

Short Communication

The Effect of the cAMP Analogue, dbcAMP, on Proliferation and Apoptosis of Rabbit Oviductal Cells*

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It was previously documented that cyclic AMP (cAMP)-dependent intracellular mechanisms can be involved in control of reproductive processes, and pharmacological regulators of these mechanisms could be practically used to improve rabbit fertility (SIROTKIN *et al.* 2008; SIROTKIN *et al.* 2010a; CHRENEK *et al.* 2012). Changes in fertility could be due to changes in oviductal functions. The aim of our study was to examine the involvement of cAMP-dependent intracellular mechanisms in control of oviductal cell functions, in particular the influence of dbcAMP, a cAMP agonistic analogue, on proliferation and apoptosis of cultured oviductal cells. For this purpose, we compared the expression of markers of proliferation (PCNA, cyclin B1) and apoptosis (bax and bcl-2) in the oviduct epithelial cells isolated from rabbits, whose ovarian and oviductal cycle was induced by gonadotropins alone or in combination with dbcAMP (50 µg/animal) by using immunocytochemistry. It was observed that dbcAMP administration caused an increase in the proportion of cells containing PCNA, but not cyclin B1, bax or bcl-2. Higher expression of PCNA, but not cyclin B1, in the dbcAMP-treated group suggests that the dbcAMP administration can stimulate oviductal cell proliferation, probably promoting transition of cells from G0 to G1 and S-phase of the cell cycle. No influence of dbcAMP administration on regulators and markers of apoptosis (pro-apoptotic – bax and anti-apoptotic – bcl-2) suggests that dbcAMP is probably not involved in the control of apoptosis in rabbit oviduct epithelial cells. The involvement of cAMP-dependent intracellular mechanisms in control of oviduct functions is assumed in this study. This is the first demonstration that dbcAMP can stimulate proliferation of the oviduct epithelial cells without influencing their apoptosis.

Key words: dbcAMP, rabbit, oviduct, proliferation (PCNA, cyclin B1), apoptosis (Bax, caspase 3).

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The involvement of cyclic nucleotide/cyclic adenosine monophosphate (cAMP)-dependent intracellular mechanisms in control of reproductive processes is well documented. Pharmacological regulators of cAMP and its effector, a cAMP-dependent protein kinase A (PKA), can promote ovarian cell proliferation (CHEADLE *et al.* 2008; VIEGAS *et al.*

2008; SIROTKIN *et al.* 2010b), either stimulate (SIROTKIN *et al.* 2010b; AMSTERDAM *et al.* 2003) or inhibit (VIEGAS *et al.* 2008) ovarian cell apoptosis, stimulate release of hypothalamic GnRH (POON *et al.* 2008) and control secretion of ovarian hormones (SIROTKIN *et al.* 2010b; AMSTERDAM *et al.* 2003; HILLIER & TETSUKA 1997; MAKAREVICH

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et al. 2004; SIROTKIN 2005; PELUSO 2006; CHRENEK *et al.* 2010). Activation of cAMP-dependent intracellular mechanisms via administration of phosphodiesterase inhibitors can increase the number of ovulations, embryos and offspring in rats (MCKENNA *et al.* 2005) and rabbits (CHRENEK *et al.* 2008; SIROTKIN *et al.* 2010a). Similar effects on rabbit reproduction (increase in number of ovulations, *Corpora lutea*, oocyte and embryo yield, weaned pups) were shown for the synthetic cAMP cell-permeable analogue, N⁶,2'-dibutyryl adenosine 3'5'-cyclic monophosphate (dbcAMP, [CHRENEK *et al.* 2012; BALAZI *et al.* 2012]).

The stimulatory action of cAMP regulators on fertility and number of pups can be mediated by the action of these regulators on ovarian hormones (SIROTKIN *et al.* 2010b; CHRENEK *et al.* 2010) and/or on functions of the oviduct, which play a key role in oocyte fertilization and embryo development. The oviduct produces substantial amounts of cAMP and PKA (MAKAREVICH & SIROTKIN 1997; ORIHUELA *et al.* 2003). Moreover, regulators of cAMP/PKA can influence ovum transport through the oviduct (ORIHUELA *et al.* 2003), secretory functions of the oviduct epithelial cells and their response to GH, IGF-I (MAKAREVICH & SIROTKIN 1997) and estrogen (ORIHUELA *et al.* 2003) action. Development and activity of the oviduct depend on oviduct cell proliferation, apoptosis and equilibration of these processes. The inhibitory influence of 3-isobutyl-methyl-xanthine (IBMX), an indirect stimulator of cAMP-dependent intracellular mechanisms (phosphodiesterase blocker), on rabbit oviduct cell proliferation and apoptosis has been reported (SIROTKIN *et al.* 2010b). The action of a direct regulator of these mechanisms, for example the cAMP agonistic analogue dbcAMP, on the oviduct cell proliferation and apoptosis has not been studied.

The aim of our study was to examine the influence of dbcAMP, a cAMP agonistic analogue, on proliferation and apoptosis of oviductal cells. For this purpose, we compared the expression of markers of proliferation (PCNA, cyclin B1 [JONES & KAZLAUSKAS 2001; MAGA & HUBSCHER 2003]) and apoptosis (bax and bcl-2 [HARADA & GRANT 2003; DEGLI ESPOSTI 2004]) in oviduct epithelial cells isolated from rabbits whose ovarian and oviductal cycle was induced by gonadotropins alone or together with dbcAMP.

Material and Methods

Animal manipulation

Animal *in vivo* experiments were described previously (SIROTKIN *et al.* 2008; SIROTKIN *et al.*

2010a; CHRENEK *et al.* 2012). Briefly, rabbit females 4 months of age kept in cages under standard conditions at the rabbit farm of Animal Production Research Centre Nitra, Slovak Republic, were treated three days before mating with pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und Werfft AG., Vienna, Austria, 100 IU/animal) followed after 72 h by human chorionic gonadotropin (hCG, Werfacher, Alvetra und Werfft AG, 200 IU/animal). Control animals were i.m injected only with these gonadotropins, whilst experimental females received gonadotropins together with dbcAMP (Biolog Life Science Institute, Bremen, Germany, 50 µg/animal). All substances (0.7 ml solution of gonadotropin with or without dbcAMP) were injected intramuscularly. At 19-20 h after mating and artificial insemination by freshly diluted sperm all females were euthanized by decapitation and their oviducts were collected for subsequent cell isolation and culture. The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a.

Preparation, culture and processing of oviductal cells

Ampullary parts of oviducts were washed in PBS with 1% antibiotic-antimycotic solution (Sigma), and epithelial cells were flushed out by sterile PBS using a syringe. Thereafter, the oviduct cell suspension was passed through a steel sieve to separate large pieces and cellular fragments. The cell filtrate was washed three times in a PBS solution and the oviduct epithelial cells were purified from the cell debris and blood cells by centrifugation in a gradient of Percoll (Sigma) according to the manufacturer's instructions. Cells were aspirated from the Percoll fraction and rinsed twice in a sterile incubation medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic-antimycotic solution, all from Sigma, St. Louis, USA). After the final centrifugation (10 min. at 200 g), the cells were resuspended in the same incubation medium. The cell concentration was determined with a haemocytometer and then adjusted to 1×10^6 cells/ml by dilution with the incubation medium. Cell viability was determined by Trypan blue staining and found to be in the range of 80-90%. Aliquots of oviduct epithelial cell suspension were placed into Lab-Tek chamber-slides (Nunc, Inc., Naperville, USA, 0.3 ml/well) and incubated at 37°C and 5% CO₂ in humidified air. After 2 days of culture, the chamber-slides were washed 3 times in an ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 20 min, washed in PBS (2 x 5 min), ethanol (70%: 5 min, 80%: 10 min, 96%: 2 x 10 min, 100%: 10 min) and kept

in 100% ethanol at -18°C until immunocytochemical analysis.

Immunocytochemical analysis

Intracellular PCNA, cyclin B1, bax and bcl-2, widely used for evaluation of apoptosis and proliferation in reproductive organs (CHEADLE *et al.* 2008; AMSTERDAM *et al.* 2003), were detected in the oviduct epithelial cells plated onto chamber-slides, using immunocytochemistry. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against human PCNA, cyclin B1, bax and bcl-2 (which cross-reacts with corresponding mouse, rat, rabbit, human and chicken antigens; all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:100) were used as directed by the manufacturer. For the visualization of a primary antibody, a secondary polyclonal porcine IgG labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:2000) and DAB-reagent (Roche Diagnostics Corporation, IN, USA; 10%) were used. These cells were mounted into glycerol mounting medium Glycergel (DAKO Corp., Carpinteria, CA, USA). The presence of antigens within the cells was checked by light microscopy. Cells treated with a secondary antibody but omitting the primary antibody were used as negative controls.

Statistics

Each experiment was performed on 6 control animals and 6 animals treated with dbcAMP. All cells collected from either control or experimental animals were pooled and processed together. Each *in vitro* group (cells collected from either control or dbcAMP-treated animals) was represented by four wells of chamber-slides. Each chamber slide was used for immunocytochemical analysis of one protein to enable adequate comparison of cells obtained from control and treated animals in one slide. The proportions of cells containing specific immunoreactivity were calculated from inspection of at least 1000 oviductal cells per well. The data shown are means of values obtained in 3 separate experiments by using separate pools of oviductal cells. Significant differences between the treatments were evaluated by one-way ANOVA followed by a paired t-test using Sigma Plot 11.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences from control at $P < 0.05$ were considered as significant.

Results and Discussion

Immunocytochemical analysis demonstrated the presence of markers of both proliferation (PCNA,

cyclin B1) and apoptosis (bax and bcl-2) in the oviduct epithelial cells. Markers of proliferation accumulated predominantly in cell nuclei, although in some cells PCNA and cyclin B1 immunoreactivity were also observed in the cytoplasm. Markers of mitochondrial apoptosis were observed mainly in the cell cytoplasm. The presence of the proliferation marker (PCNA) and of apoptosis (bax) in cultured rabbit oviduct epithelial cells is illustrated by Fig. 1.

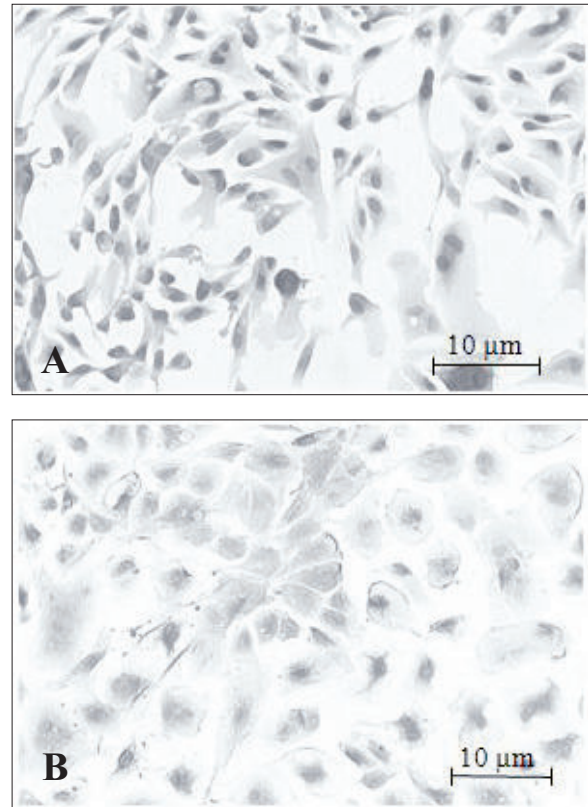


Fig. 1. Monolayer of epithelial cells isolated from oviducts of rabbits treated with dbcAMP ($50 \mu\text{g}/\text{animal}$). Markers of proliferation PCNA (A) and bax (B) are stained with DAB (brown). Immunocytochemistry and light microscopy, $\times 400$.

The proportion of the oviduct cells containing visible PCNA was significantly higher in the group of animals treated with dbcAMP (n percentage of cells containing cyclin B1 (Fig. 2b), bax (Fig. 3a) and bcl-2 (Fig. 3b) were found.

The observed formation of a cell monolayer, the exclusion of Trypan blue, and the presence of markers of both proliferation and apoptosis in cells after culture suggest that the culture of oviduct epithelial cells was viable and suitable for testing the influence of the studied preparation. A higher proportion of cells containing PCNA in the dbcAMP-treated group suggests that dbcAMP administration can promote oviduct cell proliferation. Since PCNA is a marker of the G1 and S-phase of the cell cycle (MAGA & HUBSCHER

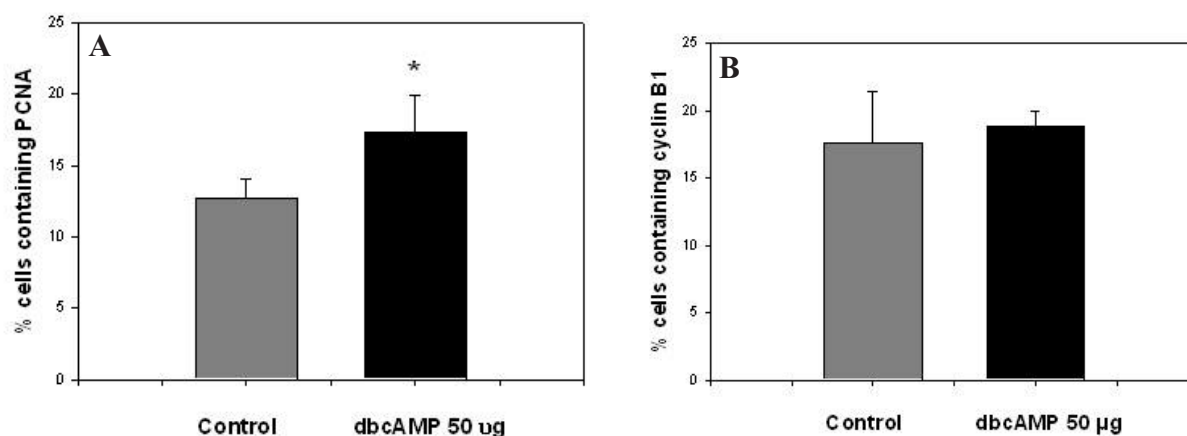


Fig. 2. Effect of dbcAMP injections (50 µg/animal) *in vivo* on subsequent expression of proliferation-related substances PCNA (A) and cyclin B1 (B) in isolated rabbit oviduct epithelial cells cultured *in vitro*. Immunocytochemistry. Values are means±S.E.M., * – significant ($P < 0.05$) differences with control (no dbcAMP treatment).

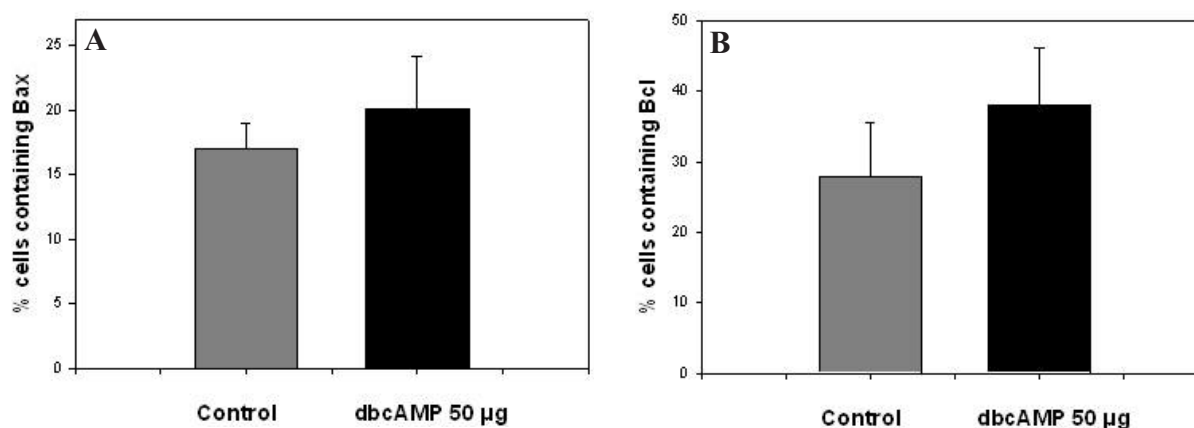


Fig. 3. Effect of dbcAMP injections (50 µg/animal) *in vivo* on subsequent expression of apoptosis-related substances bax (A) and bcl-2 (B) in isolated rabbit oviduct epithelial cells cultured *in vitro*. Immunocytochemistry. Values are means±S.E.M.

2003), it might be suggested that dbcAMP promotes cell proliferation via transition of cells from G0 to G1 and S-phase. The administration of dbcAMP tended to increase the percentage of cells containing cyclin B1, a marker of the G2 phase of the cell cycle (JONES & KAZLAUSKAS 2001), but these changes were statistically insignificant. This suggests that dbcAMP can influence oviductal cell proliferation targeting mainly G1 and S, but not the G2 phase of the cell cycle. Our observations are in line with previous reports on the ability of cAMP to promote human (CHEADLE *et al.* 2008), rat (VIEGAS *et al.* 2008) and rabbit (CHRENEK *et al.* 2012; SIROTKIN *et al.* 2010b) ovarian cell proliferation. On the other hand, they do not correspond to a previous observation (SIROTKIN *et al.* 2010b) of the ability of IBMX, a cAMP- and cGMP-specific phosphodiesterase inhibitor, to suppress apoptosis in cultured rabbit oviduct cells. The discrepancy in influence of IBMX and dbcAMP on ovarian cell

proliferation may be due to different mechanisms of action of these molecules: IBMX can promote accumulation of both cAMP and cGMP, whilst dbcAMP is a direct stimulator of cAMP-dependent protein kinase A.

Furthermore, the dbcAMP-induced increase in the expression of proliferation, but not in apoptosis, suggest that dbcAMP changes the balance between proliferation and apoptosis, determining growth, development and functional activity of each organ. The present observations indicate the involvement of cAMP-dependent intracellular mechanisms in control of oviductal function. Finally, they provide the first demonstration that dbcAMP can stimulate proliferation of oviduct epithelial cells without affecting apoptosis.

Our observations did not demonstrate a significant influence of dbcAMP administration on regulators and markers of apoptosis (pro-apoptotic bax and anti-apoptotic bcl-2, (HARADA & GRANT 2003;

DEGLI ESPOSTI 2004). This model demonstrates that dbcAMP is probably not substantially involved in control of apoptosis in rabbit oviduct epithelial cells. These observations are not in line with previous reports on either the anti-apoptotic (VIEGAS *et al.* 2008; SIROTKIN *et al.* 2010b) or pro-apoptotic (AMSTERDAM *et al.* 2003) action of phosphodiesterase (PDE) inhibitors in rat, human and rabbit ovarian cells and on the anti-apoptotic action of the PDE inhibitor IBMX on rabbit oviduct cells (SIROTKIN *et al.* 2010b). As mentioned previously, the application of PDE inhibitors non-specific for cAMP can provide less reliable results than those obtained using the cAMP analogue dbcAMP. This model demonstrated that cAMP/PKA-dependent intracellular mechanisms can be involved in the control of oviduct cell proliferation, but not of apoptosis.

The mechanism(s) of dbcAMP action on the oviductal cells requires further explanation. One such mechanism may involve changes in GnRH/gonadotropin release, reception or action. cAMP is a promoter of Gn-RH production (POON *et al.*, 2008) and mediator of its action (SIROTKIN *et al.* 1994; RAMAKRISHNAPPA *et al.* 2005). GnRH/gonadotropins are known stimulators of progestagens (PELUSO 2006) and estrogens (DOUGHERTY & SANDERS 2005), the classical activators of oviductal growth and development. The stimulatory action of dbcAMP administration on release of these steroid hormones by ovarian cells has been demonstrated in our previous experiments (CHRENEK *et al.* 2010).

The mechanisms of dbcAMP action on oviductal cells require further studies. Nevertheless, the present experiment is the first showing the involvement of the cAMP-dependent signaling pathway in control of oviductal functions. Moreover it demonstrates that dbcAMP can stimulate proliferation of oviduct epithelial cells (probably via transition from G0 to G1 and S-phase of cell cycle) without affecting their apoptosis. This action can promote growth and development of the oviduct and improve the fertility of rabbits observed in our previous experiments (SIROTKIN *et al.* 2008; SIROTKIN *et al.* 2010a; CHRENEK *et al.* 2012; BALAZI *et al.* 2012).

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References

AMSTERDAM A., SASSON R., KEREN-TAL I., AHARONI D., DANTES A., RIMON E., LAND A., COHEN T., DOR Y., HIRSH

L. 2003. Alternative pathways of ovarian apoptosis: death for life. *Biochem. Pharmacol.* **66**: 1355-1362.

BALAZI A., SIROTKIN A. V., CHRENEK P. 2012. Activators of protein kinase A and oxytocin affect rabbit reproduction. *Cent. Eur. J. Biol.* **7**: 973-979.

DEGLI ESPOSTI M. 2004. Mitochondria in apoptosis: past, present and future. *Biochem. Soc. Trans.* **32**: 493-495.

DOUGHERTY D. C., SANDERS M. M. 2005. Estrogen action: revitalization of the chick oviduct model. *Trends Endocrinol. Metab.* **16**: 414-419.

HARADA H., GRANT S. 2003. Apoptosis regulators. *Rev. Clin. Exp. Hematol.* **7**: 117-138.

HILLIER S. G., TETSUKA M. 1997. Role of androgens in follicle maturation and atresia. *Baillieres Clin. Obstet. Gynaecol.* **11**: 249-260.

CHEADLE C., NESTEROVA M., WATKINS T., BARNES K. C., HALL J. C., ROSEN A., BECKER K. G., CHO-CHUNG Y. S. 2008. Regulatory subunits of PKA define an axis of cellular proliferation/differentiation in ovarian cancer cells. *BMC Med Genomics* **1**: 43.

CHRENEK P., GROSSMANN R., SIROTKIN A. V. 2010 The cAMP analogue, dbcAMP affects release of steroid hormones by cultured rabbit ovarian cells and their response to FSH, IGF-I and ghrelin. *Eur. J. Pharmacol.* **640**: 202-205.

CHRENEK P., PIVKO J., MAKAREVICH A. V., KUBOVIČOVÁ H., BALAZI A., SIROTKIN A. V. 2012. The cAMP analogue, dbcAMP can stimulate rabbit reproductive functions. I. Effect on ovarian folliculogenesis, ovulation and embryo production. *Acta Veterinaria (Beograd)* **62**: 227-237.

JONES S. M., KAZLAUSKAS A. 2001. Growth factor-dependent signaling and cell cycle progression. *FEBS Lett.* **490**: 110-116.

MAGA G., HUBSCHER U. 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J. Cell. Sci.* **116**: 3051-3060.

MAKAREVICH A. V., SIROTKIN A. V. 1997. The involvement of the GH/IGF-I axis in the regulation of secretory activity by bovine oviduct epithelial cells. *Anim. Reprod. Sci.* **48**: 197-207.

MAKAREVICH A. V., SIROTKIN A. V., GENIESER H. G. 2004. Action of protein kinase A regulators on secretory activity of porcine granulosa cells *in vitro*. *Anim. Reprod. Sci.* **81**: 125-136.

MCKENNA S. D., PIETROPAOLO S. D., TOS E. G., CLARK A., FISCHER D., KAGAN D., BAO B., REDRESE P. J., PALMER S. 2005. Pharmacological inhibition of phosphodiesterase 4 triggers ovulation in follicle-stimulating hormone-primed rats. *Endocrinology* **146**: 208-214.

ORIHUELA P. A., PARADA-BUSTAMANTE A., CORTÉS P. P., GATICA C., CROXATTO H.B. 2003 Estrogen receptor, cyclic adenosine monophosphate, and protein kinase A are involved in the nongenomic pathway by which estradiol accelerates oviductal oocyte transport in cyclic rats. *Biol. Reprod.* **68**: 1225-1231.

PELUSO J. J. 2006 Multiplicity of progesterone's actions and receptors in the mammalian ovary. *Biol. Reprod.* **75**: 2-8.

POON S. L., AN B. S., SO W. K., HAMMOND G. L., LEUNG P. C. 2008. Temporal recruitment of transcription factors at the 3',5'-cyclic adenosine 5'-monophosphate-response element of the human GnRH-II promoter. *Endocrinology* **149**: 5162-5171.

RAMAKRISHNAPPA N., RAJAMAHENDRAN R., LIN Y. M., LEUNG P. C. 2005. GnRH in non-hypothalamic reproductive tissues. *Anim. Reprod. Sci.* **88**: 95-113.

SIROTKIN A. V. 2005 Control of reproductive processes by growth hormone: extra- and intracellular mechanisms. *Vet. J.* **170**: 307-317.

- SIROTKIN A. V., CHADIO S., CHRENEK P., XYLOURI E., FOTOPOULOUY H. 2010a. Phosphodiesterase inhibitor 3-isobutyl-methyl-xanthine affects ovaria morphology and stimulates reproduction in rabbits. *Eur. J. Inflammation* **8**: 173-179.
- SIROTKIN A. V., CHRENEK P., ČURLEJ J., ZÁHRADNÍKOVÁ M. 2008. Effect of 3-isobutyl-1-methyl-xanthine on rabbit superovulation and egg recovery. *Slovak J. Anim. Sci.* **41**: 57-59.
- SIROTKIN A. V., CHRENEK P., CHADIO S., XYLOURI E., FOTOPOULOUY H., MAKAREVICH A. V. 2010b. Phosphodiesterase inhibitor 3-isobutyl-methyl-xanthine affects rabbit ovaries and oviduct. *Eur. J. Pharmacol.* **643**: 145-151.
- SIROTKIN A. V., NITRAY J., KOLENA J., BULLA J. 1994. Cooperation between LH-RH and LH in the direct action on the ovary: LH-RH stimulation of LH/hCG receptors, basal and LH-induced cAMP and cGMP release by porcine granulosa cells in vitro. *Cell Signal.* **6**: 135-140.
- VIEGAS L. R., HOJMAN E., BEATO M., PECCI A. 2008. Mechanisms involved in tissue-specific apoptosis regulated by glucocorticoids. *J. Steroid Biochem. Mol. Biol.* **109**: 273-278.