., 2006). Primary antibodies used were the goat anti-AMH antibody (anti-Müllerian hormone, 1:500, Santa Cruz Biotechnology, CA, USA), the rabbit anti-3βHSD antibody (1:500; Santa Cruz Biotechnology), the rabbit anti-mVASA antibody (1:500; Abcam, Cambridge, UK), and the goat anti-GATA4 antibody (1:200 Santa Cruz Biotechnology). The primary antibody was detected by incubation with an appropriate biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) followed by incubation with avidin–biotin peroxidase complex (Vector Laboratories). Then, 3,3′-diaminobenzidine (DAB; Vector Laboratories) was used as the chromogen and haematoxylin was used as the nuclear counterstain. Negative controls were incubated with goat or rabbit IgG (data not shown). All counts were performed blind using Histolab analysis software (Microvision Instruments, Evry, France).

Detection of apoptosis: TUNEL and caspase 3 activity
Deparaffinized sections were hydrated, permeabilized with Proteinase K and in situ end labelling of nuclear DNA fragmentation was performed as described in the instruction manual for terminal deoxynucleotidyltransferase-mediated deoxyUTP nick-end labelling (TUNEL; FragEL kit, Calbiochem, VWR, West Chester, PA, USA). Negative controls were free of terminal deoxynucleotidyltransferase. The Caspase 3/7 Glow assays were performed according to the manufacturer’s instructions (Promega).

Leydig cell morphometry
The percent volume occupied by the Leydig cell nuclei was counted in 25 randomly-selected areas per animal (n = 4 from different litters per group), using a 441 point square lattice at × 400 magnification. Because the Leydig cell nucleus is spherical, the volume of the nucleus of a single Leydig cell was calculated from the equation for the volume of a sphere (4/3 × π × r³, where r is the radius of the nucleus of the cell). The total number of Leydig cells per testis was determined from the percentage of the testis volume occupied by Leydig cell nuclei and divided by the mean Leydig cell nuclei volume. Individual Leydig cell volumes were obtained from the nucleus volume and the nucleus/cytoplasm ratio. For each animal, 40–80 Leydig cells per testis section were measured. The mean volume of the Leydig cells was calculated as previously described (Yamamoto et al., 2001).
Testosterone and lactate assays

Testosterone concentrations were determined in duplicate by direct radioimmunoassay as previously described (Habert and Picon, 1984). Lactate concentrations were determined according to a commercial spectrophotometric assay (Lactate Pap, Bio Merieux, Marcy-L’Etoile, France) with a detection limit of 0.07 mmol/l.

Statistical analysis

All values are expressed as means ± SEM. The statistical significance of the difference between the mean values for the treated and untreated testes from the same fetus cultured in vitro was evaluated using the paired Student’s t-test. For data obtained from in vivo experiments, the unpaired Student’s t-test was used to evaluate the difference between the two treatments, saline or metformin administration.

Results

Effects of metformin on in vitro culture of human fetal testes

We first looked at the consequences of metformin stimulation on testosterone, an important hormone secreted during testis development and involved in masculinization. Because metformin is frequently associated with an increase in lactate concentrations in different in vitro and in vivo models and because lactate is also known to be an energy substrate for germ cells, intratesticular lactate levels were also measured. Increasing concentrations of metformin stimulated lactate production in a dose-dependent manner from 500 μM (P < 0.01; Fig. 1A) and the maximal metformin concentration increased lactate secretion about 4.5-fold in the culture medium. Conversely, at the therapeutic dose (50 μM), testosterone concentrations in the culture medium
were reduced by almost 45% ($P < 0.05$) and by 60% at the high dose of metformin (5 mM, $P < 0.01$; Fig. 1B).

**Effects of metformin on in vitro culture of mouse fetal testes**

These effects of metformin on fetal testis activity in human were compared with those in mouse. We used mouse fetal testes recovered at the similar period of differentiation (13.5 dpc; Livera et al., 2006; Supplementary data 1A) and cultured in the same conditions as human fetal testes.

As in the human testis, the lactate concentration was significantly increased (on average by 4-fold, $P < 0.01$) by metformin at 500 μM and 5 mM doses ($P < 0.001$; Fig. 1C). The measurement of mRNA levels of the lactate dehydrogenase enzyme (LDH) which converts pyruvate, the final product of glycolysis, to lactate, after a 5 mM metformin exposure also showed a significant 2-fold increase (mRNA relative quantity metformin/control = 2.08-fold increase; 1.17–3.72; $P < 0.05$). The testosterone secretion by mouse fetal testes after 3 days of culture was reduced by metformin in a dose-dependent manner (Fig. 1D). Nevertheless, no effect of metformin was observed at the therapeutic dose of 50 μM, in contrast to the human testis culture.

**In vitro effect of metformin treatment on steroidogenesis in mouse fetal testis**

Staining with 3β-HSD has revealed no apparent morphological alteration of the Leydig cell population (5 mM, data not shown). Therefore, to complete these in vitro effects on mouse fetal testis, we analysed the expression of genes involved in testosterone biosynthesis. Three key enzymes of steroidogenesis (Cyp11a1, Cyp17a1, 3βHSD), an important cholesterol carrier, Star, and the gonadotrophin receptor (LH/CG receptor) showed a large and significant reduction (by 60–80%, $P < 0.05$) in the messenger RNA levels after a 3-day exposure to metformin (5 mM; Fig. 2). The messenger RNA level of insulin-like-3 (INSL3), a small peptide essential for normal testis descent and produced by Leydig cells, was also dramatically decreased (by 90%, $P < 0.001$) after metformin treatment (5 mM; Fig. 2).

**In vitro effect of metformin on germ and Sertoli cells development in mouse fetal testis**

Because metformin influences androgen production, we have tested the effects of the treatment on germ cells and Sertoli cells (their nurse cells). The mRNA level of mouse homolog Drosophila Vasa (Vasa), a gonocyte marker (Tanaka et al., 2000) was not altered by metformin treatment (5 mM). Regarding Sertoli cell markers, mRNA levels of Sox9, and desert hedgehog homolog (Dhh), were not modified by a 5 mM metformin treatment in mouse fetal testis (Fig. 3A). However, the mRNA and protein levels of anti-Mullerian hormone (AMH), another Sertoli cell marker, appeared to be reduced in mouse fetal testis treated by metformin for 3 days as determined by PCR ($P = 0.06$) and immunohistochemistry (Fig. 3A and B).

**In vivo consequences of metformin treatment during early gestation on fetal and neonatal testes of offspring in mice**

To test the in vivo relevance of these in vitro data, metformin (300 mg/kg/day, mimicking the dose needed in man to obtain a therapeutic effect) or saline was administered by one daily oral gavage starting on Day 0.5 of pregnancy until E16.5 (i.e. 3 days after the stage used for in vitro explant culture). Testes were either removed at E16.5 (short-term exposure analysis, Supplementary data 1B) or analysed on neonates on Day 1 post-partum to investigate possible long-term effects (Supplementary data 1C).

Metformin treatment had no effect on the size of the litermates (control: 11 ± 0.8; metformin: 10.9 ± 0.8, $P = 0.9$) or on the male/female ratio (control: 48.9 ± 3.9; metformin: 48.6 ± 4.6, $P = 0.9$). However, metformin induced a testicular volume decrease at 16.5 dpc and this was confirmed at 1 dpp (Fig. 4A, B). At 1 dpp, the diameters of seminiferous tubules were significantly smaller in metformin-exposed males compared with the controls (control: 82 ± 0.7 μm versus metformin: 74.7 ± 0.5 μm, $P < 0.001$). The reduction in size was not due to an increase in cell death, as supported by measurement of TUNEL-positive cells (percentage of seminiferous tubules with at least one TUNEL-positive nucleus, control: 7.2 ± 1.6% versus metformin: 4.1 ± 0.9%; $n = 6$ testis in three different litters per treatment, $P = 0.11$) and by quantification of the caspase-3/7 activity by luminescent caspase-Glo 3/7 assay (control 260.4 ± 31.1 relative luminescence unit (RLU)/mg protein of testis, $n = 21$; versus metformin: 230.0 ± 20.7 RLU/mg protein of testis, $n = 26$, $P = 0.2$).

In contrast to the in vitro results, metformin did not significantly affect the intratesticular lactate concentration (compared with saline exposure; Fig. 4C).
In vivo consequences of a metformin treatment on steroidogenesis and on Leydig cell populations in fetal and newborn testis

Compared with the in vitro results, testicular testosterone contents in male newborns exposed in utero to metformin were not significantly decreased, even though in the short-term the testosterone concentration was lower than that in the controls ($P = 0.05$; Fig. 5A) and was associated with a significant decrease in the number of Leydig cells per testis ($P < 0.05$) in male fetuses exposed to metformin (Fig. 5B). This reduction was not observed at birth (Fig. 5B). To complete this analysis, the mean volume of the Leydig cells was not modified in either case, suggesting no hypoplasia (Fig. 5C).

In vivo consequences of a metformin treatment on Sertoli and germ cell populations in fetal and newborn testis

Finally, in agreement with the limitation of the testis growth, metformin exposure in utero reduced the Sertoli cell population (GATA4 positive cells) at 16.5 dpc ($P < 0.01$) and this decrease was still present at birth ($P < 0.05$; Fig. 6A). Despite a strong interaction of

---

**Figure 3** Effects of metformin on the expression of germ and Sertoli cell markers in murine fetal testis in culture. (A) After 72 h of culture of fetal mice testis in the absence or presence of metformin (5 mM), levels of mRNA-encoding germ cells marker (Vasa) and Sertoli cell markers (Sox9, Dhh, AMH) were measured by qRT–PCR. Results (mean ± SEM; $n = 3$ independent experiments with 2–6 testes per condition) were normalized by a reference gene, β-actin, as the endogenous control. Results are presented as a percentage of the control testes. (B) Representative microphotographs of AMH immunostaining after 72 h of culture in the absence (control) or presence of 5 mM metformin.
Sertoli cells with germ cells (Petersen and Soder, 2006), no effect was observed on the population of germ cells (Fig. 6B). Moreover, a culture of mouse Sertoli cells prepared from neonate pups (1 dpp) and incubated with increasing doses of metformin showed a significant and dose-dependent decrease in cell proliferation compared with the control (P, 0.01; Fig. 6C).

**Discussion**

The results described in this study have shown that metformin can decrease testosterone production in vitro and probably in vivo during exposure, and that in vivo testicular size and the Sertoli cell population, which is absolutely required for germ cell development, were reduced. Because human tissue is too precious and not easily available, and the measurement of the effects of metformin exposure on human fetal testes is not feasible, we have compared the human and mouse organotypic system and combined in vitro and in vivo approaches to better evaluate the consequences of testicular exposure to metformin.

Organotypic system cultures using human and mouse testis demonstrated similar effects of metformin on testosterone and lactate production. However, the human testis is more sensitive to metformin than is the mouse testis. Hence, only a 50 μM of metformin concentration in human culture (the concentration measured in patients’ blood) leads to a 45% decrease in testosterone secretion, whereas 500 μM of metformin is needed in mouse culture to obtain a 20% decrease.
testosterone secretion is unaffected by addition of mono-(2-ethylhexyl) phthalate in human fetal testes, a large reduction in androgen secretion has been measured in mouse and rat fetal testes (Lambrot et al., 2009; Lehraiki et al., 2009; Chauvigne et al., 2011). These results highlight the need for rigorous comparisons between humans and rodents. However, the *in vitro* effects of metformin in mouse and human fetal gonads fit well with the *in vivo* effects described after metformin administration to pregnant mice. Furthermore, we have to note that metformin pharmacodynamics may be affected by pregnancy-related changes such as modification in renal filtration or enhanced insulin resistance, so the elimination half-life of metformin could be a little longer (Hebert et al., 2009) and higher therapeutic doses could be required (Eyal et al., 2010). These observations suggest that blood levels of metformin could be higher in pregnant than in non-pregnant women, and may increase the fetal exposure.

An important consequence of metformin exposure *in utero* was a decrease in the testicular size associated with a reduction in the Leydig and Sertoli cell population at 16.5 dpc. This could be due to a reduction in cell proliferation but not to an increase in cell death as attested by the absence of difference in TUNEL-positive cells or activity of caspase 3 between the two groups. Inhibition of cell proliferation induced by activation of AMPK is now established in different cell types, including metformin (Jones et al., 2005; Rattan et al., 2011). In rat granulosa cells, the activation of AMPK resulted in an increase in p27kip expression, leading to a cell cycle arrest (Kayampany and Menon, 2009), suggesting that metformin may inhibit tests growth by AMPK activation.

During fetal testis development, two main events take place: steroi-dogenesis and gametogenesis. Although the mechanism of metformin action is still unclear, the impact of metformin on steroid production has been reported in women and in cultures of ovarian cells from several species (human, rodents, cows and goats). Hence, stimulation by metformin reduces estradiol, progesterone and even androgen production by human thecal cells (Attia et al., 2001; Tosca et al., 2007). In adolescent girls with precocious puberties, metformin administration reduces androgen excess (Ibanez et al., 2011). Here we reported that metformin exposure decreased androgen production in fetal testis cultured *in vitro* and slightly decreased androgen production in vivo during mouse pregnancy. In mice, fetal testis cultured for 3 days, as in the ovarian cell culture cited previously, metformin targeted the gene expression of proteins involved in steroid production (Cyp11a1, 3βHSD, aromatase, Star) as shown by qRT–PCR analysis, or at the protein activity level. Indeed in rat granulosa cells, metformin reduced estradiol and progesterone after only 3 h of stimulation (Tosca et al., 2006). Moreover, at 16 dpc, the reduced Leydig cell population in the testes from fetuses exposed to metformin is another factor to limit the intratesticular testosterone content.

In addition, the reduction of testosterone production by the fetal testis may have a significant impact on the development of the male reproductive tract, as has been shown Welsh et al. (2008). They identified in rats a programming window during which androgen action is essential for masculinization of the reproductive tract. Based on the timing in rats, the programming window in humans is likely to be between 8 and 14 weeks after fertilization (Welsh et al., 2008).

Since metformin decreased testosterone secretion by human fetal testicular tissues collected during the first trimester of pregnancy *in vitro*.

---

**Figure 6** Analysis of fetal and neonatal germ cells and Sertoli populations after a maternal metformin exposure. (A) Number of Sertoli cells revealed by GATA-4 immunostaining and (B) number of germ cells revealed by mVASA immunostaining were evaluated. Results are expressed as a percentage of control values and shown as the mean ± SEM. (C) Sertoli cell proliferation was evaluated *in vitro* by BrdU incorporation in cultures of 1 dpp male mice Sertoli cells, in the presence (50 μM to 5 mM) or absence of metformin. Results of *n* = 3 independent experiments. Results are represented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group.

---

decrease in testosterone release. This difference in sensitivity between the two culture models (mouse and human) has already been observed for other chemical molecules, despite similar experimental conditions (Lambrot et al., 2006a). For example, the human fetal germ cells are more sensitive to uranium and cadmium than mouse germ cells (Angenard et al., 2010; Angenard et al., 2011). While
at the therapeutic dose detected in blood, we can suggest an alteration of masculinization following metformin treatment during pregnancy.

This question arises especially as intratesticular testosterone levels and Leydig cell populations appeared to be reduced in mice at E16.5 after in utero metformin exposure. The absence of an effect on testosterone production in newborns treated during the first two-thirds of their fetal life is not aberrant. Indeed, the treatment was stopped 7 days before birth. This could be in accordance with the observation that in humans, maternal and newborn androgen and estrogen levels are not affected at birth after the administration of metformin during pregnancy. Only SHBG, a protein which binds sex steroid, has been measured to be higher in metformin-exposed newborns (Carlsen and Vanky, 2010). In our model, no alteration in testis descent was observed in newborn mice treated in utero, even though testosterone is decreased and Ins3 mRNA is greatly inhibited by metformin in mouse testicular explants in vitro. Given the results, it would be interesting to measure the anogenital distance and the development of the male tract in adult males, as this is considered as a measure of fetal androgen action.

As explained in the results, metformin is frequently described as being associated with an increase in lactate production. Indeed, metformin has been shown to cause a shift towards anaerobic respiration leading to increased lactate output by tissues. In the human and mouse organotypic testis culture, metformin (500 μM) was shown to increase lactate production 2- to 4-fold. In addition, after metformin administration to pregnant female mice, the fetal intratesticular lactate concentrations were also increased 1.3–1.5-fold but without reaching statistical significance. In the same way, Richardson et al. (2009) described in human granulosa cells a marked increase in lactate production in response to 500 μM metformin exposure. In accordance with this production, we found a direct 2-fold increase in the mRNA level of the lactate dehydrogenase LDH, which catalyses the interconversion of pyruvate to lactate. A similar stimulation of the LDH expression has also been described in rat Sertoli cells after a stimulation by another AMPK inducer, the aminoimidazole carboxamide ribonucleotide (Galaro et al., 2007). Nevertheless, the effects on testicular function of an alteration in lactate production before the blood testis barrier establishment, occurring during puberty, seem not to be known.

Interestingly, the Sertoli cell population determines spermatogenesis efficiency (Petersen and Soder, 2006). Indeed, there is a strong correlation between the number of Sertoli cells, the number of germ cells, sperm production and finally the testicular volume. At least two hypotheses can be proposed to explain the modification of Sertoli cell number observed in this study. It could be due to a decrease in stimulation by follicle-stimulating hormone (FSH), an important hormone controlling Sertoli cell proliferation, either as a decrease in FSH secretion by the pituitary or FSH sensitivity at the gonad levels. Recently, Kayampilly and Menon (2012) have reported that activation of AMPK in rat granulosa cells inhibits FSH-mediated ERK activation and induces a reduction in granulosa cell proliferation. Other studies have demonstrated that metformin treatment can reduce GnRH neuron activity and reduces LH and FSH secretion induced by GnRH (Tosca et al., 2011). Alternatively, and more probably, metformin can directly affect Sertoli cell function as suggested by the 1 dpp Sertoli cell culture, where metformin induced an inhibition of cell proliferation in a dose-dependent manner and by a reduction in AMH expression in organotypic mice cultures. Hence, analyses in adult mice should be done to determine if sperm production, correlated with the Sertoli cell population, is affected by the fetal metformin exposure, although the number of mouse germ cells did not seem to be affected in our study.

Finally, recent data have shown that the use of insulin sensitizer and metformin during the first 12 weeks of gestation, or longer, reduces the development of gestational diabetes and does not influence the health of babies, and no obstetric complications or congenital anomalies were described (Glueck, et al., 2004). However, a recent review mentions an increase in the proportion of premature births in women treated with metformin, compared with those treated with insulin (Wensel, 2009). In conclusion, despite the apparent efficacy of the oral metformin treatment in the human patient, which is a more user friendly alternative to the present treatment, our data have shown some alterations in the fetal testicular function due to metformin at concentrations equal to or 10 times higher than therapeutic concentrations. Hence, epidemiological studies should be carried out to determine the possible consequences of these alterations on adult fertility.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank Vanaique Guillory and all the staff of the animal facilities for expert animal care and A. Gouret for secretarial assistance. We would also like to thank the staff of the Department of Obstetrics and Gynaecology of the Antoine Béclère Hospital (Clamart, France), V. Muczynski, T. N’Tumba-Byn and M. Elzaiat for their help with the laboratory work, S. Messiaen for immunohistochemistry advice and C. Racine and J. Bernardino-Sgherri for helpful discussions.

Authors’ roles

P.T., D.M., C.L. and P.F. designed the study, interpreted the results, contributed to the writing and editing of the paper and gave final approval. E.G. and J.D. collected data and carried out cell culture. R.H. and V.R.-F. interpreted the results, contributed to the writing and editing of the paper. N.F., S.P. and R.F. prepared samples, collected data.

Funding

P.T. is a PhD student supported by the Ministère de l’Enseignement Supérieur et de la Recherche. This work was supported by the Université Paris Diderot-Paris 7, Commissariat à l’énergie atomique (CEA), Institut National de la Santé Et de la Recherche Médicale (INSERM), Institut National de la Recherche Agronomique (INRA) and was a part of the national programme, FERTINERGY, funded by the French National Research Agency. All the authors have nothing to declare.
Conflict of interest

None declared.

References


Attia GR, Rainey WE, Carr BR. Metformin directly inhibits androgen production in human testicular cells. Fertil Steril 2001;76:517–524.


Kayampilly PP, Menon K. Follicle-stimulating hormone inhibits adenosine S'-monophosphate-activated protein kinase activation and promotes cell proliferation of primary granulosa cells in culture through an Akt-dependent pathway. Endocrinology 2009;150:2929–2935.


Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome—an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 2001;16:972–978.


Thatcher SS, Jackson EM. Pregnancy outcome in infertile patients with polycystic ovary syndrome who were treated with metformin. *Fertil Steril* 2006;85:1002–1009.


