

## Ketoconazole Inhibits Ovulation as a Result of Arrest of Follicular Steroidogenesis in the Rat Ovary

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### ABSTRACT

**OBJECTIVE:** Ketoconazole (KCZ) is a known inhibitor of steroidogenic P450 enzymes in the adrenal cortex and the gonads. Previous studies examined the potential clinical use of KCZ for attenuation of ovarian response to gonadotropin treatments. This study aimed to use the superovulating rat model to explore the effect of KCZ on ovarian steroidogenesis, follicular function, and development toward ovulation.

**METHODS:** Prepubertal rats were treated with equine chorionic gonadotropin (eCG)/human CG (hCG) resulting in multiple follicular development and ovulation. The effect of KCZ on this model was examined by administration of KCZ-gel formula and subsequent analyses of ovarian steroidogenesis, rate of ovulation, morphometric assessments of follicular parameters, and cell-specific steroidogenic maturation of the treated ovaries.

**RESULTS:** When applied shortly before gonadotropin stimulation, KCZ markedly reduced ovarian progesterone, androstenedione, and estradiol levels down to 18.7, 36.5, and 19.0%, respectively ( $P < 0.001$ ). A single KCZ-gel administration of 6, 12, and 24 mg/rat resulted in reduction of ovulated ova/ovary down to  $8.6 \pm 4.9$ ,  $5.1 \pm 4.3$ , and  $2.4 \pm 3.2$ , respectively, as compared to  $13.6 \pm 4.4$  ova found in the oviduct of control-gel-injected animals ( $P < 0.001$ ). An alternative protocol made use of small KCZ doses injected in non-gel formula (5 mg/dose/8 hours), commenced with the eCG administration and terminated 24 hours later; this treatment readily inhibited the ovulation rates to  $6.6 \pm 6.6$  as compared to  $16.5 \pm 4.1$  ova/ovary in the control group ( $P < 0.01$ ). By contrast, KCZ failed to inhibit ovulation if administered 24 hours after eCG injection. Anovulation by KCZ resulted from arrest of follicular development at the stage of 800–840  $\mu\text{m}$  Graafian follicles as compared to 920  $\mu\text{m}$  of peri-ovulatory follicles (OFs) observed in the control group,  $P = 0.029$ . In addition, absence of CYP11A1 expression was evident in the granulosa cell layers of the growth-arrested follicles, which also lacked mucified mature cumulus cell complexes.

**CONCLUSION:** These results suggest that KCZ-mediated inhibition of follicular maturation probably results from impaired steroidogenesis at early phase of follicular development toward ovulation. Hence, attenuation of folliculogenesis by KCZ may be harnessed to modulate gonadotropin-ovarian stimulation in fertility treatments.

**KEYWORDS:** rat ovary, ketoconazole, inhibited steroidogenesis, anovulation, follicular development, CYP11A1, cumulus cells

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### Introduction

Ketoconazole (KCZ) is a widely used antimycotic drug known to inhibit steroidogenic P450 enzymes in the adrenal cortex and gonads.<sup>1</sup> Therefore, this drug has been applied to attenuate

steroidogenesis under pathological conditions such as Cushing's syndrome,<sup>2</sup> prostate cancer,<sup>3</sup> and hirsutism.<sup>4</sup> Another exaggerated steroid hormone production is encountered in fertility treatments where excessive response to gonadotropin



stimulation may lead to multifollicular development occasionally culminating in ovarian hyperstimulation syndrome.<sup>5</sup> In this regard, we and others examined the potential use of the drug to attenuate ovarian follicular recruitment during ovarian hyperstimulation.<sup>6–8</sup> Indeed, these studies showed reduced ovarian steroidogenesis; yet, the impact of KCZ on ovarian folliculogenesis was less clear.

In the present study, we aimed to establish an animal model to explore the potential outcomes of KCZ administration on follicular development during gonadotropins hyperstimulation. The superovulated rat model seemed suitable for our purpose, because gonadotropin stimulation of the immature ovary triggers the growth of a large cohort of follicles that synchronously progress through maturation and ovulation. This study shows that KCZ not only inhibits ovarian steroidogenesis but also arrests ovulation subsequent to impairment of follicular functions and development.

## Materials and Methods

**Materials.** Equine chorionic gonadotropin (eCG 600; Gestyl) was purchased from Intervet (Angers, France) and human CG (hCG; Pregnyl) from Organon Special Chemicals (West Orange, NJ). Pure KCZ (R41,400, Janssen Pharmaceutica, Inc.) base powder was a gift from Abic Ltd. Pharmaceutical and Chemical Industries (Netanya, Israel).

**Animals.** Intact, prepubertal female Wistar-derived rats (25 days old, weight  $75 \pm 2.6$  g) were obtained from Harlan (Jerusalem, Israel), housed in specific pathogen-free (SPF) air-conditioned rooms illuminated for 16 hours per day, and were provided with water and rat chow ad libitum. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols had the approval of the Institutional Committee on Animal Care and Use, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem (Jerusalem, Israel). The Hebrew University of Jerusalem is Association for Assessment and Accreditation of Laboratory Animals Care (AAALAC) approved.

Multiple follicular developments were induced by sc injection of animals with eCG (15 IU) at 10:00 am. After 52 hours, ovulation was induced by injection of hCG (4 IU, sc), and at the indicated time-points, the animals were sacrificed by cervical dislocation and the ovaries were removed and trimmed free of fat.

**Ovulation rates.** Approximately 18 hours after the administration of hCG (corresponding to the day of estrus in cycling animals), the rats were sacrificed by cervical dislocation and the oviduct ampullae were immediately excised and squeezed under a dissecting microscope to express the ovulated oocyte–cumulus complexes.

### Follicular size and function.

**Morphometric analysis.** Ovarian paraffin sections were prepared by immersion-fixation in 4% sodium phosphate-buffered paraformaldehyde, pH 7.4. After dehydration and embedding

in glycomethacrylate (Sorvall, DuPont, Wilmington, DE), 4- $\mu$ m-thick serial sections were prepared using a Sorvall Porter-Blum microtome (J-4). The ovaries were serially sectioned, and every 12th section was mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) and photographed under a Zeiss Ultraphot microscope (Carl Zeiss, New York, NY). The number of follicles and their maximal diameter taken in sections traversing the oocyte–cumulus complex was measured manually using micrometer-scaled ruler.

**Immunofluorescence staining.** Ovarian cryosections were prepared as previously described.<sup>9</sup> Briefly, the ovaries were removed, embedded in O.C.T.<sup>TM</sup> compound (Tissue-Tek, Miles Scientific, Naperville, IL), and underwent freezing on copper stab immersed in liquid N<sub>2</sub>. Cryostat sections (6  $\mu$ m) were thawed on polylysine (5 mg/mL; Sigma P1524, St. Louis, MO)-coated slides and fixed for five minutes in acetone at  $-20^{\circ}\text{C}$ . After washing with PBS, each section was incubated with 30  $\mu$ L of diluted polyclonal rabbit antiserum to rat P450scc<sup>10</sup> (1:30 in 0.1% Tween-20 in PBS, TBS) for 10 minutes at room temperature. Following  $\times 3$  PBS washes (2.5 minutes), the sections were similarly incubated with secondary antibody conjugated to Rhodamine Red-X (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1:50 in TBS. The sections were then washed, covered with 90% glycerol solution in PBS, and observed by inverted fluorescence microscope Zeiss Axiovert S100 equipped with DVC 1310C camera. The relative immunofluorescence intensities of the functional cell layers (theca, granulosa, cumulus, and theca-interstitial) were qualitatively compared within given tiled micrographs. Shown are representative ovarian sections ( $n = 3–6$  per treatment).

**Steroid hormones measurements.** Ovaries were removed, rapidly trimmed free of fat, and homogenized in phosphate-buffer saline, pH 7.2. The steroid content was extracted by ether. After evaporation to dryness, the steroids were redissolved in RIA buffer, and progesterone (P), androstenedione (A), and 17 $\beta$ -estradiol (E2) were determined by the Coat-A-Count<sup>®</sup> RIA method, using a solid phase <sup>125</sup>I-labeled P, A, and E2 (DPC method; Diagnostic Products Corporation, Los Angeles, CA). The sensitivity assays for P, A, and E2 were 0.05 ng/mL, 0.04 ng/mL, and 8 pg/mL, respectively. The intra-assay and inter-assay coefficients of variation for P, A, and E2 assays were 7.5, 5.0, 5.3% and 7.2, 10.0, 6.4%, respectively. No cross-reactivity of KCZ could be observed with any steroid measured by RIA.

**KCZ administration.** As KCZ is known to have a short serum half-life of two to eight hours,<sup>11</sup> the drug was administered sc to prepubertal (25 days) rats in a slow-release form prepared in cellulose gel. Stock solution of KCZ was prepared as follows: KCZ base powder (350 mg) was dissolved in 2.8 mL HCl 0.5 N and then further diluted with sterile PBS. The acidic pH was titrated to pH 3.3 by NaOH (0.1 N) to give a final concentration of 25 mg/mL. For preparation of

the drug in 2% cellulose, 20 mL of the latter KCZ solution was mixed with cellulose powder (600 mg) and gelation was allowed under gentle stirring. The homogenous yellowish gel with KCZ, or the clear control gel without KCZ (vehicle), was then injected sc, 1 mL/rat. For repeated injections of KCZ, stock solution of the drug was dissolved 1:1 (v/v) in polyethylene glycol-400 (PEG; Sigma-Aldrich P-202398, Saint Louis, MO) to a final concentration of 12.5 mg/mL (pH 5.3). Then, doses of 5 mg KCZ/400  $\mu$ L (66.6 mg/kg) were injected sc every eight hours.

**Statistical analysis.** Data are reported as means  $\pm$  SD. All statistical analyses were performed by using the Statistical Package for the Social Sciences 20.0 (SPSS version 20.0 for Windows). Mann-Whitney test was used to compare ovarian levels of progesterone, androstenedione, and estradiol of KCZ-treated animals and controls and the effect of KCZ on follicular size groups. The effect of KCZ administration on ovulations in KCZ-treated animals and controls was compared by one-way analysis of variance (ANOVA) with post hoc analysis by Bonferroni test.  $P < 0.05$  was considered statistically significant for all tests.

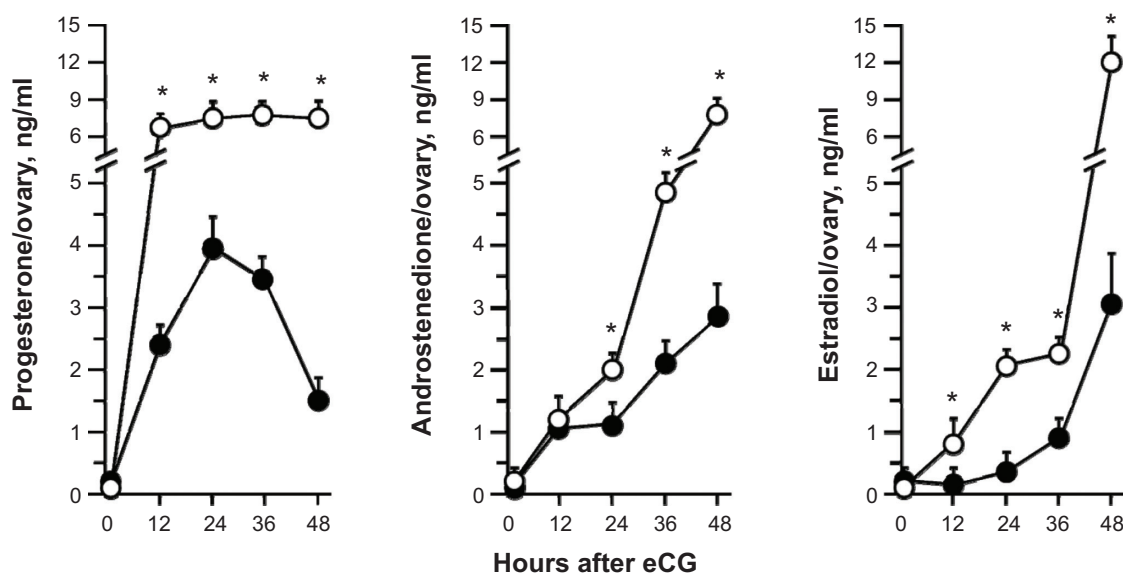
## Results

Following eCG administration to control animals, the levels of progesterone, androstenedione, and estradiol rose dramatically (40–50 fold) as expected in this superovulated model of synchronized follicular development toward ovulation (Fig. 1). When a single dose of KCZ was administered before eCG treatment, the levels of progesterone, androstenedione, and 17 $\beta$ -estradiol markedly reduced when compared to control

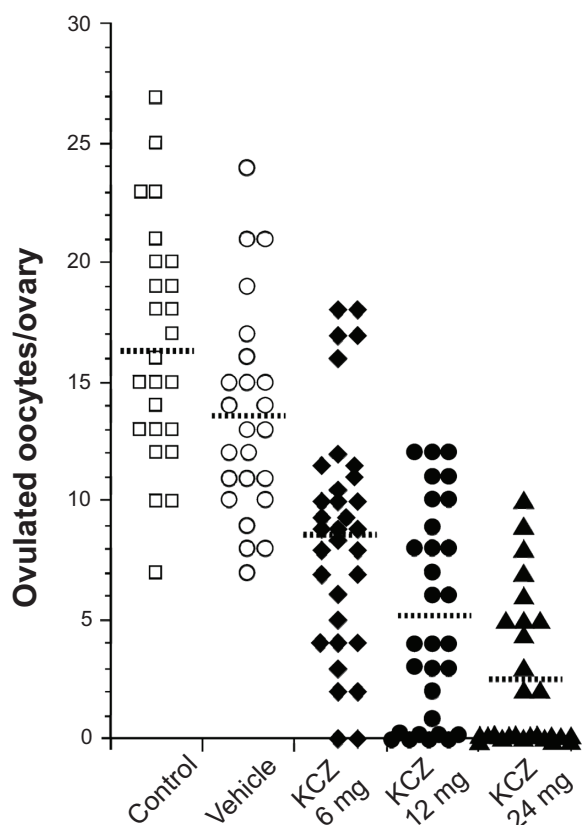
animals without KCZ (Fig. 1). These results confirmed the efficacy of the slow-release of the KCZ formula on the ovarian P450 enzymes *in vivo*.

As KCZ inhibited ovarian steroidogenesis, we further questioned if the drug can affect the rate of ovulation in the eCG/hCG-treated superovulating rat model.<sup>12</sup> For this aim, follicular development toward ovulation was induced by eCG/hCG administration to prepubertal rats. In control animals, 7–27 oocytes ( $16.3 \pm 5.1$ ) ovulated after hCG administration (Fig. 2). Ovulation was slightly inhibited (not statistically significant) in vehicle-treated animals, whereas KCZ markedly inhibited ovulation dose dependently; more than 50% of the animals did not ovulate at all when received 24 mg of KCZ before hormone treatment.

To characterize the events associated with KCZ-mediated anovulation, we examined the follicular development by exploring morphological and functional follicular parameters. For this purpose, peri-ovulatory ovaries were collected from eCG-/hCG-primed animals at 12:00 am ie two hours before ovulation. By and large, the maximal sizes of the leading follicles in KCZ-treated animals were smaller than their counterpart peri-ovulatory follicles (OFs) in the drug-free control animals (Fig. 3A). Moreover, the cumulus complexes in the largest follicles of the KCZ-treated animals were not mucified as normally observed in peri-OFs (Fig. 3C). Quantification of follicular size in serial thin paraffin sections throughout the entire ovary (see Materials and Methods) revealed that as expected, eCG/hCG treatment of prepubertal animal promoted development of multiple follicles toward ovulation (Fig. 4). When comparing between KCZ treatment



**Figure 1.** Inhibition of ovarian progesterone, androstenedione, and estradiol by slow-releasing KCZ-gel. Prepubertal rats (25 days old,  $n = 4$ ) received at 2:00 am injection of KCZ-gel (24 mg KCZ mixed in 1 mL of cellulose gel, solid circles). The following morning at 10:00 am, each rat received eCG (15 IU). Control animals were treated with eCG and cellulose gel without KCZ (vehicle, open circles). At the indicated time-points, ovaries were collected for extraction and steroids were measured by RIA. Shown are mean  $\pm$  SD of the indicated steroids. **Note:** \* $P < 0.001$  (vehicle vs KCZ).



**Figure 2.** Slow-releasing KCZ-gel dose-dependent inhibited ovulation. Groups of 12–17 prepubertal rats (total  $n = 71$ ) received at 02:00 am sc injections of 6, 12, and 24 mg KCZ-gel (solid rhomboids, circles, and triangles, respectively) and eCG as described in Figure 1. After 52 hours, hCG was administered (4 IU). Control animals were either treated with eCG/hCG alone (open squares), or gonadotropins and cellulose gel without KCZ (vehicle, open circles). Shown is the number and means (horizontal dashed line) of ovulated oocytes. Not significant (control vs vehicle);  $P < 0.05$  (KCZ (24 mg) vs KCZ (6, 12 mg));  $P < 0.001$  (control, vehicle vs KCZ (6, 12, 24 mg)).

and control ovaries, the size and the number of secondary follicles or smaller ones (50–630  $\mu\text{m}$ ) were not statistically different (Fig. 4). However, the number of the large follicles (700–920  $\mu\text{m}$ ) differed between the studied groups ( $P = 0.029$ ). Within this size range, KCZ limited the development of the follicle to sub-maximal size of 800–840  $\mu\text{m}$ , which attained a maximal peri-ovulatory size of 920  $\mu\text{m}$  in the eCG/hCG-treated control animals. All of the latter follicles contained mucified cumuli, whereas none of the KCZ-affected follicles progressed to cumulus expansion.

In an attempt to better define the time interval through which the presence of KCZ is effective to inhibit ovulation, we administered multiple injections of 5 mg each during different time-windows of follicular development following eCG administration. For this protocol and being aware of the rapid clearance rate of KCZ in the circulation,<sup>11</sup> we did not use the slow-releasing KCZ-gel formula, but rather administered

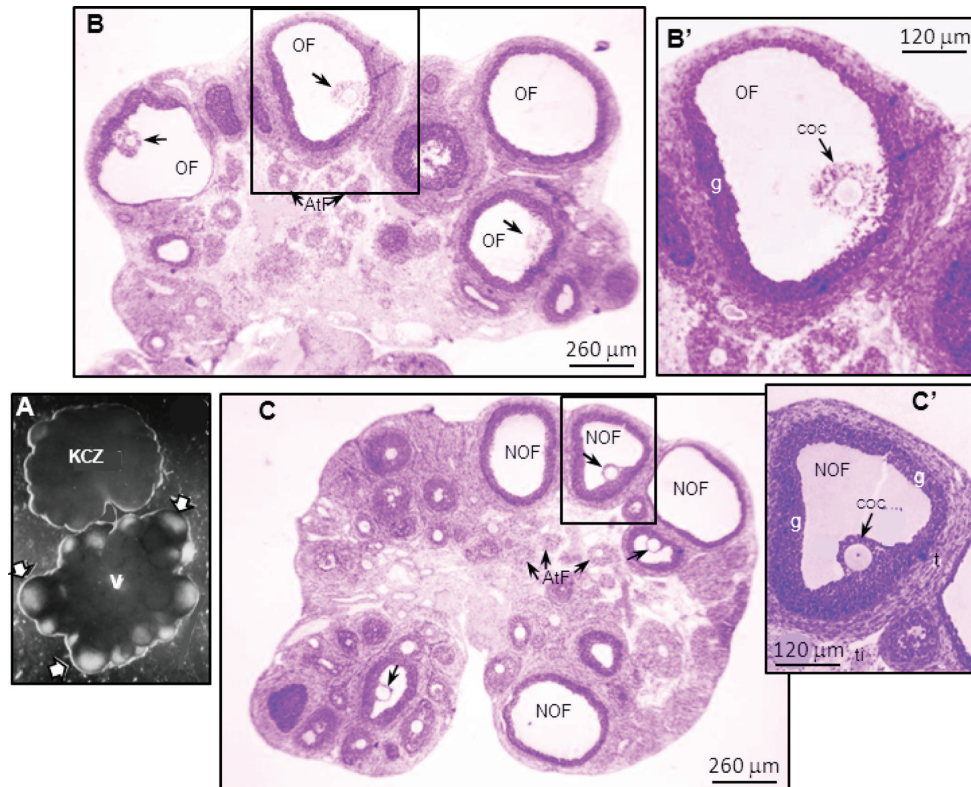
the drug dissolved in PEG (see Materials and Methods). Figure 5A shows that vehicle-treated animals ovulated an average of  $16.5 \pm 4.1$  oocytes per ovary. By contrast, when onset of gonadotropin treatment commenced eight hours after the first KCZ administration, ovulation was progressively arrested down to ~20% (Figs. 5B–D). By contrast and quite unexpectedly, KCZ failed to inhibit ovulation if onset of the drug administration commenced 24 hours after eCG treatment.

In light of the notion that expression of CYP11A1/P450<sub>scc</sub> in the granulosa cell compartment is an essential marker of functional maturity in peri-OFs,<sup>12</sup> we sought to examine the effect of KCZ on this cytochrome level in eCG/hCG-treated animals examined in ovarian sections retrieved shortly before ovulation (Fig. 6). In naive ovary, relatively low level of CYP11A1 was noted only in the androgen producing cells of theca interstitial and some theca cell layers (Figs. 6A, A'). Peri-ovulatory ovaries contained large follicles ready for ovulation endowed with CYP11A1 expression in all the functional cell types including the granulosa and cumulus complexes (Figs. 6B, B'). Again, consistent with our previous notion, CYP11A1 was not expressed in granulosa and cumulus cells of non-ovulatory follicles (NOF) even if attained a large size. Similarly, KCZ treatment resulted in lack of CYP11A1 expression in the granulosa–cumulus compartments in all of the largest follicles in such ovaries (Figs. 6C, C'). Worth mentioning is the observation that treatment with KCZ did not prevent the expression of CYP11A1 in the cell layers of theca interna and theca-interstitial cells.

## Discussion

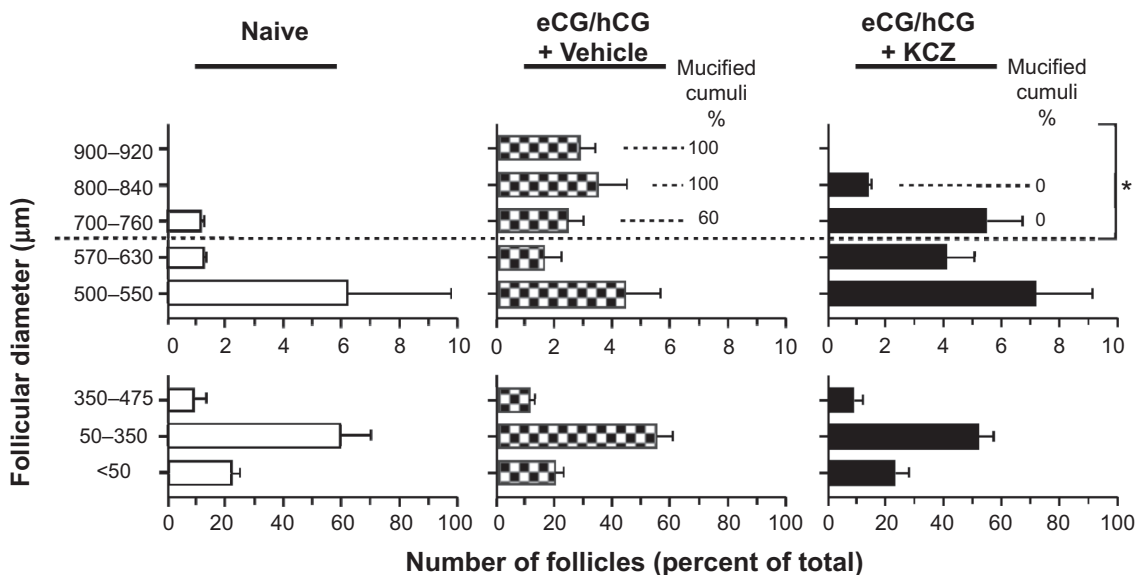
KCZ is a known inhibitor of the ovarian steroidogenic P450s, namely CYP11A1, CYP17A1, and CYP19A1.<sup>13–20</sup> The present study using the superovulating rat model shows that KCZ is also effective in vivo while exerting inhibitory effect on ovarian steroidogenesis induced by eCG/hCG treatments. Furthermore, despite the fact that the half-life of KCZ in the circulation is relatively short,<sup>11</sup> administration of a gel formula of the drug before the gonadotropin stimulation maintained its inhibitory effect for 48 hours of the eCG treatment. This observation suggests that the KCZ-gel acts in a slow-release fashion.

The effect of KCZ inhibition of ovarian steroid production is also reflected in follicular development. Our study clearly indicates that KCZ indeed inhibits ovulation probably by dual effects on follicular functions. First, KCZ attenuates the activity of the steroidogenic cytochromes<sup>13,15–17</sup> that is in agreement with earlier studies showing that other inhibitors of steroidogenesis, such as aminoglutethimide and cyanoketone, inhibited ovulation in eCG/hCG-treated rats.<sup>21</sup> Second, KCZ profoundly arrested development of the Graafian follicles and interfered with the expected expression of granulosa cells



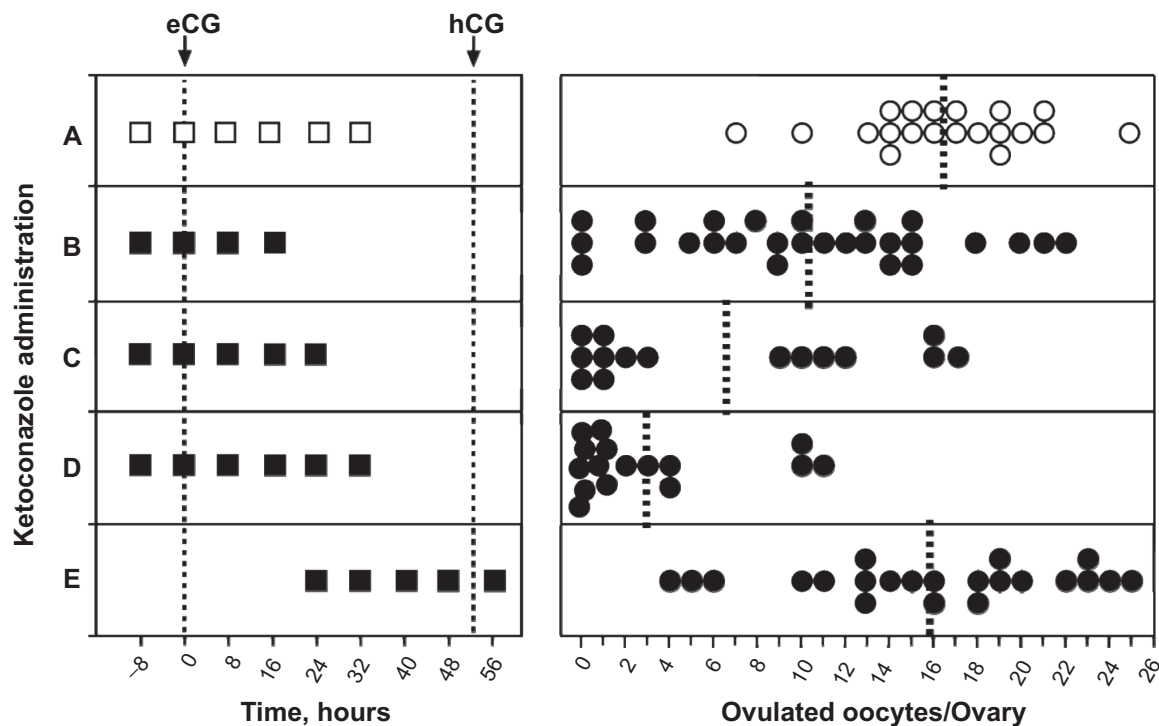
**Figure 3.** KCZ arrests development of OFs. Prepubertal rats ( $n = 4$ ) received eCG/hCG with KCZ-gel (24 mg) or without (vehicle) as described in Figure 2. Two hours before ovulation (12:00 am), the animals were sacrificed and ovaries were collected for histological analysis (**B, C**); (**B', C'**) they are high magnification of the depicted respective area. (**A**) Representative ovarian micrographs from KCZ-treated and vehicle (V) animals. (**B**) H&E-stained paraffin section of vehicle-ovary depicts large peri-OFs containing mucified cumulus–oocyte complexes (COCs) (arrows). (**C**) In KCZ-treated animals, the ovary contains large NOF with typical non-mucified compact COCs (arrows).

**Abbreviations:** AtF, atretic follicle; g, granulosa.



**Figure 4.** Effect of KCZ administration on morphometrical parameters of follicular development. KCZ-gel (24 mg), or vehicle, was administered to prepubertal rats ( $n = 4$ ) before onset of eCG/hCG treatment as described in Figure 2. Naive animals ( $n = 2$ ) did not receive any gonadotropins. Two hours before ovulation (00:00), ovaries were retrieved and prepared for histological morphometrical analyses. Shown is size distribution of follicular diameter (percent of total/ovary). Bars and error bars represent means  $\pm$  SD from four ovaries.

**Note:** \* $P = 0.029$  (naive vs eCG/hCG + vehicle, eCG/hCG + KCZ at follicular diameter  $>700 \mu\text{m}$ ).



**Figure 5.** KCZ treatment at the early stage of follicular development inhibits ovulation. **(A)** At the indicated time-points (open squares, left panel), vehicle was administered to prepubertal rats ( $n = 10$ ) while eCG was administered at time 0 followed by hCG administration 52 hours later. Shown is the number and mean of ovulated oocytes (open circles and vertical dashed line, respectively, on right panel). **(B–D)** Consecutive KCZ doses (5 mg each) were administered ( $n = 30$  animals) at the indicated time-points (solid squares), and the eCG/hCG-induced ovulations (solid circles) were monitored. **(E)** Onset of KCZ administrations (5 mg each) started at 24 hours after eCG treatment ( $n = 11$  animals). Not significant (a vs e);  $P < 0.01$  (a, e vs b, c, d; d vs b).

CYP11A1. Yet, the theca layers of these arrested follicles were fully endowed with high levels of CYP11A1 suggesting distinct patterns of differentiation that differ in those two cell types. Finally, the KCZ-arrested follicles did not respond to hCG administration as evidenced by containing compact cumulus complexes lacking mucification.

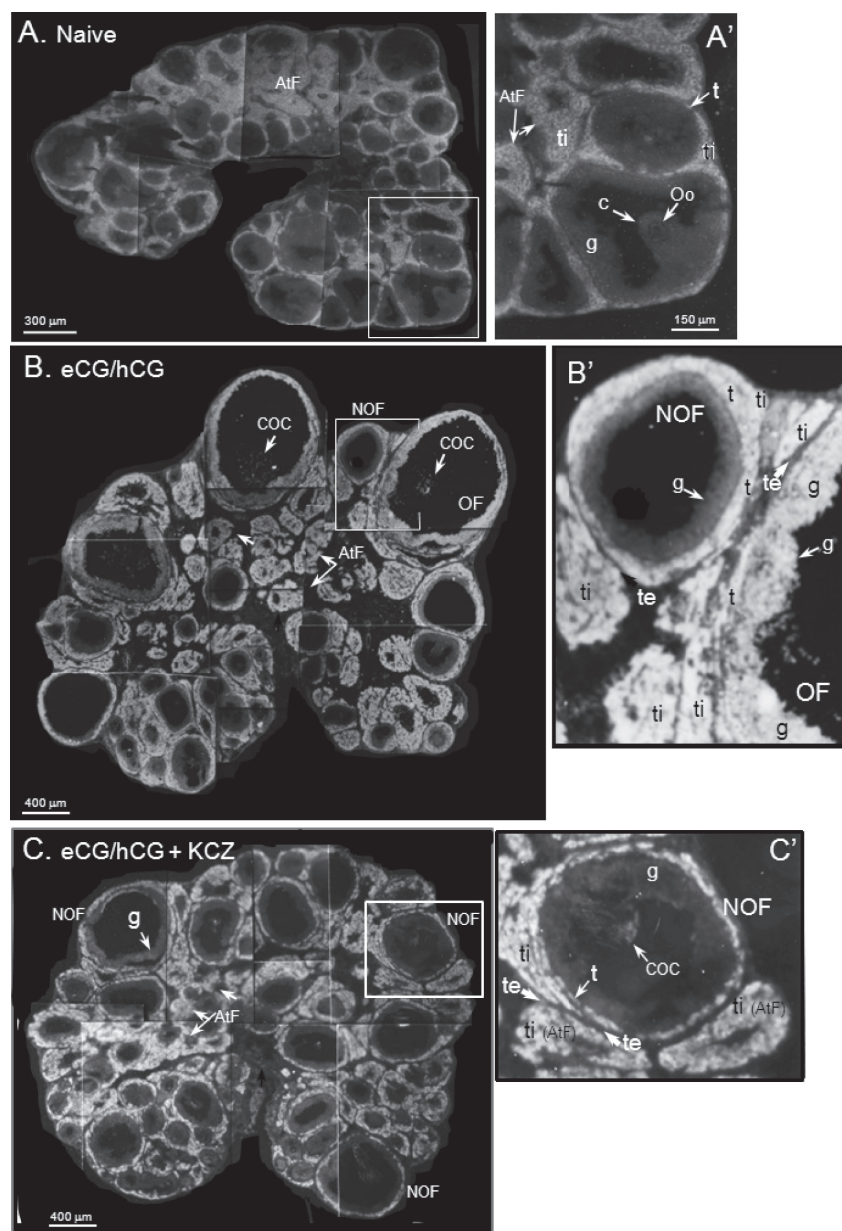
The mechanism underlying the impairment of gene expression in the granulosa and the cumulus cell types by KCZ is yet to be elucidated. In this regard, this study suggests that timing of KCZ application during follicular development plays a critical role in the ability of the drug to affect ovulation, for which KCZ should be placed ahead of gonadotropin administration. If so, what events during early follicular phase could be affected by KCZ interrupted steroidogenesis? It is known that a successful induction of follicular development by FSH requires low basal amounts of LH to support the selection of small antral follicles to enter the preovulatory stage.<sup>22</sup> The target cell-type for this tonic stimulation of LH is the theca-interstitial cells that constitutively produce androgens known to potentiate FSH induction of granulosa cells CYP11A1, CYP19A1, and LH-receptors.<sup>23,24</sup> Thus, it is tempting to speculate that KCZ inhibition of rather non-follicular androgen synthesis in theca-interstitial cells constitutes, in fact, the

predominant KCZ interruption of the ovarian response to eCG (LH + FSH). Further support in favor of this notion is that inhibition of steroidogenesis at mid-follicular phase during which the estradiol levels peak did not have any effect on ovulation. These results in the animal model are also consistent with the study by Parsanezhad et al where KCZ administration to PCOS patients at the very onset of gonadotropins treatment resulted in attenuation of folliculogenesis.<sup>8</sup> Similarly, our previous observations in PCOS patients showed that treatment with KCZ as early as the first day of gonadotropin stimulation attenuated the follicular response more than initially intended.<sup>6</sup>

In summary, this animal study shows that *in vivo* inhibition of ovarian steroidogenesis by KCZ affects a series of follicular development markers indicative of the drug potency to modulate gonadotropin-ovarian stimulation in fertility treatments.

#### Author Contributions

MG planned and executed the experiments and was responsible for data interpretation and writing of the manuscript. JO helped in planning, supervised the work, and participated in data analysis and interpretation. All authors reviewed and approved the final manuscript.



**Figure 6.** Inhibitory effect of KCZ on follicular maturation induced by gonadotropins. Equine CG was administered to prepubertal rats ( $n = 6$ ) followed by hCG. Seven hours later, ovaries were cryosectioned and processed for immunohistochemistry using CYP11A1 antiserum, as shown in representative images (A–C). (A'–C') are high magnification of the depicted respective area. (A) Untreated naive ovary shows basal expression of CYP11A1 in some of the theca (t) cells and theca-interstitial (ti) cells but not in the granulosa (g) and cumulus (c) compartment of all follicles. (B) eCG/hCG-induced peri-ovulatory ovary shows expression of CYP11A1 in granulosa, mucified COC, and theca of OF, but not in smaller NOF. (C) KCZ-gel (24 mg) was administered eight hours before eCG/hCG treatment, and ovaries were processed as described in (B). Note lack of CYP11A1 in granulosa cells and COC of large NOF. Yet, high CYP11A1 expression is noted in the theca and theca-interstitial cells.

**Abbreviations:** Oo, oocyte; te, theca externa cells; AtF, atretic follicle.

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