

Does 2-hydroxyflutamide Inhibit Apoptosis in Porcine Granulosa Cells? — An *In Vitro* Study

Malgorzata DUDA¹⁾, Malgorzata DURLEJ¹⁾, Malgorzata KNET¹⁾,
Katarzyna KNAPCZYK-STWORA¹⁾, Zbigniew TABAROWSKI²⁾ and Maria SLOMCZYNSKA¹⁾

¹⁾Department of Endocrinology, Institute of Zoology, Jagiellonian University, 30-387 Krakow, Poland

²⁾Department of Experimental Hematology, Institute of Zoology, Jagiellonian University, 30-387 Krakow, Poland

Abstract. In mammalian ovaries, the majority of follicles are lost before ovulation by atresia. This degenerative process is initiated or caused by granulosa cell apoptosis. To reveal the androgen-dependent mechanism of selective follicular atresia, the culture model system for agonism and antagonism of the androgen receptor has been established. We examined the influence of an androgen receptor antagonist, 2-hydroxyflutamide (2-Hf), on the incidence of apoptosis in cultured porcine granulosa cells. They were incubated (6 and 12-h) in the presence of testosterone (T, 10^{-7} M), 2-Hf (1.7×10^{-4} M) or both T and 2-Hf (T+2-Hf), and then analyzed by flow cytometry with fluorescein labelled annexin V. To better imitate *in vivo* conditions, the intact porcine follicles (6–8 mm in diameter) have been incubated in an organ culture system with the addition of the same factors. Sections obtained from follicles fixed after culture were stained with hematoxylin and eosin, and the presence of apoptosis-related DNA strand breaks was evaluated by the TUNEL method. Estradiol and progesterone concentrations in the culture media were measured by radioimmunoassays. The addition of T or 2-Hf to the culture media caused an increase in the number of apoptotic granulosa cells, while treatment with T+2-Hf decreased it in both *in vitro* and organotypic models. Follicles cultured with the addition of T or 2-Hf exhibited morphological changes indicating follicular atresia. Granulosa estradiol secretion was considerably stimulated by T+2-Hf. The highest increase in follicular estradiol secretion was observed after the anti-androgen addition. In both granulosa and follicular cultures, the production of progesterone declined in the presence of T or 2-Hf but increased after their simultaneous addition. In conclusion, androgen receptor antagonist 2-Hf attenuates induction of granulosa cell apoptosis in the presence of a high T level. The nature of this protective mechanism as yet is unknown and requires further research.

Key words: Apoptosis, Granulosa cells, 2-hydroxyflutamide, Ovarian follicles, Pig

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The ovary plays two distinct functions in female reproduction: it produces germ cells (oocytes) and provides a proper hormonal environment for ovarian follicles undergoing cycles of growth and development induced by gonadotropins [1–3]. Although gonadotropins are predominant regulators of follicular development, it is now clear that there is a wide range of additional regulators of follicular maturation produced within the ovarian tissue such as steroids and peptide hormones, as well as ovarian growth factors. They act by paracrine mechanisms to modulate, either to amplify or attenuate, the functions of gonadotropins [4]. Amongst the several hundred thousand primordial follicles present in the mammalian ovary, only 1% of them develop to the preovulatory stage and finally ovulate [5]. The remaining ones will be eliminated via a degenerative process called atresia [6, 7]. Atresia occurs at all stages of follicular growth and development; however, the early and middle antral stage porcine follicles are the most susceptible to degeneration [8]. Although its hormonal and molecular mechanisms are still largely unknown, it was shown that follicular selection predominantly depends on granulosa

cell apoptosis [9–11]. Therefore, it is important to identify the antiapoptotic/proapoptotic factors preventing granulosa cell apoptosis. Previous reports clearly showed that FSH and/or IGF-1 critically regulate the function of granulosa cells, including cell survival via the PI3K-Akt pathway [12]. Hickey *et al.* [13] have considered the interactions between androgens, FSH and IGF-1 in porcine antral follicles *in vitro*. They observed that androgens stimulated proliferation and inhibited progesterone secretion depending on follicle size and the presence of FSH and/or IGF-1. Furthermore, many reports indicated that androgens upregulate the expression of FSH receptor mRNA [14, 15]. Numerous researchers have attempted to indicate the primary trigger of apoptotic stimuli and the intracellular signal transduction pathway engaged in granulosa cell apoptosis during follicular atresia.

Many factors have been examined to elucidate their contribution to granulosa cell apoptosis induction in porcine ovarian follicles [16–19]. Previous studies have shown that gonadal steroids can modulate the incidence of granulosa apoptosis [20, 21]. The effects of androgens on granulosa cells apoptosis have been described in rat ovaries [22]. Androgens regulate a variety of ovarian functions by serving as a substrate for estrogen synthesis, targeting the androgen receptor (AR) or eliciting non-genomic mechanisms [23]. Androgens originating in the theca cells feed granulosa cells to produce estradiol [24]. In turn, estradiol stimulates follicular growth, increases ovarian weight,

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Correspondence: M Duda (e-mail: duda.m@op.pl)

enhances the mitotic index of granulosa cells and inhibits granulosa cell apoptosis. The expression of AR in granulosa cells of various species of mammals makes these cells responsive to androgens [25–27]. However, when androgen production exceeds a certain level, follicular development is inhibited rather than stimulated. Thus, *in vivo* treatment with androgens caused a dose- and time-dependent decrease in ovarian weight [28] and an increase in morphological signs of atresia [29]. Furthermore, testosterone treatment enhanced apoptotic DNA fragmentation in rat granulosa cells of early antral and preantral follicles, antagonizing the effect of estrogen [30]. Androgens appear to be atretogenic in rodents but may stimulate ovarian growth in primates [31]. The most compelling evidence that androgens may stimulate early stages of follicular growth comes from the observation that treatment of monkeys treated *in vivo* with a high dose T for 3–10 days it exerts a marked growth-promoting effect on small and medium sized follicles [32].

Therefore, to investigate the incidence of apoptosis in porcine granulosa cells, the androgen receptor antagonist 2-hydroxyflutamide (2-Hf) was used. 2-Hf is a potent, nonsteroidal antiandrogen that has been reported to lack other agonistic or antagonistic hormonal properties [33]. It binds to the AR and competitively inhibits the binding of testosterone and dihydrotestosterone [34, 35]. The current study was undertaken to investigate whether androgens or antiandrogens exert proapoptotic effects on granulosa cells and when/if they are involved in follicular atresia.

Materials and Methods

Animals

Porcine ovaries were obtained from Polish Landrace sows at a local slaughterhouse and placed in a cold phosphate-buffered saline (PBS; pH 7.4, PAA The Cell Culture Company, Dartmouth, MA, USA) containing Antibiotic/Antimycotic Solution (AAS 10 µl/ml; PAA The Cell Culture Company). Ovaries were transported to the laboratory within 30 min and rinsed twice with sterile PBS supplemented with antibiotics. In each experiment, ten ovaries from five animals were selected for cell isolation. Assuming that each ovary yielded 3–5 follicles, the total number of follicles varied from 30 to 50. The phase of the estrous cycle was determined according to the established morphological criteria [36]. Medium follicles (6–8 mm in diameter), classified by morphometric criteria as healthy [37, 38], were selected for cell and organ cultures. Briefly, follicles were dissected free from the ovarian stroma and separately classified under a microscope. Healthy follicles were characterized by a well-vascularized follicular wall and the clarity of the follicular fluid. Early atretic and atretic follicles were traversed by few or no blood vessels, and the surface of the follicles was opaque with the progression of atresia.

This procedure was chosen to minimize the variability between tissues and animals.

Granulosa cell preparation and culture

The isolation of granulosa cells (GCs) was performed according to the technique developed in our laboratory [39]. GCs were scraped from the follicular wall with round-tip ophthalmologic tweezers. After collection, GCs were washed several times in PBS and recovered

by low speed centrifugation (90× *g* for 10 min). Cell viability was tested by the trypan blue exclusion test (mean ± SD: 92% ± 3%). The cells were seeded in 6-well culture plates (Nunc, Kamstrup, Denmark) at an initial density of 8×10⁵ cells/ml. Control cultures were carried out in McCoy's 5A medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, PAA The Cell Culture Company). Experimental cultures were carried out in McCoy's 5A medium with the addition of testosterone (T) (10⁻⁷ M), 2-Hf (1.7×10⁻⁴ M) or both T and 2-Hf (T+2-Hf). After 6 and 12 h of culture, all media were collected and stored at -20 C for radioimmunoassay of estradiol and progesterone. All experiments were performed in quadruplicate (four wells) in five separate cultures (n=5 independent experiments).

Annexin V-FITC test for apoptosis

Examination of phosphatidylserine translocated to the external leaflet of cell membranes was used to evaluate the frequency of apoptosis in granulosa cells populations [40]. Four groups of granulosa cells (control, treated with T, 2-Hf or T+2-Hf, respectively) after 6 and 12 h of culture were analyzed. Cells were centrifuged and washed with Annexin V Binding Buffer (51-66121E, BD Biosciences Pharmingen, San Diego, CA, USA). Annexin V (AV) conjugated with fluorescein (FITC) (51-65874X, BD Biosciences Pharmingen) and propidium iodide (PI) (51-66211E, BD Biosciences Pharmingen) were added to all groups to distinguish between living (AV-/PI-), apoptotic (AV+/PI-) and necrotic cells (AV+/PI+) or cell debris (AV-/PI+). After being stained for 30 min, the samples were analyzed in a flow cytometer (352052, BD Falcon™, Franklin Lakes, NJ, USA). Excitation of the DNA-associated PI was accomplished with an argon-ion laser tuned to 488 nm and operated using 15 mW in the standard FACSCalibur flow cytometer configuration. Fluorescence intensity of FITC (520 nm) was detected using an FL1 detector, whereas PI intensity (617 nm) was detected using an FL3 detector. On the basis of dependence between counts received from the FL3 and FL1 detectors, findings were calculated using the WinMDI 2.8 software.

Follicle culture and preparation for morphological and TUNEL analysis

Whole follicles (n=36, 6–8 mm in diameter) isolated from porcine ovaries were cultured on a filter disk on a triangular stainless steel grid over a well of McCoy's 5A medium supplemented with 10% FBS and AAS (5 µl/ml) [41]. Follicles during organ culture were randomly assigned to treatment groups with the addition of T (10⁻⁷ M), 2-Hf (1.7×10⁻⁴ M) or both T and 2-Hf (T+2Hf). All culture media were collected after 6 and 12 h and stored at -20 C for further estradiol and progesterone level analysis, whereas follicles (n=3/each group) were fixed in 4% paraformaldehyde, subsequently dehydrated in an increasing gradient of ethanol and embedded in Paraplast (Sigma-Aldrich, St. Louis, MO, USA). Sections of 5 µm in thickness were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich), deparaffinized and rehydrated through a series of decreasing alcoholic solutions. For morphology, routine hematoxylin-eosin staining (H&E) was performed.

TUNEL assay

The presence of apoptosis-related DNA strand breaks in sections

of follicles was evaluated by TUNEL assay using an *In situ* Cell Death Detection kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, the 5 µm paraffin tissue sections were pretreated with 10 µg/ml Proteinase K solution (Promega, Madison, WI, USA) for 15 min at 37 °C. Next, samples were rinsed in PBS, immersed for 10 min in 3% H₂O₂ in methanol at room temperature to quench endogenous peroxidase activity and incubated with 5% bovine serum albumin (BSA, Sigma-Aldrich) for 20 min to block non-specific binding sites. Thereafter, sections completely rinsed in PBS were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase and fluorescein labeled nucleotide mixture) for 60 min at 37 °C in a humidified atmosphere in the dark. Next, sections were rinsed in PBS, and mounted in VectaShield medium for fluorescence (Vector Labs, Burlingame, CA, USA) and viewed under a Zeiss confocal laser scanning microscope LSM510 (GmbH, Jena, Germany).

Sections from each follicle were viewed under a 40× objective and scored by an observer blinded to the treatment groups. For each follicular cross section, all cross-sectional granulosa cell profiles (100) were counted and then the number of TUNEL-positive cells noted for each cross-sectioned follicle.

Radioimmunoassay

Samples of the culture media were analyzed for estradiol and progesterone content using radioimmunoassay [42, 43]. Estradiol-17β was determined using [2,4,6,7-³H] estradiol (specific activity 104 Ci/mmol; Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK) as a tracer and rabbit antibody against estradiol-17-O-carboxymethylxime-BSA (a gift from Prof Roman Rembiesa, Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland). The lower limit of sensitivity of the assays was 5 pg. Cross-reaction was 1% with keto-estradiol-17β, 0.8% with estrone, 0.8% with estriol, 0.01% with testosterone and less than 0.1% with major ovarian steroids. Coefficients of variation within and between assays were below 4% and 7.5%, respectively. Progesterone was measured using [1,2,6,7-³H] progesterone (specific activity 96 Ci/mmol; Amersham, GE Healthcare) as a tracer and an antibody induced in sheep against 11α-hydroxyprogesterone succinyl-BSA (a gift from Prof Brian Cook, University of Glasgow, Glasgow, Scotland). The lower limit of sensitivity of the assays was in the order of 20 pg. Coefficients of variation within and between assays were below 5.0% and 9.8%, respectively.

Statistical analysis

All annexin V-FITC and TUNEL test data were analyzed statistically using the Statistica 5.1 software (StatSoft, Tulsa, OK, USA) by one-way analysis of variance (ANOVA). Statistical significances were set at $P < 0.05$. All radioimmunological data are expressed as means ± SEM from at least three independent experiments ($n = 3$). Each experiment was performed in quadruplicate, resulting in at least 12 observations. Significant differences in steroid medium concentration between the control and experimental cultures were assayed by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Detection of granulosa cell apoptosis

In granulosa cells that originated from all groups after 6 h of culture, flow cytometry with annexin V revealed only a negligible number of apoptotic cells ($0.6 \pm 0.4\%$). Almost 100% of cells were healthy and alive. This analysis showed that $41.85 \pm 1.51\%$ of granulosa cells originating from 12-h cultures of the control group exhibited phosphatidylserine at the external leaflet of cell membranes. In the presence of T, the fraction of annexin V-positive cells increased ($P < 0.05$) to $44.78 \pm 0.42\%$. With the addition of 2-Hf, a substantial increase in the number of apoptotic cells was observed ($45.96 \pm 0.70\%$). The opposite effect was observed when T+2-Hf was added to granulosa cell cultures. The percentage of annexin V-positive cells decreased to $40.9 \pm 0.7\%$ (Fig. 1).

Morphology of porcine follicles

Routine staining with H&E showed no changes in follicular morphology of porcine follicles after 6 h of incubation. However, the addition of an AR agonist or antagonist to the culture media of porcine follicles incubated for 12 h caused changes indicating follicular atresia (Fig. 2).

TUNEL assay

To study the incidence of apoptosis in follicles (after 6 and 12 h of culture), we employed a more definitive protocol that determines genomic DNA fragmentation, the TUNEL assay (Fig. 3). Only few apoptotic cells were observed in follicles after 6 h of culture in comparison to follicles fixed after 12 h of culture. We found that in control follicles and follicles cultured under the influence of 2-Hf for 6 h, around one third of granulosa cells underwent apoptosis as defined by the TUNEL assay ($27 \pm 2\%$ and $34 \pm 2\%$, respectively). The number of TUNEL-positive granulosa cells was significantly higher in follicles cultured with the addition of T ($75 \pm 3\%$). On the other hand, TUNEL-positive cells were very sparse ($9 \pm 2\%$) in granulosa cells of follicles supplemented with T+2-Hf. After 12 h of culture, we observed $60 \pm 2\%$ of TUNEL-positive granulosa cells in control follicles. The administration of T and 2-Hf to the culture media significantly increased ($P < 0.05$) the numbers of apoptotic cells ($91 \pm 1\%$ vs. $75 \pm 2\%$ respectively), while T+2-Hf significantly decreased the percentage of TUNEL-positive granulosa cells ($52 \pm 2\%$).

Thirty-six follicles for each time point of follicle culture were tested, and comparisons by ANOVA revealed significant differences among the four groups ($P < 0.05$). Therefore, the TUNEL assay showed clear differences in the number of apoptotic cells in follicles cultured in the presence of an androgen or antiandrogen (Fig. 4).

Estradiol and progesterone media concentration

Estradiol secretion was considerably stimulated by both T (1.6-fold) and T+2-Hf (1.4-fold) added to porcine granulosa cells when estimated after 12 h of culture. The addition of 2-Hf to the culture media caused only a slight increase in estradiol secretion. In contrast, T as well as 2-Hf decreased granulosa progesterone secretion (1.1-fold and 3.1-fold, respectively). In turn, T+2-Hf-treated cells increased progesterone secretion (1.4-fold; $P < 0.05$; Fig. 5B). A similar tendency

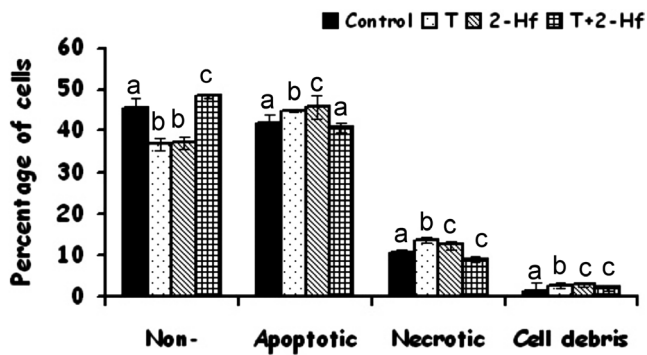


Fig. 1. Mean percentage of non-apoptotic, apoptotic and necrotic cells and cell debris after 12 h of granulosa cell culture treated with T, 2-Hf and T+2-Hf. Data are means $\pm$ SEM of five independent experiments. The statistical differences were assayed by an Anova test ($P < 0.05$). T, testosterone; 2-Hf, 2-hydroxyflutamide; T+2-Hf, testosterone + 2-hydroxyflutamide.

in steroid production was observed in 6-h cultures of granulosa cells.

Interesting was that the highest increase (2-fold) in estradiol secretion was observed after the addition of 2-Hf to the follicles cultured for 6 and 12 h ($P < 0.05$, Fig. 5C).

During the 12 h of follicular culture, progesterone secretion was lower than during the first 6 h, which was not the case for follicles cultured with the addition of T+2-Hf. Testosterone and 2-Hf significantly inhibited progesterone production by porcine follicles, while T+2-Hf stimulated significantly follicular progesterone production in both 6 and 12 h cultures.

Discussion

In the present study, we obtained the results indicating that the androgen receptor antagonist 2-hydroxyflutamide in the presence

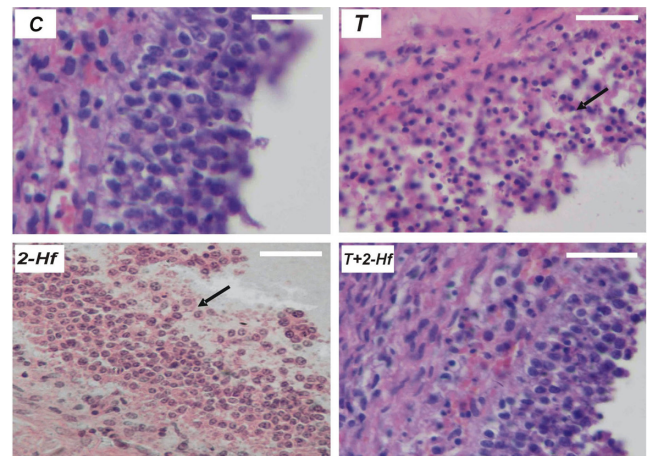


Fig. 2. Sections of pig follicles cultured with T, 2-Hf and T+2-Hf (incubated for 12 h) (H&E). Arrows indicate dispersion of granulosa cells and granulosa cell layer detachment from the follicle wall. C, control; T, testosterone; 2-Hf, 2-hydroxyflutamide; T+2-Hf, testosterone + 2-hydroxyflutamide. Bar=50 μ m.

of a high level of androgens attenuates induction of granulosa cell apoptosis and may act as an antiapoptotic/survival factor.

Sows have a complete estrus cycle with a clearly defined follicular and luteal phase. In the porcine ovary, there are many follicles of different stages and sizes because of the lack of follicular waves, unlike domestic ruminants [45]. Apoptosis is induced in granulosa cells located in the inner surface of the granulosa layer but not in the cumulus cells and oocytes in the early stages of atresia [44], which was confirmed by data collected in our study. Therefore, it can be ascertained that atresia of porcine follicles is primarily initiated by granulosa cell apoptosis.

The present experiments indicated that the granulosa apoptosis

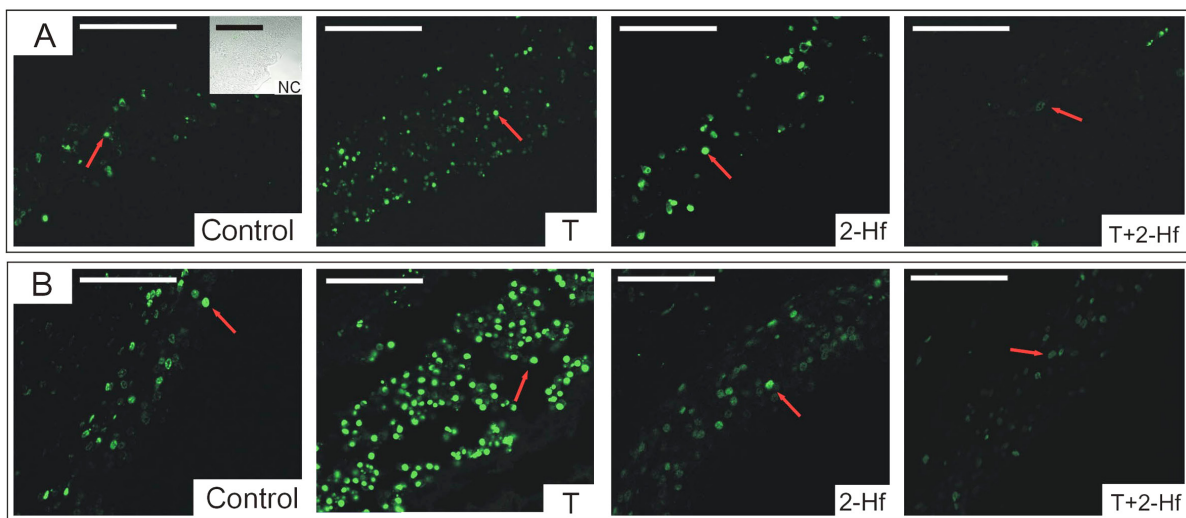


Fig. 3. Apoptosis in the porcine follicles after 6-h (A) and 12-h (B) organ cultures. Arrows show green stained apoptotic cells in the granulosa cell layer. T, testosterone; 2-Hf, 2-hydroxyflutamide; T+2-Hf, testosterone + 2-hydroxyflutamide; NC, negative control. Bar=50 μ m

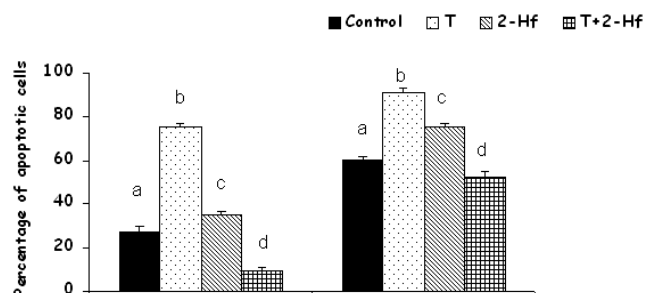


Fig. 4. Mean percentage of apoptosis in 6 and 12 h follicular organ cultures treated with T, 2-Hf and T+2-Hf. Data are means $\pm$ SEM of three independent experiments. The statistical differences were assayed by an ANOVA test ($P < 0.05$). T, testosterone; 2-Hf, 2-hydroxyflutamide; T+2-Hf, testosterone + 2-hydroxyflutamide.

profile observed in cultured whole antral follicles was similar to that found for cultured granulosa cells. Moreover, the proportion of apoptotic granulosa cells increased with the time of culture, which is in agreement with the findings of Maillet *et al.* [45]. Lin and Rui [37] showed that the rate of cell apoptosis in porcine small follicles cultured for 24 hours was higher than that of cells isolated from large and medium ones, suggesting that follicular apoptosis may be regulated in a stage-specific manner. In our granulosa and follicular culture system, supplementation with T or 2-Hf significantly stimulated granulosa cell apoptosis and also in whole follicles increased the

percentage of granulosa cells that became apoptotic. Porcine granulosa cells conform to type II apoptotic cell death, which relies on the mitochondrion-dependent signaling pathway [10]. In this type of apoptosis, two proapoptotic proteins, Bid and Bax, play a crucial role in intracellular signal transduction [46]. In AR-positive prostate cancer cells, it has been demonstrated that androgens and AR promote stress-mediated apoptosis via augmentation of Bax translocation to the mitochondria and upregulation of Noxa protein expression [47]. Moreover, it has been shown that another nonsteroidogenic androgen antagonist, casodex [48], induces apoptosis in AR-positive human prostate tumor cells line LNCaP, suggesting that androgen may be required for cell survival [49]. This is in agreement with our findings. Interestingly, simultaneous addition of T+2-Hf to the culture media of both granulosa cells and whole follicles suppressed granulosa apoptosis. Such an antiapoptotic effect of this antiandrogen together with a high concentration of testosterone might be explained by the presence in porcine granulosa cells of two intracellular inhibitory proteins (cellular FLICE-like inhibitory protein short and long form, cFLIP_S and cFLIP_L) [50]. These proteins directly inhibit the activation of procaspase-8 or inhibit two adaptor proteins (FADD) that initiate apoptosis by procaspase-8 activation [51, 52]. Gao *et al.* [53] demonstrated how androgen can determine survival and apoptosis of prostate gland cells. Specifically, in the presence of androgens, AR in the nucleus acts as a transcriptional activator for expression of the c-FLIP gene, the function of which is to inhibit apoptosis. If androgens are depleted by chemical or surgical castration, however, this protective function is abolished, resulting

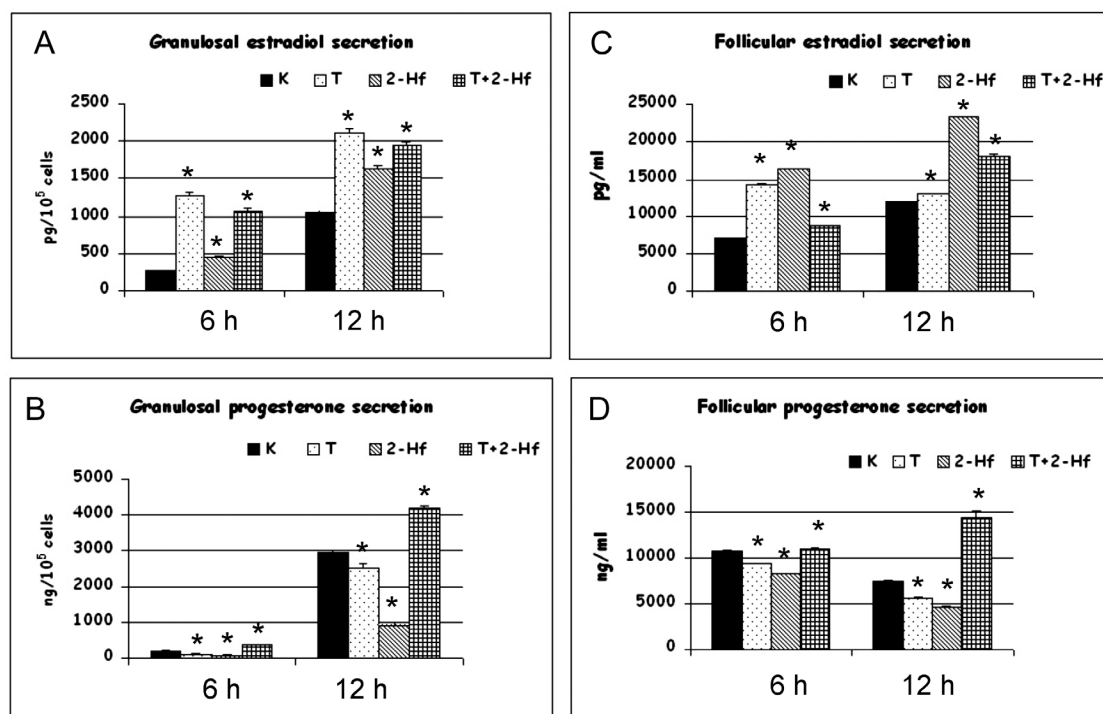


Fig. 5. Estradiol (A, C) and progesterone (B, D) (mean $\pm$ SEM) secretion by granulosa cells and whole follicles cultured for 6 and 12 h ($n=3$ independent experiments). The statistical differences between control and experimental cultures were determined by the Student's *t*-test. Asterisks indicate statistically significant differences ($*P < 0.05$). C, control; T, testosterone; 2-Hf, 2-hydroxyflutamide; T+2-Hf, testosterone + 2-hydroxyflutamide.

in apoptosis in the prostate gland. We believe that intracellular inhibitor proteins (c-FLIP), which are also strongly expressed in granulosa cells, may be powerful candidates for an antiapoptotic/survival factor in pigs. Unfortunately, little is still known about their actual role in the regulation of granulosa cell apoptosis and follicular atresia in pigs. More research is needed to explain how the expression of cFLIP in granulosa cells during follicular growth, development and atresia is controlled. Previous findings have shown that interleukin-6 upregulates cFLIP_L expression in human granulosa tumor cell-derived KGN cells [54, 55] and thus acts as a survival factor. However, there is no confirmed information on what the initial trigger is that controls cFLIP expression. Granulosa cell apoptosis is likely to be regulated by a sophisticated balance of pro-apoptotic and anti-apoptotic factors. The mechanisms of death ligand-receptor signaling should be determined to entirely define the mechanism of granulosa cell apoptosis.

In this study, the process of atresia was accompanied by a reduction in estradiol secretion by granulosa cells under 2-Hf influence in comparison with granulosa cells cultured under the influence of exogenous T. The addition of T caused the highest increase in granulosa estradiol secretion, which might have arisen from its metabolism to estradiol. Quite the opposite situation was observed in whole follicle cultures, where the highest estradiol level was observed after the addition of 2-Hf. In view of the finding that in sows, theca cells also take part in follicular estradiol production [56], it is likely that follicle apoptosis and atresia affected only granulosa estrogen secretion. The primary cause for decreased estradiol production in atretic follicles has been ascribed to the decreased aromatase enzyme activity and reduced expression of cytochrome P450 aromatase mRNA [57]. The increase in estradiol secretion by cultured follicles exposed to 2-Hf may represent some persistence of thecal aromatase before it fades out in later stages of atresia.

The response of the ovarian follicle to the combined effect of survival and death promoting factors determines its ultimate fate: follicular atresia or ovulation. Progesterone is one of the factors that is induced by LH and that has been reported to act as an antiapoptotic factor in luteinized rat and human granulosa cells [58, 59]. The current study demonstrated a significant increase in granulosa progesterone production after 12 h of culture, which was not the case for follicular progesterone production, where a significant decrease was observed. The increase in progesterone production by granulosa cells during the 12 h of culture is characteristic of cells undergoing luteinization [60] as they start to express the characteristics of granulosa-luteal cells. Moreover, in our experiment, culture of intact follicles or granulosa cells progesterone production in response to T or 2-Hf decreased similarly. This was accompanied by morphological signs of atresia, such as the collapsed follicles, dissolving follicular wall and pyknosis of granulosa cells. This corresponded also with the results received from the annexin V-FITC test and TUNEL method revealing apoptotic cells. This is in agreement with Henderson *et al.* [61], who found that granulosa cells isolated from non-atretic cow follicles cultured under the influence of testosterone secreted diminishing amounts of progesterone. This confirms that follicles used in our cultures, both granulosa and follicular, were healthy and non-atretic. Simultaneous addition of T and 2-Hf to the culture media of granulosa cells and intact follicles caused a significant

increase in progesterone production. This corresponded with results of apoptosis tests performed in cultured granulosa cells as well as in whole follicles, where a lower incidence of apoptosis was observed. Perhaps a high level of androgens together with estrogens produced by granulosa cell aromatization of these androgens have a physiological role in limiting progesterone production by granulosa cells during follicular development in pigs.

In conclusion, our present studies indicate that androgens play a critical role in selective apoptosis of granulosa cells during porcine follicular atresia. Further studies are considered necessary to determine which ligand-receptor system dominantly causes or regulates granulosa cell apoptosis and in which stage androgens determine the mechanism of apoptosis-signal transmission in granulosa cells during follicular atresia.

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