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Effect of punicalagin on proliferation of porcine ovarian granulosa cells *in vitro*

Účinok punicalagínu na proliferáciu ovariálnych granulóznych buniek ošípaných *in vitro*

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Abstract

Punicalagin is a major component responsible for pomegranate's (*Punica granatum*) antioxidant properties. Punicalagin is the predominant ellagitannin of *Punica granatum* and present in two isomeric forms: punicalagin α and β. Punicalagin is metabolised to ellagic acid (antioxidant) and microorganisms present in colon can metabolize ellagic acid to urolithins. The aim of *in vitro* study was to examine the effect of punicalagin on mitochondrial activity and markers of proliferation in porcine ovarian granulosa cells. The 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 compound – punicalagin. MTT assay and immunocytochemistry were used in this study. Stimulatory influence of punicalagin on the mitochondrial activity of ovarian granulosa cells at concentrations 1 μg*ml^{-1} was found. Punicalagin (at 1 μg*ml^{-1}) had a significant ($P < 0.05$) impact on the presence of proliferative markers cyclin B1 (increase) and PCNA - proliferating cell nuclear antigen (decrease) in porcine ovarian granulosa cells. These results suggest dose-dependent effect of punicalagin on cell proliferation. Further verification of possible role of punicalagin in proliferation is therefore needed.

Keywords: granulosa cells, proliferation, punicalagin, viability

Abstrakt

Punicalagín je hlavnou antioxidačnou zložkou granátového jablka. Punicalagín je hlavný ellagitanín vyskytujúci sa v granátovom jablku (*Punica granatum*) v dvoch izomérnych formách: α a β. Punicalagín je metabolizovaný na kyselinu elágovú (antioxidant) a mikroorganizmy tráviaceho traktu metabolizujú kyselinu elágovú až na urolitíny Cieľom *in vitro* štúdie bolo zaznamenať vplyv punicalagínu na

mitochondriálnu aktivitu a markery proliferácie v ovariálnych granulóznych bunkách ošípaných.. Ovariálne granulózne bunky ošípanej boli kultivované 24 h bez prídavku (kontrolná skupina) a s rôznymi prídavkami (0,01; 0,1; 1; 10 a 100 μg*ml^{-1}) punicalagínu – komponentu granátového jablka. Stimulačný efekt punicalagínu na mitochondriálnu aktivitu ovariálnych granulóznych buniek bol zaznamenaný pri koncentrácii 1 μg*ml^{-1} Punicalagín (pri koncentrácii 1 μg*ml$^{-1)}$ mal signifikantný ($P < 0.05$) vplyv na prítomnosť oboch markerov proliferacie - cyklínu B1 a PCNA, pri koncentrácii 1 μg*ml^{-1}. Tieto výsledky naznačujú vplyv rôznych dávok punicalagínu na bunkovú proliferáciu. Je potrebné vykonať ďalšie experimenty pre dosiahnutie ucelených záverov.

Kľúčové slová: granulózne bunky, proliferácia, punicalagín, viabilita

Introduction

Cell-cycle progression is dependent on highly ordered events controlled by cyclins and cyclin-dependent kinases (Cdks) (Hartwell and Weinert, 1989). The cyclin B1/Cdk1 complex is shown to specifically regulate the entry into mitosis (Hunt, 1989, Nurse, 1990). Cyclin B1/Cdk1 is involved in the integration of mitochondrial fission with the onset of G2/M transition (Taguchi et al., 2007), and a substantial amount of cyclinB1/Cdk1 remains in the cytoplasm in mitosis and active cyclinB1/Cdk1 kinase is found both in the cytoplasm and nucleus during prophase (Gavet and Pines, 2010).

Proliferating cell nuclear antigen (PCNA) plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery (Kelman, 1997). One of the well-established functions for PCNA is its role as the processivity factor for DNA polymerase δ and ε. PCNA interacts with proteins involved in cell-cycle progression which are not a part of the DNA polymerase apparatus (Kelman, 1997). Some of these interactions have a direct effect on DNA synthesis while the roles of several other interactions are not fully understood (Kelman, 1997).

Punicalagin (PG) and ellagic acid (EA) are responsible for antioxidant activity and healthy benefits of pomegranates. Punicalagin (isomers α and β) are the major component responsible for pomegranate´s antioxidant and health benefits (Tyagi et al., 2012). Punicalagin or pomegranate's juice or extract have influenced proliferation and apoptosis of the cancer of breasts, prostate, colon, lung or skin (Syed et al., 2007). Punicalagin and ellagic acid could regulation of cyclins A and B1, cell - cycle, arrest in S phase and induction of apoptosis via intrinsic pathway through Bcl - XL down - regulation with mitochondrial release of cytochrome c into the cytosol, activation of initiator caspase 9 and effectors caspase 3 by the colon cancer cells (Larrosa et al., 2006).

The aim of *in vitro* study was to observe the effect of punicalagin on mitochondrial activity and cellular markers of proliferation in porcine ovarian granulosa cells.

Materials and methods

Preparation, culture and processing of granulosa cells from ovaries

Ovaries of prepubertal gilts were obtained after slaughter at a local abattoir. Porcine ovaries were obtained from healthy Slovakian White gilts without visible reproductive abnormalities. The ovaries were transported to the laboratory in containers at 4 °C and washed with sterile physiological solution. The follicular fluid was aspirated from 3–5 mm follicles. The granulosa cells (GCs) were centrifuged for 10 min at 200 x g followed by washing in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal bovine serum (Sigma, St.Louis, Mo, USA) and 1% antibiotic–antimycotic solution (Sigma, St.Louis, Mo, USA) at a final concentration of 106 cells/mL (as quantified by a haemocytometer). Portions of the cell suspension were dispensed to 16 - and 96 - wells culture plates. The well plates were incubated at 37 °C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (5 days), at this point, the medium was renewed and ovarian GCs were incubated with the same supplements (DMEM/F12 1:1 medium, 10% fetal bovine serum, 1% antibiotic-antimycotic solution), without (control) or with punicalagin treatment (0.01, 0.1, 1, 10, 100 μg*ml^{-1}) (Sigma Aldrich, St. Louis, MO, USA, purity ≥ 98%) for 24 h. After 24 h of culture the media from wells were removed and the culture media from well plates were aspirated and kept at –80 °C for a subsequent assay.

Viability of granulosa cells assessed by trypan blue solution

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 blue was gently mixed and incubated 5 minutes 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 cells x 100%) (Packova and Kolesarova, 2016).

MTT assay

The methodology is according to Zhu et al. (2012), but team of authors optimized it for specific conditions. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (Sigma, St. Louis, USA) was diluted in complete culture medium (on shaker 1 hour at 37 °C in the dark) to a final concentration 0.5 mg*ml^{-1}. Ovarian granulosa cells from porcine were cultivated in 96-wells plates until creating a 75% confluent monolayer. The medium was removed after creating of monolayer and changed to 200 μl (per each well) fresh complete cultivated medium – 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), 1% antibiotic–antimycotic solution (Sigma Aldrich, St. Louis, MO, USA) and without (control group) or with punicalagin (Sigma Aldrich, St. Louis, MO, USA, purity ≥ 98%) at concentration 0.01, 0.1, 1, 10, 100 μg*ml^{-1} for 24 h. The medium was removed and added medium with MTT (100 μl per each well) for 4 hours at 37 ºC and 5% CO_2 in the dark. Tetrazole was reduced to insoluble formazan in living cells. The reaction was stopped with isopropyl alcohol (2-propanol, CentralChem, Bratislava, Slovak Republic) (200 μl per

each well) for 15 - 20 minutes by gently shaking. Absorbance of each well was measured at 570 nm and 620 nm with ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland).

Immunocytochemistry

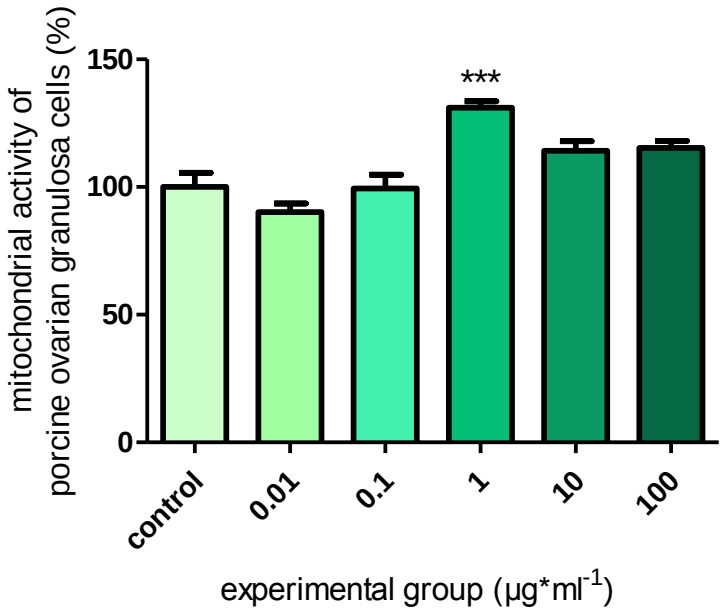
Signaling substances within GCs plated on chamber slides were detected by immunocytochemistry methods (Osborn and Isenberg, 1994). The ImmunoCruz Staining System and primary mouse monoclonal antibodies against PCNA and cyclin B1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as directed by the manufacturer at a dilution of 1:500. Visualization of the primary antibody binding sites was achieved with a secondary polyclonal antibody against mouse immunoglobulins (Igs), labeled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000). Chamber slides stained with peroxidase/diaminobenzidine (DAB) (Roche Diagnostics Corporation, IN, USA, 10%) reagent were mounted with Glycergel (DAKO, Carpinteria, CA, USA) mounting medium. The counting of the percentage of cells containing specific immunoreactivity was done by light microscopy (Kolesarova et al., 2010).

Statistical analyses

The results were evaluated by One Way ANOVA test by statistical program GraphPad Prism 5 Demo (GraphPad Software Incorporated, San Diego, California, USA). The values are presented as average ± SEM.

Results

In the *in vitro* study punicalagin was studied as possible regulator of proliferation of ovarian granulosa cells. Viability of the cells assessed by trypan blue was 75%. The study was focused on mitochondrial activity and presence markers of proliferation, cyclin B1 and PCNA.



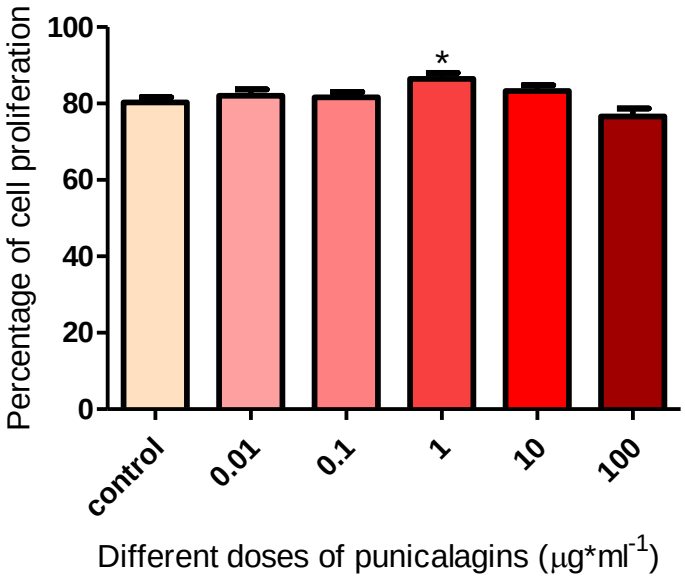
*** Significant (P < 0.001) differences among the control and experimental groups. The results were evaluated by One Way ANOVA

***Preukaznosť (P < 0.001) výsledkov bola vyhodnocovaná medzi kontrolnou skupinou a experimenálnymi skupinami. Výsledky boli vyhodnotené pomocou One Way ANOVA

Figure 1. Effect of punicalagin (µg*ml^{-1}) on mitochondrial activity (%) of porcine ovarian granulosa cells.

Obrázok 1. Vplyv punicalagínu na mitochondriálnu aktivitu ovariálnych granulóznych buniek ošípanej.

Mitochondrial activity was evaluated by MTT. Punicalagin stimulated mitochondrial activity of porcine ovarian granulosa cells (Figure 1.). Similarly a significant (P < 0.001) stimulation of presence of cyclin B1 was found by punicalagin treatment at concentration 1 µg*ml^{-1} (figure 2). This result suggests a dose-dependent effect of punicalagin on presence of proliferative marker cyclin B1.



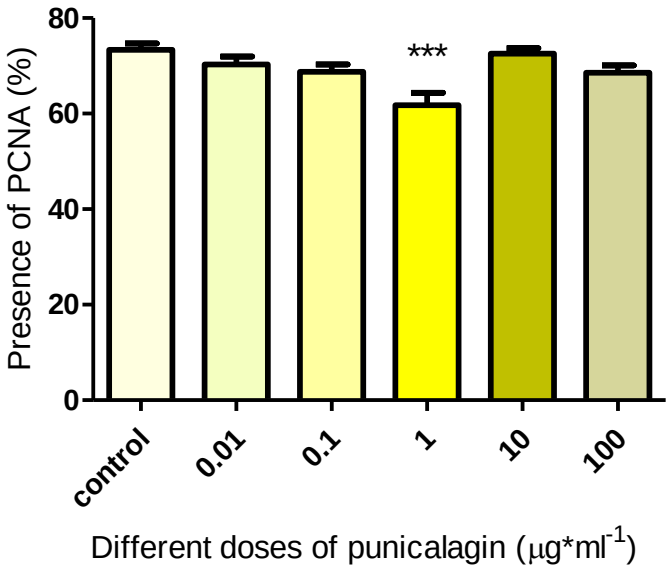
* Significant (P < 0.05) differences among the control and experimental groups. The results were evaluated by One Way ANOVA

*Preukaznosť (P < 0.05) výsledkov bola vyhodnocovaná medzi kontrolnou skupinou a experimenálnymi skupinami. Výsledky boli vyhodnotené pomocou One Way ANOVA

Figure 2. Effect of punicalagin (μg*ml^{-1}) on presence of proliferative marker (%) - cyclin B1 - in porcine ovarian granulosa cells.

Obrázok 2. Vplyv punicalagínu na prítomnosť markeru proliferácie – cyklínu B1 – v ovariálnych granulóznych bunkách ošípanej.

On the other hand proliferating cell nuclear antigen - PCNA was significantly (P < 0.001) decreased at concentration 1 μg*ml^{-1} (Figure 3). Similarly the result suggests dose-dependent effect of punicalagin on presence of proliferative marker PCNA.



***Significant (P < 0.001) differences among the control and experimental groups. The results were evaluated by One Way ANOVA

***Preukaznosť (P < 0.001) výsledkov bola vyhodnocovaná medzi kontrolnou skupinou a experimenálnymi skupinami. Výsledky boli vyhodnotené pomocou One Way ANOVA

Figure 3. Effect of punicalagin (μg*ml^{-1}) on presence of proliferative marker (%) - PCNA - in porcine ovarian granulosa cells.

Obrázok 3. Vplyv punicalagínu na prítomnosť markeru proliferácie – PCNA – v ovariálnych granulóznych bunkách ošípanej.

Discussion

Punicalagin are present in pomegranate and other fruits. Punicalagin has been reported to be mainly responsible for the antioxidant capacity of pomegranate. These experiments are designed to study the effects of punicalagin on ovarian granulosa cells *in vitro*. Little is known about the fate of ellagitannins in animals or humans and only a low concentration of PG has been shown in plasma when a bolus dose was provided (Cerdá et al., 2003). PG or part of punicalagin – elagic acid, was described as a possible effector of cell cycle. Punicalagin could inhibit proliferation of human tumor cells (oral, colon or prostate) and have a proapoptotic effect (Jemal et al., 2011; Gil et al., 2000). In *in vitro* study mitochondrial activity and markers of proliferation of ovarian granulosa cells were examined. Larrosa et al. (2006) described time-dependent and dose-dependent influence of punicalagin on adrenocarcinoma cells Caco-2 by using the mitochondrial pathway. According to Larrosa et al. (2006) 24 h incubation with PG had a positive effect on cell proliferation. Similarly the study has used a 24 h incubation of ovarian granulosa cells with PG *in vitro*. Mitochondrial activity of porcine ovarian granulosa cells was a significantly ($P < 0.001$) increased at a concentration of 1 μg*ml^{-1}. Previous study described concentration 10 μg*ml^{-1} for antioxidant properties and concentration 100 μg*ml^{-1} for apoptotic effect (Seeram et al., 2005). PG exhibited strong anti-

proliferative activity against the human lung, breast and cervical cancer cell lines (Aqil et al., 2012). The study describes pro-proliferative activity of PG on porcine ovarian granulosa cells *in vitro*. Stimulatory effect of PG was demonstrated by MTT assay. Similarly a significant ($P < 0.05$) stimulation of cyclin B1 in porcine ovarian granulosa cells was found. Cyclin B1 is a critical point of mitosis (Larrosa et al., 2006). In general, uncontrolled expression of cyclins and/or Cdks leads to either tumorigenesis or cell cycle arrest (Le and Richardson, 2002). The mechanism of action included down-regulation of cyclins B1 – in different cancer cell lines (Larrosa et al., 2006). On the other hand, inhibitory impact of PG in case of PCNA was noted. PCNA plays important roles in nucleic acid metabolism. The protein is essential for DNA replication, is involved in DNA excision repair, has been suggested to be involved in chromatin assembly, and in several instances has been shown to be involved in RNA transcription (Kelman, 1997). Further verification of possible roles of punicalagin in proliferation is therefore needed.

Conclusion

Punicalagin as a bioactive compound of pomegranate has shown its influence on cell functions. This research was focused on possible effects of punicalagin on porcine ovarian granulosa cells *in vitro*. These results suggest a dose-dependent effect of punicalagin on cell proliferation. Further verification of possible role of punicalagin in proliferation is necessary.

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