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DOSE-RESPONSE OF PORCINE OVARIAN GRANULOSA CELLS TO AMYGDALIN TREATMENT COMBINED WITH DEOXYNIVALENOL

Marek Halenár, Marína Medveďová, Nora Maruniaková, Dagmara Packová, Adriana Kolesárová*

Address(es): Ing. Marek Haklenár,
Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.

*Corresponding author: halenarmarek@gmail.com

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ABSTRACT

Amygdalin is one of many nitrilosides, which are natural cyanide-containing substances abundant in the seeds of apricots, almond, peaches, apples, and other rosaceous plants. It is a controversial anti-tumor natural product that has been used as an alternative cancer drug for many years. On the other hand, one of the most widely distributed mycotoxin contaminating food and animal feed is deoxynivalenol (DON). Deoxynivalenol has adverse effects on humans, animals, and crops that result in illnesses. The aim of the *in vitro* study was to investigated the effect of natural substance amygdalin at the selected doses (1, 10, 100, 1000, 10 000 μg/mL) in combination with deoxynivalenol (1000 ng/mL) on secretion of steroid hormones (progesterone and estradiol) by ovarian granulosa cells (GCs) from cyclic pigs. Our results showed that the releasing of progesterone and estradiol by ovarian granulosa cells was affected by amygdalin plus DON addition. The secretion of progesterone by ovarian GCs was significantly ($P \leq 0.05$) affected by administration of both compounds in all experimental groups. Similarly, estradiol releasing by GCs was significantly ($P \leq 0.05$) increased in experimental groups with amygdalin (10, 100 and 10 000 μg/mL) plus DON (1000 ng/mL) addition. Amygdalin treatment combined with DON caused increase of steroid hormones release by ovarian granulosa cells. Our findings suggest possible involvement of these natural substances (amygdalin and deoxynivalenol) in the regulation process of steroidogenesis. In conclusion, results from this experiment contribute to knowledge about interaction between two different natural compounds and their positive or negative interferences with ovarian functions.

Keywords: Amygdalin, deoxynivalenol, steroid hormones, ovarian granulosa cells

INTRODUCTION

Natural plant compound as amygdalin is still a major part of traditional medicine. Amygdalin (D-mandelonitrile-β-D-gentiobioside, Fig. 1), sometimes termed to as a Vitamin B_{17}, is found in the seeds of apricots, almonds, peaches and other plants (**Fukuda *et al*., 2003; Chang *et al*., 2006; Nabavizadeh *et al*., 2011**). This controversial natural substance is composed of two molecules of glucose, one of benzaldehyde, which induces an analgesic action, and one of hydrocyanic acid, which is an anti-neoplastic compound. It has been reported that amygdalin canbe used in medicine for effectively prevent and treat cancers, chronic inflammation, migraine, relieve fever and pain (**Yan *et al*., 2006, Fukuda *et al*., 2003, Zhou *et al*., 2012**). However, the Food and Drug Administration (FDA) has not approved amygdalin as a cancer treatment owing to insufficient clinical evidence of its efficacy and potential toxicity. Despite the failure of clinical tests to demonstrate the anticancer effects of amygdalin in the U.S.A. and in Europe, amygdalin continues to be manufactured and administered as an anticancer therapy in northern Europe and Mexico (**Chang *et al*., 2006; Kwon *et al*., 2010**).

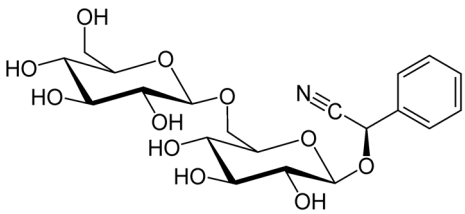

Figure 1 Chemical structure of amygdalin

Deoxynivalenol (DON) is one of the most important and occurring *Fussariu m*mycotoxin (**Łazicka and Orzechowski, 2010; Klem *et al*., 2007**). Occurrence of thismycotoxin is mainly in grains such as wheat, barley and maize (**Creppy, 2002**). DON could be rapidly absorbed after oral administration passively throughout the gastrointestinal tract and actively in the kidneys, liver, muscle, adipose tissue and reproductive tissues. Thus, mycotoxin exposure that alters granulosa cells steroid hormone production may also alter oocyte development, ovulation, reproductive tract function and pregnancy outcome (**Medveďová *et al*., 2011**).

Steroid hormones, such as progesterone and estradiol are produced by ovarian cells and both are substantial for normal ovarian cycles (**Hagan *et al*., 2008; Arnhold *et al*., 2009**), contribute to regulation of ovarian follicular development and remodelling (**Mahajan, 2008**).

In the present study, we observed the potential effects of both natural compounds amygdalin and deoxynivalenol on secretion of steroid hormones (progesterone and estradiol) by porcine granulosa cells from cyclic ovaries.

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Ovaries from cyclic pigswere obtained from healthy Slovakian White gilts without obvious reproductive abnormalities. Isolated ovaries were transported to the laboratory in containers at 4 ºC and washed in sterile physiological solution. Follicular fluid was aspirated from 3-5 mm follicles. Granulosa cells 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 haemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1ml/well; for Enzyme Linked ImmunoSorbent 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 similar 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, Sigma-Aldrich, St. Louis, Mo, USA) combined with deoxynivalenol (1000 ng/mL) (Romer Labs

Division Holding GmbH, Tulln, Austria) for 24h. Experimental group P1/E1 represents culture medium with amygdalin (1 µg/mL) plus deoxynivalenol (1000 ng/mL), other groups represent culture medium with amygdalin P2/E2 (10 µg/mL), P3/E3 (100 µg/mL), P4/E4 (1000 µg/mL) and P5/E5 (10 000 µg/mL) plus in each group deoxynivalenol (1000 ng/mL). 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 were assayed using ELISA (Dialab, Wiener Neudorf, Austria) according to the manufacturer´s instructions.

Statistical Analysis

Each experimental group was represented by four culture wells of granulosa cells. Assay of hormone level in the incubation media was performed in duplicate.Significance of differences between the control and experimental groups were evaluated by one-way ANOVA and t-testusing statistical software Sigma Plot 11.0 (Jandel, Corte Madera, USA). The data are expressed as means ± SD. Differences were compared for statistical significance atthe P – level less than 0.05 ($P \leq 0.05$).

RESULTS

Release of progesterone by porcine ovarian granulosa cells

The secretion activity of granulosa cells from cyclic porcine ovaries after addition of natural compounds amygdalin (1, 10, 100, 1000, 10 000 µg/mL) combined with DON (1000 ng/mL) was determined (Figs. 2, 3). The release of steroid hormone progesterone by ovarian granulosa cells was significantly ($P \leq 0.05$) stimulated after amygdalin administration in combination with deoxynivalenol in all experimental groups (P1, P2, P3, P4, P5) compared to control group without addition of both natural compounds (Fig. 2).

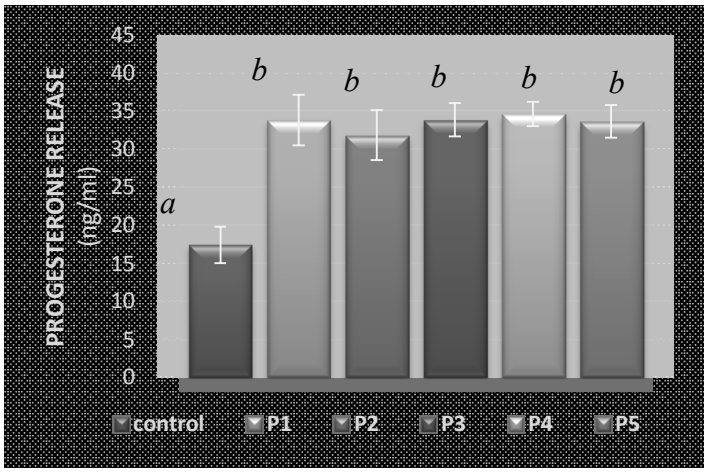

Figure 2 The effect of amygdalin in combination with deoxynivalenol on progesterone release by porcine ovarian granulosa cells
Control represents culture medium without amygdalin and deoxynivalenol addition. Experimental group P1 represents culture medium with amygdalin (1 µg/mL) plus deoxynivalenol (1000 ng/mL), other groups represent culture medium with amygdalin P2 (10 µg/mL), P3 (100 µg/mL), P4 (1000 µg/mL) and P5 (10 000 µg/mL) plus in each group deoxynivalenol (1000 ng/mL). Signs a,b denote values significantly different from control group ($P \leq 0.05$) evaluated by one-way ANOVA and t-test.ELISA.

Release of estradiol by porcine ovarian granulosa cells

The release of estradiol by ovarian granulosa cells after addition of amygdalin (1, 10, 100, 1000, 10 000 µg/mL) in combination with DON (1000 ng/mL) was detected. Significant ($P \leq 0.05$) stimulation of the estradiol release by ovarian granulosa cells was detected in experimental group E2, E3, E4 and E5 compared to control group without addition of both natural substances. On the other hand, no significant ($P \leq 0.05$) differences in estradiol release by porcine ovarian granulosa cell were found out between control group without administration of these compounds and experimental group E1 with the lowest dose (1 µg/mL) of amygdalin treatment combined with DON (1000 ng/mL).

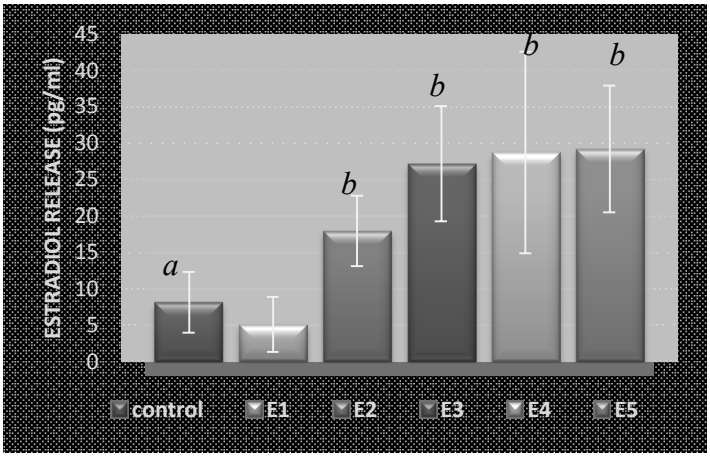

Figure 3 The effect of amygdalin in combination with deoxynivalenol on progesterone release by porcine ovarian granulosa cells
Control represents culture medium without amygdalin and deoxynivalenol addition. Experimental group E1 represents culture medium with amygdalin (1 µg/mL) plus deoxynivalenol (1000 ng/mL), other groups represent culture medium with amygdalin E2 (10 µg/mL), E3 (100 µg/mL), E4 (1000 µg/mL) and E5 (10 000 µg/mL) plus in each group deoxynivalenol (1000 ng/mL). Signs a,b denote values significantly different from control group ($P \leq 0.05$) evaluated by one-way ANOVA and t-test. ELISA.

DISCUSION

In the present *in vitro* study, possible response of porcine ovarian granulosa cells to amygdalin addition in combination with deoxynivalenol (DON) was examined. Our isolated ovarian granulosa cells were able to survive, growth in culture and release hormonal substances (progesterone and estradiol) after experimental addition of natural substances amygdalin and DON. The results from our investigation demonstrate that the release of both steroid hormones (progesterone, estradiol) was influenced by natural product amygdalin in combination with mycotoxin deoxynivalenol.
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 (**Zhou et *al*., 2012**).Recent study showed preventive and therapeutic effects of amygdalin on absolute alcohol-induced gastric ulcer in rats. The results of this study showed that amygdalin protected gastric mucosa from alcohol-induced gastric ulcer. This gastroprotection was mediated via gastric mucosal nitric oxide production and TNF-α suppression (**Nabavizadeh *et al*., 2011**).
Previous studies examined the effects of natural compounds on different parts of animal reproductive system (**Kolesárová *et al*., 2012a; 2012b; 2011; Tanyildizy and Bozkurt, 2004; Yasui *et al*., 2003; Randel *et al*., 1992**). Our recent *in vitro* investigation showed that the release of steroid hormone progesterone by granulosa cells from cyclic and non-cyclic porcine ovaries was not affected by the amygdalin addition (1, 10, 100, 1000, 10 000 µg/mL) (**Halenár *et al*., 2013a**). But on the other hand, amygdalin (at 10 000 but not at 1, 10, 100, 1000 µg/mL) combined with DON (1000 ng/mL) significantly ($P \leq 0.05$) stimulated the release of steroid hormones progesterone and estradiol by granulosa cells from non-cyclic porcine ovaries (**Halenár *et al*., 2013b**).The results from this study showed that amygdalin combined with deoxynivalenol caused significant ($P \leq 0.05$) stimulation of progesterone release by granulosa cells from cyclic porcine ovaries in all experimental groups. Similarly, release of estradiol by GCs from cyclic porcine ovaries were affected by addition of amygdalin (10, 100, 1000, 10 000 µg/mL) in combination with DON (1000 ng/mL), but not in experimental group with the lowest dose (1 µg/mL) of amygdalin.
Steroid hormones, such as progesterone and estradiol are produced by ovarian cells and both play irreplaceable role in ovarian cycles (**Hagan *et al*., 2008; Arnhold *et al*., 2009**), contribute to regulation of ovarian follicular development and remodeling (**Mahajan, 2008**).The possible impact of different naturally cyanide-containing substances on the male reproductive system, focused on sperm motility and morphological abnormality in bull sperm, was observed previously(**Tanyildizy and Bozkurt, 2004**). Many studies have described the dose-dependent effects of different mycotoxins on the secretion activity of porcine (**Medveďová *et al*., 2011, Maruniaková *et al*., 2013, Ranzenigo *et al*., 2008**) and rats ovarian cells (**Kolesárová *et al*., 2011**). Steroid secretion by porcine ovarian granulosa cells after deoxynivalenol addition was examined by **Medveďová *et al*. (2011)**. The release of progesterone by porcine ovarian granulosa cells was stimulated by DON treatment at the doses 1000 ng/mL but not at 10 and 100 ng/mL. Previous data also indicated dose-depended effects of DON on ovarian granulosa cells (**Ranzenigo *et al*., 2008**). Exposure to toxic concentrations of deoxynivalenol, resveratrol and their combination on the release of progesterone by porcine ovarian granulosa cells was studied by **Kolesárová *et al*. (2012a)**. Results from this *in vitro* study suggested that

reproductive toxicity of animals induced by a mycotoxin – deoxynivalenol can be inhibited by a protective natural substance - resveratrol.
There is now considerable evidence suggesting that production of steroid hormones by ovarian granulosa cells *in vitro* is affected by addition of some natural compounds like amygdalin co-incubated with trichothecene deoxynivalenol.

CONCLUSION

Potential effects of reportedly anti-tumor natural substances amygdalin combined with mycotoxin deoxynivalenol on secretion of steroid hormones by granulosa cells from porcine cyclic ovaries was demonstrated in this study. Amygdalin treatment combined with DON caused increase of steroid hormones release by ovarian granulosa cells. In conclusion, our findings suggest possible involvement of these natural substances (amygdalin and deoxynivalenol) in the regulation process of steroidogenesis.

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