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REGULAR ARTICLE

THE EFFECT OF CURCUMIN ON SECRETORY ACTIVITY, PROLIFERATION AND APOPTOSIS OF THE PORCINE OVARIAN GRANULOSA CELLS

Attila Kádasi[1], Alexander V. Sirotkin[2], Nora Maruniaková[1], Adriana Kolesárová[1], Jozef Bulla[1], Roland Grossmann[3]*

Address: [1]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[2]Institute for Genetics and Reproduction of Farm Animals, Animal Production Research Centre Nitra - Lužianky, Slovak Republic

[3]Department of Functional Genomics and Bioregulation, Institute of Animal Genetics, Mariensee, 31 535 Neustadt, Germany

*Corresponding author: attila.kadasi@gmail.com

ABSTRACT

The aim of this *in vitro* study was to examine the effect of natural plant (*Curcuma longa*) molecule curcumin on secretory activity, proliferation and apoptosis of porcine granulosa cells. The secretion of steroid hormones (progesterone, testosterone), accumulation of PCNA (marker of proliferation) and bax (marker of apoptosis) in granulosa cells of swine ovaries after curcumin treatment at the doses 0, 1, 10, 100 $\mu g.mL^{-1}$ was determined by RIA and immunocytochemistry. It was observed that, addition of curcumin stimulated progesterone (at doses 1 and 10 $\mu g.mL^{-1}$, but not 100 $\mu g.mL^{-1}$) and testosterone at (100 $\mu g.mL^{-1}$ but not 1 and 10 $\mu g.mL^{-1}$) release. The number of cells contained PCNA was down-regulated by curcumin administration (at dose of 10 $\mu g.mL^{-1}$, but not of 1 and 100 $\mu g.mL^{-1}$). Bax expression was stimulated by curcumin at all doses added. Our results suggest a direct effect of curcumin on ovarian functions: steroidogenesis, proliferation and apoptosis. This could suggest antireproductive properties of curcumin in swine ovaries.

Keywords: curcumin, progesterone, testosterone, proliferation, apoptosis, porcine granulosa cells

INTRODUCTION

Reproduction is a key and the most complicated biological process in existence and maintaining of species. Humanity used the power of herbs to suppress or promote fertility **(Harat *et al.*, 2008)**. Consummation of herbs can positively influence improving the menstrual cycle in women **(Ushiroyama *et al.*, 2001)**, strengthening endometrium, improving blood supply and circulation of uterine and ovary, promoting growth and development of follicle **(Xia *et al.*, 2004)**.

One of the plant often used in folk medicine is *Curcuma longa.* In this herb curcumin has the highest proportion **(Aggarwal BB *et al.*, 2007)**. Curcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-eptadiene-3,5-dione **(Nadkarmi, 1976).** Curcumin has apoptotic, anti-proliferative, anti-oxidant, and antiangiogenic properties **(Steward *et al.*, 2008).** Among effects of *Curcuma longa* extract is reduction of progesterone (P) secretion in mature follicle of swine granulosa cells **(Nurcahyo and Kadarsih, 2003)**. This progestin is essential for normal ovarian cycle of females **(Hagan *et al.*, 2008)**, regulate the function of corpus luteum **(Gregoraszczuk, 1994)**. Is produced by ovarian granulosa cells **(Sirotkin and Luck, 2008; Kolesárová *et al.*, 2010; Sirotkin, 2011).** Another hormone produced in ovary is testosterone **(Delort *et al.*, 2009).** Testosterone (T) as well as (P) are necessary as a precursor for the synthesis of estrogen **(Mindnich *et al.*, 2004). Khatimah and Kadasir (2003)** in their study did not found the effect of curcumin on (T) release.

Curcumin can down-regulate proliferation of ovarian granulosa cells **(Hanif *et al.*, 1997).** In human, curcumin addition of food diminished proliferation human ovarian cancer cell **(Shi *et al.*, 2006).** Opposite proces to proliferation is apoptosis. Curcumin addition down-regulated the follicular cell amount through apoptotic pathway in murine ovary **(Voznesenska *et al.*, 2010)**, but induces cell apoptosis via caspase activation **(Bhaumik *et al.*, 1999)** or by decreasing of the expression of bcl-2 and p53 **(Zheng *et al.*, 2004). Choudhury *et al.* (2002)** established that curcumin induced apoptosis in non-ovarian tumor cells via a p53-dependent pathway in which Bax is the downstream effector of p53.

Effect of curcumin has been performed "in vivo" in different animal species or human, on other organs as ovary. Only **Nurcahyo and Kadarsih (2003)** have studied the effect of

curcumin on steroidogenesis (P release not T secretion), proliferative activity and apoptosis in cultured porcine granulosa cells. We wanted to refute or confirm the results of previous study, compare the results from our and other experiments made on the different species. Effect of proliferation, apoptosis and production of steroid hormones may be different in mice and pigs. T release due to curcumin has not been studied in porcine granulosa cells.

The aim of our study was to examine the effect of curcumin treatment at doses 1, 10 and 100 $\mu g.mL^{-1}$ on secretory activity of swine granulosa cells (secretion of P and T) and examination of markers of proliferation (PCNA expression) and apoptosis (bax expression).

MATERIAL AND METHODS

Isolation and culture of granulosa cells

Granulosa cells were collected from the ovaries of prepubertal (100-120 day old) Slovakian White gilts, 100-120 days of age, after slaughter at a local abattoir. Ovaries were transported to the laboratory at 4°C and washed in sterile physiological solution. Follicular fluid was aspirated from 3-5 mm follicles and granulose 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 24-well culture plates (Nunc^{TM}, Roskilde, Denmark, 1 mL suspension/well; for RIA) or 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µL/well, for immunocytochemistry). Both, the plate wells and chamber slides were incubated at 37°C and 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 2 mL culture medium in 24-well plates (medium for RIA) or 200 µL/medium in 16-well chamber slides, (cells for immunocytochemistry) as described previously.

After medium replacement experimental cells were cultured in the presence of curcumin (Changsha Sunfull Bio-tech. Co, Hunan China) alone at concentrations of 1, 10 and 100 $\mu g.mL^{-1}$. Curcumin was dissolved in culture medium immediately before their addition to the cells. Controll cells were cultured in vehicle alone.

After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24°C to await RIA. 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 held 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 stannig 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.

Immunoassay

Concentrations of P4 and T were determined in 25-100 μL samples of incubation medium by RIA. The concentrations of P4 and T were assayed using Radioimmunoassay (RIA) according to the manufacturer's instructions. All RIAs were validated for use in samples of culture medium.

Statistical analysis

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

RESULTS AND DISCUSSION

Steoidogenesis (RIA)

Secretion of steroid hormones was detected by radioimmunoassay (Table 1). Doses 1 and 10 μg.mL^{-1} of curcumin stimulated the P4 secretion, while the highest dose (100 μg.mL^{-1}) did not affect this secretion. The dose of curcumin at 100 μg.mL^{-1} significantly increased the T secretion but lower concentrations (1 and 10 μg.mL^{-1}) did not influenced it.

Table 1 The secretion of steroid hormones in the porcine granulosa cells treated and not treated with curcumine (RIA).

Supplement	P4 secretion ng/10^6 cells/day	T secretion pg/10^6 cells/day
Curcumine 0 μg.mL^{-1} (control)	145±6.88	787±82.9
Curcumine 1 μg.mL^{-1}	250±5*	596±91.8
Curcumine 10 μg.mL^{-1}	250±5*	549±88.4
Curcumine 100 μg.mL^{-1}	62.1±6.9	1203.28±47.7*

Legend: All the values represent P or T release, means ± SEM, * - significant (P<0.05) differences with control (cells not treated with curcumin).

Proliferation and apoptosis (Immunocytochemistry)

Table 2 The percentage of granulosa cells containing markers of proliferation (PCNA) and apoptosis (bax) in the porcine granulosa cells treated and not treated with curcumine, (imunocytochemistry assay).

Supplement	% of cells contained	
	PCNA	bax
Curcumine 0 μg.mL^{-1} (control)	51±1.43 (1404)	49.88±1.72 (1980)
Curcumine 1 μg.mL^{-1}	50.5±3.43 (867)	58.25±1.31* (949)
Curcumine 10 μg.mL^{-1}	42.83±1.49* (743)	66.13±2.37* (886)
Curcumine 100 μg.mL^{-1}	44.71±2.56 (823)	71.0±3.07* (921)

Legend: All the values represent % of cells containing particular antigen, means ± SEM, * - significant (P<0.05) differences with control (cells not treated with curcumin). In the brackets is a number of counted cells.

The results of immunocytochemistry are showed in Table 2.

In our study the dose of 10 μg.mL^{-1} curcumin significantly decreased the PCNA expression. Other doses (1 and 100 μg.mL^{-1}) did not affect the proliferation. Number of porcine granulosa cells containing bax was improved by curcumin at 1, 10 and 100 μg.mL^{-1}.

This study demonstrated effect of curcumin addition on porcine granulosa cells. Curcumin stimulated the release of P. Our data did not correspond the result of **Nurcahyo and Kadarsih (2003)**, who found diminished effect of curcumin treatment on secretion of this progestin on porcine granulosa cells isolated from large mature follicles. The diffrences in curcumin effect observed in our experiments and experiments of **Nurcahyo and Kadarsih (2003)** could be explained by different source of ovarian cells. **Nurcahyo and Kadarsih (2003)** performed their experiment on mature porcine ovaries, while we worked with granulosa cells from young noncyclic swine ovaries. In our experiment, T release was stimulated by curcumin. This is the first finding, that curcumin can influence not only P4 but also androgen output. Both P4 and T have antiproliferative and proapoptotic properties, therefore they can suppress growth of ovarian follicles. Therefore, it might be hypothesized, that curcumin through promotion of P4 and T can inhibit porcine ovarian development. This hypothesis was supported by the ability of curcumin to affect markers of ovarian cell proliferation and apoptosis.

In our experiment, curcumin addition significantly decreased PCNA expression in granulosa cells. These data suggest, that curcumin can inhibit proliferation of swine ovarian cells. The number of granulosa cells containing bax was increased after curcumin addition. It suggests, that curcumin induced apoptosis of ovarian granulosa cells from gilts. Our results confirmed the study of **Nurcahyo and Kadarsih (2003)**, who found, that curcumin addition reduced PCNA expression in porcine granulosa cells. The proapoptotic activity of this plant supplement, observed in our experiment, confirms report of **Chen and Huang (1998)** and **Zheng *et al.*, (2004)**, who observed increased apoptosis in human ovarian cells after *in vitro* curcumin treatment.

In our study we found that curcumin can directly suppress the accumulation of proliferative peptide PCNA and promote the expression of apoptotic peptide bax. Therefore it is possible that it can suppress synthesis of DNA during cell proliferation and promote the programmed cell death. This effect of curcumin can be due to its ability to promote release of P4 and T, those have antiproliferative and proapoptotic activity. Physiological influence of curcumin on ovarian granulosa cells could be important practical viewpoint. It is not to be

excluded the curcumin may used in the regulation of pig reproductive function (ovarian folliculogenesis, oocyte maturation and ovulation), including fertility and treatment of reproductive disorders.

CONCLUSION

The present study suggest a possible stimulatory effect of curcumin on the release of progesterone and testosterone, inhibitory impact on proliferation (accumulation of PCNA) and stimulatory influence on apoptosis (accumulation of bax) on granulosa cells of porcine ovary. Our results suggest a direct effect of curcumin on steroidogenesis, proliferation and apoptosis in porcine ovaries. Our study is the first evidence between curcumin treatment and its increased effect on testosterone release. Taken together, these data suggest that curcumin can suppress porcine reproductive (ovarian) function.

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