DO PUNICALAGINS HAVE POSSIBLE IMPACT ON SECRETION OF STEROID HORMONES BY PORCINE OVARIAN GRANULOSA CELLS?

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ABSTRACT

Punicalagin is a major component responsible for pomegranate's antioxidant properties. Punicalagin is the predominant ellagitannin of *Punica granatum* and present in two isomeric forms; punicalagin α and β. Punicalagins are metabolised to ellagic acid (antioxidant) and microorganisms present in colon can metabolize ellagic acid to urolithins – each other substances could be responsible for effect on cell intracellular mechanism. The aim of our study was to observe the effect of punicalagins on secretion of steroids hormones – progesterone and 17β-estradiol. Ovarian granulosa cells were cultivated during 24h without (control group) and with various doses (0.01, 0.1, 1, 10 and 100 µg.ml^{-1}) of pomegranate compounds – punicalagins. Steroid hormones progesterone and 17β-estradiol were evaluated by ELISA. Obtained results showed that the secretion of progesterone and 17β-estradiol by ovarian granulosa cells was not significantly (P≥0.05) influenced by punicalagins. In this pilot study no effects of punicalagins on porcine ovarian granulosa cells were found.

Keywords: Progesterone, 17β-estradiol, granulosa cells, punicalagins, steroidogenesis

INTRODUCTION

Punicalagins are ellagitannins in which gallagic and ellagic acid are linked to a glucose molecule (Cerdá *et al.* 2003). Punicalagin has two isomeric forms in pomegranate: α and β. Chemical name of punicalagin is 2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose (Tyagi *et al.* 2012). Punicalagins are phenolic compounds which are responsible for the main antioxidant activity of pomegranate (*Punica granatum*, Punicaceae) (Syed *et al.* 2007). Punicalagin levels are widely variable in pomegranate juice and can range from as low as 0.014-1.5g.l^{-1} depending on the fruit cultivar as well as processing and storage conditions (Syed *et al.* 2007). Pomegranate peels and pulps have a higher total phenolic and antioxidant activity than juice (Gözlekci *et al.* 2011; Elfalleh *et al.* 2011).

Punicalagin is a large molecule and knowledge about the fate of ellagitannins (include punicalagins) in human or animals is very sparse. In a recent study by Cerdá *et al.* (2003) the metabolism of punicalagins was described in rat. Punicalagins or metabolites of punicalagins were detected in faeces, urine and plasma. Native punicalagins were identified in plasma and urine, but at a very low concentration. The main metabolites present in biological fluids are those derived from punicalagins by hydrolysis and future conjugation. Rat microflora is able to metabolise ellagic acid derivatives to produce 6H-dibenzo[b,d]pyran-6-onemetabolites (urolithins) (Cerdá *et al.* 2003). Punicalagins were metabolized and/or absorbed. This means that most of the ingested punicalagins have to be transformed to known (punicalin, ellagic acid, gallagic acid) or unknown metabolites or accumulated in tissue (Cerdá *et al.* 2003). This *in vitro* study was focused on the secretion of steroid hormones, progesterone and 17β-estradiol, by porcine ovarian granulosa cells after punicalagin administration.

MATERIAL AND METHODS

Material

Ovaries (n=12 per experiment) of Slovakian White gilts were obtained from healthy animals without visible abnormalities. All experimental animals are kept under standard conditions at slaughterhouse in Myjava. Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution.

Isolation of granulosa cells

Ovarian granulosa cells were used in this *in vitro* study. The suspension of the cells was centrifuged for 10 min at 200xg (to divide the follicular liquid from granulosa cells), washed in sterile DMEM/F12 1:1 medium with 10% fetal bovine serum and 1% antibiotic–antimycotic solution. The washing was repeated two or three times as needed. Viability of cells was determined using trypan blue. Ovarian cells were divided into and cultured in 24–well culture plates. The well plates were incubated at 37.5 ºC and 5% CO$_2$ in humidified air until a 75% confluent monolayer (5 days). At this point, the medium (1 ml per well) was removed and ovarian cells were incubated in a fresh medium with 10% fetal bovine serum, 1% antibiotic–antimycotic solution and without (control group) or with punicalagins (Sigma Aldrich, St. Louis, MO, USA) at 0.01, 0.1, 1, 10, 100 µg.ml^{-1}. The cultures were kept for 24 h. Following cultivation the media were aspirated from the well plates and analysed for steroids hormones.

Viability of granulosa cells

Viability of granulosa cells was assessed by trypan blue solution (0.4%). A reference sample (500 µl) was detracted from cell suspension. Trypan blue was added (100 µl) into the test tube with suspension in a ratio of 5:1. The suspension with trypan blues was gently mixed and incubated 5 min at room temperature. The total cells, vital and death cells were accounted using a haemocytometer from minimum 10 fields and the percentage of vital cells was assessed using a formula (vital cells/total cellsx100%).

ELISA (Enzyme-linked immunosorbent assay)

Quantification of hormones (progesterone and 17β-estradiol) was performed after exposure of punicalagins by enzyme linked immunosorbent assay (ELISA). The principle of this colorimetric method is a series of competitive reactions between antigens (hormones) in the sample with horseradish enzyme-labelled antigen for binding to the limited number of antibody sites within a 96-well microplate (Grainer, Germany). ELISA assays (Dialab, Wiener Neudorf, Austria) were performed according to the manufacturer's instructions. After 1 h incubation (37 ºC, 95 % air atmosphere, 5 % CO$_2$) the bound/free separation was performed by a simple solid-phase washing. The enzyme substrate (H$_2$O$_2$) and the TMB-Substrate were added. After the appropriate time was elapsed for maximum colour development, the enzyme reaction was stopped and the absorbancies were

determined. Hormone concentration in the sample was calculated based on a series of calibrators. The colour intensity was inversely proportional to the hormone concentration in the sample. The absorbance was determined at a wavelength 450 nm using a microplate ELISA reader (Thermo Scientific Multiskan FC, Vantaa, Finland). The results were evaluated by One Way ANOVA. Intra- and inter-assay coefficients for progesterone were ≤4 % and ≤9.3 %. For 17β-estradiol intra- and inter-assay coefficients were ≤9 % and ≤10 %. Sensitiveness for progesterone was 0.05 ng.ml^{-1} and 8.68 pg.ml^{-1} for 17β-estradiol..

RESULTS AND DISCUSSION

In our previous *in vitro* study punicalagins were described as a possible effector in the processes of ovarian steroidogenesis (**Packová *et al.,* 2015**). **Nagata *et al.* (2007)** have shown that pomegranate juice is a potent inhibitor of CYP2C9 and CYP3A enzymes – these enzymes belong to cytochrome p450 group; and are responsible for cholesterol, steroid and lipid metabolism or synthesis. This study was focused on steroid hormones and the influence of punicalagins on the secretion of steroid hormones – progesterone and 17β-estradiol. Fig.1 describes the secretion of progesterone as the first important hormone of steroid synthesis. Punicalagin had no significant (P≥0.05) impact on secretion of progesterone. Similarly, **Ming *et al.* (2014)** described a significantly decreased progesterone in the samples (prostate cancer cell lines – 22RV1 and LNCaP) treated with pomegranate extracts. However in our previous study, punicalagin at 100 µg.ml^{-1} increased the progesterone secretion by rabbit ovarian cells (**Packová *et al.,* 2015**).
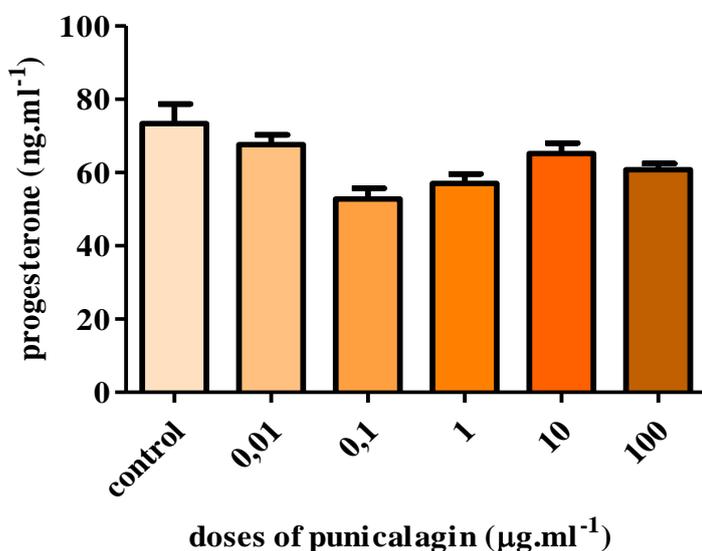
Figure 1 Effect of punicalagins (µg.ml^{-1}) on progesterone (ng.ml^{-1}) secretion by porcine ovarian granulosa cells. Non-significant (P≥0.05) differences among the control and experimental groups. The results were evaluated by One Way ANOVA.

The polyphenol from fermented pomegranate juice, pericarp and oil were shown to affect a blockade of endogenous active estrogen biosynthesis with subsequent inhibition of aromatase activity (**Toi *et al.* 2003**). Fig. 2 shows secretion of 17β-estradiol by porcine ovarian granulosa cells after application of different doses of punicalagin, but these results were not significant (P≥0.05). Recently, **Ming *et al.* (2014)** used pomegranate extracts on prostate cancer cells to suggest that steroid biosynthesis might favour the backdoor pathway over the classical Δ4 and Δ5 pathways. They described the effect of pomegranate extract on androgen biosynthesis pathways specifically using two prostate cancer cell lines and prostate cancer mouse model. According to our previous study punicalagins - like mainly ellagitannins of pomegranate, had influence on secretion of 17β-estradiol. Punicalagins at 10 µg.ml^{-1} significantly (P<0.005) reduced the secretion of 17β-estradiol by rabbit ovarian fragments (**Packová *et al.,* 2015**). Using lyophilized fresh pomegranate juice, **Kim *et al.* (2002)** reported that phenols from pomegranate inhibit 55% estrogen activity by normal human breast epithelial cells. Punicalagins or its derivatives (ellagic acid etc.) might have a possible effect on the process of ovarian steroidogenesis (**Packová *et al.* 2015**). **Hong *et al.* (2008)** showed that pomegranate protectants (polyphenols) may be effective against prostate cancer by down regulation of genes involved in androgen synthesis. Crushed and dried seed produce oil with 80% punicic acid, the 18-carbon fatty acid, along with isoflavone genistein, the phytoestrogen coumestrol and the sex steroid estrone (**Syed *et al.* 2007**).
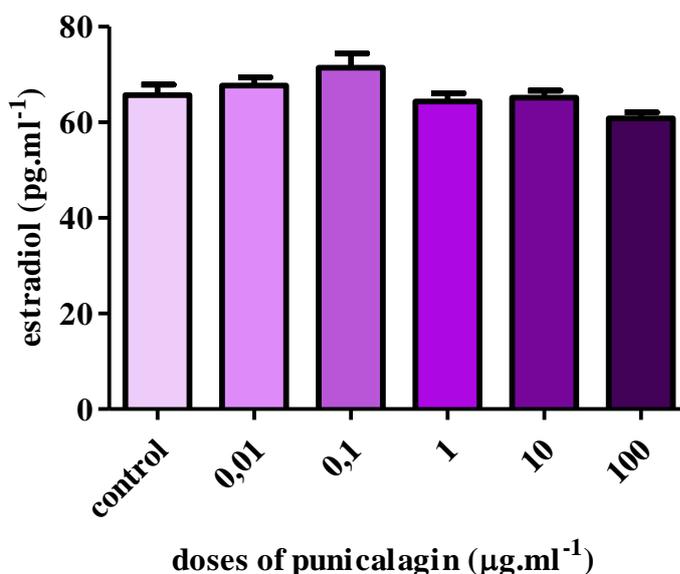
Figure 2 Effect of punicalagin (µg.ml^{-1}) on 17β-estradiol (pg.ml^{-1}) secretion by porcine ovarian granulosa cells. Non-significant (P≥0.05) differences among the control and experimental groups. The results were evaluated by One Way ANOVA test.

CONCLUSION

The research was focused on possible effects of punicalagins on porcine ovarian granulosa cells. Punicalagins or its derivatives (ellagic acid etc.), which were used in the present study might have no effect on steroidogenesis by porcine ovarian granulosa cells, but our previous studies with rabbit ovarian fragments have shown that punicalagin affects thesecretion of steroid hormones (progesterone and 17β-estradiol). Further research is necessary for a complex conclusion. There are more questions related to the effect of punicalagins or compounds from pomegranate on regulation of ovarian cells.

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