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REGULAR ARTICLE

EFFECT OF dbcAMP ON PROLIFERATION AND APOPTOSIS OF PORCINE GRANULOSA CELLS *in vitro*

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ABSTRACT

Cyclic nucleotide cAMP and its target protein kinase A (PKA) dependent intracellular mechanisms can play an important role in regulation of ovarian cell function and in mediating gonadotropin action on these cells. The aim of the present study was to examine the effect of cAMP analogue, dibutyryl cyclic adenosine monophosphate (dbcAMP) (0; 0.1; 1 and 10 µg/ml) or FSH (0; 0,01; 1 IU/ml) on proliferation and apoptosis of porcine granulosa cells in vitro. Indices of cell apoptosis (expression of apoptotic peptide bax) and proliferation (expression of proliferation-associated peptide PCNA) within ovarian granulosa cells were analysed by immunocytochemistry. It was observed that accumulation of PCNA was increased by dbcAMP and FSH at all doses added. The occurrence of bax was also stimulated by dbcAMP after exposition (at 0,1 and 1 µg/ml, but not at dose 10 µg/ml) and by FSH (at all doses added). The stimulatory effect of both dbcAMP and FSH on both ovarian cell apoptosis and proliferation suggest, that these substances may promote ovarian follicular cell turnover. The similarity of dbcAMP and FSH effect may indicate that FSH can affect ovarian functions

via cAMP-dependent intracellular mechanisms. The present data may provide new tools to regulate human and animal reproductive processes via cAMP-dependent mechanisms.

Keywords: dbcAMP, proliferation, apoptosis, porcine granulosa cells, cAMP/PKA

INTRODUCTION

Cyclic nucleotide cAMP and its target, protein kinase A, are intracellular mediators of hormone action on ovarian cells **(Sirotkin, Makarevich and Grosmann, 2010)**. The cAMP/PKA-dependent intracelular mechanisms plays an important role in regulation of mitosis **(D´Angiolella *et al.,* 2001)**, meiosis **(Sato, Matsuo and Miyamoto, 1990)**, proliferation **(Shawa *et al.,* 2002; Cheadle *et al.,* 2008)**, apoptosis **(Amsterdam *et al.,* 2003)**, release of hormones **(Makarevich, Sirotkin and Genieser, 2004; Sirotkin 2005; Chrenek, Grossmann and Sirokin, 2010)** and growth factors **(Makarevich and Sirotkin, 2000)** in ovarian cells natural and hormone-induced ovulation **(Sirotkin, Makarevich and Grosmann, 2010)**.

Follicle-stimulating hormone (FSH) can promote granulosa cells proliferation and apoptosis **(Sirotkin *et al.,* 2008; Sirotkin *et al.,* 2012; Jiang *et al.,* 2003; Cuiling *et al.,* 2005)** and therefore to induces ovarian folliculogenesis via cAMP/PKA **(Hunzicker-Dunn and Maizels, 2006)**.

N6,2'-dibutyryladenosine 3'5'-cyclic monophosphate (dbcAMP) is a synthetic analogue of cAMP **(Gordon, Gochenauer and Michael, 2001; Fang *et al.,* 2012)**. The administration of dbcAMP resulted in reduction of progesterone and testosterone but not of estradiol release in rabbits **(Chrenek, Grossmann and Sirokin 2010). Sirotkin (1996)** observed the inhibitory effect on vasopressin and oxytocin secretion in porcine granulosa cells. dbcAMP treatment increased number of corpora lutea, ovulation, number of oocyte, zygotes and embryo yield and development in rabbits (**Chrenek *et al.,* 2012; Balazi, Sirotkin and Chrenek, 2012)**. Furthermore, dbcAMP stimulated proliferation of granulosa cells in chicken **(Yoshimura and Tamura, 1991)**, increased proliferation of granulosa cells and the oocyte in mice **(Carroll, Whittingham and Wood, 1991). Bagg *et al.* (2009)** did not observed effect of dbcAMP on the cAMP content in cumulus–oocyte complex and cumulus expansion of pigs. dbcAMP was described as apoptotic in granulosa cells of rat **(Aharoni *et al.,* 1995)** and rabbits **(Maillet *et al.,* 2002)** but as a survival factor in rat follicles **(Chun *et al.,* 1996)**.

Therefore dbcAMP looks to be a potential regulator of reproduction, but its influence on basic ovarian functions (proliferation, apoptosis a. o.) in others species, especially in farm animals such as pigs, has not been examined yet. Only **Sirotkin (1996)** investigated the effect of dbcAMP on endocrine response of porcine granulosa cells, whilst the influence of dbcAMP on porcine granulosa cells proliferation and apoptosis has not been studied yet. Furthermore it remains unknown whether cAMP-dependent intracellular mechanism can mediate the effect of gonadotropins on porcine ovaries.

The aim of this study is to examine the effect of dbcAMP on porcine granulosa cells proliferation (PCNA expression) and apoptosis (bax expression) and to compare its effects with effects of FSH.

MATERIAL AND METHODS

Isolation and culture of granulosa cells

Ovaries of non-cycling pubertal gilts, about 180 days of age, were obtained after slaughter at a local abattoir. They were washed several times in sterile 0.9% NaCl and 95% alcohol. Granulosa cells were aspirated by syringe and sterile needle from follicles 3–5 mm in diameter and granulosa cells isolated by centrifugation for 10 min at 200g. Cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/ml medium. Portions of the cell suspension were dispensed to 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µl/well) and incubated at 37.5 °C in 5 % CO_2 in humidified air until 60-75% confluent monolayer was formed (3-5 days), at which point the medium was renewed. Further culture was performed in 200 µl of the medium in the same chamber slides, as described previously.

After medium replacement experimental cells were cultured in the presence of dbcAMP (Biolog Life Science Institute, Bremen, Germany) alone at concentrations of 0,1;1 and 10 µg/ml or bovine FSH (Sigma-aldrich spol s.r.o., St. Louis, MO, USA) alone at concentration 1; 10 and 100 IU/ml. dbcAMP and FSH was dissolved in culture medium immediately before their addition to the cells. Control cells were cultured without dbcAMP or FSH. After removing the medium from chamber slides, cell were washed in ice-cold PBS (pH

7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and kept at 4°C to await immunocytochemistry.

Immunocytochemical analysis

Following washing and fixation, the cells were incubated in the blocking solution (1% of goat serum in phosphate-buffered saline - PBS) at room temperature for 1 h to block nonspecific binding of antiserum. Afterwards, the cells were incubated in the presence of monoclonal antibodies against either PCNA (marker of proliferation) and bax (marker of apoptosis) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) for 2 h at room temperature at overnight at 4°C. For the detection of binding sites of primary antibody, the cells were incubated in secondary swine antibody against mouse IgG labeled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by staining with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany).

Following DAB-staining, the cells on chamber-slides were washed in PBS, covered with a drop of Glycergel mounting medium (DAKO, Glostrup, Denmark); then coverslip was attached to a microslide. Cellular presence and localization of PCNA and bax positivity in cells was proved on the basis of DAB-peroxidase brown staining. A ratio of DAB-HRP-stained cells to the total cell number was calculated.

Statistical analysis

Significant differences between the experiments were evaluated using Student´s T-test and one/two-way ANOVA followed by paired Wilcoxon-Mann Whitney test, by using Sigma Plot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Analysis of porcine granulosa cells revealed detectable levels of PCNA and Bax within the cells, either without or after exposure to dbcAMP and FSH. Imunocytochemical analysis showed that the addition of dbcAMP alone significantly increased the percentage of cells containing PCNA (marker of proliferation) at all doses (0,1µg/ml, 1µg/ml, 10µg/ml) and

significant increase in the percentage of cells containing Bax (marker of apoptosis) at doses 0,1µg/m, 1µg/ml, but not in group treated with 10µg/ml of dbcAMP in comparison to control (Table 1).

Table 1 The percentage of granulosa cells containing markers of proliferation (PCNA) and apoptosis (bax) in the porcine granulosa cells cultured with and without dbcAMP

Treatment, dose	% of cells containing	
	PCNA	BAX
dbcAMP 0 µg/ml (control)	14,8±5,96 (331)	17,2±5,56 (641)
dbcAMP 0,1 µg/ml	25,2±7,69* (119)	32,5±4,93* (160)
dbcAMP 1 µg/ml	33,8±6,93* (240)	29,7±6,85* (125)
dbcAMP 10 µg/ml	35,5±4,32* (648)	18,2±5,93 (171)

Legend: Values shown are mean % of cells containing particular antigen, ±SD (standard deviation), * differences statistically significant compared to the control group at $p<0,05$, analysed cell number is indicated in brackets

Addition of FSH alone significantly increased the percentage of cells containing PCNA at doses: 0,01 IU/ml and 1 IU/ml, and also significant increase the percentage of cells containing Bax at doses: 0,01 IU/ml and 1 IU/ml (Table 2).

Table 2 The percentage of granulosa cells containing markers of proliferation (PCNA) and apoptosis (bax) in the porcine granulosa cells cultured with and without FSH

Treatment, dose	% of cells containing	
	PCNA	BAX
FSH 0 IU/ml	14,8±2,7 (331)	17,2±3,8 (641)
FSH 0,01 IU/ml	26,8±9,2* (253)	35,0±3,5* (100)
FSH 1 IU/ml	32,5±9,0* (120)	46,0±9,4* (104)

Legend: Values shown are mean % of cells containing particular antigen, ±SD (standard deviation), * differences statistically significant compared to the control group at $p<0,05$, analysed cell number is indicated in brackets

The results of our experiment demonstrate that dbcAMP affects granulosa cells proliferation and apoptosis, thereby it influences ovary function. Our study showed significant

stimulation of proliferation of porcine granulosa cells by dbcAMP. Promoted proliferation after dbcAMP treatment was previously described in chicken **(Yoshimura and Tamura, 1991)** and mice granulosa cells **(Carroll, Whittingham and Wood, 1991)**. The dbcAMP-induced stimulation of follicular development up to ovulation **(Chrenek *et al.* 2012)** and increased corpus luteum development after exposure to dbcAMP **(Balazi, Sirotkin and Chrenek 2012),** what could be due to increased ovarian cell proliferation, was reported in rabbits.

Furthermore, our experiment demonstrated the stimulatory influence of dbcAMP on porcine granulosa cells apoptosis. Previously, dbcAMP was described as apoptotic in granulosa cells of rats **(Aharoni *et al.,* 1995)** and rabbits **(Maillet *et al.,* 2002)**, but as a survival factor in cultured antral rat follicles **(Chun *et al.,* 1996)**. **Chrenek *et al.* (2012)** reported promoted cystic, but not luteinization-associated atresia of rabbit ovarian follicles after exposure to dbcAMP at high dose. The administration at medium dose enhanced cystic and luteinization-associated atresia, but did not affect reproductive parameters. These results are in line with results of our study, where dbcAMP induced apoptosis of granulosa cells after treatment at low and medium doses, but not at high dose.

Nevertheless, our observations are the first evidence, that cAMP-dependent intracellular mechanisms can be involved in promotion of apoptosis in porcine ovaries. Furthermore, we may conclude, that stimulation of apoptosis via dbcAMP is dose-dependent. The stimulatory effect of dbcAMP at low and middle doses on both, ovarian cell apoptosis and proliferation suggest, that it may promote ovarian follicular cell turnover. On the other hand, dbcAMP, when given at high dose promoted proliferation but not apoptosis. It indicates that high amounts of cAMP can enhance proliferation, apoptosis rate and promote ovarian follicular growth. Such increased cAMP production can occur during preovulatory gonadotropin surge.

FSH is claimed to induce ovarian folliculogenesis via cAMP/PKA intracellular mechanism **(Hunzicker-Dunn and Maizels, 2006)**. In our experiment, we observed increase in both, apoptosis and proliferation after FSH exposure. The present observations confirm our previous reports of FSH action on porcine granulosa cells **(Sirotkin *et al.,* 2008; Sirotkin *et al.,* 2012)**. The similarity of FSH and dbcAMP action suggests that FSH could promote ovarian cell proliferation, apoptosis and subsequent folliculogenesis probably via cAMP/PKA dependent intracellular mechanisms.

Taken together, our results suggest, that dbcAMP and its related cAMP/PKA-dependent intracellular mechanisms are able to induce proliferation and apoptosis and thereby

increase turnover and multiplication of granulosa cells, what could lead to stimulation of folliculogenesis. dbcAMP can effect ovary function and could be used for regulation of animal reproductive processes. Furthermore, our observations are the first evidence that dbcAMP can affect not only apoptosis, but also proliferation of porcine granulosa cells, suggesting that dbcAMP can be potentially useful for improvement of porcine reproduction. This suggestion should be however validated by further in vivo studies.

CONCLUSION

Our study confirmed the involvement of dbcAMP in control of basic ovarian function and demonstrated its stimulatory effect on proliferation and dose-dependent increase of apoptosis in porcine granulosa cells. This effect of dbcAMP is similar to that of FSH and could be liable for stimulation of turnover in granulosa cells and stimulated folliculogenesis probably via FSH-cAMP/PKA system. It is not to be excluded, that dbcAMP could to be a potential regulator of reproduction in farm animals including pigs.

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