Effect of aromatase inhibitor (fadrozole) on proliferation, estradiol production and telomerase activity in pig granulosa cells *in vitro*

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ABSTRACT: The objective of the present work was to study the effect of a nonsteroidal aromatase inhibitor (fadrozole) on proliferation, estradiol production, aromatase expression and telomerase activity (TA) in pig granulosa cells (GC) from small (1–2 mm) and large (5–7 mm) follicles. The cells were treated with fadrozole for 48 h and 72 h in basal and FSH-stimulated conditions. Fadrozole caused a decrease ($P < 0.05$) of ^{3}H-thymidine incorporation in granulosa cells derived from small (1–2 mm) and large follicles (5–7 mm). The proliferative potential of small-follicle GC was significantly higher ($P < 0.01$) under all culture conditions. Estradiol production was suppressed ($P < 0.01$) in both granulosa cell populations cultured in the presence of fadrozole for 48 and 72 h. Fadrozole caused a decrease ($P < 0.05$) of aromatase gene expression in small-follicle granulosa cell incubated for 72 h and in large-follicle GC after 48 h of culture. Large-follicle GC were characterized by a higher ($P < 0.01$) level of estradiol production and aromatase gene expression. Telomerase activity decreased ($P < 0.05$) in large-follicle granulosa cells incubated in the presence of an aromatase inhibitor for 72 h. The TA level in large-follicle granulosa cells was higher ($P < 0.01$) in comparison to small-follicle GC in all culture conditions after 72 h of incubation. The results of the present study suggest the important role of telomerase in the process of follicular growth and development.

Keywords: granulosa cells; aromatase inhibitor; proliferation; telomerase; estradiol

During the growth process of ovarian follicles, granulosa cells acquire an enhanced responsivness to FSH and LH and begin to produce estradiol. Estrogens, acting in an autocrine and paracrine way, are important factors controlling the proliferation and differentiation of granulosa cells (Drummond and Findlay, 1999). As a result of the action of gonadotrophins and estrogens, a phase of rapid proliferation begins which leads to the formation of large preovulatory follicles (Richards, 1975). The effect of estrogens on granulosa cell proliferation *in vitro* is not clear. It was observed that the proliferative potential of rat granulosa cells *in vitro* is stimulated by estradiol and FSH (Bendell and Dorrington, 1991; Bley et al., 1997). Estradiol applied alone had no effect on the proliferative potential of bovine granulosa cells cultured *in vitro* (Langhout et al., 1991). On the contrary, the proliferation of pig granulosa cells from small and large follicles decreased as a result of estradiol stimulation (Ranson et al., 1997).

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We previously determined the presence of telomerase activity in pig granulosa cells obtained from small and large antral follicles (Tománek et al., 2008). However, the mechanisms of TA regulation in granulosa cells are still largely unknown. There is evidence that telomerase activity is under the control of estrogens in some tissues, being a target of these steroids. A stimulating effect of estrogens on TERT (Telomerase reverse transcriptase) expression was shown in the human ovarian epithelium (Misiti et al., 2000). Telomerase activity was also detected in the human endometrium, and its level correlated with estrogen changes, being the highest in the follicular phase of the estrus cycle (Kyo et al., 1997; Yokoyama et al., 1998). Application of estradiol to female rats prevented decrease of telomerase activity in granulosa cells (Yamagata et al., 2002). The role of estrogens in telomerase activity regulation in pig granulosa cells is not fully understood. In our previous study (Tománek et al., unpublished), using antiestrogens, we determined the possible role of estrogen receptors in telomerase activity regulation in pig granulosa cells *in vitro*.

The level of telomerase activity strongly correlates with the proliferation and differentiation status of the cell (Greider, 1998; Bayne and Liu, 2005). Lavranos et al. (1999) showed that the level of telomerase activity was highest in the granulosa cells the least differentiated in lineage (from preantral follicles). On the other hand, the activity of the enzyme was the lowest in granulosa cells derived from large antral follicles.

The conversion of androgen to estrogen is mediated by aromatase. Theca cells synthesize androgens, which are then aromatised by granulosa cells. In pigs, theca cells also express aromatase and have the ability to convert androgens to estrogens (Conley et al., 1997).

Aromatase inhibitors represent tools to study the role of aromatase and estrogens in the development and growth of estrogen-dependent cancers (Miller, 2003). Previously, a specific aromatase inhibitor was used to study the aromatase activity and estradiol secretion by porcine corpora lutea at various stages of the luteal phase (Gregoraszczuk, 1996; Gregoraszczuk and Oblonczyk, 1996).

Since the effect of estrogens on telomerase activity regulation in pig granulosa cells is not fully known, in the present study we applied a specific aromatase inhibitor (fadrozole) to clarify the role of the aromatization process in the regulation of telomerase activity in pig granulosa cells obtained from small (1–2 mm) and large (5–7 mm) follicles. Inhibition of estrogen synthesis could help to determine whether estrogens play a role in telomerase activity regulation in pig granulosa cells. The efficiency of fadrozole as an estrogen synthesis inhibitor was determined by the measurement of estradiol secretion and the aromatase gene expression level in two populations of pig granulosa cells. Due to the tight correlation between telomerase activity and the process of cell proliferation, we also determined the effect of fadrozole on ^{3}H-thymidine incorporation.

MATERIAL AND METHODS

Isolation of porcine granulosa cells and *in vitro* culture conditions

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermo-container filled with phosphate-buffered saline (PBS) within 30 min. In the laboratory the ovaries were placed in sterile PBS supplemented with an antibiotic-antimycotic solution (Sigma, UK) for 20 min. Individual follicles of the desired size (small antral 1–2 mm and large antral 5–7 mm) were isolated as described earlier (Tománek et al., 2008). The number of live granulosa cells in suspension was estimated using of 0.25% trypan blue (Sigma, USA) in PBS and by counting in a hematocytometer. The viability of granulosa cell suspension after trypsyn/DNAse treatment was 85–90%. The cells were seeded onto 4- or 24-well Nunclon Delta culture microplates (Nunc, Denmark) in a density of 1–1.5 × 10^5 of living cells/well and cultured as a proliferating monolayer in a DMEM/F12 medium supplemented with an insuline-transferrin-selenium mixture (ITS-X, 1 ml/100 ml) and gentamicin (all Gibco, BRL). 2% of fetal bovine serum (FBS, Gibco, BRL) was added in order to facilitate plating. Cell cultures were performed at 37°C in a humidified-atmosphere of 5% CO_2 in a Sanyo MCO-175M incubator. The cells were cultured for 48 h and 72 h in the presence of hormonal supplements.

Hormonal supplements

The granulosa cells were cultured in the presence of the non-steroidal aromatase inhibitor CGS16949A (fadrozole) (Novartis, Switzerland) added to the culture medium in a concentration of 5μM.

The follicle stimulating hormone (pFSH-I-1 Iod/Bio, AFP 10640D, NIADDK-NIH, Torrance, CA, USA) was used in a concentration of 50 ng/ml to induce steroidogenesis in the granulosa cells. Testosterone (Steraloids Inc., USA) in a concentration of 10^{-8}M was added as an aromatase substrate.

Cell proliferation assay

To determine the granulosa cell proliferation after 48 and 72 h of *in vitro* culture, the newly synthesized DNA in the cell cultures was measured by incorporation of ^{3}H-thymidine using the technique of TCA precipitation and liquid scintillation counting as described earlier (Vacková et al., 2003).

Estradiol production analysis

The induction and stimulation of estradiol production in granulosa cell *in vitro* cultures were evaluated by radioimmunoassay of estradiol 17-β in a culture medium using RIA techniques (described earlier in Petr et al., 1991). Radioligand estradiol 6-/0-karboxymethyl/-oximino/2-/^{125}-I/jodhistamin was obtained from Amersham (A.P., Prague, Czech Republic). The lower detection limit was 0, 066 ng per ml. The intra-assay and inter-assay coefficient of variation was 5, 92% and 8, 87%, respectively.

Telomerase activity assay

Preparation of granulosa cells

In vitro cultured granulosa cells were prepared as follows: after 48 and 72 h of *in vitro* culture, the culture media from individual wells were removed and stored at –20°C for RIA. The cells were washed with a serum-free medium and PBS and detached from the well using Accutase (Chemicon, USA). The cells were then transferred to Eppendorf tubes and centrifuged at 4°C and 3 500 G for 15 min. Pellets of the cells were stored at –80°C until use.

TRAP (Telomeric Repeat Amplification Protocol) assay

At the time of the telomerase activity assay, cell lysates from *in vitro* cultured cells were prepared in a CHAPS buffer. Briefly: cell pellets of cultured granulosa cells (2×10^5) and the positive control provided in the kit were resuspended with 200 µl of

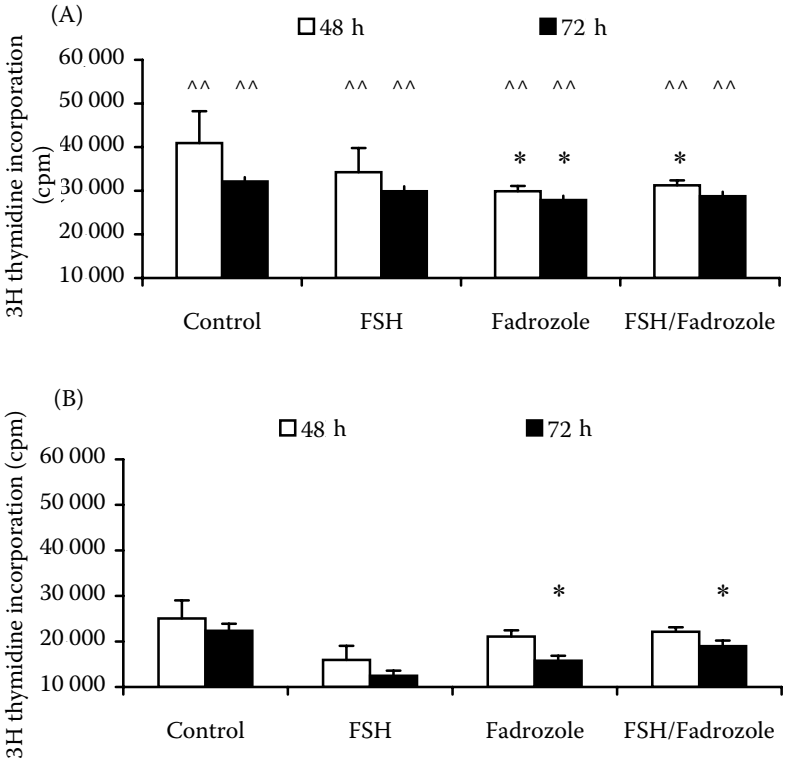

Figure 1. Proliferation of porcine granulosa cells derived from small (1 to 2 mm) (A) and large (5–7 mm) (B) follicles assessed by incorporation of ^{3}H thymidine after 48 and 72 h of *in vitro* culture without additives (control) and in the presence of FSH (50 ng/ml) and fadrozole (5µM) applied individually and in combination; each bar represents mean ± SEM for 4 experiments ($n = 4$) performed in triplicates; *denotes means significantly different from control ($P < 0.05$); ^^denotes significant differences between small and large follicle GC ($P < 0.01$)

1X CHAPS Lysis Buffer. The protein concentration was measured spectrophotometrically.

A modified protocol based on the TRAPEZE Telomerase Detection Kit (Chemicon, USA) was applied to evaluate telomerase activity. The method on which the kit is based employs a phenomenon first described by Kim et al. (1994), i.e., addition by telomerase of the six-nucleotide repeat sequence GGTTAG to the template, which results in the formation of an electrophoretic ladder. The modification consisted in the fluorescent labeling of the germinal sequence of the primer. We used an internal telomerase assay standard and the fluorescent type-specific TS primer AATCCGTCGAGCAGAGTT-6-FAM™ (Applied Biosystems, USA). Telomerase extension and PCR were performed in a Biometra T radient PCR thermocycler (Biometra, Germany). Telomerase activity was expressed as TPG/mg protein Total Product Generated) per milligram of protein.

Real time RT-PCR quantification of the level of P450 aromatase mRNA

Total RNA was isolated from the cultured cells using 6100 Nucleic Acid PrepStation and Total RNA Chemistry (Applied Biosystems (ABI), USA) and quantified by measuring absorbance at 260 nm. 50 µl of RNA was transcribed into cDNA using the cDNA Archive Kit (ABI). Glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene. Aromatase primers (forward, 5'-CTGACCGTCTGTGCCGATT-3'; reverse, 5'GCGGACCTCCT-3') and MGB probe (TET – AATCACCAAGCACCTGGA) and glyceraldehyde 3-phosphate dehydrogenase primers (forward, 5'-GAGCATCTCCTGACTTCCAGTTTC-3'; reverse, 5' CCTAAGCCCCTCCCCTTCT-3') and MGB probe (VIC- ATCCCAGACCCCC) were designed using the Primer Express 3.0 Program (ABI). Quantification of aromatase was performed using the ABI 7500 Fast Real-Time PCR System. The PCR mix composition for one probe was as follows: 1 µl of cDNA, 500 nM of forward and reverse primers, 250 nM of TaqMan®MGB probe, 5 µl of 1XTaqMan®Fast Universal MasterMix, NoAmpErase®UNG (Applied Biosystems, USA), water to a volume of 10 µl. The PCR reaction was continued for 40 cycles after the initial denaturation at 95°C for 20 s. Each cycle of PCR consisted of 5 s of denaturation at 95°C and 30 s of annealing at 60°C.

Statistical analysis

The data were obtained from 4 experiments with different pools of small- and large-follicle granulo-

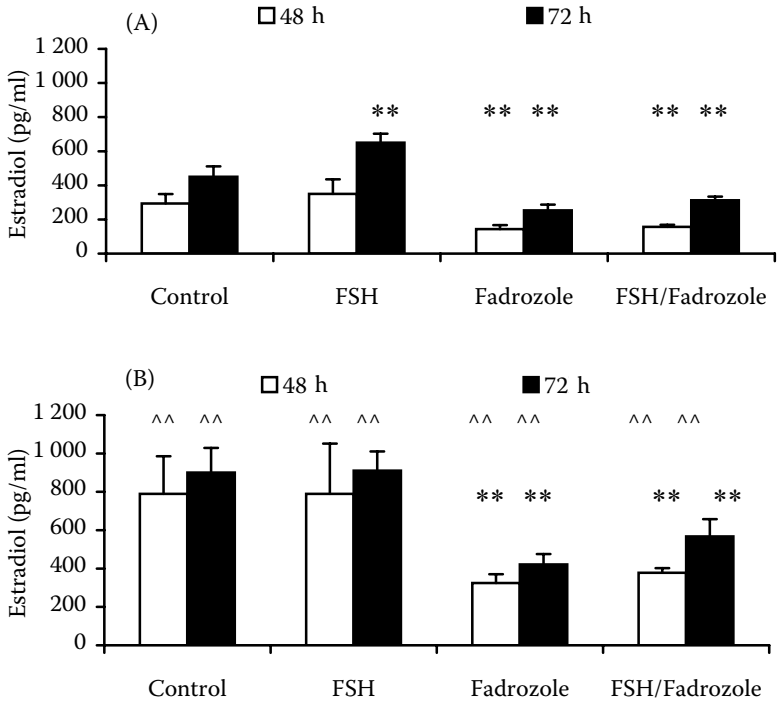

Figure 2. Estradiol secretion by porcine granulosa cells derived from small (1 to 2 mm) (A) and large (5–7 mm) (B) follicles assayed by RIA after 48 and 72 h of *in vitro* culture without additives (control) and in the presence of FSH (50 ng/ml) and fadrozole (5µM) applied individually and in combination; each bar represents mean ± SEM for 4 experiments (n = 4) performed in triplicates; **denotes means significantly different from control ($P < 0.001$); ^^ denotes significant differences between small and large follicle GC ($P < 0.01$)

sa cells. Each experiment consisted of three repetitions per treatment. The data are presented as means ± SEM. All data were analyzed using the SAS Programme (SAS 2001, SAS System for Windows, Release 8.2 (TS2M0), SAS Inst., Inc., Cary, NC, USA). ANOVA was used to determine the significance of differences of FSH and the fadrozole effect, applied alone and in combination, on proliferation, estradiol production, aromatase expression and telomerase activity of small-follicle and large-follicle granulosa cells *in vitro*. Differences with a probability of $P < 0.05$ were considered significant.

RESULTS

Small-follicle and large-follicle granulosa cell proliferative potential *in vitro (*Figure 1A, B) summarises the measurements of newly synthesized DNA in granulosa cells at the intervals of 48 and 72 h of culture. As presented in Figure 1A, the level of incorporated radioactivity was higher in small-follicle GC in comparison with large-follicle GC in all culture conditions. Fadrozole applied alone decreased ^{3}H-thymidine incorporation in the small-follicle granulosa cells after 48 and 72 h of culture (Figure 1A), and in the large-follicle granulosa cells incubated for 72 h (Figure 1B). In comparision with the control, fadrozole decreased the level of ^{3}H-thymidine incorporation in small-follicle GC cultured for 48 h and in large-follicle GC incubated for 72 h in FSH-stimulated conditions (Figure 1).

Estradiol production

Granulosa cells obtained from small and large follicles continued to secrete estradiol when cultured *in vitro*. Significantly higher ($P < 0.01$) levels of estradiol were measured in large-follicle granulosa cell vs. small-follicle granulosa cell cultures in all treatments (Figure 2).

Fadrozole effectively inhibited ($P < 0.01$) estradiol secretion in small- and large-follicle granulosa cells cultured in basic as well as in FSH-stimulated conditions (Figures 2). FSH-stimulated estradiol production only in small-follicle granulosa cell cultures after 72 h (Figure 2).

Aromatase gene expression

The level of aromatase gene expression was significantly ($P < 0.01$) higher in large-follicle granulosa cells vs. small-follicle granulosa cells in all treatments. FSH stimulated aromatase expression in small-follicle, granulosa cell cultures after 72 h

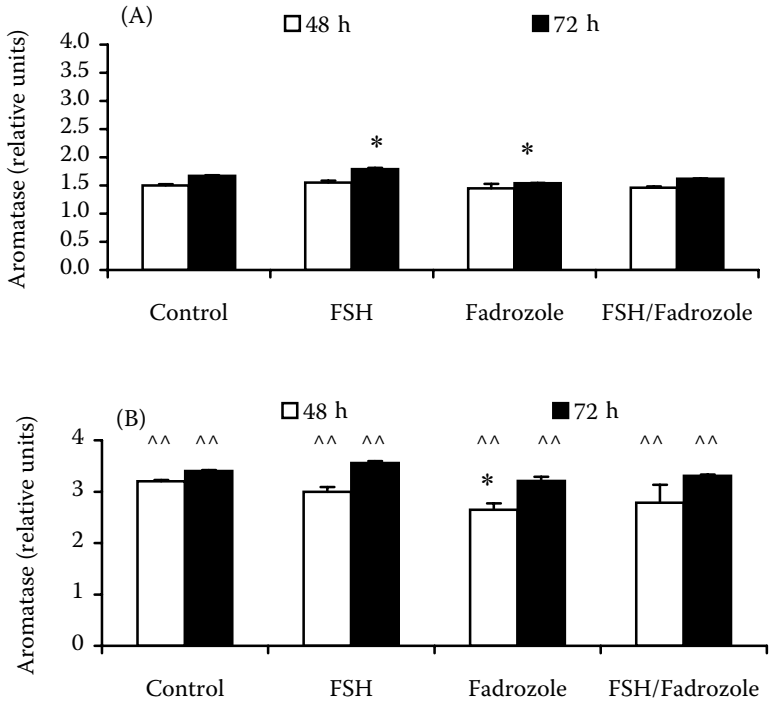

Figure 3. Aromatase gene expression in porcine granulosa cells derived from small (1–2 mm) (A) and large (5–7 mm) (B) follicles assessed by Real Time RT PCR after 48 and 72 h of *in vitro* culture without additives (control) and in the presence of FSH (50 ng/ml) and fadrozole (5μM) applied individually and in combination; each bar represents mean ± SEM for 4 experiments ($n = 4$) performed in triplicates; *denotes means significantly different from control ($P < 0.05$); ^^denotes significant differences between small and large follicle GC ($P < 0.01$)

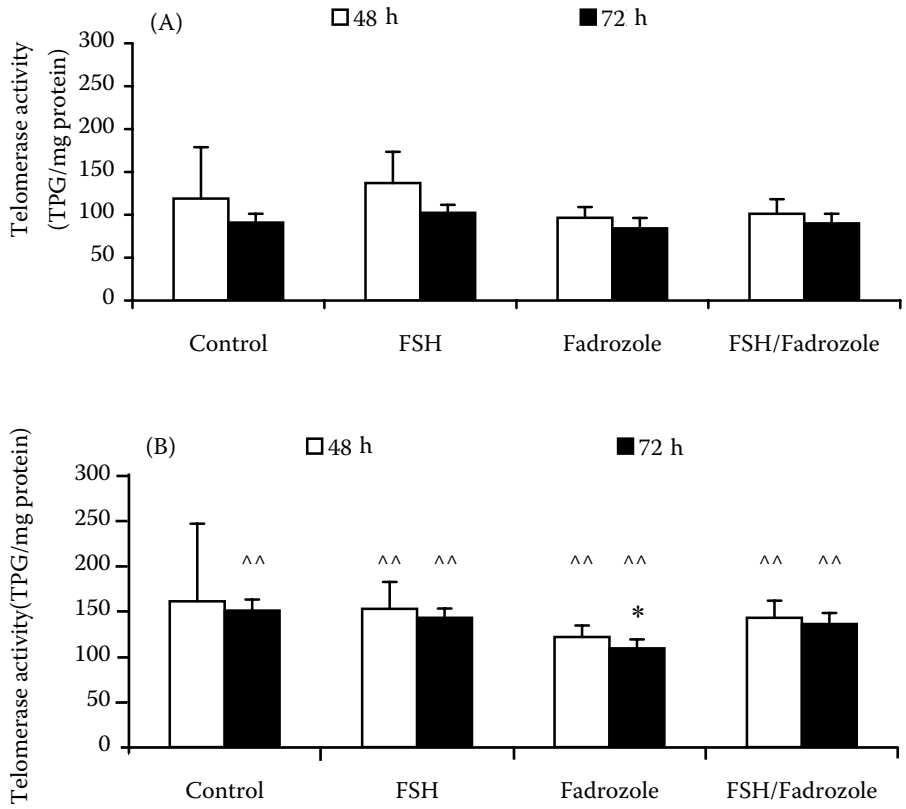

Figure 4. Telomerase activity in porcine granulosa cells derived from small (1–2 mm) (A) and large (5–7 mm) (B) follicles assayed using TRAPEZE Telemerase® Detection Kit after 48 and 72 h of *in vitro* culture without additives (control) and in the presence of FSH (50 ng/ml) and fadrozole (5μM) applied individually and in combination; each bar represents mean ± SEM for 4 experiments (n = 4) performed in triplicates; *denotes means significantly different from control ($P < 0.05$); ^^denotes significant differences between small and large follicle GC ($P < 0.01$)

(Figure 3A). Fadrozole decreased ($P < 0.05$) aromatase expression in small-follicle granulosa cells after 72 h (Figure 3A) and in large-follicle granulosa cells cultured *in vitro* for 48 h (Figure 3B).

Telomerase activity in small- and large-follicle granulosa cells *in vitro*

Fadrozole decreased ($P < 0.05$) telomerase activity in large-follicle granulosa cells after 72 h of culture (Figure 4). In FSH-treated cells, the level of telomerase activity did not differ significantly from the control (Figure 4). The level of telomerase activity was higher in large-follicle granulosa cells incubated under control conditions as well as in the presence of FSH and fadrozole applied alone for 72 h and in combination after 48 h of culture (Figure 4).

DISCUSSION

Estrogens play a key role in the growth and development of ovarian follicles. However, their influence on proliferation and telomerase activity in granulosa cells cultured *in vitro* is still not fully understood. In the present study, the specific aromatase inhibitor fadrozole was used to determine the role of estrogens in the regulation of telomerase activity and proliferation of pig granulosa cells derived from small (1–2 mm) and large (5–7 mm) follicles.

Estradiol production and the expression of aromatase are the basic markers of functional maturation of granulosa cells (Hirhfield, 1991). In the present work, granulosa cells obtained from small and large follicles continued to synthesize estradiol during 72 h of *in vitro* culture. However, the level of estradiol production by large-follicle granulosa cells was significantly higher in all culture conditions in comparison with small-follicle GC. These observations indicate that large-follicle GC are much more advanced in the differentiation process than their small-follicle counterparts. The higher level of estradiol production in large-follicle GC was accompanied by higher expression of the aromatase gene. Interestingly, in this work the stimulating effect of FSH on estradiol production and aromatase expression was observed only in small-follicle granulosa cells. This observation indicates the important role of FSH in enhancing the process of terminal differentiation of granulosa cells *in vitro*. It is well documented that granulosa cells luteinize spontaneously during *in vitro* culture in

the presence of serum (Picton et al., 1999). The lack of stimulating effect of FSH on estradiol synthesis in large-follicle granulosa cells may be explained by their luteal phenotype, which is reached much more quickly due to its advanced differentiation status at the beginning of the culture in comparison with small-follicle granulosa cells. Similar results were obtained by Picton et al. (1999), who noticed the inhibiting effect of FSH on aromatase gene expression in long-term pig granulosa cell cultures. This effect was accompanied by reduced estradiol production and increased progesterone secretion, indicating cellular luteinisation. On the contrary, the stimulating effect of FSH on aromatase expression in granulosa cells *in vitro* was previously demonstrated in rats (Fitzpatrick and Richards, 1991; Escamilla-Hernandez et al., 2008).

In the present study, fadrozole applied at a concentration of 5μM significantly decreased the estradiol secretion in small and large pig GC cultured in basal and FSH-stimulated conditions. This suggests the presence of active aromatase in GC of both populations and demonstrates the high efficiency of fadrozole as an estradiol synthesis inhibitor in pig granulosa cells. Similar results, showing the high potency of fadrozole with regard to the inhibition of the aromatase enzyme in porcine granulosa and luteal cells, were obtained earlier by Gregoraszczuk and Oblonczyk (1995). However, the authors tested lower doses of the inhibitor (0.1, 0.5, 1.0μM), and its inhibiting effect was dose dependent. In our study, the relatively high concentration of fadrozole (5μM) did not have any negative, toxic influence on the cultured cells. We did not observe any signs of apoptosis (data not shown) in cells cultured for 72 h.

In the present work, fadrozole decreased aromatase gene expression in small-follicle GC after 72 h and in large-follicle GC after 48 h of culture. This observation suggests that fadrozole may exert its inhibiting effect not only on the protein level but also on the aromatase gene expression level. This effect is interesting if we take into account that fadrozole belongs to a group of second-generation nonsteroidal aromatase inhibitors with the mechanism of action being tight binding to aromatase at the heme moiety of the active enzyme (Miller, 2003). On the basis of our observation, we assume that the mechanism of fadrozole action in pig granulosa cells may differ from that observed in other types of cells with active aromatase (Younus and Vandenberg, 2005) and may also involve some events on the molecular level.

It was shown that estrogens cause induction of the cell cycle and lead to shortening of the G1 stage (Brunner et al., 1989). In the present study, inhibition of estrogen synthesis by fadrozole decreased the proliferative potential of small- and large-follicle granulosa cells. Since inhibition of aromatase activity led to a decrease of ^{3}H-thymidine incorporation, we assume that in our model estradiol had a stimulating effect on granulosa cell proliferation. Our results differ from those obtained by Ranson et al. (1997), who noticed the inhibiting effect of estradiol on porcine granulosa cell proliferation derived from small and large follicles. According to the authors, those results indicate that estrogens acting in a paracrine and autocrine manner encourage differentiation and are most likely regulators of the later stage of antral follicle development. On the other hand, our results are supported by the data of Bendell and Dorrington (1991) and Bley et al. (1997), who noticed the stimulating effect of estrogens on rat granulosa cells. However, in our experiments, the observed inhibition of cell proliferation in the presence of fadrozole may also be attributed to testosterone added to the cultures as an aromatase substrate. From this point of view, our results agree with those obtained by Ranson et al. (1997). The authors observed the inhibiting effect of testosterone on thymidine incorporation in small- and large-follicle GC. Similar results were obtained by Hickey et al. (2005), who noticed that androgen applied alone had little effect on DNA synthesis in porcine mural granulosa cells, but significantly enhanced the mitogenic effect of growth differentiation factor 9 (GDF9).

In the present study, granulosa cells derived from small follicles were characterised by higher proliferative potential in all culture conditions in comparison with their large-follicle counterparts. This observation confirms our previous results (Tománek et al., 2008), demonstrating the higher proliferative potential of less differentiated granulosa cells.

In the present study, fadrozole decreased telomerase activity in large-follicle granulosa cells cultured for 72 h. This observation suggests that estrogens regulate telomerase activity in granulosa cells advanced in their differentiation process. Similar results were obtained by Yamagata et al. (2002), who observed a higher level of telomerase activity in healthy, preovulatory follicles as a result of estradiol

application to female rats. Estradiol produced by growing follicles is an antiatretic factor, increasing the survivability of the follicular cells. The level of its synthesis significantly decreases in the atretic follicles (Billig et al., 1993). The involvement of estradiol in stimulating telomerase activity in large follicles may be an important mechanism protecting granulosa cells against atresia.

In small-follicle granulosa cells we did not observe any effect of fadrozole on telomerase activity. This observation agrees with the results of our previous study (unpublished) aimed at determining the effect of estradiol acting via estrogen receptors on telomerase activity in pig granula cells. We did not observe changes in telomerase activity as a result of aniestrogen application, whereas proliferative potential significantly decreased.

Due to the important role of telomerase in the cell cycle, the activity of the enzyme strongly correlates with the proliferative potential of the cells (Bayne and Liu, 2005). It was shown that granulosa cells with high proliferative potential were characterised by a higher telomerase activity level in comparision with those which were more differentiated (Lavranos et al., 1999).

In the present work, the enhanced proliferative potential of granulosa cells from small follicles was not accompanied by higher telomerase activity. On the other hand, we observed higher telomerase activity in large-follicle granulosa cells characterised by a high level of estradiol production. On the basis of this observation, we conclude that telomerase activity in pig granulosa cells is not necessarily linked only to cell proliferation but that some regulatory mechanisms of the differentiation process may also be involved.

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REFERENCES

Bayne S., Liu J.P. (2005): Hormones and growth factors regulate telomerase activity in ageing and cancer. Molecular and Cellular Endocrinology, 240, 11–22.

Bendell J.J., Dorrington J. (1991): Estradiol-17-beta stimulates DNA-synthesis in rat granulosa cells action mediated by transforming growth factor beta. Endocrinology, 128, 2663–2665.

Billig H., Furuta I., Hsueh A.J. (1993): Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology, 133, 2204–2212.

Bley M.A., Saragueta P.E., Baranao J.L. (1997): Concerted stimulation of rat granulosa cell deoxyribonucleic acid synthesis by sex steroids and follicle-stimulating hormone. Journal of Steroid Biochemistry and Molecular Biology, 62, 11–19.

Brunner N., Bronzert D., Vindelov L.L., Rygaard K., Spang-Thomsen M., Lippman M.E. (1989): Effect on growth and cell cycle kinetics of estradiol and tamofixen on MCF-7 human breast cancer cells grown *in vitro* and in nude mice. Cancer Research, 49, 1515–1520.

Conley A., Corbin J., Smith T., Hinshelwood M., Liu Z., Simpson E. (1997): Porcine aromatases: studies on tissue-specific, functionally distinct izozymes from a single gene? Journal of Steroid Biochemistry and Molecular Biology, 61, 407–413.

Drummond A., Findlay J. (1999): The role of estrogen in folliculogenesis. Molecular and Cellular Endocrinology, 151, 57–64.

Escamilla-Hernandez R., Little-Ihrig L., Orwig K.E., Yoe J.M., Chandran U., Zeleznik A.J. (2008): Constitutively active protein kinase a qualitatively mimics the effects of follicle-stimulating hormone on granulosa cell differentiation. Molecular Endocrinology, 22, 1842–1852

Fitzpatrick S.L., Richards J.S. (1991): Regulation of cytochrome P450 aramatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. Endocrinology, 129, 1452–1462.

Greider C.V. (1998): Telomerase activity, cell proliferation, and cancer. The Proceedings of the National Academy of Science of the United States of America, 6, 95, 90–92.

Gregoraszczuk E.L. (1996): Large and small cells of the porcine corpus luteum: Differential capacity to secrete estradiol and aromatize exogenous androgen during mid-and late phase. Experimental and Clinical Endocrinology and Diabetes, 104, 278–283.

Gregoraszczuk E.L., Oblonczyk K. (1996): Effect of specific aromatase inhibitor on oestradiol secretion by porcine corpora lutea at various stages of the luteal phase. Reproduction Nutrition Development, 36, 65–72.

Hickey T.E., Marrocco D.L., Ritter L.J., Norman R.J., Gilchrist R.B., Armstrong D.T. (2005): Androgens augment the mitotic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. Biology of Reproduction, 73, 825–832.

Hirshfield A.N. (1991): Development of follicles in the mammalian ovary. International Review of Cytology, 124, 43–101.

Kim N.W., Piatyszek M.A., Prowse K.R., Harley C.B., West M.D., Ho P.L., Coviello G.M., Wright W.E., Weinrich S.L., Shay J.W. (1994): Specific association of human telomerase activity with immortal cells and cancer. Science, 266, 2011–2015.

Kyo S., Takakura M., Kohama T., Inoue M. (1997): Telomerase activity in human endometrium. Cancer Research, 57, 610–614.

Langhout D.J., Spicer L.J., Geisert R.D. (1991): Development of a culture system for bovine granulosa cells effects of growth hormone, estradiol and gonadotropins on cell proliferation, steroidogenesis and protein synthesis. Journal of Animal Science, 69, 3321–3334.

Lavranos T.C., Mathis J.M., Latam S.E., Kalionis B., Shay J.W., Rodgers R.J. (1999): Evidence for ovarian granulosa stem cells: Telomerase activity and localization of the telomerase ribonucleic acid component in bovine ovarian follicles. Biology of Reproduction, 61, 358–366.

Miller W.R. (2003): Aromatase inhibitors: Mechanism of action and role in the treatment of breast cancers. Seminars in Oncology, 30, 3–11.

Misiti S., Nanni S., Fontemaggi G., Cong Y.S., Wen J.P., Hirte H.W., Piaggio G., Sacchi A., Pontecorvi A., Bacchetti S., Farsetki A. (2000): Induction of hTERT Expression and telomerase activity by estrogens in human ovary epithelium cells. Molecular and Cellular Biology, 20, 3764–3771.

Petr J., Tománek M., Fulka J. Jr., Míka J., Jílek F. (1991): Effects of GnRH on preovulatory endocrinology and oocyte maturation in PMSG-superovulated cows. Animal Reproduction Science, 24, 37–52.

Picton H.M., Campbell B.K., Hunter M.G. (1999): Maintenence of oestradiol production and expression of cytochrome P450 aromatase enzyme mRNA in long-term serum-free cultures of pig granulosa cells. Journal of Reproduction and Fertility, 115, 67–77.

Ranson E.J., Picton H.M., Hunter M.G. (1997): Effects of testosterone and oestradiol on (H-3)-thymidine incorporation by porcine granulosa and theca cells. Animal Reproduction Science, 47, 229–236.

Richards J.S. (1975): Estradiol receptor content in rat granulosa cells during follicular development. Endocrinology, 97, 1174–1184.

Tománek M., Chronowska E., Kott T., Czerneková V. (2008): Telomerase activity in pig granulosa cells proliferating and differentiating *in vitro*. Animal Reproduction Science, 104, 284–298.

Vacková I., Engelová M., Marinov I., Tománek M. (2003): Cell cycle synchronization of porcine granulosa cells in G1 stage with mimosine. Animal Reproduction Science, 77, 235–245.

Yamagata Y., Nakamura Y., Umayahara K., Harad A., Takayama H., Sugino N., Kato H. (2002): Changes in telomerase activity in experimentally induced atretic follicles of immature rats. Endocrine Journal, 49, 589–595.

Yokoyama Y., Takahashi K., Morshita S., Hashimoto M., Niwa K., Tamaya T. (1998): Telomerase activity in the human endometrium throughout the menstrual cycle. Molecular Human Reproduction, 4, 173–177.

Younus J., Vandenberg T.A. (2005): A practical overview of aromatase inhibitors in postmenopausal women with hormone receptor-positive breast cancer. Bulletin of Cancer, 92, 39–44.

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