

Effect of mitotane on mouse ovarian follicle development and fertility

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Abstract

Mitotane (MTT) is an adrenolytic drug used in advanced and adjuvant treatment of adrenocortical carcinoma, in Cushing's disease and in ectopic syndrome. However, knowledge about its effects on the ovary is still scarce. The purpose of this study is to investigate the effect of MTT on the ovary using *in vivo* and *in vitro* models. The study was performed in CD1 mice and in the COV-434 human ovarian granulosa cell line. We examined ovarian morphology, follicle development, steroidogenesis and procreative function in mice and the effect of MTT on cell growth *in vitro*. Our results revealed that treatment of CD1 mice with MTT induces a decrease in early antral follicles with a subsequent increase in the secondary follicles, measured by the increased levels of anti-Mullerian Hormone ($P < 0.05$) and decreased levels of FSH receptor ($P < 0.05$). Moreover, we observed a significant decrease in *Cyp11a1* ($P < 0.01$) and *Cyp17a1* ($P < 0.001$) mRNA level in MTT-treated animals. Ovulation, induced by PMSG/hCG stimulation, was also significantly impaired, with a reduction in the number of ovulated oocytes ($P < 0.01$) and fewer corpora lutea in treated animals. Likewise, the mating experiment demonstrated a delay in the time of conception as well as fewer pups per litter in MTT-treated mice ($P < 0.05$). Experiments performed on the COV-434 cell line showed a significant inhibition of growth followed by apoptosis ($P < 0.01$). In conclusion, our study highlights the key points of ovarian folliculogenesis affected by MTT and demonstrates impairment of the ovulation process with a negative impact on conception, which is nevertheless preserved.

Key Words

- ▶ mitotane
- ▶ adrenocortical carcinoma
- ▶ ovary
- ▶ fertility

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Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine cancer with a poor prognosis (Else *et al.* 2014). Its incidence ranges from 1 to 2 million people per year (Else *et al.* 2014). ACC incidence is bimodal with a first peak in the first decade and another in the fourth or fifth decade (Else *et al.* 2014). However, despite the rarity of this cancer, it is more

common in females with a sex ratio of 1.5, and it occurs in women of childbearing age (Else *et al.* 2014). The clinical presentation of ACC is frequently characterized by overt Cushing's syndrome (Fassnacht *et al.* 2011, Else *et al.* 2014). Fertility and the resulting pregnancy rarely occur in Cushing's syndrome because this condition includes

symptoms of amenorrhea, oligomenorrhea, infertility and high spontaneous abortion rate (Lindsay *et al.* 2005, Abiven-Lepage *et al.* 2010). Management of ACC is still a challenge for endocrinologists, and fertility linked to clinical and hormonal therapy has scarcely been investigated. At the moment, surgery is the most useful weapon in the treatment of this unpredictable cancer (Fassnacht *et al.* 2011, Stigliano *et al.* 2016) and its hormonal features. Adjuvant therapy in ACC is supported by frequent observations of locoregional or distant metastases in at least one-third of patients after radical adrenalectomy (Stigliano *et al.* 2016). Despite several therapeutic strategies that have been tested in the treatment of the ACC, the only drug that has demonstrated some effectiveness in countering the tumor growth is mitotane (1,1-dichloro)-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane or *o',p'*-DDD; MTT) (Stigliano *et al.* 2016). MTT is a compound derived from the insecticide DDT and is able to inhibit 11 β -hydroxylase and P450 side-chain cleavage (P450_{sc}) in the mitochondria of steroidogenic cells (Touitou *et al.* 1978). Therefore, its action results in a subsequent block of cortisol synthesis with a reduction in steroid levels in the plasma and urine (Baudry *et al.* 2012). Although the effect of MTT on adrenal tissue has been investigated, little is known about the steroidogenic effect on ovarian function. There is still no information regarding the effects of MTT on ovarian physiology, and there are only few data on childbearing potential and pregnancy outcome. Currently, endocrinologists cannot provide indications on the steps to be taken in those cases in which the patient wishes to become pregnant after illness. In this study, we tested the effect of MTT on murine ovarian cells, and we used a human ovarian cell line as an *in vitro* model to examine the effects of the pharmacokinetics and toxicity of MTT on female gonads.

Materials and methods

Animals

CD1 mice (Charles River) were housed under controlled temperature (25°C) and light conditions (12h light/day) with access to food and water *ad libitum*. Immature animals, at twelve days of age, were treated for up to 18 days with 0.152 mg/kg MTT (Sigma-Aldrich). The drug was resuspended in dimethyl sulfoxide (DMSO) and administered in a final volume of 20 μ L per day per animal via intraperitoneal injection. Healthy mice were treated with the same volume of the vehicle alone (DMSO).

The animals were weighed daily and killed at the end of treatment by cervical dislocation. All animal procedures were approved by the Local Ethics Committee for Animal Research.

The ovaries were quickly removed, fixed in Bouin's liquid (for morphological evaluation) or 4% paraformaldehyde (PFA, for immunofluorescence study), embedded in paraffin, serially sectioned at 6 μ m and stained with carmalum or processed for immunofluorescence.

To study the RNA, after 18 days of treatment, whole ovaries were removed, punctured with a 25-gauge needle and gently pressed to release granulosa cells (GCs) from the largest follicles. The residual tissue (theca/interstitial cells; TI) was gently scraped to eliminate adherent GCs, transferred to fresh medium and washed vigorously. GCs and TI cells were centrifuged at 250g for 5 min and resuspended in lysis buffer for RNA extraction (RNeasy Kit, Qiagen SpA).

Morphological studies

We performed follicle counts on every fifth section of the entire ovary. Growing follicles were categorized as follows: monolaminar follicles with one layer of cuboidal GCs; bilaminar follicles, surrounded by two complete layers of cuboidal GCs; preantral follicles with several layers of GCs and no antrum; early antral follicles with vacuolization of the intercellular spaces among GCs; antral follicles with a fluid-filled cavity, the antrum (Guglielmo *et al.* 2011). In the animals treated with PMSG/hCG to induce ovulation, we performed corpora lutea counts on every fifth section of the entire ovary.

Immunofluorescence

For PCNA (Proliferating Cell Nuclear Antigen) detection, sections obtained from the ovaries of control and treated mice were mounted on Polysine-TM slides (Menzel-Glaser, Braunschweig, Germany). For antigen retrieval, the slides were transferred to a plastic Coplin jar containing citrate buffer (pH 6.0, 10 mmol/L) and then heated in a microwave oven three times for 5 min each. The sections were incubated with 1 M glycine at pH 7.5 for 30 min and then in a blocking solution of 1 \times phosphate-buffered saline (PBS) containing 1% w/v bovine serum albumin (BSA; Sigma-Aldrich) and 5% v/v normal goat serum (Sigma-Aldrich) to minimize non-specific binding. They were then incubated for 20 h at 4°C with 1:200 anti-PCNA rabbit polyclonal IgG (ab2426-1, Abcam). After extensive

washes in PBS, the sections were incubated for 2 h at room temperature with 1:400 Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Invitrogen-Life Technologies). Nuclei were stained using 1:2000 iodide nuclear fluorescent dye 642/661, TOPRO-3 (Molecular Probes, Invitrogen-Life Technologies), and examined by confocal microscope. In control samples, the primary antibody was substituted with rabbit pre-immune serum.

RNA extraction, reverse transcription and real-time PCR

RNA was isolated using a silica gel-based membrane spin column (RNeasy Kit, Qiagen SpA). Aliquots of RNA were assessed for purity and quantification using a Nanodrop ND-100 (Thermo Fisher Scientific). Total RNA (4 µg) was reverse transcribed in a final volume of 20 µL using the M-MLV Reverse Transcriptase kit (Invitrogen-Life Technologies) according to the manufacturer's instructions. cDNA was stored at -20°C. The presence of specific transcripts was evaluated by SYBR Green real-time PCR on an Applied Biosystems 7500 real-time PCR system equipped with 96-well optical reaction plates using SYBR Green Universal PCR Master Mix (Euroclone, Milan, Italy), by adding 0.3 µmol/L of each specific primer to a total volume of 20 µL reaction mixture according to the manufacturer's recommendations. Negative controls contained water instead of first-strand cDNA. Each sample was normalized to its content. Final results are expressed as arbitrary units (a.u.). The primers used are shown in Table 1.

Mating protocol

Four-week-old mice were injected daily with MTT for 4 weeks; control mice were treated with vehicle alone (DMSO) for the same time. After the last injection, female mice were mated with untreated males of proven fertility (two females to one male) for one month. Mating was confirmed by the formation of the vaginal plug. The females were separated until the birth of the litter.

Finally, we evaluated the number of mating days and pups per litter.

Cell cultures

The human ovarian granulosa carcinoma cell line COV-434, derived from a human granulosa cell tumor, was kindly provided by Dr S H van der Burg (Department of Clinical Oncology, University Medical Center at Leiden, the Netherlands). Cells were authenticated by ATCC by STR analysis. COV-434 cells were cultured in RPMI medium (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% v/v fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza Group Ltd). Cells were cultured in a 37°C incubator in an atmosphere of 5% CO₂ in humidified air. For all conditions, cell monolayers were subcultured to 100 mm dishes (0.9 × 10⁶ cells/each), and 24 h after seeding, cells were treated with MTT (5–50 µM). All experiments were repeated at least three times, and each experimental sample was seeded in triplicate.

Trypan blue analysis

Cell number was determined using a hemocytometer, and viability was assessed by the cells' ability to exclude trypan blue. After trypsinization, cells were suspended in PBS and mixed with an equal volume of 0.4% w/v trypan blue in PBS, and the percentage of stained cells was determined.

TUNEL assay

The induction of apoptosis was evaluated by TUNEL assay (Roche Diagnostics) using Flow Cytometry (FCM). All cells were harvested and washed in Dulbecco's Phosphate-Buffered Saline (DPBS) (Lonza Group Ltd). Nearly 500,000 cells per sample were centrifuged and then fixed in 4% formaldehyde (Sigma-Aldrich) in DPBS for 30 min at RT. Finally, samples were washed in DPBS and then incubated with a permeabilizing solution of 0.1% v/v Triton X-100 (Sigma-Aldrich) in 0.1% w/v sodium citrate

Table 1 Primers sequences used in qRT-PCR reaction.

Transcripts	Forward	Reverse
<i>Amh</i>	5'-GCAGGCCCTGTTAGTGCTAT-3'	5'-TCAGGGTGGCACCTTCTCT-3'
<i>Fsh-r</i>	5'-ACAGGGTCTTCCTCTGCCAA-3'	5'-TTTCTCCAGGTCCCAAATCC-3'
<i>Cyp11a1</i>	5'-GGTCCACTCCTCAAAGCCA-3'	5'-GGTCCACTCCTCAAAGCCA-3'
<i>Cyp17a1</i>	5'-GCCAGAGAAGTGCTCGTAA-3'	5'-AATACCAGCTCCGGTGACAG-3'
<i>β-Actin</i>	5'-TGTGATGGTGGGAATGGGTCAGAA-3'	5'-GCTTCTCTTTGATGTCACGCACGATT-3'

(Sigma-Aldrich) for two minutes on ice. Apoptosis was assessed using the In Situ Cell Death Detection Kit, Peroxidase (POD) (Roche Diagnostics), according to the manufacturer's instructions. Positive controls were incubated with 300U DNase for 10min at RT, and the negative control was incubated without the enzyme solution. Samples were then measured at different times (24 and 48h) after MTT treatment using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA).

Western blotting analysis

Total protein extracts were obtained by suspension of cellular pellets in 100µL of extraction buffer containing 1M NaCl, 1M Hepes (pH 7.6), 2M MgCl₂, 0.1M CaCl₂, 0.1M Na₂PO₄, 0.1M phenylmethylsulfonyl fluoride and protease inhibitors and were sonicated on ice, clarified by centrifugation at 20,000g and stored at -80°C. Protein concentrations were determined by the Bradford colorimetric assay (Bio-Rad Laboratories). Fifty micrograms of total protein were electrophoresed in a 10% w/v polyacrylamide gel in the presence of SDS under reducing conditions and transferred onto a nitrocellulose membrane. Blots were blocked for 1h at room temperature with 5% w/v nonfat dry milk in PBS buffer containing 0.1% v/v Tween 20 (T-PBS) (Bio-Rad Laboratories). Treated and untreated cells were incubated with 1:200 anti-cyclin E rabbit polyclonal IgG (sc-198), 1:200 anti-pro-caspase goat polyclonal IgG (sc-1225), 1:200 anti-PARP mouse monoclonal IgG (sc-74470), 1:250 anti-CDK2 (cyclin-dependent kinase 2) mouse monoclonal IgG (sc-6248) (Santa Cruz Biotechnology) or 1:10,000 anti-vinculin rabbit polyclonal IgG (V4139) (Sigma-Aldrich).

To visualize the antigens, membranes were washed with T-PBS and incubated for 1h with the appropriate peroxidase-conjugated secondary antibody (1:4000) (anti-rabbit A0545, anti-mouse A9044 or anti-goat A8919, Sigma-Aldrich) diluted in T-PBS with 5% w/v nonfat dry milk for 1h, and then washed with T-PBS for 1h (all at room temperature). Bound antibodies were visualized using enhanced chemiluminescent detection reagent (ECL) (Thermo Fisher Scientific). Bands were analyzed with the ImageJ (Image Processing and analysis in Java) software program.

Statistical analyses

All experiments were repeated at least three times, and each experiment was performed at least in duplicate.

Statistical analyses were performed using ANOVA followed by the Tukey–Kramer test for comparisons of multiple groups or a two-tailed *t*-test when comparing data derived from two groups. Values with $P < 0.05$ were considered statistically significant.

Results

Mitotane interferes with the development of ovarian follicles

Twelve-day-old prepubertal female mice were treated intraperitoneally with 0.152mg/kg/day MTT or vehicle alone (DMSO) for 17 days. At the end of the 17 days of treatment, the ovaries were retrieved for morphological examination. Macroscopic evaluation of ovaries indicated that treatment with MTT did not produce major alterations in the growing follicles (Fig. 1A).

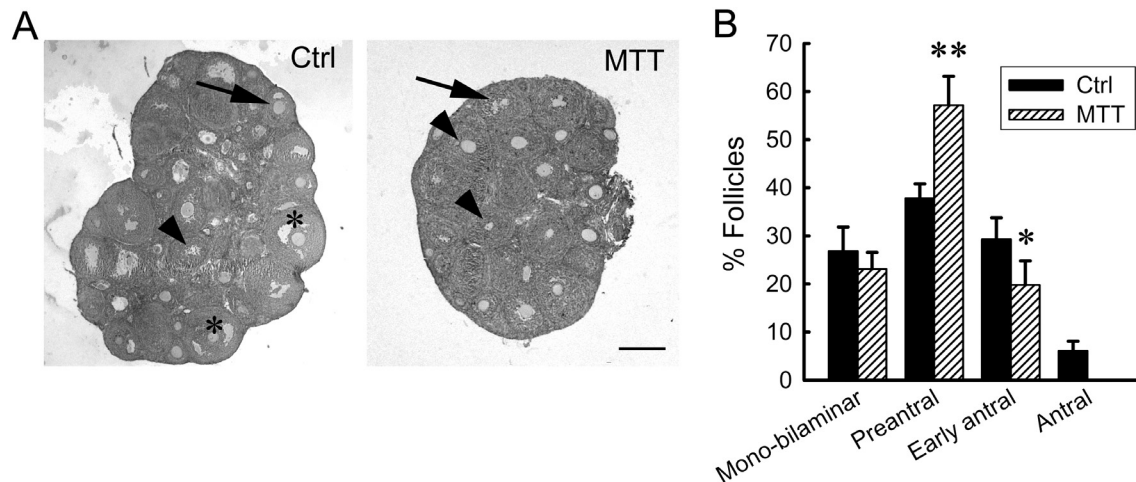
However, in MTT-treated animals, we observed a decrease in the number of early antral follicles (19% in treated vs 28% in untreated mice, $P = 0.05$), a corresponding significant increase in the number of preantral follicles (57% in treated mice vs 38% in untreated, $P < 0.001$), and no antral follicles, suggesting a decreased rate of growth in response to the treatment (Fig. 1B).

To investigate the effect of MTT on ovarian cell proliferation, we performed an immunofluorescence analysis with the proliferation marker PCNA on sections of ovaries obtained from control animals and from mice treated for 17 days with MTT. As shown in Fig. 2, control ovaries showed more proliferating GCs in the growing follicles than ovaries from treated animals (44% of control).

Effect of mitotane on *Amh* and *Fsh-R* expression levels in GCs

In order to validate the efficacy of the GC and TI cell dissection method, we evaluated by real-time PCR the presence of mRNA for specific markers of GCs and TI cells, *Fsh-R* and *Cyp17a1* mRNA, respectively, in the two cell populations obtained from the untreated mice. As shown in Fig. 3A, *Fsh-R* mRNA was found mainly in the GC population, while *Cyp17a1* mRNA was found exclusively in the TI population. These data suggested that in TI cells a low contamination by GCs is present, whilst in the GC population the contamination by TI cells is absent or very low.

To evaluate the effect of MTT on the expression levels of granulosa cell-specific genes implicated in follicle development, such as *Fsh-R* and *Amh*, the mRNA of GCs

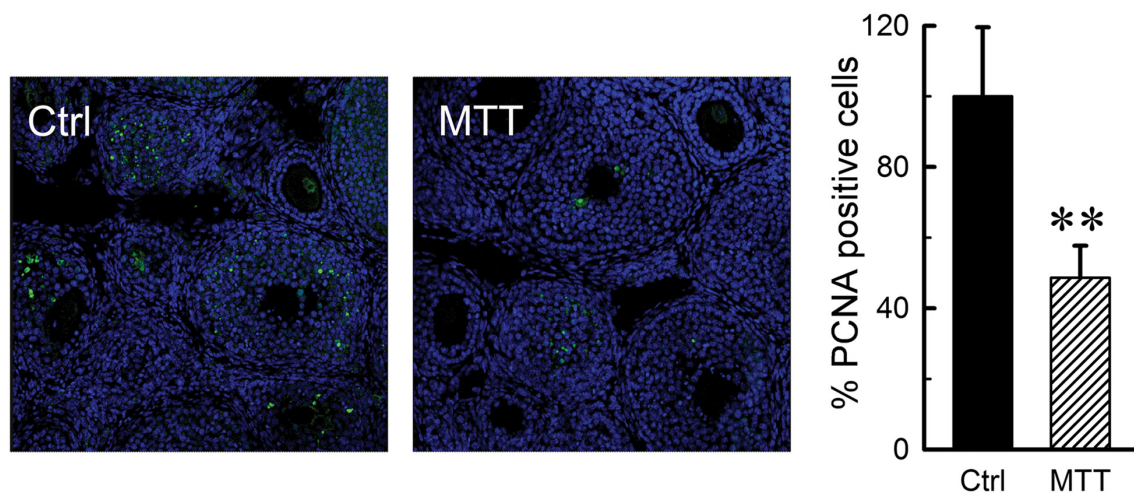
**Figure 1**

Effects of mitotane on follicular growth and maturation in ovaries removed from untreated (Ctrl) or treated animals (MTT). (A) Representative histological sections of ovaries removed after 18 days of treatment from vehicle alone- (Ctrl) and mitotane-treated (MTT) mice. Histological evaluation of ovaries showed no evident morphological changes in treated animals compared to controls. In MTT-treated animals, we observed numerous preantral follicles (arrow heads), a significant decrease in the number of early antral follicles (arrows) and no antral follicles (asterisk). Scale bar = 200 μ m. (B) Counts of follicles at different stages of maturation, categorized as follows: mono-bilaminar follicles with one/two layers of cuboidal GCs, preantral follicles with several layers of GCs and no antrum, early antral follicles with vacuolization of the intercellular spaces among GCs, antral follicles with a fluid-filled cavity, the antrum. Data are expressed as mean \pm s.d. from three independent experiments carried out on a total animal number of Ctrl = 18 and MTT = 18. In MTT-treated animals, we observed a decrease in the number of early antral follicles (19% in treated vs 28% in untreated mice, $P=0.05$), a corresponding increase in the number of preantral follicles (57% in treated mice vs 38% in untreated, $P<0.001$), and no antral follicles. Statistical analysis was performed using ANOVA followed by the Tukey–Kramer test. * $P<0.001$ vs respective Ctrl.

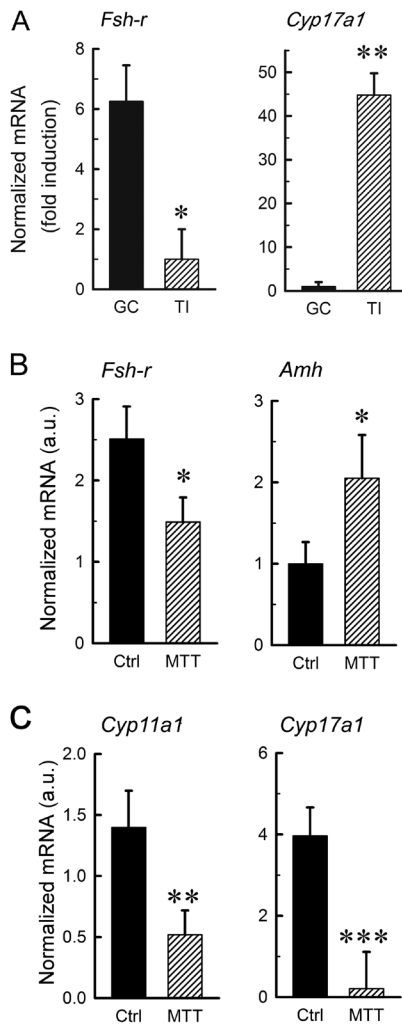
isolated from the ovaries of mice treated for 17 days with MTT or vehicle alone was analyzed by real-time PCR. We observed significantly lower levels of *Fsh-R* mRNA (67% of control, $P<0.05$) and higher levels of *Amh* (137.5% of control, $P<0.05$) in the GCs of treated animals (Fig. 3B).

Mitotane affects *Cyp11a1* and *Cyp17a1* steroidogenic gene expression

We examined the possible effects of MTT on mRNA expression of two steroidogenic enzymes involved in

**Figure 2**

Effect of mitotane on cell proliferation in ovaries removed from untreated (Ctrl) or treated animals (MTT). Effect of MTT treatment on PCNA immunoreactivity in control and treated ovaries. Representative images of immunofluorescent PCNA staining in sections of ovaries from mice untreated (Ctrl) and MTT treated (MTT). Scale bar = 100 μ m. The graph shows the mean \pm s.d. of PCNA positive GCs per field. In the presence of MTT treatment, PCNA immunoreactivity was significantly decreased, whereas control ovaries showed a higher number of PCNA positive cells in almost all the growing preantral, early antral follicles. Statistical analysis was performed using the Student's *t*-test; ** $P<0.01$ vs Ctrl.

**Figure 3**

Effects of mitotane on gene expression in ovaries removed from untreated (Ctrl) or treated animals (MTT). (A) Ovaries were collected from control mice; GCs and TI cells were separated as described in 'Materials and methods' section. Total RNA extracted from isolated granulosa and TI cells was subject to real-time PCR using primers specific for *Fsh-R*, and *Cyp17a1*, specific markers for GCs and TI cells, respectively. Each sample was normalized to its β -actin content. Final results are expressed as arbitrary units (a.u.) and are represented as the mean \pm s.d. of three independent cell preparations. Statistical analysis was performed using the Student's *t*-test; * $P < 0.01$ and *** $P < 0.001$ vs respective Ctrl. (B) Expression of *Fsh-R* and *Amh* mRNA in mouse GCs and (C) *Cyp17a1* and *Cyp11a1* in TI cells, detected by real-time PCR. Ovaries were collected from control (Ctrl) or MTT-treated (MTT) mice, GCs and TI cells were separated as described in 'Materials and methods' section. Total RNA extracted from isolated granulosa and TI cells, was subject to real-time PCR using primers specific for *Fsh-R*, *Amh*, and P-450scc (*Cyp11a1*) and 17 α -hydroxylase (*Cyp17a1*), respectively. Each sample was normalized to its β -actin content. Final results are expressed as arbitrary units (a.u.) and are represented as the mean \pm s.d. of three independent experiments with a total animal number of Ctrl=15 and MTT=18. Statistical analysis was performed using the Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs respective Ctrl. We observed significantly lower levels of *Fsh-R* mRNA and higher levels of *Amh* in GCs obtained from MTT-treated mice while the expression levels of both *Cyp11a1* and *Cyp17a1* mRNA were significantly decreased in TI cells from the same animals.

crucial steps of steroid synthesis: cholesterol side-chain cleavage enzyme, a mitochondrial enzyme commonly referred to as P450scc (*Cyp11a1*) that catalyzes the conversion of cholesterol to pregnenolone, and 17- α -hydroxylase (*Cyp17a1*), a key enzyme in the biosynthesis of sex hormones that is localized in endoplasmic reticulum of theca cells.

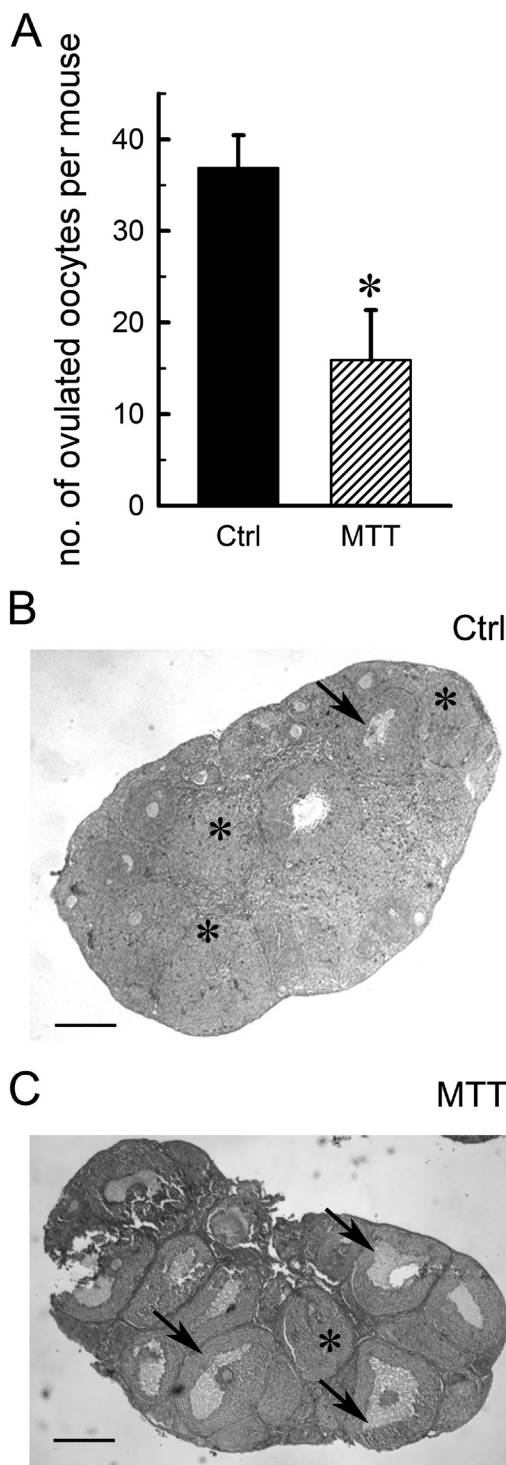
The transcripts of both genes were significantly affected by treatment with MTT. In particular, *Cyp11a1* was significantly lower in MTT-treated mice than in untreated mice (64%, $P < 0.01$). A very strong difference was observed in the *Cyp17a1* expression levels. In fact, MTT-treated mice showed a reduction of 95% ($P < 0.001$) with respect to untreated mice (Fig. 3C).

Effect of mitotane treatment on female fertility

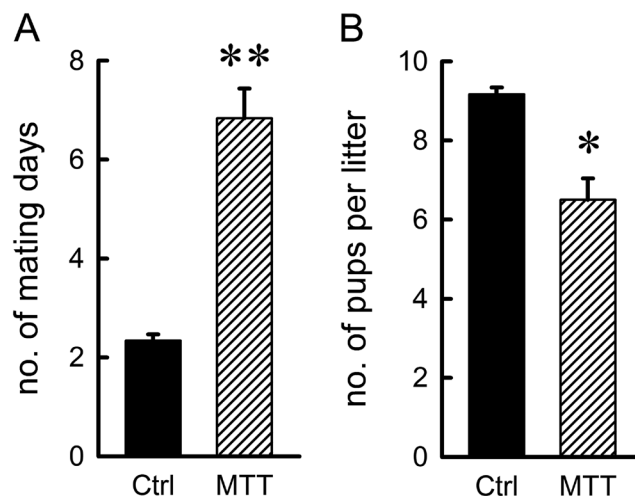
To assess the impact on fertility of treatment with MTT, after 17 days of treatment, we induced ovulation by PMSG/hCG injection. Ovulated oocytes were collected from oviducts 16h after hCG injection. In the treated females, we observed a significant decrease in the number of ovulated oocytes (approximately 57% decrease, $P < 0.01$) (Fig. 4A). The number of corpora lutea in the whole ovaries were counted in serial sections through the entire ovary. As expected, histological examination of ovarian sections showed the presence of fewer corpora lutea in treated mice than in control mice (16.85 ± 1.8 and 6.75 ± 2.4 per ovary in Ctrl and MTT-treated animals, respectively; $P < 0.01$) (Fig. 4B and C). In treated animals, we also found more antral follicles with healthy oocytes and expanded cumuli (Fig. 4C).

To assess whether treatment with MTT affects female fertility, 30-day-old females were treated for 30 days with the drug, and at the end of the treatment, females were mated with healthy males of proven fertility. MTT administration resulted in an increase in the number of days required for breeding (mating days) from 2.3 ± 0.2 to 6.8 ± 1.6 ($P < 0.01$) (Fig. 5A) and in a decrease in the main number of pups per litter from 9.2 ± 0.3 to 6.5 ± 1.4 offspring/female ($P < 0.05$) (Fig. 5B).

In order to investigate if MTT treatment withdrawn could restore female fertility, 12 mice were treated with MTT as described above and 12 mice were injected with vehicle alone as a control. After 18 days of treatment we induced ovulation in 5 mice/group. As expected we observed a significant reduction in the number of ovulated oocytes in MTT-treated animals (Fig. 6A; $P < 0.001$). The remaining mice were left in their cages without

**Figure 4**

Effect of mitotane treatment on female fertility. Prepubertal mice were treated for 18 days with vehicle alone (Ctrl, $n=10$) or mitotane (MTT, $n=12$) before PMSG/hCG induction of ovulation. (A) Mean \pm s.d. of numbers of ovulated oocytes/mouse. Midline histological sections of ovaries removed from control (B) and treated (C) animals stained with carmalum and showing corpora lutea (examples of corpora lutea in each section are marked with asterisks) and antral follicles (arrows). Scale bars, 200 μ m. * $P<0.01$ vs Ctrl.

**Figure 5**

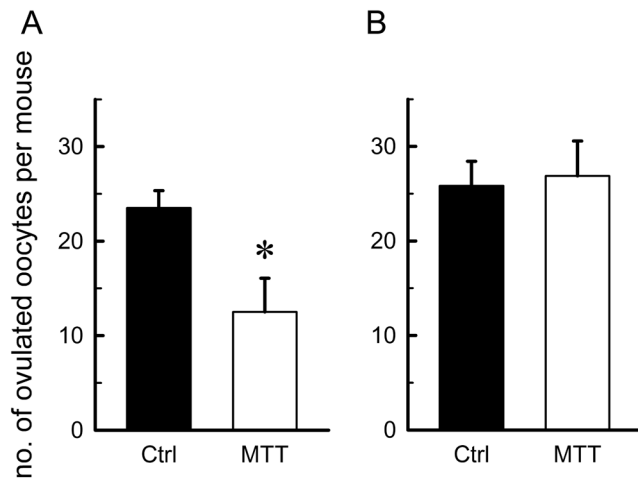
Effect of mitotane treatment on reproduction. (A) Graph of the housing time with the male required to yield pregnancy in control (Ctrl, $n=10$) and mitotane-treated (MTT, $n=10$) mice. (B) Mean litter size per pregnant mouse in control (Ctrl) and mitotane-treated (MTT) females. Values are expressed as mean \pm s.d. Statistical significance was calculated by Student's *t*-test. * $P<0.05$, ** $P<0.01$ vs respective Ctrl.

any treatment. After 25 days, ovulation was induced by PMSG/hCG and the oocytes in the tuba counted. As shown in Fig. 6B, MTT withdrawn completely restored ovulation efficiency.

Impact of mitotane on cell proliferation and cell cycle in the COV-434 ovarian cell line

To investigate the pharmacological significance of MTT on the proliferation of COV-434 cells, the dose–response was measured. We observed a dose- and time-dependent inhibition of cell proliferation in treated cells compared with non-treated cells as early as 24 h (44%) after MTT treatment, reaching 72% of inhibition 72 h after an MTT dose of 50 μ M (Fig. 7A). This concentration was selected for subsequent experiments. To study the protein involved in regulating the cell cycle, Western blot analysis was performed. As evidenced in Fig. 7B, CYCLIN E and CDK2 were significantly downregulated 48 h (90%, $P<0.001$) after MTT treatment.

To characterize cell death, we performed a TUNEL assay: after 24 h, an increase in the level of apoptosis (19%; $P<0.01$) was observed in treated cells with respect to control samples. This difference increased progressively, and a 60% increase in apoptosis of treated cells was observed after 48 h, while levels did not increase over time in the control sample (6%, physiological level) ($P<0.01$) (Fig. 8A). To confirm the

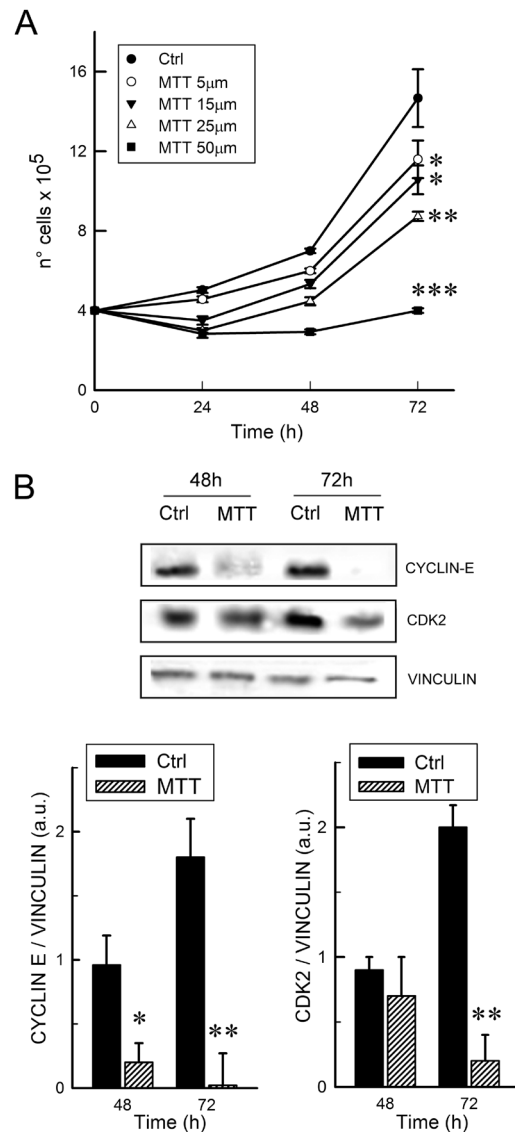
**Figure 6**

Effect of mitotane withdrawn on female fertility. Prepubertal mice were treated for 18 days with vehicle alone (Ctrl, $n=12$) or mitotane (MTT, $n=12$). At the end of treatment, 5 mice/group were injected with PMSG/hCG to induce ovulation. While the remaining mice (Ctrl, $n=7$; MTT, $n=7$) were left with no treatment and 25 days later ovulation was induced. (A) Mean \pm s.d. of numbers of ovulated oocytes/mouse after 18 days of treatment. (B) Mean \pm s.d. of numbers of ovulated oocytes/mouse 25 days after drug withdrawn.

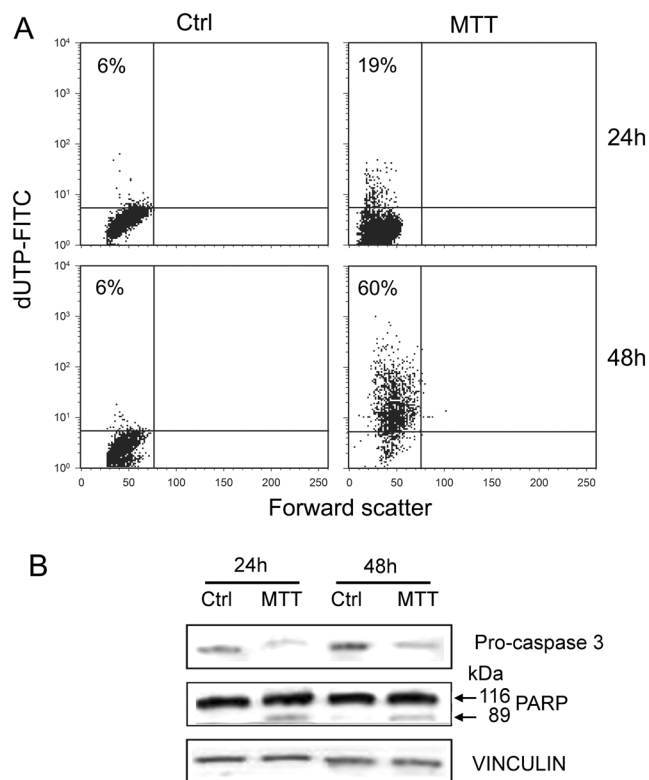
data obtained, the expression levels of proteins directly involved in the apoptotic process, pro-caspase 3 and PARP, an important target of caspase-3, were evaluated. In treated cells, we indeed observed a dramatic decrease in pro-caspase-3 (51% at 48h; $P<0.01$) after treatment (Fig. 8A). The demonstration of the cleaved form of PARP (fragments of 116 and 89 kDa) confirmed caspase-3 activation (Fig. 8B).

Discussion

ACC is an insidious endocrine tumor associated with a poor prognosis and a high risk of recurrence (Else *et al.* 2014). In most cases, this cancer affects female patients of reproductive age (Fassnacht *et al.* 2011, Else *et al.* 2014). The reason for higher incidence in women is still unknown, but recent findings indicate a pivotal role of estrogens in the pathogenesis of the ACC (Sirianni *et al.* 2012). Currently MTT, an isomer of the insecticide DDT with adrenolytic activity, is the main compound used in therapy for advanced ACC. It inhibits enzymatic activity in the mitochondria of steroidogenic cells, leading to a reduction in cortisol levels (Lehmann *et al.* 2013). Therapeutic MTT concentrations ranging from 14 to 20 mg/L are associated with anti-neoplastic activity (Kerkhofs *et al.* 2013), with an average response rate of up to 55% (Baudin *et al.* 2001). The recent ESMO guidelines on management of adrenal cancer recommend

**Figure 7**

Effect of mitotane treatment on COV-434 cell growth. (A) MTT induces cell growth inhibition on COV-434 cells. The cells were treated with MTT at the indicated concentrations (5, 15, 25 and 50 μ M) for different times. Cell number was determined at each time point as described in 'Materials and methods' section. The figure shows a dose- and time-dependent inhibitory effect on cell number. Cell growth inhibition progressively increases and, at the highest concentration at 72 h, reaches 72% of inhibition after MTT treatment. The results represent the mean \pm s.d. of three independent experiments done in duplicate. A comparison of the individual treatment was conducted by using ANOVA followed by the Tukey-Kramer *post hoc* test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs Ctrl. (B) Western blot analyses of cell cycle regulatory proteins, cyclin E and Cdk2, were performed on 50 μ g of total proteins extracted from COV 434 cells treated with vehicle alone (Ctrl) or with 50 μ M MTT for 48h and 72h. Vinculin was used as a loading control. Densitometric absorbance values were normalized by their respective vinculin values and are presented as arbitrary units (a.u.). Values are expressed as mean \pm s.d. of three independent experiments. Statistical significance was calculated by Student's *t*-test. The results of Western blots show a downregulation of cyclin E and Cdk2 at both indicated times. * $P<0.05$, ** $P<0.01$ vs respective Ctrl.

**Figure 8**

Induction of apoptosis by mitotane treatment in COV-434 cells. (A) Cells were treated for 24 and 48 h with 50 μ M mitotane (MTT) or vehicle alone (Ctrl), the apoptotic process was examined using the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling) and the quantification of the TUNEL labeling was evaluated by flow cytometric analysis as described in 'Materials and methods' section. After MTT treatment, the percentage of apoptotic cells was 19% at 24 h and the cell death increased at 48 h reaching 60% dUTP (Terminal deoxynucleotidyltransferase-mediated deoxyuridine 5-triphosphate). (B) Western blot analyses of apoptotic proteins pro-caspase 3 and PARP were performed on 50 μ g of total proteins extracted from COV 434 cells treated with vehicle alone (Ctrl) or with 50 μ M MTT for 24 h and 48 h. Vinculin was used as a loading control. The figure shows the decrease of pro-caspase3 content and the cleavage of PARP protein after MTT treatment at all the indicated times. * $P < 0.05$, ** $P < 0.01$ vs respective Ctrl.

the use of MTT in an adjuvant setting in patients with stage III ACC with Ki-67 >10% and incomplete (R1) or unrecognizable (Rx) surgical resection (Berruti *et al.* 2012). As demonstrated, MTT affects the enzymatic cascade in steroidogenic cells (Lehmann *et al.* 2013), potentially including ovarian tissue. This feature is particularly important considering the increased incidence of ACC in females and, therefore, the greatest impact of therapy with MTT. Further thought must be given to the question of treatment with a drug interfering with steroidogenesis in a woman of childbearing age. The lack of data in the literature on this topic probably results from the rarity of this tumor but particularly from the high incidence of

mortality due to the aggressiveness of ACC. Currently, clinical endocrinologists are frequently faced with the need to make a decision about the treatment of female patients of reproductive age suffering from ACC. Open issues to be addressed are related to impaired primordial follicle reserve and damage to the steroidogenesis machinery in the ovary. Data on this topic are very scarce, except for a few case reports regarding pregnancy in patients with ACC treated with MTT (Kotteas *et al.* 2012, Tripto-Shkolnik *et al.* 2013).

We used *in vivo* and *in vitro* models to study the effect of MTT on ovarian functions. The *in vivo* treatment of prepubertal female mice caused a significant reduction in growth compared with controls without affecting animal viability (data not shown). Although the ovaries of treated animals did not show evident morphological damage compared to controls (Fig. 1A), we observed fewer mature follicles in the ovaries of the treated animals. In those ovaries, we found no antral follicles and fewer early antral follicles with a subsequent increase in the number of secondary follicles, demonstrating an impediment to physiological follicular maturation (Fig. 1B). Indeed, the immunofluorescence analysis demonstrated decreased expression of PCNA in sections obtained from the gonads of treated animals compared to those sections obtained from control mice, supporting the data that show fewer proliferating GCs in the growing follicles of ovaries from MTT-treated mice than in ovaries from untreated animals (Fig. 2).

On the other hand, the significant reduction in FSH receptor (*Fsh-R*) mRNA expression and the increased expression of *Amh* evident in the GCs obtained from treated animals would be related to a block in the early maturation stage with inhibited progression toward the final follicular maturation stage (Fig. 3B, $P < 0.05$). It has been demonstrated that AMH is able to not only inhibit primordial follicle recruitment into the pool of growing follicles but also decrease the responsiveness of growing follicles to FSH (Durlinger *et al.* 2002). Interestingly, a very recent study showed an increase in AMH levels during treatment with MTT, although the data were from only three patients aged 33–45 affected by Cushing's disease (Salenave *et al.* 2015).

The administration of MTT *in vitro*, in COV-434, a human granulosa cell line, showed a dose-dependent reduction in cell number, and, at the highest concentration of 50 μ M, a significant inhibition of growth (Fig. 7A), supposed by the reduction of CYCLIN E and the corresponding CDK2 catalytic subunit (Fig. 7B), followed

by apoptosis of up to 60% after 48 h of treatment (Fig. 8A). Because the blood therapeutic concentration of MTT has been identified to be 14–20 mg/L (43.75–62.5 μ M) in ACC patients and is detected for considerably longer than 24 h in the blood, an MTT concentration of greater than 50 μ M suggests possibly higher apoptosis *in vivo* as well.

MTT has always been considered an adrenolytic drug able to impair steroidogenesis, resulting in focal degeneration in the fasciculata and reticular zona of the adrenal cortex (Bergental *et al.* 1960). Recently, several studies have noted the effect of MTT on mitochondrial machinery. In a previous study, performed in the H295R cell line, we demonstrated in a total cell extract or in a mitochondria-enriched fraction that MTT treatment interfered with the expression of D-3-PGDH isoforms and adrenodoxin reductase (AdR), enzymes strongly involved in the mechanism of redox potential and electron transfer from NADPH to ferredoxin, which provides electrons to the mitochondrial P450 (CYP) cytochromes (Stigliano *et al.* 2008). Another study by Poli *et al.* demonstrated that the cytotoxic effect of MTT seems to be mainly mediated by a specific mitochondrial disruption, leading to the impairment of steroidogenesis (Poli *et al.* 2013). Mitochondrial machinery plays a crucial role in the initiation of steroidogenesis through StAR, which transports cholesterol to the inner mitochondrial membrane by presenting it to key enzymes, such as CYP11A, responsible for the biosynthesis of pregnenolone, the precursor for all steroid hormones. As shown in Fig. 3B, the expression level of *Cyp11a* was significantly lower in ovaries treated by MTT, thus showing that MTT affects the first step of steroidogenesis, potentially impairing the synthesis of all steroids (Fig. 3C; $P < 0.05$). Furthermore, the significant reduction in *Cyp17a1* mRNA (Fig. 3C; $P < 0.01$) shows an inhibitory effect on the biosynthesis of androgens along the steroidogenesis pathway. Both results demonstrate fairly complete inhibition of steroidogenesis by MTT. These data are in accord with several reports regarding the effect of MTT on the adrenal cortex (Touitou *et al.* 1978, Lehmann *et al.* 2013, Poli *et al.* 2013). It is commonly observed that MTT therapy prevents clinical hyperandrogenism in women with Cushing's disease (Baudry *et al.* 2012). Moreover, Salenave *et al.* demonstrated a reduction in testosterone and its immediate precursor Δ 4-androstenedione in premenopausal women affected by ACC and Cushing's disease and treated chronically with MTT (Salenave *et al.* 2015). This result provides evidence of impairment of steroidogenesis, along with a reduction in the number of ovulated oocytes per mouse in MTT-treated

mice (approximately 57%; $P < 0.01$) after PMSG/hCG stimulation, with an expected decrease of corpora lutea upon histological analysis.

To further evaluate the effect of MTT administration on female fertility, we treated adult fertile female mice for 30 days with MTT, and at the end of treatment, we mated them with untreated male mice of proven fertility. The increase in the number of days needed to become pregnant in female mice treated with MTT (Fig. 5A, $P < 0.01$), as well as the decrease in the numbers of pups per litter compared with the untreated controls (Fig. 5B, $P < 0.05$), confirmed that MTT interferes with ovarian fertility. Notably, the fact that treated mice were able to become pregnant, although to a lesser extent and after longer times than untreated animals, suggests that the ovary can recover after withdrawal of the treatment. Twenty-five days after the interruption of treatment, we demonstrate, indeed, that the animals showed a complete recover in terms of number of ovulated oocytes per mouse (Fig. 6B). The results of our experiments are in agreement with recent case reports (Kotteas *et al.* 2012, Tripto-Shkolnik *et al.* 2013) and with an extensive study that included 17 cases of pregnant women affected by ACC, recruited through the multicenter and international databases provided by the European Network for the Study of Adrenal Tumors (ENSAT) (de Corbiere *et al.* 2015). Furthermore, in our mating experiments, we found no malformations or signs of adrenal insufficiency in the offspring of mice treated with MTT, in agreement with the findings of the clinical ENSAT retrospective study (de Corbiere *et al.* 2015).

Therefore, MTT on the one hand inhibits follicle growth, along with some typical proteins produced in the GCs during follicle development, such as the FSH-R; on the other hand, it interferes with ovarian steroidogenesis at the level of the theca cells. Both mechanisms contribute to the failure of follicular progression. These data are very preliminary and require further confirmation of the different ovarian cell lineages (theca and GCs) as well as confirmation in a clinical setting. It is worth recalling that the current clinical data do not provide information related to the plasma MTT concentration in women who carried out a pregnancy. For our *in vitro* results, we considered this essential information. In the COV-434 cell line, we have shown that the inhibition of growth of GCs is directly proportional to the drug concentration. Although *in vitro* data requires validation in the clinical setting, it appears very likely that maintaining the plasma MTT level at the upper limits of the therapeutic window may result in major follicular depletion and increased apoptosis in the GCs.

In conclusion, MTT treatment at therapeutic concentrations seems to interfere with follicular development

and endocrine ovarian activity without inducing irreversible changes in the gland. However, a higher drug concentration might lead to increased apoptosis of GCs. Interestingly, data from mating experiments demonstrated that MTT therapy increases the time of breeding but does not preclude the possibility of procreative functions. Although other experimental evidence is needed, MTT was not correlated with an appreciable evidence either of malformations or the risk of adrenal insufficiency in the offspring of treated mice.

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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