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ORIGINAL ARTICLE

Exposing cultured mouse ovarian follicles under increased gonadotropin tonus to aromatizable androgens influences the steroid balance and reduces oocyte meiotic capacity

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Abstract Acquisition of oocyte developmental competence relies on the well-controlled events accompanying antral follicular development. Elevated basal androgen levels, as in PCOS, potentially affect oocyte quality. Current experiments in an in vitro follicle bioassay studied dose-effects of androstenedione and testosterone on FSH and hCG stimulated antral follicle growth and meiotic maturation. The addition of either androgens altered follicle's endogenous production of androstenedione, testosterone, estradiol, and progesterone and affected the oocyte's capacity to resume meiosis. Exposure to 200 nM androstenedione induced an increased production of testosterone and estradiol. Exposure to a concentration of ≥200 nM testosterone induced elevated levels of estradiol and progesterone. Significant dose-dependent negative effects on polar body extrusion were seen at concentrations of ≥200 nM of either androgen. In addition, chromosome displacement on the metaphase plate was observed in oocytes obtained from androstenedione-treated follicles. Follicles exposed to a combination of 25 mIU/ml FSH and 3 mIU/ml hCG and elevated aromatizable androgens altered the steroid production profile, affected the follicular development and impaired oocyte meiotic competence.

Keywords PCOS · Follicle culture · Meiosis · Androgens · Steroidogenesis

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Introduction

Androgens play a dual developmentally dependent role in follicular development and oocyte maturation [1, 2]. Hyperandrogenism is commonly perceived to be negative as it is associated with the syndrome of polycystic ovaries (PCO), anovulation, and early pregnancy loss [3]. Studies in primate and murine follicular function made it clear, however, that at certain well-defined stages of follicular growth, androgens are essential for the development of the ovarian follicle [4–7]. More specifically, during antral follicular stages, an increase of androgens can have opposite effects on follicle fate depending on whether or not they are converted to estrogens by aromatase. Estrogens produced from androgens within antral follicles promote follicular growth, while androgen effects mediated by the androgen receptor (AR) result in follicular demise [8–13].

Good oocyte/embryo quality after assisted reproductive technologies (ART) in human was found to be related to a less androgenic environment (testosterone values, in follicular fluid, lower than 10 μg/l at ovum pick up) [14, 15], moreover estrogenized follicles are required for producing healthy oocytes [16]. This decreased androgen tonus is the result of physiological changes in the follicle: an increased aromatase activity, inducing increased SHBG levels and resulting in decreased free testosterone levels. In addition, the normal testosterone metabolization by 5α-reductase to the more potent form, dihydrotestosterone (DHT) is decreased possibly as a result of the increased intrafollicular levels of progesterone, estrone, and estradiol [17].

Patients undergoing conventional IVF are usually exposed to elevated levels of gonadotropins, which stimulate the development of FSH dependent small ovarian antral follicles. Depending on the patient's endocrine

background, these hyperstimulation protocols can be accompanied by elevated androgens, which have been suggested to exert negative effects on follicle, oocyte and early conception [18]. Data from IVF clinics also suggest that a hyperandrogenic follicular environment, as in patients with PCO, can lead to a decreased oocyte and embryo quality [19, 20]. In addition to the fact that PCO patients are difficult to stimulate for ART and that the procedure is not without danger for OHSS, some reports also suggest a higher frequency of oocyte maturation problems, which may compromise the long-term viability of the conceptus [21, 22].

In order to unravel the follicle stage-dependent role of androgens in the process of folliculogenesis, many studies have already been conducted, mainly in rodent and other mammal models. FSH combined with non-aromatizable androgens, DHT, androsterone, and 3α-Diol (DHT derivative) were, for instance, demonstrated to stimulate DNA synthesis of rat granulosa cells in culture [23]. Hickey et al. [24] later showed that combinations of FSH, DHT, and IGF-1 increased cumulus cell proliferation in small porcine antral follicles. Actions of steroids on oocyte meiotic resumption had also been demonstrated, i.e., testosterone had been proven to induce oocyte meiotic reinitiation in murine [25, 26] and porcine [27], via a non-genomic pathway.

Androgen receptor knock-out mice (AR−/−) demonstrate that androgens are important for antral growth during follicular development [12, 28]. Other studies have shown that the AR is down-regulated in mural granulosa from the pre-ovulatory follicles, allowing most of the androgens to be consumed by P450 aromatase, thereby shifting the balance toward growth instead of atresia [10, 11].

Experimental PCO animal models (rat, mouse, and monkey) have attempted to reproduce specific or systemic signs of the PCO syndrome (PCOS); yet, an appropriate model comprising the combined features of PCOS in human is still missing (for a review, see Singh [29]).

Even though it has been previously suggested that elevated androgen levels within a follicle is rather a sign of follicular atresia [16], the effects of elevated androgens upon healthy growing follicles have not been previously studied. Using a well characterized in vitro mouse folliculogenesis model [30], the aim was to culture a synchronously developing cohort of follicles and expose them (during their entire antral growth stage) to a sustained increased gonadotropin tonus, as is the case during the hyperstimulation protocol for human IVF, while additionally exposing them to a dose range of aromatizable androgens (androstenedione or testosterone). The concentration of the major secreted steroids was monitored throughout in vitro folliculogenesis and their relation to the outcome of meiotic maturation was studied.

Results

Morphological analysis of cultured follicles

Control versus "Plus" condition

Antral follicles cultured under elevated gonadotropin ("Plus Medium" condition) developed normally and had the same survival rate as control; nevertheless, there was a remarkable morphological difference: a more diffuse antral-like cavity (Fig. 1a, b).

In vitro hyperandrogenism

In vitro hyperandrogenism was induced (concurrently with the elevated gonadotropins treatment) either by administering 20 or 200 nM of androstenedione, or 20 nM, 200 nM, or 2 μM testosterone to "Plus" medium-treated follicles. The androgen-supplemented conditions did not affect the survival of the follicles compared to controls.

Follicles grown under different androgen regimens did not show major morphological changes in comparison to follicles grown in "Plus" condition. Only follicles grown under the highest testosterone concentration (2 μM) showed an atypical antral-like cavity, abundantly filled with granulosa cells with no clearly distinguishable cumulus cells (Fig. 1d).

Oocyte nuclear maturation assessment

Androstenedione-treated oocytes

Nuclear maturation of oocytes grown in control condition (Basal medium) was as follows: 90% of oocytes reinitiated meiosis (GVBD + PB) and 83% extruded the first polar body (PB). After "Plus" treatment, 93% of the oocytes reinitiated meiosis and 63% extruded the first PB (not significantly different than the control condition). Meanwhile, oocytes from follicles treated with "Plus" and "Vehicle" did not show a significant difference when compared to "Plus" alone ($P < 0.05$). In total, at least 50 follicles per treatment condition were included in the analysis.

Oocytes from follicles treated with 20 or 200 nM androstenedione showed a dose-dependent decrease in the rate of first PB extrusion, respectively, 51 and 32%, with the latter significantly different from "Plus" and/or the vehicle-treated group (Fig. 2a; $P < 0.05$).

Testosterone-treated oocytes

In a next set of experiments ($n =$ at least 50 follicles), all oocytes grown under "Plus" treatment reinitiated meiosis

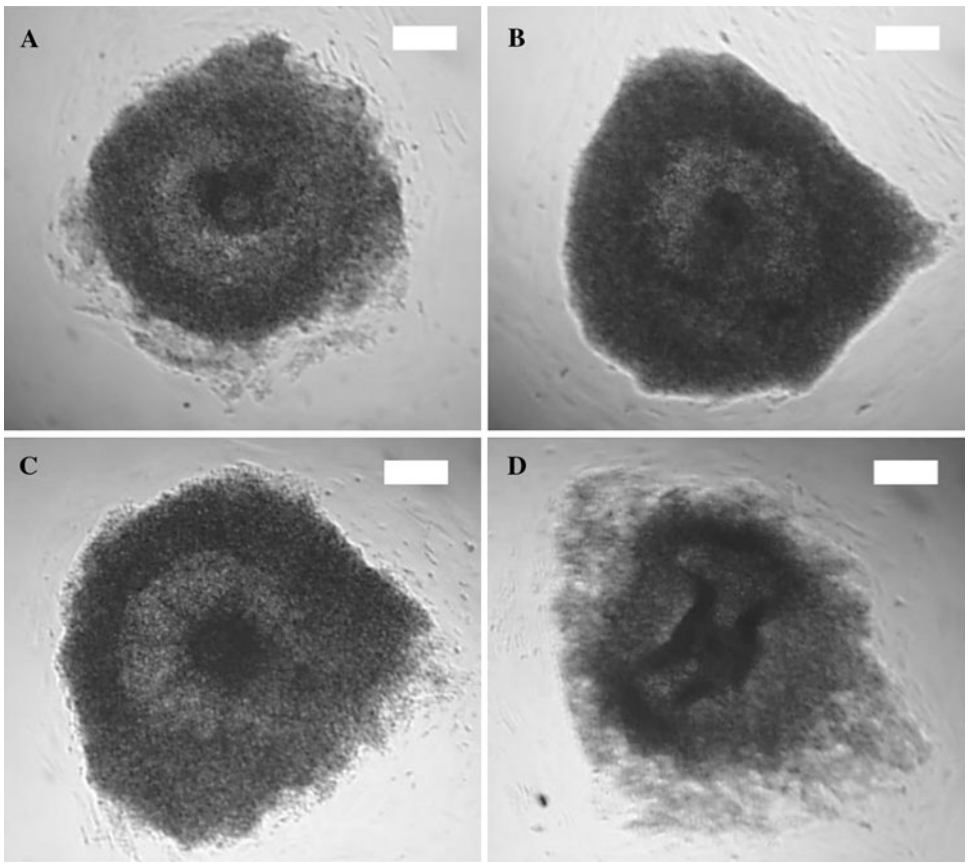

Fig. 1 Representative follicles on D12 of in vitro development. **a** Follicle from control group. **b** Follicle from the group exposed to elevated levels of gonadotropins. **c** Follicle from the group treated with elevated gonadotropins, supplemented by 200 nM androstenedione. **d** Follicle from the group treated with 2 μM testosterone. *Scale bar* 200 μm

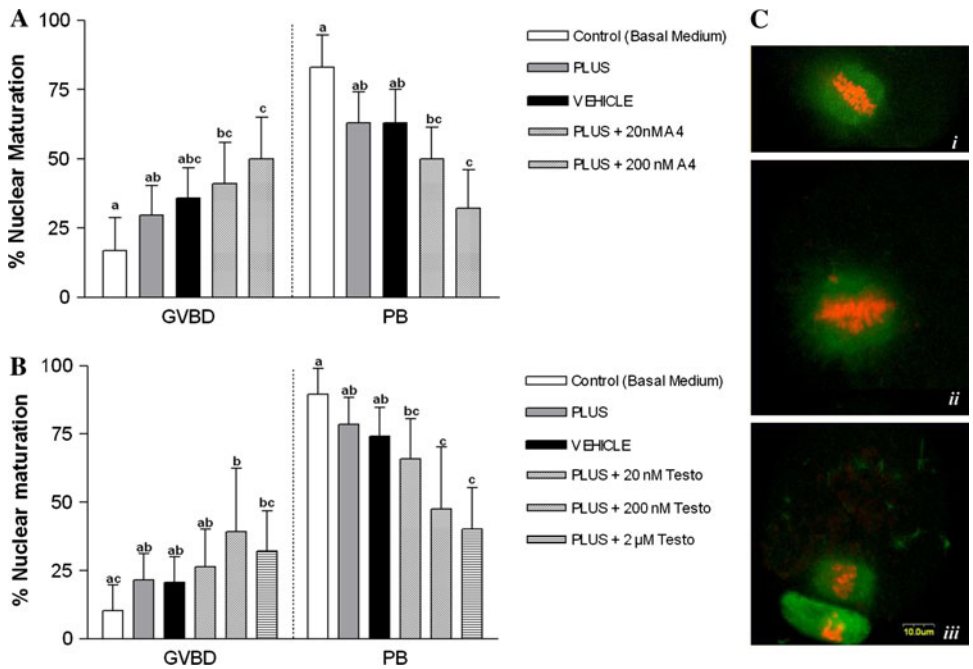

Fig. 2 Nuclear maturation stages 18 h after stimulation with hCG/EGF in androstenedione (**a**) and testosterone (**b**) treated groups; *bars left of the dotted line* are proportion of GVBD; *bars right of the dotted line* are proportion of PB. Vehicle control contains 0.1% DMSO. *Different letters* state statistically significant differences ($P < 0.05$). At least 50 follicles per treatment were included in the analysis. (**c**) Representative images of oocytes cultured in 500 nM androstenedione, obtained by CLSM: (*i*) normal metaphase I oocyte; (*ii*) metaphase I oocyte depicting chromosomal misalignment; (*iii*) metaphase II oocyte depicting chromosomal misalignment (To note the numerous microtubule asters in the cytoplasm)

and 78% extruded the first PB. No significant difference between this group and either the control condition (Basal medium) or the vehicle-treated group was observed.

Depending on the concentration, oocytes from follicles treated with 20 nM, 200 nM, or 2 μM testosterone showed a steady dose-dependent decrease in PB extrusion, respectively, 66, 48, and 40%. The 200 nM and 2 μM groups showed a significant difference when compared to the vehicle-treated follicles (Fig. 2b; $P < 0.05$).

Chromosomal analysis in oocytes

Because of the decreased rate of PB extrusion in oocytes exposed to high androgen levels, some GVBD (metaphase I) arrested and metaphase II oocytes were analyzed by confocal laser scanning microscopy (CLSM). Ten out of 15 analyzed GVBD oocytes showed chromosomal misalignment. Moreover, four out of 12 metaphase II oocytes also showed chromosomal misalignment. Examples of chromosomal misalignment can be observed in Fig. 2c.

Assessment of steroid production of cultured follicles

Overall, follicles grown under the high gonadotropin condition ("Plus" or "Vehicle"), and especially those supplemented by increased androgens ("Plus + Androgens") secreted more steroids than untreated follicles. Conditioned media were pooled per plate ($n = 8$–10 follicles) and analyzed for 6–9 plates from each treatment. For the sake of clarity, steroidal profile for follicles grown under the Basal and Plus condition are excluded from the graphs.

Addition of androgens androstenedione and testosterone to the culture medium positively correlated with levels of measured testosterone and estradiol on Day 12 of culture (Table 1).

Androstenedione-treated follicles

Absolute steroid concentration in conditioned media Values of steroids from follicles treated with 20 nM androstenedione showed no significant differences when compared to "Vehicle" treated follicles at any of the time-points analyzed. Although the follicles treated with 200 nM androstenedione showed increased levels for the three monitored steroids (testosterone, estradiol, and progesterone), the testosterone concentrations from the androstenedione treatment were significantly higher than in "Vehicle" condition on D9, D12, and D13. Similarly, the estradiol absolute values in the 200 nM androstenedione condition became significantly different from the "Vehicle" condition on D9, D12, and D13 (Fig. 3a).

Table 1 Correlation coefficient between the different measured steroids in an in vitro fully grown follicles (after 12 days of culture), regardless of the treatment (Spearman correlation analysis)

Spearman *r*	Testosterone	Estradiol
Adding androstenedione		
Androstenedione	0.6600 ($P < 0.0001$)	0.6393 ($P < 0.0001$)
Testosterone		0.5652 ($P < 0.0001$)
Adding testosterone		
Androstenedione	0.8905 ($P < 0.0001$)	0.6108 ($P < 0.0001$)
Testosterone		0.7464 ($P < 0.0001$)

Relative steroid production The second way of assessing the production of steroids was to calculate the relative difference in concentration between consecutive time points (on D6, D9, D12, and D13 of culture) (Fig. 3b).

The highest relative increase in steroids production occurred from D6 to D9. For all steroids, in any condition, values on D12 did not differ considerably from those on D9; the relative production of steroids at this specific point therefore remained lower than five times (increase) and close to the one-time value.

At 18 h after the ovulatory stimulus, a general observation for all treatments was that progesterone concentration had increased by a factor of 100. This increase was accompanied by a steep decrease in estradiol (Fig. 3b).

Testosterone-treated follicles

Absolute steroid concentration in conditioned media Values of steroids from follicles treated with 20 nM testosterone showed no significant differences when compared to "Vehicle" treated follicles at any of the time-points analyzed. In follicles treated with 200 nM or 2 μM testosterone, estradiol, and progesterone values tend to increase in a dose-dependent fashion estradiol absolute values were significantly higher than in "Vehicle" condition after treatment with 200 nM and 2 μM testosterone on D12 and after 2 μM on D13. Furthermore, by D12, follicles treated with 2 μM testosterone produced progesterone to a concentration significantly higher than in "Vehicle" condition (Fig. 4a).

Treatment with 2 μM testosterone induced high levels of androstenedione at every time-point. To note, these values were even higher than those obtained after addition of the highest androstenedione concentration.

Relative steroid production Spent media were analyzed for presence of steroids on D6, D9, D12, and D13 of culture and a comparison was made between two consecutive time-points (Fig. 4b).

Overall and in accordance with the results of the previous experiment, the highest increase in steroids production occurred from D6 to D9, with minimal increase from D9 to D12. Androstenedione values significantly increased

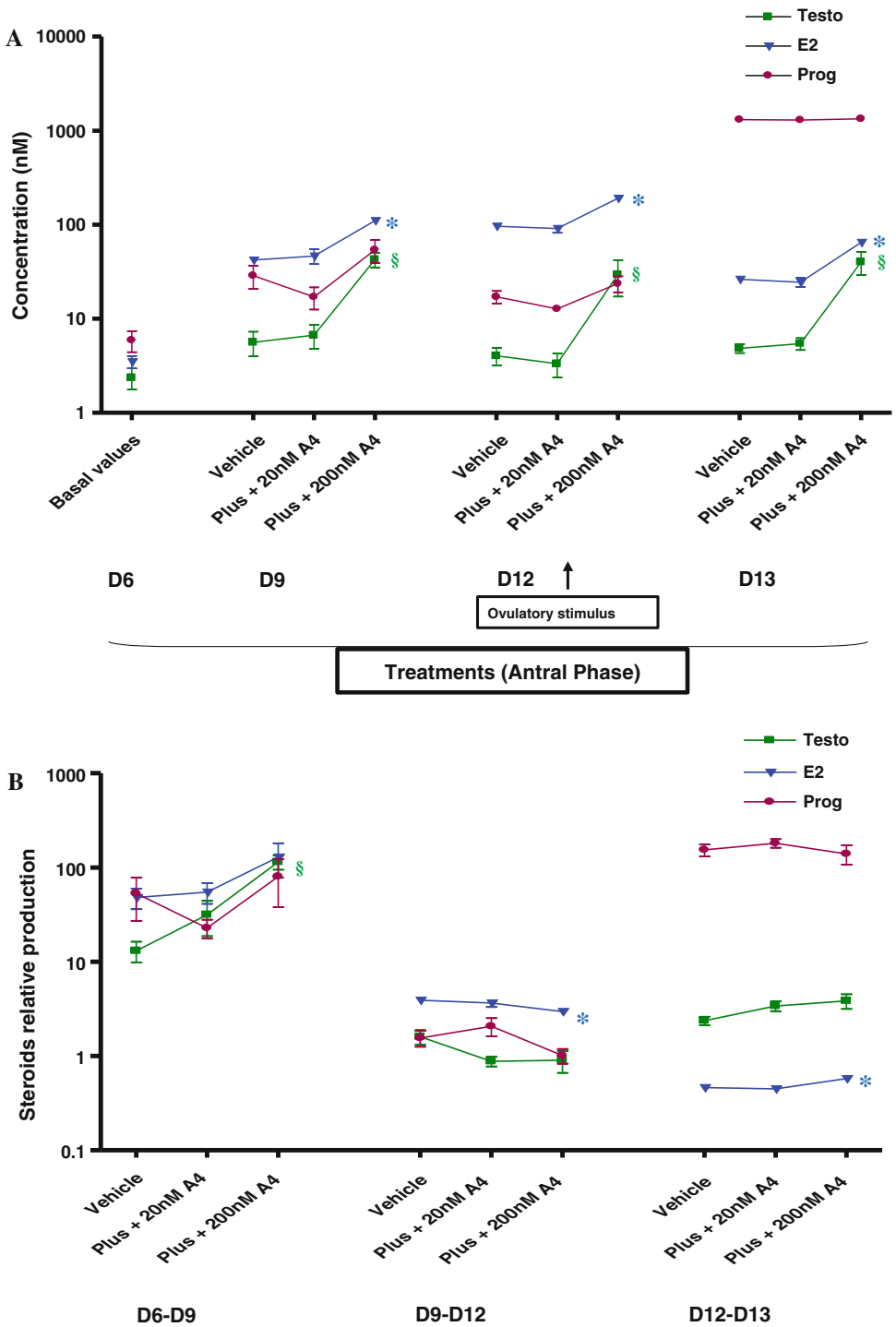

Fig. 3 Steroidal profile in follicles cultured in 20 or 200 nM of androstenedione; **a** Steroids absolute values. Concentrations of testosterone (T), estradiol (E2), and progesterone (Prog) on Day 6 (D6), Day 9 (D9), Day 12 (D12), and Day 13 (D13) of treatment. Each *dot* represents the mean ± SEM of nine plates containing 8–10 follicles each. **b** Relative production of steroids. Changes (relative production, calculated as described in "Materials and methods" section) in testosterone (T), estradiol (E2), and progesterone (Prog) profiles from Day 6 to Day 9 (D6–D9), from Day 9 to Day 12 (D9–D12) and from Day 12 to Day 13 (D12–D13), during treatment with 20 or 200 nM of androstenedione. Each *dot* represents the mean ± SEM of the relative production for nine plates (containing 8–10 follicles each). Statistical analysis was done by one-way ANOVA (Tukey post-test). Series connected between different conditions by a line have the sole purpose to visually emphasize trends. **a** § Shows statistical significant difference for testosterone concentration from the Vehicle group, $P < 0.05$. * Shows statistical significant difference for estradiol concentration from the Vehicle group, $P < 0.001$. **b** § Shows statistical significant difference for testosterone concentration from the Vehicle group, $P < 0.001$. * Shows statistical significant difference for estradiol concentration from the Vehicle group, $P < 0.05$

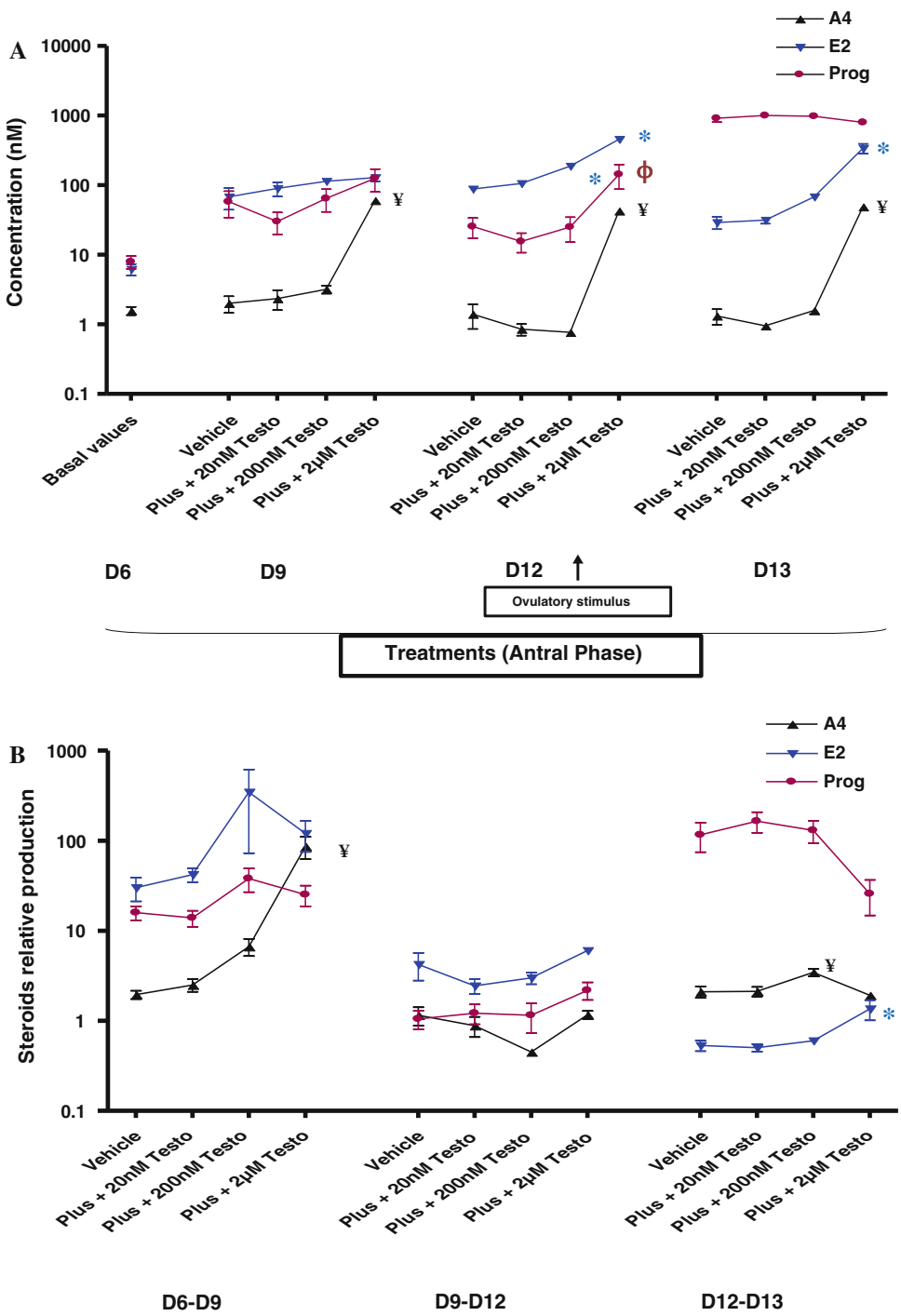

Fig. 4 Steroidal profile of follicles cultured in 20 nM, 200 nM, or 2 μM of testosterone; **a** Steroids absolute values. Concentrations of androstenedione (A4), estradiol (E2), and progesterone (Prog) on Day 6 (D6), Day 9 (D9), Day 12 (D12), and Day 13 (D13) of treatment. Each *dot* represents the mean ± SEM of six plates containing 8–10 follicles each. **b** Relative production of steroids. Changes (relative production, calculated as described in "Materials and methods" section) in androstenedione (A4), estradiol (E2), and progesterone (Prog) profiles from Day 6 to Day 9 (D6–D9), from Day 9 to Day 12 (D9–D12) and from Day 12 to Day 13 (D12–D13), during treatment with 20 nM, 200 nM or 2 μM of testosterone. Each *dot* represents the mean ± SEM of the relative production for six plates (containing 8–10 follicles each). Statistical analysis was done by one-way ANOVA (Tukey post-test). Series connected between different conditions by a line have the sole purpose to visually emphasize trends. **a** ¥ Shows statistical significant difference for androstenedione concentration from the Vehicle group, $P < 0.001$. * Shows statistical significant difference for estradiol concentration from the Vehicle group, $P < 0.05$. ϕ Shows statistical significant difference for progesterone concentration from the Vehicle group, $P < 0.05$. **b** ¥ Shows statistical significant difference for androstenedione concentration from the Vehicle group, $P < 0.01$. * Shows statistical significant difference for estradiol concentration from the Vehicle group, $P < 0.05$

from D6 to D9 at the highest tested concentration of testosterone. As with androstenedione-treated follicles, it was observed that steroid values on D12 did not considerably differ from D9 values for every condition.

After ovulatory stimulus, progesterone concentrations increased approximately 100 times. As for androstenedione experiments, estradiol values at this point were also considerably lower than D12 for every condition.

Although progesterone and estradiol kinetics (D12–D13) were similar for all the treatments, the increase in progesterone levels from D12 to D13 under treatment with 2 μM testosterone was less pronounced when compared with the rest of the treatments. Accordingly, follicles treated with 2 μM testosterone had the lowest decrease in estradiol production (compared to vehicle-treated follicles, $P < 0.01$) (Fig. 4b).

In accordance with the androstenedione absolute values, under treatment with 2 μM testosterone, there was a tremendous increase in androstenedione concentration (from D6 to D9). Given that 2 μM testosterone seemed to induce an aberrant (i.e., non-physiological) environment with increased levels of androstenedione and an altered response to ovulatory stimulus, it was decided not to include this condition in the further analysis.

Steroids and meiotic resumption

Although the addition of androgens (either androstenedione or testosterone) show a dose-dependent detrimental effect on meiosis, there was, however, no significant correlation (Spearman correlation analysis) between meiotic resumption (PB rate) and any of the analyzed steroids in conditioned medium, which was consistent in every treatment (data not shown).

Discussion

Protocols for assisted reproduction techniques (ART) rely on a sufficiently large number of good quality oocytes by using exogenous administration of gonadotropin hormones (controlled ovarian stimulation, for a review, see Macklon et al. [31]). Superovulated patients have an increased pregnancy rate when androgen levels in serum and follicular fluid are kept in the lower reference ranges [8, 9]. Latter authors suggested that elevated androgen levels might interfere with oocyte/embryo or endometrium quality. Few studies have been able to demonstrate that borderline increased androgen concentrations can induce a direct effect on follicle/oocyte development and maturation rather than being an epiphenomenon of follicle atresia [8, 9, 32, 33]. Hyperandrogenism is a condition that prevails in part of the anovulatory WHO type II patient group [34]. Androgen tonus is amplified by superovulation in PCOS patients, who also have a higher expression of the AR in granulosa, as initially suggested by studies in monkeys [35] and later confirmed by Catteau-Jonard et al. in human [36]. From these in vivo studies, the question still remains unanswered as to how near physiological intrafollicular androgen concentrations might exert effects that influence oocyte/embryo quality. Indeed, the harmful effects of an hyperandrogenic condition during ART stimulation might be blurred by the occurrence of inappropriate LH rises, which unsuspectedly trigger meiotic maturation. Here, we studied the extent to which increasing levels of two androgens, androstenedione and testosterone, could affect meiotic resumption and how near physiological androgen supplementation, under a constant gonadotropic hyperstimulation tonus, could alter the steroidal balance in the follicle environment.

The possibility of growing individual follicles, in vitro, in relatively large amounts, under standardized, near physiological conditions, allows us to show the effects of an unbalanced endocrine environment on follicle growth and oocyte maturation. As the primary aim was to mimic ovarian stimulation conditions in vivo, in vitro-grown follicles at the early antral stage (Day 6) were exposed to recombinant gonadotropins at an FSH/LH ratio that mimics a classical menotropin-induced stimulation protocol [37]. It was decided to use hCG as LH-bioactive component as this molecule is generally used to spike for LH bioactivity to the requested 75 IU per ampoule on a menotropin preparation [38].

Our results demonstrate that the increased FSH/LH tonus (approximately three times the normal basal FSH concentration) induces follicular growth and that the granulosa cells seem to proliferate more than those in basal condition (Fig. 1a, b). Although under increased gonadotropin exposure the oocyte was able to resume meiosis after the ovulatory stimulus (hCG/EGF), both series of experiments mimicking hyperstimulation, reduced PB extrusion rate though not significantly, even in absence of androgens. Supplementation by androgens from Day 6 induced morphologically more profuse granulosa cell layer around the oocyte (Fig. 1c, d). Increased proliferation of granulosa cells in the presence of FSH, androgens, and oocyte secreted factors (OSF) has been previously documented by others in porcine [21, 22, 39].

Elevated levels of androgens androstenedione and testosterone negatively affected meiotic resumption ($P < 0.05$) and had a clear influence on the steroidal microenvironment of the cultured follicles. Oocytes treated either with androstenedione or testosterone showed a lower potential to reinitiate meiosis. Eighteen hours after the ovulatory stimulus, both androgens supplements induced a supplementary decrease in PB extrusion rates. Statistical significance compared to either "Plus" or "Vehicle" control was reached only in the highest concentrations, i.e., 200 nM of androstenedione and 200 nM and 2 μM of testosterone. Also, a moderate number of oocytes (17.8, 11.7, and 27.3%, respectively) remained blocked at the GV stage (not shown in Fig. 2) and there was an increased proportion of oocytes arrested during meiosis (GVBD oocytes unable to extrude the first PB). Analysis of chromosomal alignment of GVDB arrested oocytes showed that many of them do not have chromosomes correctly aligned in the equator of the meiotic spindle (Fig. 2c *i*, *ii*); similarly metaphase II oocytes also showed chromosomes outside the metaphase plate (Fig. 2c *iii*). GVBD arrested and metaphase II oocytes obtained from our follicle culture system barely show chromosomal misalignment (13 and 14%, respectively) [40] or spindle abnormalities (2–6%) [41]. These findings suggest that high androgen levels during folliculogenesis may impair spindle assembly during oocyte meiosis.

Androgens added to a background of increased FSH/LH further increase the steroid levels within the follicle. As expected, both androstenedione and testosterone additions were metabolized into other steroids (in part testosterone, estradiol, as seen on the Figs. 3 and 4). The estradiol increase could be explained by upregulation of the main enzyme complex cytochrome P450 Aromatase (P450arom) [42]. Progesterone levels tended to increase dose dependently with androgen concentrations. Progesterone accumulation as a result of treatment with androgens has been previously shown in follicle [43] and granulosa cell [44] cultures.

When androstenedione was added to the culture, testosterone and estradiol levels were moderately, though significantly, correlated with androstenedione levels, suggesting that androstenedione was driving the increase in testosterone and estradiol in the medium. Similarly, estradiol levels were positively correlated with testosterone levels (Table 1).

Testosterone additions to the culture medium induced high levels of estradiol that were positively correlated with the increase in testosterone. Introducing 2 μM testosterone in medium (on Day 6) induced an unexpected increase in androstenedione levels and a drop in estradiol (NS). Assuming that this atypical/un-physiological environment could only be found in exceptional pathologies, we found it less relevant in the context of our study; therefore, the 2 μM testosterone condition was not further included in the subsequent analyses.

The applied 200 nM testosterone is slightly higher than the normal physiological concentration (∼50–150 nM testosterone) seen in human [45], ovine [46], and porcine graafian antral follicles [47]. Similarly 200 nM androstenedione is also higher than the physiological levels [47].

In the current study, the selection of the androgen concentrations tested was based on the values determined in follicular fluids from patients at oocyte retrieval after ART, in a large prospective multicenter study [48].

In an attempt to find a relation between steroid composition/changes in medium and meiotic resumption, a series of correlation analyses of steroids and PB rates were performed (not shown). These analyses on a large number of data points ($n = 78$ samples) revealed that none of the analyzed steroids (alone) was found to be associated with the decreased meiotic maturation rates. Similarly, the relation between androgen/estradiol ratios, reflecting aromatase activity, and PB rate was also assessed, however, no clear relationship could be found either.

In conclusion, the in vitro culture approach of intra-ovarian follicles allowed to demonstrate that elevated androgen levels on top of increased gonadotropin tonus during antral follicular development significantly alter the steroidal micro-environment of the follicle, and have an impact on the ability of oocytes to resume meiosis. In the absence of a strong correlation between the currently measured steroids and nuclear maturation outcome, the molecular mechanisms which affect the oocyte meiotic spindle assembly need to be further investigated.

Materials and methods

Animal model

Mice used for these experiments were housed and bred according to national legislation and with the consent of the committee for animal experimentation of the Vrije Universiteit Brussel (Project number: 06-535-1).

Follicle culture

Ovarian follicles from pre-pubertal mice (13-day-old, F1 hybrids: C57Bl/6j × CBA/ca) were mechanically isolated and cultured for 13 days in a modified protocol from Cortvrindt & Smitz [30]. Briefly, animals were sacrificed by cervical dislocation, ovaries were retrieved from the abdominal cavity and early pre-antral follicles (with diameters between 110 and 130 μm) were dissected out in Leibovitz L-15 (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS), containing 100 IU/ml penicillin, 100 μg/ml streptomycin (penicillin/streptomycin-mix, Invitrogen). Only follicles with distinguishable theca cells (observed under high magnification on the basal membrane) were used for culture.

Follicle culture was carried out in two steps; first, follicles were grown from the early pre-antral stage to the early antral stage (6 days of culture). Follicles were individually cultured (1 follicle/well) in 75 μl of basal culture medium in 96-well microtiter plates (Costar, Belgium). Basal culture medium was composed of α-MEM glutamax (Invitrogen), 5% FBS (Invitrogen), 5 ng/ml insulin, 5 μg/ml apo-transferrin, 5 ng/ml sodium selenite (all from Sigma, Bornem Belgium), 10 mIU/ml r-FSH and 10 mIU/ml r-LH (recombinant gonadotropins are purchased from Serono, Belgium); r-LH was only added at the start of the culture and was not included in the medium used for refreshment.

Second, on Day 6, most of the oocytes grown in the follicle culture model had reached the surrounded nucleolus stage (SN) [49]. This condition is quite comparable to the situation in the menstrual cycle Day 1, where a cohort of interest is being stimulated [50]. Day 6 of culture was therefore considered an appropriate time-point to start the experimental treatments. Plates containing in vitro growing follicles (6 days in culture) were distributed into one of the following treatments and further cultured for six more days.

Control

Ovarian follicles were cultured for six more days (D6–D12) in basal culture medium. Note that a dose of 10 mIU/ml r-FSH was determined to be a minimal effective dose to obtain maximal follicle survival (≥95%) in this culture model [51]. Follicle survival is defined as follicles containing a developing oocyte completely surrounded by granulosa cells and it was calculated as a percentage from plated follicles at the beginning of culture.

In vitro hyperstimulation model

In order to model an ovarian stimulation protocol like used for ART, early antral follicles (Day 6) were exposed for 6 days to a supraphysiological gonadotropin level, mimicking the use of human menotropins (containing FSH and LH bioactivity) (Fig. 5). Medium composition was similar to the basal medium, except that 25 mIU/ml FSH + 3 mIU/ml recombinant hCG was substituting the 10 mIU/ml FSH [30]. This medium is further referred to as "Plus" medium. Recombinant hCG was purchased from Serono (Serono, Belgium).

The dose of FSH used in this treatment (being three times higher than the minimal dose needed to obtain a maximal follicle survival) was chosen to model a condition of ovarian stimulation for ART. The dose of hCG was administered to provide a constant low LH bioactivity to stimulate theca cells androgenesis.

In vitro hyperandrogenism

In order to mimic a hyperandrogenic environment during ovarian hyperstimulation, follicles cultured in "Plus" medium were in addition exposed to supra-physiological concentrations of androstenedione (two doses) and testosterone (three doses) (both from Sigma, purity ≥98 and 99%, respectively; Bornem, Belgium) during the antral growth phase (D6–D12). For androstenedione, concentrations of 20 and 200 nM (final) were applied. For testosterone, concentrations of 20 nM, 200 nM and 2 μM (final) were used.

Androgens were first dissolved in DMSO and further diluted in culture medium. A "Vehicle" control was included in every experiment. Final DMSO concentration in culture was ≤0.1%. Once the steroids had been added to the medium, target values were confirmed by radioimmunoassay (see further details on the assay).

Follicle cultures were carried out at 37°C, 5% CO_2, 100% humidity. Medium refreshments were performed every 3 days by replacing 30 μl of spent media by fresh

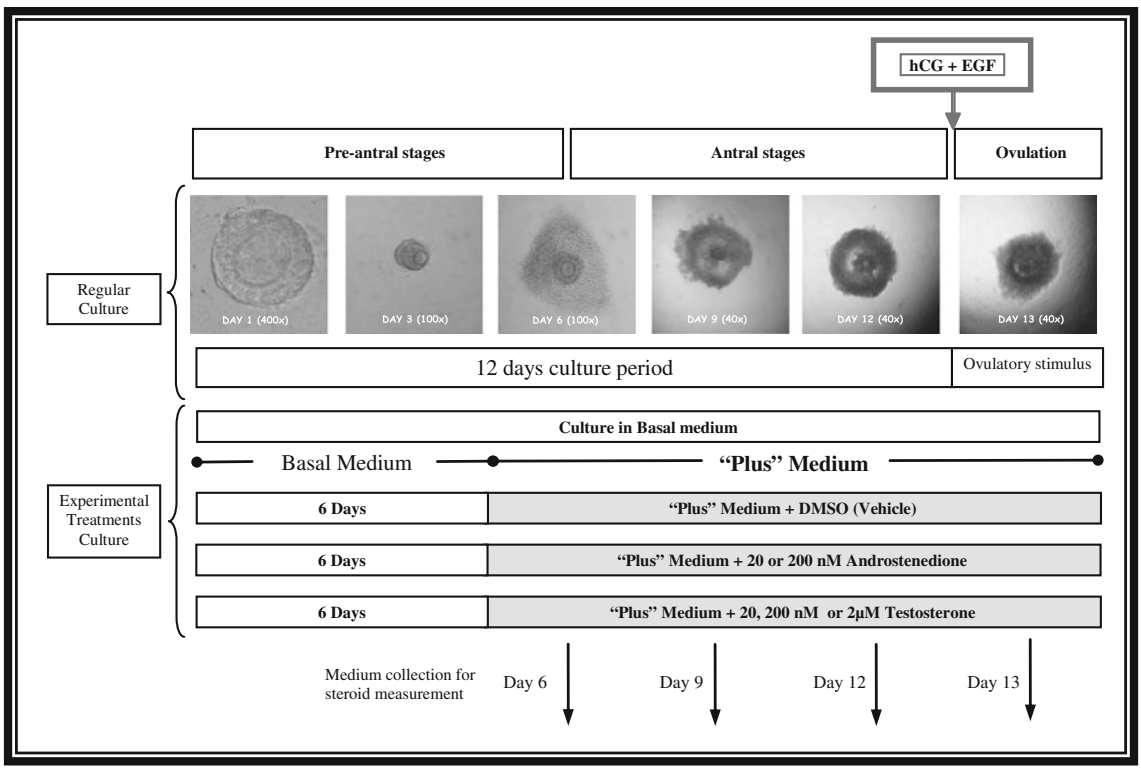

Fig. 5 Scheme showing the experimental design. Ovarian follicles were cultured for 6 days in basal medium (10 mIU/ml FSH) until reaching an early antral stage, then follicles were exposed to "Plus" medium (25 mIU/ml FSH + 3 mIU/ml hCG). "Plus" medium resembles controlled ovarian hyperstimulation protocols using human menotropin therapy

medium (also containing the respective androgen components).

Assessment of oocyte nuclear maturation

On Day 12 of culture, follicles of all treatments were stimulated for oocyte maturation (resumption of meiosis) with 1.2 IU human chorionic gonadotropin (hCG; Ares-Serono, Geneva Switzerland) + 0.65 nM epidermal growth factor (EGF; Roche Diagnostics, Mannheim Germany); 18 h after the ovulatory stimulus, the oocytes were denuded by drawing the cumulus oocyte complexes (COCs) in and out of a fine glass pipette, and oocytes were assessed for nuclear maturation. Oocyte nuclear maturation stages were visualized under an inverted microscope equipped with a Hoffman modulation contrast system (Nikon, Tokyo, Japan). Nuclear maturation was scored as GV (presence of a germinal vesicle), GVBD (germinal vesicle not visible), or PB (first PB was extruded). Percentages of nuclear maturation were calculated per plate. Results are expressed as mean ± SEM, averaged for three independent experiments (in total, 6–9 plates per treatment, with 8–10 follicles per plate).

Chromosomal analysis in oocytes

For evaluation of androgen effects on oocyte chromosomal alignment, follicles were exposed for 6 days to gonadotropins and 500 nM androstenedione.

Oocytes were fixed and stained as previously described by Lenie et al. [40]. Briefly, spindles were stained by sequentially incubating the oocytes (at least 45 min) with a monoclonal mouse anti-α-tubulin (Sigma-Aldrich, Bornem, Belgium), 1:100, and 45 min with Alexa Fluor 488-polyclonal goat anti-mouse antibody, 1:200 (Molecular Probes, The Netherlands). The chromosomes were stained with ethidium-homodimer-2, 1:2,000 (Molecular Probes, The Netherlands) (incubation time: 15 min). Antibody dilution and intermediary washing steps were performed with a wash-block solution (PBS containing 0.02% sodium azide, 0.2% milk powder, 2% normal goat serum (NGS), 1% BSA, 0.1 M glycine, and 0.01% Triton X-100) and all incubations were carried out at 37°C. Stained oocytes were analyzed under a Fluoview confocal microscope system (Olympus IX 70) equipped with an Argon-Krypton laser (488–568 nm) and band pass filter 510–540 and long pass filter 610 to visualize Alexa Fluor 448 and ethidium-homodimer-2, respectively.

Assessment of steroid production of cultured follicles

Culture medium from individual wells (only those containing an intact follicle structure which made it to the end of culture) was pooled per plate for the controls and each of the treatments on Days 6, 9, and 12 (D6, D9, and D12); additionally, medium was also collected 18 h after ovulatory stimulus (D13). Spent medium was stored at −20°C until measurements of androstenedione, testosterone, estradiol, and progesterone had been performed.

All immunoassays had also been slightly adapted to permit their use on conditioned medium. Androstenedione was measured by direct radioimmunoassay from Biosource (androstenedione-RIA-CT, BioSource Europe, Nivelles, Belgium) with a sensitivity of 70 ng/l and a total imprecision profile CV <10% for concentrations between 100 and 7,000 ng/l. Testosterone was measured by direct immunoassay from Orion Diagnostica (RIA, ORION Diagnostica; Espoo, Finland). The analytical sensitivity of this assay is 0.07 μg/l and the total imprecision profile CV <10% is observed for concentrations from 0.1 to 50 μg/l. 17β-Estradiol (E2) production was measured by direct radioimmunoassay from clinical assays (DiaSorin, Sorin Fueter, Brussels, Belgium) with an analytical sensitivity of 10 ng/l and a total imprecision profile <10% CV. Progesterone (P) secretion was determined both before and after maturation stimulus by direct radioimmunoassay from Cisbio (Cisbio international, Gif-sur-Yvette cedex, France). The progesterone assay has an analytical sensitivity of 0.06 μg/l and a total imprecision profile <10% CV.

Regarding specificity of the assays, cross-reactions as stated by the manufacturer were minimal, so that specificity could be guaranteed. Experimental cross-reactivity assays showed that in experiments where androstenedione was added to culture media, testosterone was detected at low levels in refreshment media (on average 12.9 μg testosterone for 1 mg of added androstenedione). Similarly, in experiments where testosterone was added to culture media, androstenedione was also detected (on average 1.6 μg androstenedione for 1 mg of added testosterone).

The steroids profiles were represented in two ways, allowing us to provide additional insight in the temporal changes: (a) representation of the absolute values (i.e., the concentration), comparing the steroid values obtained by immunoassays; (b) evaluation of their relative production (over time). Relative production was calculated using the following formula: DayB/[(DayA)(0,6)], where "DayA" and "DayB" refer to two consecutive refreshment days and Day A is corrected by a dilution factor due to medium refreshment. This value was calculated for each plate and for the culture period D6–D9, D9–D12, and D12–D13.

A statistical evaluation of the possible relationship between the different steroid levels and the meiotic capacities of their corresponding oocyte was also carried out.

Statistical analysis

Nuclear maturation (GV, GVBD, or PB) rates were compared among the treatments. Analysis of the percentages of meiotic stages was done by using a generalized linear mixed model with plate nested into experiment as random factors and a logic link.

Analysis of either steroid concentration (absolute values on D9, D12, or D13) or production (relative production over time; D6–D9, D9–D12, or D12–D13) was performed independently, using one-way ANOVA, with Tukey as post-test.

Correlation of levels of produced steroids with added androstenedione and testosterone (regardless of the treatment) was assessed by Spearman correlation analysis.

In order to assess the relationship between the different steroid levels and the meiotic capacities, Spearman correlation analysis was performed.

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