Animal welfare, etológia és tartástechnológia

Animal welfare, ethology and housing systems

Volume 9 Issue 3

Különszám/Special Issue

Gödöllő

2013

THE EFFECT OF RESVERATROL ON PORCINE OVARIAN GRANULOSA CELL FUNCTIONS

Attila Kádasi[*1], Adriana Kolesárová[1], Nora Maruniaková[1], Roland Grossmann[3], Richard Alexa[4], Aneta Štochmaľová[4], Alexander V. Sirotkin[2]*

[1] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Department of Animal Physiology Tr. A. Hlinku2, 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 Science, Mariensee, 31535 Neustadt, Germany;
[4]Constantine the Philosopher University in Nitra, Faculty of Natural Sciences, Department of Zoology and Anthropology,
*: attila.kadasi@gmail.com

Abstract

The aim of our study was to elucidate the role of resveratrol in control of ovarian functions. For this purpose, we have examined its effect (at the doses 0, 1, 10, 100 µg/ml) on proliferation, apoptosis and steroidogenesis in porcine ovarian granulosa cells *in vitro*. Accumulation of PCNA (marker of proliferation), BAX (marker of apoptosis) and the release of steroid hormones (progesterone and testosterone) were analysed by immunocytochemistry and RIA respectively. It was observed, that resveratrol addition decreased the percentage of proliferative (PCNA-positive) and increased the percentage of apoptotic (BAX-positive) cells at all used doses (1, 10 and 100 µg/ml). On the other hand decreased progesterone release (at all doses of resveratrol) and stimulated testosterone release (at 10 and 100 µg/ml but not at 1 µg/ml of resveratrol) by porcine granulosa cells was detected. Our results suggest a direct effect of resveratrol on proliferation, apoptosis and steroidogenesis in porcine ovaries. Taken together, these data suggest that resveratrol can affect reproductive (ovarian) functions of pigs.

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

Introduction

Phytoalexins are antimicrobial substances under natural conditions not found in plants. Their creation occurs in plants attacked by pathogens (Dixon and Paiva, 1995). Resveratrol (RSV) was chemically 3,4',5-trihydroxystilbene and is one of the natural phytoalexins (Hain et al., 1990). It is a naturally occurring phytoestrogen and antioxidant that is found in grapevines, but also in soft fruits and hazelnut (Frémont, 2000). RSV is well known for its phytoestrogenic, antioxidant properties, growth-inhibitory and apoptosis-inducing activities (Joe et al., 2002; Jiang et al., 2005; Baur and Sinclair, 2006). It is able to inhibit PI3K/Akt Protein Kinase/ mammalian target of rapamycin (PI3K/Akt/mTOR) pathway in various types of cells (Jiang et al., 2005; Shankar et al., 2007; Bai et al., 2010). RSV is favourable for biological processes in the body to protect against cardiovascular diseases. It also has anti-cancer properties because RSV inhibits proliferation and induces apoptosis in cells at different levels (Ferry-Dumazet et al, 2002, Haider et al, 2003).

Cell proliferation is the amount of cells in culture or in the body can be divided. The extent of DNA synthesis is marker for proliferation (Wyllie et al., 1998). Involving the protein to cell proliferation include PCNA (Tomanek and Chronowska, 2006). This protein is localized in the cell nucleus (Makarevich et al., 2000; Nahryzny and Lee, 2001) and located in granulosa cells of gilts (Sanislo et al. 2001).

Apoptosis is programmed death of cells. This process eliminates unnecessary and useless cells from the body (Wyllie et al., 1998). Apoptosis is supported by group of caspases, which include BAX

(Zwain and Amato, 2001). This protein is localized especially in mitochondria (Markström et al., 2002) and located in granulosa cells of gilts (Sanislo et al. 2001).

Progesterone (P4) is an ovarian steroid produced by ovarian granulosa cells (Kolesárová et al., 2010a; Kolesárová et al., 2010b; Medvedová et al., 2011) and *corpus luteum* (Gregoraszczuk, 1992; Gregoraszczuk, 1997) of pigs and contributes to regulation of ovarian follicular development and remodelling (Mahajan, 2008). It is a local paracrine or autocrine factor regulating luteal function (Gregoraszczuk, 1992; Gregoraszczuk, 1997). This progestin is essential for normal ovarian cycle of females (Hagan et al., 2009). Another hormone produced in ovary is testosterone (T) (Delort et al., 2009). T is steroid hormone as well as P4 are necessary as a precursor for the synthesis of estrogen (Mindnich et al., 2004).

The aim of our study was to research the effect of RSV treatment at doses 1, 10 and 100 µg/ml on accumulation of markers of proliferation (PCNA) and apoptosis (BAX) and secretory activity (steroid hormones of P4 and T) of porcine ovarian granulosa cells (GCs) *in vitro*.

Material and method

Preparation, culture and processing of granulosa cells from ovaries

Granulosa cells were collected from the ovaries of prepubertal Slovakian White gilts, after slaughter at a local abattoir. After aspiration and isolation of granulosa cells, these 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 RSV (Changsha Sunfull Bio-tech. Co, Hunan China) at concentrations of 0, 1, 10 and 100 µg/ml. RSV was dissolved in culture medium immediately before their addition to the cells. 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 labelled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by stanning 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 RIA 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 followed 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

Proliferation and apoptosis (Immunocytochemistry)

The results of immunocytochemistry are showed in Tab.1. In our study all used doses of RSV significantly ($P<0.05$) decreased the percentage of cells containing PCNA. Wong et al. (2010) published diminished proliferative activity of interstitial theca cells after RSV addition at doses 30, 50, 70 and 100 µmol/ml. Antiproliferative activity of RSV in rat ovarian granulosa cells was confirmed by Ortega et al. (2012). A number of porcine granulosa cells containing BAX was improved by RSV treatment at doses 1, 10 and 100 µg/ml. Previous studies confirmed pro-apoptotic effect of RSV (at 30, 50, 70 and 100 µmol/ml) in theca cells (Wong et al., 2010) and rat ovarian granulosa cells (Ortega et al., 2012) via activation of apoptotic peptide caspase 3/7.

Tab.1.

The percentage of cells containing markers of proliferation (PCNA) and apoptosis (BAX) after RSV treatment (Imunocytochemistry)

Supplement	% of cells containing			
	PCNA		BAX	
RSV 0 µg/ml (control)	51.00±1.43	(1404)	49.88±1.72	(1980)
RSV 1 µg/ml	36.30±1.20*	(356)	67.50±1.04*	(453)
RSV 10 µg/ml	39.50±1.50*	(273)	67.00±3.34*	(472)
RSV 100 µg/ml	34.00±10.00*	(320)	66.50±2.02*	(441)

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

Steoidogenesis (RIA)

Secretion of steroid hormones was detected by radioimmunoassay (Tab. 2). In our study doses 1, 10 and 100 µg/ml of RSV decreased the P4 secretion by porcine ovarian granulosa cells. We confirmed the findings of Basini et al. (2010), who published significant reduction of P4 secretion by porcine granulosa cells after treatment of polymethoxystilben 2 – analogue of RSV. On the other hand stimulatory effect of RSV on P4 release by porcine ovarian GCs was recorded after RSV treatment at the dose of 50 µg/ml, while doses of 30 and 10 µg/mL did not affect the release of the steroid hormone (Kolesarova et al., 2012). RSV in combination with mycotoxin – deoxynivalenol (DON) at the highest doses (50 µg/ml of RSV and 5000 ng/ml of DON) stimulated P4 release by GCs. In the case of secretion of T by porcine ovarian GCs stimulatory effect of RSV (at the doses 10 and 100 µg/ml but not at 1 µg/ml) was found in our study. Decreased P4 release by rat granulosa cells after resveratrol treatment was described in previous study (Ortega et al., 2012).

Tab.2.

The secretion of steroid hormones by porcine ovarian granulosa cells after RSV treatment (RIA)

Supplement	P4 secretion ng/10^6 cells/day	T secretion pg/10^6 cells/day
RSV 0 µg/ml (control)	78.10±5.75	420.70±54.90
RSV 1 µg/ml	52.00±2.26*	496.70±27.50
RSV 10 µg/ml	50.80±3.03*	777.00±15.00*
RSV 100 µg/ml	41.40±5.90*	1932.00±41.90*

All the values represent P4 or T release, means ± SEM, *- significant (P<0.05) differences with control (cells not treated with RSV).

Conclusion

The present study suggest a possible inhibitory effect of resveratrol on proliferation (PCNA), stimulatory influence on apoptosis (BAX), inhibitory effect on the release of progesterone and stimulatory impact on testosterone release by porcine ovarian GCs. Our results suggest a direct effect of resveratrol on proliferation, apoptosis and steroidogenesis in porcine ovaries. Taken together, these data suggest that resveratrol can affect reproductive (ovarian) functions of pigs.

Acknowledgments: We would like to thank Mrs. Katarína Tóthová and Ing. Žofia Kuklová (Animal Production Research Centre in Nitra – Lužianky), to Mrs. Iris Stelter (Institute of Animal Genetics, Neustadt, Germany), to Mr. Yani Deng (Changsha Sunfull Bio-tech. Co, Hunan China) for kind of providing of resveratrol. This work was financially supported by the the Ministry of Education, Science, Research and Sport of the Slovak Republic projects no. 1/0022/13, APVV no. 0137-10, 0854-11 and APVV-0304-12, and no. 740531-OPVaV-2011/2.2/07-SORO. This publication was written during realization of the project "ZDRAVIE“ no. 26220220176 supported by the Operational Programme Research and Development funded from the European Regional Development Fund.

References

Bai, Y. et al. (2010): Resveratrol induces apoptosis and cell cycle arrest of human T24 bladder 588 cancer cells in vitro and inhibits tumor growth *in vivo*. Cancer Science, 101, 488-493.

Basini, G., Tringali, C., Baioni, L., Bussolati, S., Spatafora, C., Grasselli, F. (2010): Biological effects on granulosa cells of hydroxylated and methylated resveratrol analogues. Mol Nutr Food Res., 54, 2, 236-43.

Baur, J.A., Sinclair, D.A. (2006): Therapeutic potencial of resveratrol: the *in vivo* evidence. Natrure reviews Drug Discovery, 5, 493-506.

Delort, L., Kwiatkowski, F., Chalabi, N., Satih, S., Bignon, Y-J., Bernard-Gallon, D. J. (2009): Central Adiposity as a Major Risk Factor of Ovarian Cancer. Anticancer research, 29, 5229-5234.

Dixon, R. A., Paiva, N. L. (1995): Stress-induced phenylpropanoid metabolism. Plant Cell, 7, 1085-1097.

Ferry-Dumazet, H., Garnier, O., Mamani-Matsuda, M., Vercauteren, J., Belloc, F., Billiard, C., Dupouy, M., Thiolat, D., Kolb, J. P., Marit, G., Reiffers, J., Mossalayi, M. D. (2002): Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. Carcinogenesis, 23, 8, 1327-1333.

Frémont, L. (2000): Biological effects of resveratrol. Life Sciences, 66, 8, 663-673.

Gregoraszczuk, E. L. (1992): Interrelations between steroid hormone secretion and morphological changes of porcine corpora lutea at various periods of luteal phase. Endocrine Reg., 26, 189–194.

Gregoraszczuk, E.L. (1997): Progesterone, androgen and estradiol production by porcine luteal cell subpopulations: dependence on cell composition and periods of luteal phase. Endocrine Reg., 31, 41–46.

Hagan, R. C., Faivre, A. E., Lange, A. C. (2009): Scaffolding actions of membrane-associated progesterone receptors. Steroids, 74, 7, 568-572. ISSN 0039-128X.

Haider, U. G., Sorescu, D., Griendling, K. K., Vollmar, A. M., Dirsch, V. M. (2003): Resveratrol increases serine15-phosphorylated but transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells. Molecular Pharmacology, 63, 4, 925-932.

Hain,R., Bieseler, B., Kindl, H., Schroder, G., Stocker, R. (1990): Expression of a stilbene synthase gene in Nicotiana tabacum results in synthesis of the phytoalexin resveratrol. Plant Molecular Biology, 15, 325-335.

Jiang, H. et al. 2005. Resveratrol-induced apoptotic death in human U251 glioma cells. Molecular Cancer Therapy, 4, 554-561.

Joe, A. K. et al. (2002): Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clinical Cancer Research, 8, 893-903.

Kolesarova, A., Roychoudhury, S., Slivkova, J., Sirotkin, A., Capcarova, M., Massanyi, P. (2010a): In vitro study on the effect of lead and mercury on porcine ovarian granulosa cells. J. Environ. Sci. Health Pt. A, 45, 320–331.

Kolesarova, A., Capcarova, M., Sirotkin, A., Medvedova, M., Kovacik, J. (2010b): Cobalt-induced changes the IGF-I and progesterone release, expression of proliferation- and apoptosis-related peptides in porcine ovarian granulosa cells *in vitro*. J. Environ. Sci. Health Pt A, 42, 7, 810-817.

Kolesarova, A.; Capcarova, M.; Maruniakova, N.; Lukac, N.; Ciereszko, R. E.; Sirotkin, A.V. (2012): Resveratrol inhibits reproductive toxicity induced by deoxynivalenol. In J Environ Science and Health, part A, 2012, 47(9), 329-34.

Mahajan, D.K. (2008): Pig Model to study dynamics of steroids during ovarian follicular growth and maturation. Sourcebook of Models for Biomedical Research: Humana Press, 778 p, ISBN 978–1–58829–933–8.

Makarevich , A., Sirotkin, A., Chrenek, P., Bulla, J., Hetenyi, L. (2000): The role of IGF-I, c AMP-protein kinase A and MAP-kinase in the control of steroid secretion, cyclidnucleotide production, granulosa cell proliferation and preimplantation embryo development in rabbits. Journal of Steroid Biochemistry and Molecular Biology, 73, 123-133.

Markström, E., Svensson, E. Ch., Ruijin, S., Svanberg, B., Billig, H. (2002): Survival facators regulating ovarian apoptosis-dependence on follicle differentiation. Reproduction, 123, 23-30

Medvedova, M., Kolesarova, A., Capcarova, M., Labuda, R., Sirotkin, A. V., Kovacik, J., Bulla, J. (2011): The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells in vitro. J. Environ. Sci. Health B, 46, 3, 213-219.

Mindnich, R., Moller, G. et al. (2004): The role of 17 beta-hydroxysteroid dehydrogenases. Molecular and Cellular Endocrinology, vol. 218, 1-2, 7-20.

Nahryzny, S. N., Lee, H. (2001): Protein profiles of the Chinese hamster ovary cells in the resting and proliferating stages. Electrophoresis, vol. 22, 9, 1764-1775.

Ortega, I., Wong, D. H., Villanueva, J. A., Cress, A. B., Sokalska, A., Stanley, S. D., Duleba, A. J. (2010): Effects of resveratrol on growth and function of rat ovarian granulosa cells. Fertil Steril., 98, 6, 1563-73.

Sanislo, P., Sirotkin, A. V., Budacova, A., Florkovicova, I., Schaeffer, H. J. (2001): Úloha thrombopoetínu a rozličných proteinkináz v regulácii proliferácie, apooptózy a steroidogenézy ovariálnych buniek ošípaných in vitro. Acta fytotechnica et zootechnica, vol. 4, 2, 31-34.

Shankar, S. et al. (2007): Molecular mechanisms and therapeutic potential. Frontiers in Bioscience, 12, 4839-4854.

Tomanek, M., Chronowska, E. (2006): Immunohistochemical localization of proliferating cell nuclearantigen (PCNA) in the pig ovary. Folia Histochem. Cytobiol., 44, 269-274.

Wong, D. H., Villanueva, J. A., Cress, A. B., Duleba, A. J. (2010): Effects of resveratrol on proliferation and apoptosis in rat ovarian theca-interstitial cells. Molecular Human Reproduction, 16, 4, 251–259.

Wyllie, A. , Donahue, M. S. , Fischer, B. , Hill, D., Keesey, J., Manzow, S. (1998): Apoptosis and cell proliferation. Manheim: Boehringer, 1998, 1-5, 64-66

Zwain, I. H., Amato, P. (2001): cAMP-induced apoptosis in granulosa cells is associated with up-regulation of P53 and BAX down-regulation of closterin. Endocr Res., 27, 1-2, 233-249.