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FOLLICLE-STIMULATING HORMONE: EFFECTS AND POSSIBLE MECHANISMS OF ACTION IN RABBIT OVARIAN CELLS

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ABSTRACT

The aim of our experiment was to examine the role of follicle-stimulating hormone (FSH) in the control of rabbit ovarian functions (proliferation, apoptosis) and in the regulation of transcription factor CREB expression. Rabbit ovarian granulosa cells were cultured in the presence of FSH (0, 1, 10 and 100 ng/ml of medium). Expression of intracellular peptides – markers of proliferation (cyclin B1) and apoptosis (caspase-3, TdT) as well as the expression of CREB-1 in the cells were detected using immunocytochemistry, TUNEL and SDS PAGE- Western immunoblotting. It was observed, that FSH stimulates accumulation of cyclin B1, reduces caspase-3 and TdT and increases CREB-1 accumulation in rabbit ovarian cells. Our observations suggest, that FSH activates proliferation and inhibits apoptosis in rabbit ovarian cells, and that these effects can be mediated via cAMP/protein kinase A/CREB- or MAP kinase/CREB-dependent intracellular mechanisms.

Key words: FSH, rabbit, ovarian granulosa cells, CREB, apoptosis (caspase-3, TdT), proliferation (cyclin B1)

INTRODUCTION

Follicle-stimulating hormone (FSH) plays a key role in female reproduction regulating ovarian follicular growth and development. It acts through FSH receptors on granulosa cell membrane surface. The signal is transmitted through several intracellular mechanisms like the activation of cAMP/protein kinase A (PKA)-dependent, mitogen-activated protein kinase (MAPK)-dependent and other signalling pathways. Transcription factor CREB (cAMP response element binding protein) is one of their substrates. CREB regulates expression of some genes related to proliferation and steroidogenesis of granulosa cells (GC) (Hunzicker-Dunn and Maizels, 2006). It is known that FSH acts as anti-apoptotic agent and stimulator of GC proliferation in different animal species (Chun et al, 1996), but effect of FSH on rabbit ovarian cell proliferation and apoptosis was not sufficiently studied yet. There is evidence (Maillet et al, 2002) that increasing FSH level had no effect on apoptosis of cultured isolated rabbit GC, but it caused a decrease in the number of apoptotic GC from cultured preovulatory follicles. It was concluded, that FSH probably requires follicle integrity to exert its anti-apoptotic effect. FSH increased rabbit follicular cell proliferation evaluated by labelling with 3H-thymidine (indirect marker of S-phase of the cell cycle) (Mariana and Solari, 1993). Effect of FSH on other phases of rabbit ovarian cell cycle has not been reported yet.

The cellular mechanism, which controls apoptosis and proliferation under influence of FSH stimulation in rabbit GC is studied insufficiently. It remains unknown, whether transcription factor CREB could act as a mediator of FSH actions on these processes. There are only limited relevant information obtained on other animal species. FSH prevented apoptosis by suppressing DNA fragmentation in rat GC (Chun et al, 1996) and by reducing caspase-3 in porcine GC (Dineva et al, 2007). FSH stimulated proliferation by increasing cyclin D2 (Robker a Richards, 1998) and PCNA expression (Yu

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et al, 2005) in rat GC. In rat GC FSH altered neighter CREB mRNA nor the CREB protein amount, but rapidly increased phosphorylated CREB level (Mukherjee et al, 1996). CREB can directly regulate genes and their products involved in proliferation and survival of GC. At least CRE elements were detected in murine and human PCNA (Lee and Matthews, 1997) and cyclin D2 (Muniz et al, 2006) gene promoter indicating involvement of CREB in regulation of PCNA and cyclin D2 expression by binding to these regions. Elevated PCNA and CREB levels were found in proliferous GC of primates, whilst CREB decline was associated with drop of PCNA amount and subsequent cessation of the cell cycle (Somers et al, 1995).

In our experiment we investigated, whether proliferation, apoptosis and corresponding proliferative (cyclins, PCNA) and apoptotic (caspase-3, TdT) proteins could be controlled by FSH in rabbit ovaries, and whether CREB can be a potential mediator of FSH actions on these processes in rabbit GC.

MATERIALS AND METHODS

Isolation and culture of rabbit ovarian granulosa cells

Ovaries from New Zealand White rabbits (n = 5) of 4 months age were used. Ovaries were placed in culture dish with sterile DMEM/F12 medium (BioWhittaker, Verviers, Belgium) and dissected using multiblade knife. Tissue suspension was passed through a steel sieve to remove large ovarian fragments. The cell filtrate was washed 2 times by centrifugation (10 min, 230 g) and the pelet was resuspended in incubation medium consisting of DMEM/F12 (BioWhittaker) supplemented with 20% fetal calf serum (BioWhittaker) and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO, USA). The cell concentration was determined by haemocytometer and then adjusted to 1-1,5 x 10^6 cells/ml by dilution with the incubation medium. Thereafter the cell suspension was mixed by using magnetic stirrer and dispensed to 24-well plates (Becton Dickinson, Lincoln Park, USA; 1 ml/well) and to chamber-slides (Nange Nunc International, Naperville, USA; 200 µl/well) and cultured at 38,5°C and 5% CO_2 in humidified air for 10 days until the confluent monolayer was formed. Thereafter the medium was replaced with a fresh culture medium of the same composition supplemented with bovine FSH (kindly provided by Dr.A.P.F.Parlow, NHPP, Torrance, USA) at 0, 1, 10 a 100 ng/ml medium and the cells were cultured in the same conditions during 2 days. After culture, the medium from both well plates and chamber-slides was gently aspirated. Cells from well plates were lyzed in lysis solution consisted of 0.06260 M Tris-base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue (all from Sigma Aldrich Chemie GmbH, Steinheim, Germany) and stored at -80°C to await SDS-Western blotting and cells from chamber-slides were washed 3 times in ice-cold PBS, fixed 20 min in 4% paraformaldehyde in PBS and kept in 100% ethanol (all from AppliChem GmbH, Darmstadt, Germany) at 4°C to await immunocytochemical analysis and TUNEL.

Protein gel electrophoresis (SDS-PAGE) and Western immunoblotting

The frozen cell lyzates were thawed and subjected to electrophoresis in polyacrylamide gel (PAGE) in presence of SDS (sodium dodecyl sulfate) according to Laemmli (1970) as described previously (Sirotkin and Makarevich, 1999). Blocked membranes were probed 1 h with mouse monoclonal antibody against cyclin B1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and CREB-1 (Santa Cruz). After 1 h of incubation with primary antibody membranes were then incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG (Sevac, Prague, Czech republic) for 40 min. Positive signals were visualized using Roti-Lumin detection reagent (Carl Roth GmbH+Co, Karsruhe, Germany) and ECL Hyperfilm (Amersham Biosciences UK Ltd., Buckinghamshire, UK).

Immunocytochemical analysis

The presence of cyclin B1, caspase-3 and CREB-1 was detected in GC plated in chamber-slides using immunocytochemistry according to Osborn and Isenberg (1994) as described previously (Sirotkin and Makarevich, 1999). Proliferation, apoptosis and CREB-1 were detected in cells using mouse monoclonal antibody against cyclin B1, caspase-3 and CREB-1 (Santa Cruz). For the visualization of the binding sites of primary antibodies, a secondary polyclonal horseradish-conjugated goat antiserum against mouse IgG (Sevac) and DAB reagent (Boehringer Mannheim GmbH, Mannheim, Germany) were used. The presence of cyclin B1, caspase-3 and CREB-1 immunoreactivity and the percentage of positive-stained cells in each group were evaluated by light microscopy.

TUNEL analysis

Visualisation of apoptotic cells (containing fragmented DNA) in chamber-slides was performed by TUNEL (TdT-mediated dUTP nick end labeling) method as described previously (Sirotkin and Makarevich, 1999) using In Situ Cell Death Detection Kit and DAB reagent (Boehringer Mannheim GmbH) following instructions of manufacturer. Fixed and permeabilized cells incubated without TdT, but with secondary HRP-conjugated antibody and DAB, were used as a negative control. Cells

containing intensive TdT-positive staining in the nuclei were considered as apoptotic ones. The general cell morphology and percentage of TUNEL-positive cells in each culture was determined using light microscopy.

Significant differences between experimental and control group (without FSH) were evaluated by Student´s t-test using Sigma Plot 9.0 statistic software (Systat Software GmbH, Erkrath, Germany).

RESULTS AND DISCUSSION

TUNEL (Fig.1 and Fig.2) and immunocytochemical analysis showed, that the addition of FSH into culture medium significantly ($P<0.05$) decreased the percentage of apoptotic (TdT-positive) cells (at dose 10 ng/ml) (Fig.3) as well as the proportion of caspase-3-positive cells (at dose 1 and 10 ng/ml) (Fig.4). These results demonstrate anti-apoptotic effect of FSH on rabbit GC in relation to FSH dose. While physiological FSH concentrations (1 and 10 ng/ml) act anti-apoptotically, higher concentrations could decrease FSH receptor sensibility and activate negative feedback mechanisms. Similar observations were obtained on rat GC, in which

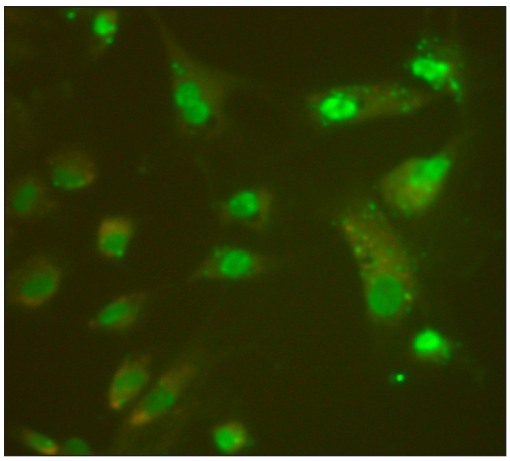

Fig.1: Expression of apoptosis (TUNEL green fluorescence staining) in nuclei of rabbit granulosa cells cultured without FSH. TdT is labelled with FITC, magnification x 400.

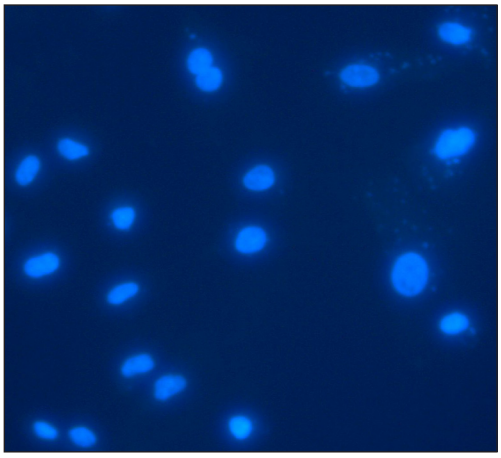

Fig.2: Lack of TUNEL staining in nuclei of negative control cells (TdT ommited). Nuclei are stained with DAPI, magnification x 400.

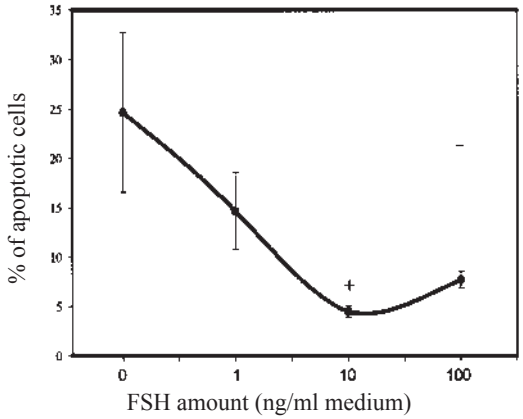

Fig.3.: Effect of FSH (0, 1, 10, 100 ng/ml) on the expression of apoptosis in cultured rabbit granulosa cells (percentage of TUNEL-positive cells) after 2 d culture in a serum-supplemented medium. Values are means ± S.E.M., + significant ($P<0.05$) difference compared with control in a serum-supplemented medium (medium without additions).

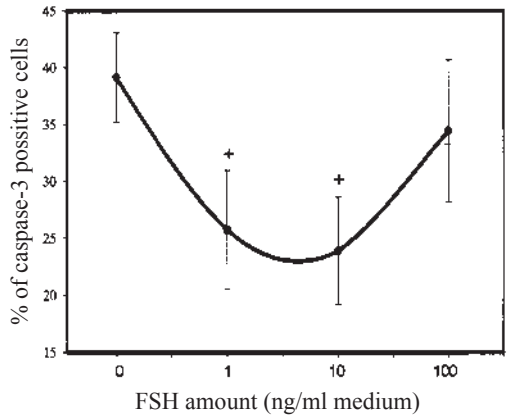

Fig.4: Effect of FSH (0, 1, 10, 100 ng/ml) on the expression of caspase-3 in cultured rabbit granulosa cells (percentage of caspase-3-positive cells evaluated by immunocytochemistry) after 2 d culture in a serum-supplemented medium. Values are means ± SEM, + significant difference ($P<0.05$) compared with control in a serum-supplemented medium (medium without FSH).

FSH reduced DNA fragmentation (Chun et al, 1996), and on porcine GC, in which FSH reduced caspase-3 accumulation (Dineva et al, 2007). Our data obtained on rabbits, together with data obtained by other authors on other species, suggest, that FSH can decrease apoptosis by blocking cytoplasmic caspase-3, an activator of apoptotic events in mitochondria and of nuclear DNase.

Results obtained by Western immunoblotting demonstrated, that FSH raised the accumulation of cyclin B1 proliferative peptide (at all doses added), (Fig.5). On the other hand, immunocytochemistry did not confirm FSH effect on the percentage of cyclin B1-positive cells. Results of Western blotting correspond to the results obtained by Western blotting on rat GC, where FSH stimulated proliferation by increasing cyclin D2 (Robker a Richards, 1998) and PCNA expression (Yu et al, 2005). Our data, together with reports concerning other species suggest, that FSH can promote cell cycle at different stages through their specific regulators (cyclins and PCNA). FSH by stimulating cyclin D2 can promote entrance of cells into G1-phase of cell cycle. In addition, by increasing PCNA, FSH can stimulate DNA replication and transition into S-phase of the cycle, and by activating cyclin B1 induce transition of cells from G2- to M-phase of the cell cycle.

The ability of FSH to promote accumulation of CREB-1 in rabbit GC was demonstrated by both immunocytochemistry (at dose 10 ng/ml) (Fig.6) and SDS-Western blotting (at all doses added) (Fig.7). These data do not correspond the data of Mukherjee et al (1996), who reported, that FSH increased not the total amount of CREB, but the amount of phosphorylated form of CREB in rat GC. These findings suggest, that FSH/CREB dependent mechanism of action may be species-specific: in rats FSH acts just through phosphorylation of CREB, whilst in rabbits FSH could act also through the increase of total CREB amount.

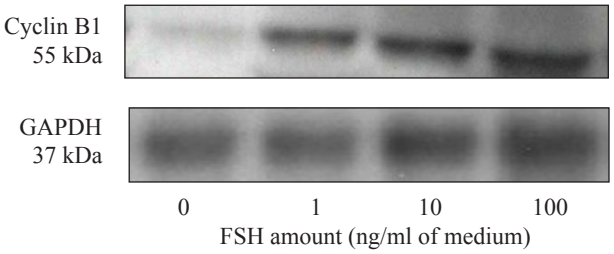

Fig.5: Cyclin B1 identified in lyzate of rabbit granulosa cells after 2 d culture in a serum-supplemented medium with FSH (1, 10, 100 ng/ml) and without FSH (control) using SDS PAGE-Western immunoblotting. Molecular weight (kDa) is indicated left. Loading control (GAPDH) is shown.

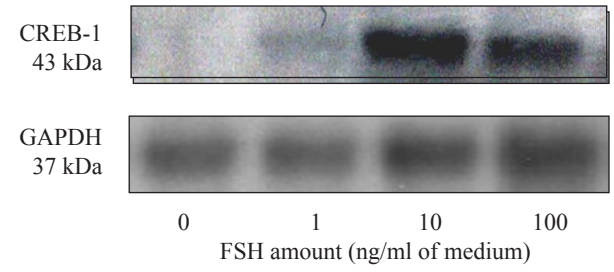

Fig.6: CREB-1 identified in lysate of porcine granulosa cells after 2 d culture in a serum-supplemented medium with FSH (1, 10, 100 ng/ml) and without FSH (control) using SDS PAGE-Western immunoblotting. Molecular weight (kDa) is indicated left. Loading control (GAPDH) is shown.

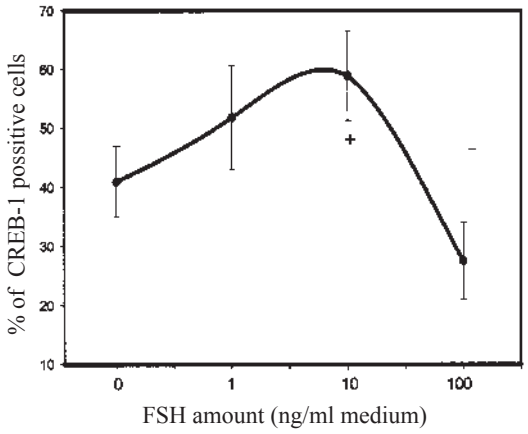

Fig.7: Effect of FSH (0, 1, 10, 100 ng/ml) on the expression of CREB-1 in cultured rabbit granulosa cells (percentage of CREB-1 positive cells evaluated by immunocytochemistry) after 2 d culture in a serum-supplemented medium. Values are mean ± SEM, + significant (P<0.05) difference compared with control in a serum-supplemented medium (medium without FSH).

In our experiments FSH also decreased apoptosis and this effect was associated with increase in CREB accumulation. It is known that FSH activates MAPK-dependent pathway, which is critical to survival and mitosis of porcine (Shiota et al, 2003) and rat (Das et al, 1996) GC. As MAPK is able to activate CREB in non-ovarian cells (Bonni et al, 1999; Costes et al, 2006), we may suggest, that FSH acts as anti-apoptotic agent by activation of MAPK/CREB in GC, too. Raising CREB

level can block caspase-3 expression and therefore prevent further apoptotic events in mitochondria and nucleus (Costes et al, 2006). Thus, available data suggest the existence of negative feedback between these proteins and that FSH can suppress apoptosis through MAPK → CREB → apoptotic peptides.

Our results demonstrate, that FSH stimulates expression of both proliferative peptides and transcription factor CREB-1 in a dose-dependent manner. Since CREB-1 can be involved in activation of PCNA, the activator of cell proliferation (Mukherjee et al, 1996; Lee and Matthews, 1997; Somers et al, 1995), it is possible, that CREB could act as a potential mediator of FSH effect on GC proliferation. In this case FSH can first promote cAMP and/or MAPK production, the known activators of CREB (Das et al, 1996). CREB, in turn could activate accumulation of different proliferative peptides (cyclin D2, PCNA and cyclin B1), which are important for transition through several phases of the cell cycle (Robker and Richards, 1998; Yu et al, 2005). Thus, GC proliferation could be activated by the following sequence of events: FSH → cAMP, PKA, MAPK → CREB → PCNA, cyclins → cell proliferation.

As present results did not provide direct evidence for CREB involvement in regulation of cell cycle and apoptosis, further investigations to confirm our suggestion, that FSH affects ovarian cell cycle and apoptosis just through CREB-1, are needed. Results of such studies can contribute to better understanding of pattern and mechanism of FSH action on ovarian cells, as well as the role of transcription factor CREB as a regulator of different reproductive processes and a mediator of FSH action on these processes. This information could be practically used for more efficient regulation of rabbit reproduction in farm conditions.

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REFERENCES

BONNI, A. – BRUNET, A. – WEST, A.E. – DATTA, S.R. – TAKASU, M.A. – GREENBERG, M.E. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. In: *Science* 1999, vol. 286, p. 1358-62.

CHUN, S.Y. – EISENHAUER, K.M. – MINAMI, S. – BILLIG, H. – PERLAS, E. – HSUEH, A.J. 1996. Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. In: *Endocrinology*, 1996 Apr, vol. 137(4), p. 1447-56.

COSTES, S. – BROCA, C. – BERTRAND, G. – LAJOIX, A.D. – BATAILLE, D. – BOCKAERT, J. – DALLE, S. 2006. ERK1/2 control phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic beta-cell survival. In: *Diabetes*, 2006 Aug, vol. 55(8), p. 2220-30.

DAS, S. – MAIZELS, E.T. – DEMANNO, D. - ST CLAIR, E. – ADAM, S.A. -HUNZICKER-DUNN, M. 1996. A stimulatory role of cyclic adenosine 3,5-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. In: *Endocrinology,* 1996 Mar, vol. 137(3), p. 967-74.

DINEVA, J. – AUGUSTOWSKA, K. 2007. Expression of atrial natriuretic Peptide, progesterone, apoptosis related proteins and caspase-3 in in vitro luteinized and leptin-treated porcine granulosa cells. In: Endocr Regul, 2007 Mar, vol. 41(1), p. 11.

LAEMMLI, U. K. 1970. Cleavage and structure of proteins during the assembly of the head of bacteriophage T4. In: *Nature*, vol. 227, p. 680-685.

LEE, B.H. – MATTHEWS, M.B. 1997. Transcriptional coactivator cAMP response element binding protein mediates induction of the human proliferating cell nuclear antigen promoter by the adenovirus E1A oncoprotein. In: *Proc Natl Acad Sci USA*, vol. 94, p. 4481–4486.

HUNZICKER-DUNN, M. – MAIZELS, E.T. 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A. In: *Cell Signal*, 2006 Sept, vol. 18(9), p. 1351–1359.

MAILLET, G. – BRÉARD, E. – BENHAIM, A. – LEYMARIE, P. – FÉRAL, C. 2002. Hormonal regulation of apoptosis in rabbit granulosa cells in vitro: evaluation by flow cytometric detection of plasma membrane phosphatidylserine externalization. In: *Reproduction*, 2002, vol. 123, p. 243–251.

MARIANA, J.C. – SOLARI, A. 1993. Proliferation of follicular cells and the effect of FSH on the onset of follicular growth in the ovary of 30-day old rabbits studied by continuous labelling with 3H-thymidine. In: *Reprod Nutr Dev*, 1993, vol. 33(1), p. 63-7.

MUKHERJEE, A. - PARK-SARGE, O-K. – MAYO, K.E. 1996. Gonadotropins induce rapid phosphorylation of the 3',5'-cyclic adenosine monophosphate response element binding protein in ovarian granulosa cells. In: *Endocrinology*, 1996, vol. 137, no. 8.

MUNIZ, L.C. – YEHIA, G. – MEMIN, E. – RATNAKAR, P.V. – MOLINA, C.A. 2006. Transcriptional regulation of cyclin D2 by the PKA pathway and inducible cAMP early repressor in granulosa cells. In: *Biol Reprod*, 2006 Aug, vol. 75(2), p. 279-88.

OSBORN, M. – ISENBERG, S. 1994. Immunocytochemistry of frozen and paraffin tissue sections. In: *Cell biology,* A Laboratory Handbook, Ed JE Celis, New York Academic Press, 1994, vol. 2, p. 361-367.

ROBKER, R. – RICHARDS, J.S. 1998. Hormone-Induced Proliferation and Differentiation of Granulosa Cells: A Coordinated Balance of the Cell Cycle Regulators Cyclin D2 and $p27^{Kip1}$, In: *Molecular Endocrinology*, 1998, vol. 12 (7), p. 924-940.

SHIOTA, M. – SUGAI, N. – TAMURA, M. – YAMAGUCHI, R. – FUKUSHIMA, N. – MIYANO, T. – MIYAZAKI,

H. 2003. Correlation of mitogen-activated protein kinase activities with cell survival and apoptosis in porcine granulosa cells. In: *Zoolog Sci.*, 2003 Feb, vol. 20(2), p. 193-201.

SIROTKIN, A.V. – MAKAREVICH, A. 1999. Growth hormone regulates secretory activity and apoptosis in cultured bovine granulosa cells through the activation of the cAMP/protein kinase A system, In: *J.Endocrinol*, 1999, vol. 163, p. 317-327.

SOMERS, J.P. – BENYO, D.F. - LITTLE-IHRIG, L. – ZELEZNIK, A.J. 1995. Luteinization in primates is accompanied by loss of a 43-kilodalton adenosine 3‘,5‘-monophosphate response element-binding protein isoform. In: *Endocrinology*, 1995 Nov, vol. 136(11), p. 4762-8.

YU, F.Q. – HAN, C.S. – YANG, W. – JIN, X. – HU, Z.Y. – LIU, Y.X. 2005. Role of ERK1/2 in FSH induced PCNA expression and steroidogenesis in granulosa cells. In: *Front Biosci,*2005 Jan, vol. 1 (10), p. 896-904.

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