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STIMULATORY EFFECT OF AMYGDALIN ON THE VIABILITY AND STEROID HORMONE SECRETION BY PORCINE OVARIAN GRANULOSA CELLS *IN VITRO*

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ABSTRACT

Amygdalin has been one of the most popular "alternative cancer cures" in many European and South American countries. Its anticancer, anti-inflammatory activity and other medicinal benefits have been known for many years. The objective of this in vitro study was to examine the potential impact of amygdalin on the cell viability and production of steroid hormone testosterone by porcine ovarian granulosa cells. Granulosa cells were isolated from porcine ovaries and subsequently cultured without (control) or with amygdalin at various doses (1; 10; 100; 1000 and 10 000 µg/mL) for 24 h. The cell viability was determined by alamarBlue™ reagent and release of testosterone was assayed by ELISA. Obtained results showed a significant ($P<0.05$) increase of testosterone secretion only at the highest dose of amygdalin (10 000 µg/mL). Other experimental doses of amygdalin did not affect the testosterone production. Moreover, amygdalin treatment strongly enhanced the viability of ovarian granulosa cells. The viability was significantly ($P<0.05$) stimulated after amygdalin treatment at all used doses, except the highest concentration (10 000 µg/mL). To conclude, application of amygdalin to culture media positively affected cell viability, but not highest dose (10 000 µg/mL), and stimulated testosterone release by porcine ovarian cell. Present results could help to reveal the potential impact of amygdalin on cellular growth, as well as its mechanism of action in processes of ovarian steroidogenesis.

Keywords: Amygdalin, cell viability, testosterone, ovarian granulosa cells

INTRODUCTION

Natural plant origin products like amygdalin are still a major part of traditional medicine More than 50% of cancer patients in Europe use complementary/ alternative medicine (CAM) instead of, or combined with, conventional therapy (**Nabavizadeh *et al.*, 2011; Huebner *et al.*, 2014**). Amygdalin has been one of the most popular "alternative cancer cures" in many European and South American countries for long period (**Chang *et al.*, 2006; Hwang *et al.*, 2008; Makarević *et al.*, 2014**).

This natural substance is occurring in the seeds of various plant species, belonging to the *Rosaceae* family, such as bitter almonds, apricots, apples and other. Its anticancer, anti-inflammatory activity and other medicinal benefits have been known for many years. This bioactive compound is composed of glucose, benzaldehyde, which induces an analgesic action, and hydrocyanic acid, which is an anti-neoplastic compound (**Fukuda *et al.*, 2003; Chang *et al.*, 2006**). β-glucosidase, one of the enzymes that catalyzes the release of cyanide from amygdalin, is present in the human small intestine and is also found in a variety of common foods (**Strugala *et al.*, 1995; Deng *et al.*, 2002**). In the late 1970s and early 1980s, amygdalin was reported to selectively kill cancer cells at the tumor site without systemic toxicity and to effectively relieve pain in cancer patients (**Ellison *et al.*, 1978**). Nowadays, there are statistics described that by 1978 more than 70,000 patients with cancers in the United States have been treated with amygdalin (**Moss, 2005; Makarević *et al.*, 2014; Qian *et al.*, 2015**). However, the use of the drug was discouraged when it was demonstrated that amygdalin is metabolized in the body to release significant amount of cyanide thus leading to cyanide poisoning (**Chandler *et al.*, 1984; Bromley *et al.*, 2005**). Numerous studies have demonstrated the beneficial properties of amygdalin and its ability to effectively induce cell death (**Zhou *et al.*, 2012; Chen *et al.*, 2013**). Nevertheless, proponents consider amygdalin a natural cancer cure, whereas opponents warn that amygdalin is ineffective and even toxic (**Makarević *et al.*, 2014**).

The endocrine signaling molecules represent source of communication between several organs, as well as specific cell populations. Testosterone, one of the steroid hormones, plays a key role in ovarian cycle, folliculogenesis, cell proliferation, and also in programmed cell death (**Graham *et al.*, 1997; Sirotkin, 2014**). Unfortunately, there is still no scientific evidence related to the potential effect of amygdalin on the healthy, non-pathologic cells.

This *in vitro* study demonstrates the potential effect of amygdalin on the cell viability and production of steroid hormone by porcine ovarian granulosa cells (GCs).

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Ovaries from non-cyclic pigs were obtained from healthy Slovakian White gilts without obvious reproductive abnormalities. The ovaries were transported to the laboratory in containers at 4 ºC and washed in sterile physiological solution. The follicular fluid was aspirated from 3-5 mm follicles. The granulosa cells (GCs) were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™, Verviers, Belgium) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at the final concentration of 10^6 cells/mL (as detected by a haemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1ml/well; for Enzyme Linked Immuno Sorbent Assay, ELISA). The well plates were incubated at 37 °C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (4-5 days), at this point, the medium was renewed and ovarian granulosa cells were incubated with the same supplements (DMEM/F12 1:1 medium, 10% fetal calf serum, without 1% antibiotic-antimycotic solution) and without (control) or with amygdalin (1, 10, 100, 1000, 10 000 µg/mL) (≥99 % purity, from apricot kernels, Sigma-Aldrich, St. Louis, Mo, USA) for 24h. After 24h of incubation the culture media from well plates were aspirated and kept at –80°C for subsequent assay. The concentrations of steroid hormones progesterone and estradiol-17β were

assayed using ELISA (Dialab, Wiener Neudorf, Austria) according to the manufacturer´s instructions.

Cell viability test

The cell viability was determined after treatment of amygdalin by alamarBlue™ reagent (BioSource International, Nivelles, Belgium) (**Bannerman *et al*., 2001; Nynca *et al*., 2009**). This assay is based on the ability of living and metabolically active cells to convert the oxidized indigo blue state of alamarBlue dye into the reduced pink state. Isolated granulosa cells were cultured in 96-well plates/100 µL at the concentration 0.1 x 10^5 cells per well (37 °C, 5% CO_2). After pre-incubation (48/72 h), the monolayer of granulosa cells was cultured for 24 hours with or without amygdalin (1, 10, 100, 1000, 10 000 µg/mL) or DMSO (as a positive control). Twenty four hours before the end of cell culture, alamarBlue™ dye was added to all wells. Thereafter, alamarBlue™ reduction was measured spectrophotometrically at 565 and 595 nm and expressed as a percentage according to the manufacturer calculations. All analyses were performed in quadruplicates.

Statistical Analysis

Each experimental group was represented by four culture wells of granulosa cells (each dose = 4 replicates, biological parallels). Assessments of hormone concentrations in the incubation media were performed in duplicates. The data are presented as means of values obtained from one experiment using separate pools of ovaries from 10–12 animals. The significance of differences between the control and experimental groups was evaluated by One-Way ANOVA (Dunnett´s multiple comparison test) using the statistical software GraphPad Prism 3.01 (GraphPad Software Inc., San Diego, CA, USA). The data are expressed as means ± SEM. Differences were compared for statistical significance at the *p*-level less than 0.05 ($P<0.05$).

RESULTS

The effect of amygdalin on viability of porcine ovarian granulosa cells in vitro

The viability of granulosa cells isolated from porcine ovaries was assayed after amygdalin application (Fig.1). Amygdalin aplication led to a significant ($P<0.05$) stimulation in viability of ovarian granulosa cells, compared to control cells. Viability was stimulated by the increasing doses of amygdalin (1, 10, 100, 1000 µg/mL). Whereas, the highest metabolic activity was detected after exposure of amygdalin at dose 1000 µg/mL. However, the highest dose of amygdalin (10 000 µg/mL) did not affect the viability of porcine ovarian granulosa cells, in comparison to untreated control cells.

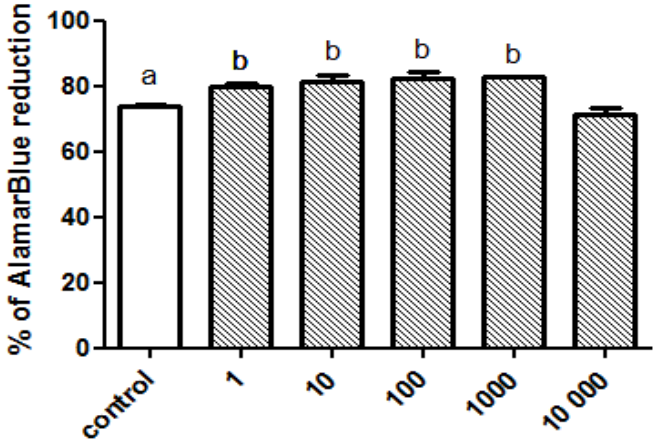

Figure 1 The viability of porcine ovarian granulosa cells incubated for 24 h without (control) or with amygdalin treatment (1, 10, 100, 1000, 10 000 µg/mL). Signs *a*, *b* denote value significantly ($P<0.05$) different from control group. Significance of differences between the groups was evaluated by One-way ANOVA (Dunnett´s multiple comparison test). The data are expressed as means ± SEM. AlamarBlue™.

The effect of amygdalin on testosterone release by porcine ovarian granulosa cells

The release of steroid hormone testosterone by ovarian granulosa cells after amygdalin addition is shown in Figure 2. Significant ($P<0.05$) stimulation of testosterone production was observed after amygdalin treatment at highest used dose (10 000 µg/mL), in comparison to control group without addition of the substance. However, other experimental doses of amygdalin did not affect the testosterone release by granulosa cells.

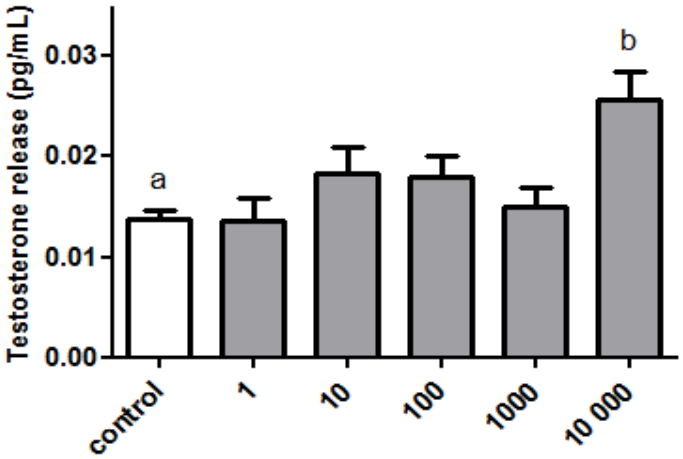

Figure 2 The effect of amygdalin on testosterone release by porcine ovarian granulosa cells. The control represents culture media without amygdalin addition; the experimental groups represent culture media supplemented with amygdalin (1, 10, 100, 1000, 10 000 µg/mL) addition. Signs *a*, *b* denote value significantly ($P<0.05$) different from control group. Significance of differences between the groups was evaluated by One-way ANOVA (Dunnett´s multiple comparison test). The data are expressed as means ± SEM. ELISA.

DISCUSSION

The present investigation suggests stimulatory impact of amygdalin on a viability of ovarian granulosa cells, as well as the production of steroid hormone testosterone *in vitro*. Granulosa cells, isolated from porcine ovaries, were able to survive, grow in culture and release the steroid hormone after the experimental addition of natural compound amygdalin. Numerous studies have reported the inhibitory action of amygdalin on the proliferation and growth of different cancer cells (**Syrigos *et al*., 1998; Chen *et al*., 2013; Qian *et al*., 2015**). However, possible impact of amygdalin on the cellular growth, differentiation and death of normal, non-pathological cells remains unknown.

In our study, amygdalin strongly enhanced the viability of ovarian granulosa cells at all doses, except the highest used dose (10 000 µg/mL). Viability of ovarian cells was stimulated by the increasing doses of amygdalin (1, 10, 100, 1000 µg/mL). Previously, cytotoxic effect of amygdalin on human prostate cancer cells was evidenced by **Chang *et al*. (2006)**. Amygdalin exhibited a dose-dependent suppression of cell viability at higher concentrations. Moreover, amygdalin is able to block the bladder cancer cell growth *in vitro* by diminishing of cellular regulators (**Makarevic *et al*., 2014**). Recent study carried out by **Nynca *et al*. 2009** demonstrated the effect of a natural phytoestrogen-daidzein on the viability of porcine ovarian granulosa cells. They found that the cell viability was not affected by daidzein application at various doses (0.5-50 µM).

Previous studies described the effect of various natural substances with protective (**Kolesárova *et al*., 2012; Halenár *et al*., 2013**) or toxic (**Ranzenigo *et al*., 2008; Medveďová *et al*., 2011; Maruniaková *et al*., 2013**) potential on the cellular processes in ovarian cells. In addition, the release of steroid hormone testosterone by ovarian granulosa cells after amygdalin application was observed in this examination. Increased secretion ($P<0.05$) of testosterone by granulosa cells was detected only in experimental group with the highest dose of amygdalin (10 000 µg/mL). The present results are in accordance with our recent examination, where a stimulatory effect of amygdalin on the release of 17β-estradiol by ovarian granulosa cells was observed, too. Amygdalin treatment (10 000 µg/mL) resulted in significant ($P<0.05$) increase of the hormone production by porcine ovarian granulosa cells (**Halenár *et al*., 2015**). Interestingly, the presence of amygdalin at selected doses did not affect the progesterone secretion by porcine ovarian GCs.

Similarly, exposure of deoxynivalenol, resveratrol and their combination on the release of progesterone by porcine ovarian granulosa cells was studied by **Kolesárová *et al*. (2012)**. Progesterone release was significantly ($P<0.05$) stimulated by resveratrol treatment at the dose 50 µg/mL, but not at 30 and 10 µg/mL. **Kádasi *et al*.** (**2012**) also reported a stimulatory effect of curcumin, a natural plant molecule, on the release of testosterone by porcine ovarian granulosa cells.

CONCLUSION

This study helps to reveal the potential impact of amygdalin on cellular growth, mechanism of action in processes of ovarian steroidogenesis. Application of amygdalin to culture media affected cell viability, but not the highest dose (10 000 µg/mL), and stimulated testosterone release by porcine ovarian granulosa cells.

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REFERENCES

Bannerman, D.D., Tupper, J.C., Ricketts, W.A., Bennet, C.F., Winn, R.K., Harlan, J.M. (2001). A constitutive cytoprotective pathway protects endothelial cells from lipopolysaccharide-induced apoptosis. J. Biol.Chem. 276(18), 14924-14932.

Bromley, J., Hughes, B.G., Leong, D.C., Buckley, N.A. (2005). Life-threatening interaction between complementary medicines: cyanide toxicity following ingestion of amygdalin and vitamin C. *Ann. Pharmacother*. 39(9), 1566-1569. http://dx.doi.org/10.1345/aph.1E634

Deng, Y., Guo, Z.G., Zeng, Z.L., Wang, Z. (2002). Studies on the pharmacological effects of saffron (Crocus sativus L.). *Chin. J. Chin. Mater. Med*. 27(8), 565-568.

Ellison, N. M., D. P. Byar., G. R. Newell. (1978). Special report on Laetrile: The NCI Laetrile Review. Results of the National Cancer Institute's retrospective Laetrile analysis. *N. Engl. J. Med*. 299, 549-552. http://dx.doi.org./10.1056/NEJM197809072991013

Fukuda, T., Ito, H., Mukainaka, T., Tokuda, H., Nishino, H., Yoshida, T. (2003). Anti-tumor promoting effect of glycosides from *Prunus persica* seeds. *Biological and Pharmaceutical Bulletin*, 26, 271-273. http://dx.doi.org/10.1248/bpb.26.271

Graham, J.D., Clarke, C.L. (1997). Physiological action of progesterone in target tissues. *Endocrine Rev*. 18, 502-519. http://dx.doi.org/10.1210/edrv.18.4.0308

Halenár, M., Medveďová, M., Maruniaková, N., Kolesárová, A. (2015). Assessment of a potential preventive ability of amygdalin in mycotoxin-induced ovarian toxicity. *Journal of Environmental Science and Health*, part B, 50, 411-416. http://dx.doi.org./10.1080/03601234.2015.1011956

Halenár, M., Medveďová, M., Maruniaková, N., Kolesárová, A. (2013). Amygdalin and its effect on animal cells. *Journal of Microbiology, Biotechnology and Food Sciences*, 2, 1414-1423. http://dx.doi.org/10.1111/jpn.12013

Huebner, J., Micke, O., Muecke, R., Buentzel, J., Prott, F.J., et al., (2014). User Rate of Complementary and Alternative Medicine (CAM) of Patients Visiting a Counseling Facility for CAM of a German Comprehensive Cancer Center. *Anticancer Res*, 34, 943-948.

Hwang, H.J., Lee, H.J., Kim, CH. J., Shim, I., Hahm, D.H. (2008). Inhibitory effect of amygdalin on lipopolyccharide-inducible TNF-α and IL-1β mRNA expression and carrageenan-induced rats arthritis. *Journal of Microbiology and Biotechnology*, 18(10), 1641-1647.

Chandler, R.F., Anderson, L.A., Phillipson, J.D. (1984). Laetrile in perspective. *Can. Pharm. J*. 117(11), 517-520.

Chang, H.K., Shin, M.S., Yang, H.Y., Lee, J.W., Kim, Y.S., Lee, M.H., Kim, J., Kim, K.H.,Kim, C.J. (2006). Amygdalin Induces Apoptosis through Regulation of Bax and Bcl-2 Expressions in Human DU145 and LNCaP Prostate Cancer Cells. *Biological and Pharmaceutical Bulletin*, 29(8), 1597-1602. http://dx.doi.org/10.1248/bpb.29.1597

Chen, Y., Ma, J., Wang, F., Hu, J., Cui, A., Wei, C., Yang, Q., Li, F. (2013). Amygdalin induced apoptosis in human cervical cancer cell line HeLa cells. *Immunopharmacology and Immunotoxicology*, 35, 43-51. http://dx.doi.org/10.3109/08923973.2012.738688

Kádasi, A., Sirotkin, A.V., Maruniaková, N., Kolesárová, A., Bulla, J., Grossmann, R. (2012). The effect of curcumin on secretory activity, proliferation and apoptosis of the porcine ovarian granulosa cells. *Journal of Microbiology, Biotechnology and Food Sciences*, 2, 349-357.

Kolesárová, A., Capcarová, M., Maruniaková, N., Lukáč, N., Ciereszko, R.E., Sirotkin, A.V. (2012). Resveratrol inhibits reproductive toxicity induced by deoxynivalenol. *Journal of Environmental Science and Health*, Part A, 47, 1329-1334. http://dx.doi.org/10.1080/10934529.2012.672144

Makarević, J., Rutz, J., Juengel, E., Kaulfuss, S., Reiter, M., Tsaur, I., Bartsch, G., Haferkamp, A., Blaheta, R.A. (2014). Amygdalin blocks bladder cancer cell growth *in vitro* by diminishing cyklin A and cdk2. *PLoS ONE*, 9(8), 1–9. http://dx.doi.org/10.1371/journal.pone.0105590

Maruniaková, N., Kolesárová, A., Kádasi, A., Medveďová, M., Halenár, M., Sirotkin, A.V., Grossmann, R., Bulla, J. (2013). Release of progesterone and testosterone by ovarian granulosa cells after addition of T-2 toxin and its combination with growth factor IGF-I. *Journal of Microbiology, Biotechnology and Food Sciences*, 2, 1864-1874.

Medveďová, M., Kolesárová, A., Capcarová, M., Labuda, R., Sirotkin, A.V., Kováčik, J., Bulla, J. (2011). The effect of deoxynivalenol on the secretory activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro*. *Journal of Environmental Science and Health Part B*, 46(3), 213-219. http://dx.doi.org/ 10.1080/03601234.2011.540205

Moss, R.W. (2005). Patient perspectives: Tijuana cancer clinics in the post-NAFTA era. *Integrative Cancer Therapies*, 4, 65–86. http://dx.doi.org/10.1177/1534735404273918

Nabavizadeh, F., Alizadeh, A.M., Sadroleslami, Z., Adeli, S. (2011). Gastroprotective effects of amygdalin on experimental gastric ulcer: Role of NO and TNF-α. *Journal of Medical Plants Research*, 5(14), 3122-3127.

Nynca, A., Jablonska, O., Slomczynska, M., Petroff, B.K., Ciereszko, R. (2009). Effect of phytoestrogen deidzein and estradiol on steroidogenesis and expression of estrogen receptors in porcine luteinized granulosa cells from large follicles. *Journal of physiology and pharmacology*, 60, 95-105.

Ranzenigo, G., Caloni, F., Cremonesi, F., Aad, P.Y., Spicer, L.J. (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science*, 107, 115-130. http://dx.doi.org/10.1016/j.anireprosci.2007.06.023

Sirotkin, A.V. (2014). Regulators of Ovarian Functions. New York: Nova Publishers , 194.

Strugala, G.J., Stahl, R., Elsenhans, B., Rauws, A.G., Forth, W. (1995). Small-intestinal transfer mechanism of prunasin, the primary metabolite of the cyanogenic glycoside amygdalin. *Human Experimental Toxicology*, 14(11), 895-901. http://dx.doi.org/10.1177/096032719501401107

Syrigos, K.N., Rowlinson-Busza, G., Epenetos, A.A. (1998). *In vitro* cytotoxicity following specific activation of amygdalin by beta-glucosidase conjugated to a bladder cancer-associated monoclonal antibody. *International Journal of Cancer*, 78(6), 712-719. http://dx.doi.org/10.1002/(SICI)1097-0215(19981209)78:6<712::AID-IJC8>3.0.CO;2-D

Qian, L., Xie, B., Wang, Y., Qian, J. (2015). Amygdalin-mediated inhibition of non-small cell lung cancer cell invasion in vitro. *Int. J. Clin. Exp. Pathol*. 8(5), 5363-5370.

Zhou, C., Qian, L., Ma, H., Yu, X., Zhang, Y., Qu, W., Zhang, X., Xia, W. (2012). Enhancement of amygdalin activated with β-D glucosidase on HepG2 cells proliferation and apoptosis. *Carbohydrate Polymers*, 90, 516-523. http://dx.doi.org/10.1016/j.carbpol.2012.05.073