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

Marek Halenár1*, Eva Tušimová1, Anna Nynca2, Agnieszka Sadowska2, Renata Ciereszko2, Adriana Kolesárová1

Address(es):
1Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinka 2, 949 76 Nitra, Slovak Republic.
2Department of Animal Physiology, University of Warmia and Mazury, Olszyn, Poland.

*Corresponding author: halenarmarek@gmail.com

ARTICLE INFO

Received 11. 12. 2015
Revised 12. 1. 2016
Accepted 21. 1. 2016
Published 8. 2. 2016

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; Makarevič 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; Makarevič 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 (Makarevič 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 °C 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 200g 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 10^5 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% CO2; 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) (Baneman 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 × 10⁵ cells per well (37 °C; 5% CO₂). 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-value 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 application 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.

**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.

**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 (Makevic 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 Kolesárová et al., 2012; Halenár et al., 2013) or toxic (Ranzenigo et al., 2008; Novedová et al., 2011; Maruniakoviá 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 deoxyxynivalenol, 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 progesterone 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.
REFERENCES


