POLYPHENOL-RICH POMEGRANATE EXTRACT AS A POTENTIAL MODULATOR OF STEROIDIOGENESIS IN HUMAN OVARIAN CELLS

Simona Baldovská¹, Katarina Michalcová¹, Marek Halenár³, Angel A. Carbonell-Barrachina², Adriana Kolesárová¹²

Address(es): prof. MSc. Adriana Kolesárová, PhD., ¹Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, phone number: +421-37-641 4119, ²University of Miguel Hernández, Faculty of Experimental Sciences, Department of Agro-Food Technology, Ctra. de Beniel, Km. 3.2, 03312, Orihuela (Alicante), Spain.

*Corresponding author: adriana.kolesarova@uniag.sk

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ABSTRACT

Pomegranate represents a rich source of phytochemicals with high medicinal value. Nowadays, many studies have shown that the pomegranate extract also possesses anti-oxidant, anti-inflammatory and anti-proliferative effects on cancer cells, thus leading to increased popularity as a functional food and nutraceuticals. The aim of the study was to determine the biological effect of dry pomegranate extract (at concentrations 5, 10, 20 and 40 µg/ml; for 24h) on the viability of ovarian cells and the secretion of steroid hormones. Cultures of human ovarian granulosa cells (HGL5) and human ovarian carcinoma cells (OVCAR-3) were used such as a model cell system. The metabolic activity was evaluated by AlamarBlue® assay, the release of steroid hormones was assayed by the ELISA method. Experimental results indicated a significant (P≤0.001) increase of proliferation in HGL5 cells after the addition of the extract at the concentrations 5, 10 and 20 µg/ml. Moreover, the number of viable OVCA-3 cells significantly (P<0.05; P<0.01; P<0.001) decreased after the addition of the extract at the concentrations 10, 20 and 40 µg/ml compared to the control. In addition, the secretion of 17β-estradiol by the HGL5 cells was significantly (P<0.05; P<0.001) increased at all used concentrations of the extract. Despite to increasing of the 17β-estradiol secretion, progesterone levels produced by the HGL5 cells were not significantly (P>0.05) affected at all used concentrations of the extract. The current study provided experimental evidence that the pomegranate extract might be a promising candidate as a potential modulator of steroidogenesis and as a potential chemoprotective agent.

Keywords: pomegranate, ovarian cells, steroid hormones, cancer

INTRODUCTION

Punica granatum L., commonly known as pomegranate, is a deciduous shrub, native to the Mediterranean region (Al-Said et al., 2009). Nowadays, pomegranate has received attention as a functional food and can be used for dietary supplement or nutraceutical and consumed as a fresh fruit or in processed juice, jam, wine, and powdered capsules that contain extracts of different pomegranate tissues (Aqil et al., 2012; Akhtar et al., 2015). Many investigators have reported a free radical scavenging and strong antioxidant properties of pomegranate polyphenols including flavonoids, flavonols, and anthocyanins (Elalleh et al., 2011). The flavonoids such as luteolin, resveratrol, and quercetin are found in the peel pomegranate extract (Choi et al., 2006; Viuda-Martos et al., 2010). Additionally, pomegranates contain hydrolysable tannins, especially ellagitannins such as punicalagin, punicalin, gallo-tannin (Gil et al., 2000; Landete et al., 2011), condensed tannins, proanthocyanins, anthocyanins (Zhang et al., 2011), organic and phenolic acids, including ellagic and gallic acid (Mousavinejad et al., 2009), sterols, triterpenoids, fatty acids, triglycerides, and alkaloids (Seeram et al., 2005). Various parts of the pomegranate fruit have been shown to exert multiple beneficial effects on human health. Clinical and preclinical studies have determined that pomegranates have anti-oxidant (Singh et al., 2014; Zhang et al., 2011), anti-inflammatory (Gonzalez-Trujano et al., 2015), anti-bacterial (Viladomiu et al., 2013), anti-cancer (Syed et al., 2013; Sharma et al., 2017), anti-obesity (Al-Mnaamar et al., 2012), and neuroprotective (Yuan et al., 2016) activities. Some plants, including Punica granatum L., contain phytoestrogens. Punic acid, kaempferol and β-sitosterol present in pomegranate have shown phytoestrogenic activity (Choi et al., 2006). Phytoestrogens are structurally similar to steroid hormone 17β-estradiol and compete with the endogenous hormone for binding to estrogen receptor, thus reducing the hormonal effect of endogenous estrogens (Papoutsis et al., 2005). The ovaries are responsible for the production of sex steroids, various growth factors, transcription factors and cytokines (Kolesarova et al., 2015). The steroid hormones are the best-known and best-characterized secretory products of the ovary (Schams and Berischa, 2002). The influence of steroid hormones, including 17β-estradiol, progesterone, and others on ovarian functions and female fertility is complex and still requires elucidation. Understanding the mechanisms which regulate ovarian steroidogenesis at the molecular and cellular level requires readily available cells for in vitro studies (Havelock, 2004). The Immortalized human granulosa cell line HGL5 has been previously described in detail (Rainey et al., 1994). The HGL5 cell line exhibits qualities consistent with primary ovarian granulosa cells, includes the ability to produce progesterone and 17β-estradiol, and offer an optimal tumor-like system for studies focused on gonadotropin-dependent proliferation, cell survival and apoptosis (Patel et al., 2009). Human ovarian epithelial carcinoma cell line, NIH: OVCAR-3 is an appropriate model system in which to study drug resistance in ovarian cancer, the presence of hormone receptors and has been previously described in detail (Hamilton et al., 1983). Therefore, selected ovarian cell lines HGL5 and OVCAR-3 can serve as useful models for studying signalling pathways and regulation of steroid biosynthesis. In this in vitro study, the effect of dry pomegranate extract on human ovarian cells, using HGL5 and OVCAR-3 cells was investigated. The objective was to examine the viability of human ovarian cells and the secretion of selected steroid hormones after the addition a number of the pomegranate extract concentrations (5, 10, 20 and 40 µg/ml).

MATERIAL AND METHODS

Cell lines

The human ovarian granulosa cell line HGL5 (ABM®, BC, Canada) were cultured in Dulbecco’s modification of Eagle medium (Sigma-Aldrich, MO, USA), supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA), and incubated in a 5 % CO₂ incubator at 37 °C until 80–90 % confluent. The cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 80–90 % confluence.
The human ovarian carcinoma cell line, NIH-OVCAR-3 was obtained from the American Type Culture Collection (ATCC®, VA, USA). The cells were cultured in culture medium RPMI1640 (Gibco-BRL, MD, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA), 1 % non-essential amino acids (Sigma Aldrich, UK), and incubated in a 5 % CO2 incubator at 37 °C. The cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 80-90 % confluence.

Pomegranate extract treatment

Pomegranate extract used in this study get from commercially purchased capsules from Spain, which contain dry extract of Punica granatum. Total polyphenols in the pomegranate powder present not than less 50 % and punicalagins (α and β) present even 30 % of the content. Prior to the experiments, pomegranate extract powder was dissolved in culture medium and diluted to the desired concentrations. Depending on the pomegranate extract treatment, the cells were cultured in plates without (control group) or with pomegranate extract at concentrations 5, 10, 20 and 40 μg/mL for 24 h.

Cell viability assay

Cell viability was evaluated using AlamarBlue™ (BioSource International, Nivelles, Belgium) assay as a suitable indicator of cellular health and viability (Bannerman et al., 2001). Briefly, the HGL5 and OVCAR-3 cells were cultured in a 96-well plate (Graener, Germany) per 100 μL at the density of 1 x 10^3 cells per well and grown overnight in a 5 % CO2 incubator at 37 °C. After pre-incubation, the cells were grown in culture for 24 hours without (control group) or with pomegranate extract (5, 10, 20 and 40 μg/mL). The AlamarBlue solution was added to each well 4 hours before the endpoint at a volume of 10 μL. The AlamarBlue reduction as a result of multiple metabolic reactions was measured spectrophotometrically by recording the absorbance at 560 nm and 590 nm using an ELISA microplate reader (Multiskan FC, ThermoFisher Scientific, Finland). For each experiment, wells containing only the AlamarBlue solution without cells were also prepared and incubated. The fluorescence measured in those was used as a background and subtracted. The results were expressed as the percentage of viable cells. Analyses were performed in three independent experiments with replicates per experiment.

ELISA (enzyme-linked immunosorbent assay)

Concentrations of secreted steroid hormones after supplementation by pomegranate extract were determined using ELISA kit (NOVATEC, Dietzenbach, Germany) according to the manufacturer’s instructions. Cells HGL5 were re-seeded in a 24-well culture plate (Graener, Germany) at a density of 1 x 10^5 cells per well and incubated in DMEM culture media (control) or with pomegranate extract (at concentrations 5, 10, 20 and 40 μg/mL) for 24 h. The release of 17β-estradiol and progesterone was measured spectrophotometrically according to the manufacturer’s instructions at a wavelength 450 nm on an ELISA microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Intra- and inter-assay coefficient for 17β-estradiol was set as ≤ 5% and ≤10%, for progesterone was set as ≤ 4% and ≤ 9.3%. The sensitiveness was 8.68 pg/mL for 17β-estradiol and 0.05 ng/mL for progesterone. The results were averaged over 3 different independent experiments with replicates per experiment.

Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using the GraphPad Prism 5 program (version 3.02 for Windows; GraphPad Software, CA, USA). Experiments were repeated three times in duplicate for experiments. One-way ANOVA along with Dunnett’s test as a follow-up test to ANOVA was performed as appropriate to determine the statistical significance. The statistical significance was established at P≤0.05, P<0.01, P<0.001.

RESULTS AND DISCUSSION

Effects of pomegranate extract on cell viability

In this in vitro study, we observed a significant (P≤0.001) increase of viable HGL5 cells after treatment of pomegranate extract at the concentrations 5; 10 and 20 μg/mL. Moreover, our results indicated significantly (P≤0.05; P≤0.01; P≤0.001) inhibited the proliferation of OVCAR-3 cells in a dose-dependent manner after pomegranate extract application at the concentrations 10; 20 and 40 μg/mL. The results are shown in the figure 1.

Figure 1 The viability of human ovarian granulosa cell line (A) and human ovarian carcinoma cell line (B) without (control) or with pomegranate extract treatment (5, 10, 20 and 40 μg/mL) for 24 h. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as means ± SEM. AlamarBlue.

Effects of pomegranate extract on the release of steroid hormones

The current study suggests that pomegranate extract could have an impact on the secretion of steroid hormones — 17β-estradiol and progesterone by the cells. The results showed a significant (P≤0.05; P≤0.001) increase of the 17β-estradiol secretion by pomegranate extract treatment at the concentrations 5; 10 and 40 μg/mL in comparison to control. On the other hand, progesterone levels were not significantly (P≥0.05) affected at all used concentrations in comparison to control after addition of pomegranate extract. The results are shown in the figure 2.

Figure 2 The release of 17β-estradiol (A) and progesterone (B) by HGL5 cells after treatment with pomegranate extract (5, 10, 20 and 40 μg/mL) for 24 h. Control represents culture medium without pomegranate extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as means ± SEM. ELISA.

Nowadays, natural biomolecules and their impact on reproductive functions have attracted a huge scientific attention (Kolesarova et al., 2011; Halenar et al., 2016; Packova et al., 2016; Roychoudhury et al., 2018). Naturally-occurring phytochemicals from dietary fruits and vegetables have received public interest for the prevention and treatment of various diseases (Modaeinama et al., 2015; Sharma et al., 2017).

The quest for effective and safe chemotherapeutic agents is urgently necessary for a view of the fact that many anticancer drugs elicit harmful side effects on normal cells. The current experiments were designed to determine the biological effects of rich-polyphenol pomegranate extract on the viability of human ovarian cells. The stimulation of proliferation of healthy human granulosa cells HGL5 by the pomegranate extract in vitro was observed.

The hormonal properties associated with punicalagin as a major phytocompound of pomegranate, described Packova et al. (2016). In previous study, the pro-proliferative effect of punicalagin on porcine ovarian granulosa cells in vitro was examined. Further studies of the potential roles of pomegranate in proliferation are therefore needed.

Many reports have shown that the pomegranate as well as juice, extract or oil exert antiproliferative, anti-inflammatory, and anti-cancer properties by modulating multiple signalling pathways (Sharma et al., 2017). In current studies, antioxidants have been examined due to their protective properties against free radical-induced damage. Clinical and experimental studies proved the relationship between a polyphenol-rich diet and reduced risk of degenerative diseases (Vauzour et al., 2010; Viuda-Martos et al., 2010).

Ovarian cancer is one of the most common malignancies in the female reproductive system with high mortality rates worldwide (Liu et al., 2017). In this study, the cell-specific and dose-dependent antiproliferative effect of pomegranate extract as a promising chemo-preventive agent was examined. The polyphenol-rich pomegranate extract inhibited the proliferation of human ovarian