ORIGINAL ARTICLE

TITLE

Antioxidative effect of dietary flavonoid isoquercitrin on human ovarian granulosa cells HGL5 *in vitro*

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SHORT TITLE

Effect of isoquercitrin on ovarian cells *in vitro*

SUMMARY

This study aimed to examine the effect of dietary flavonoid isoquercitrin on ovarian granulosa cells using the immortalized human cell line HGL5. Cell viability, survival, apoptosis, release of steroid hormones 17β-estradiol and progesterone, and human transforming growth factor-

β2 (TGF-β2) and TGF-β2 receptor as well as intracellular ROS generation were investigated after isoquercitrin treatment at the concentration range of 5 – 100 μg.ml^{-1}. It did not cause any significant change ($p>0.05$) in cell viability as studied by AlamarBlue assay in comparison to control. No significant change was observed ($p>0.05$) in the proportion of live, dead and apoptotic cells as revealed by apoptotic assay using flow cytometry. Similarly, the release of 17β-estradiol, progesterone, TGF-β2 and its receptor were not affected significantly ($p>0.05$) by isoquercitrin as detected by ELISA, in comparison to control. Except for the highest concentration of 100 μg.ml^{-1}, which led to oxidative stress, isoquercitrin exhibited antioxidative activity at lower concentration used in the study (5, 10, 25, and 50 μg.ml^{-1}) by hampering the production of intracellular reactive oxygen species (ROS), in comparison to control, as detected by chemiluminescence assay ($p<0.05$). Findings of the present study indicate an existence of the antioxidative pathway that involves inhibition of intracellular ROS generation by isoquercitrin in human ovarian granulosa cells.

KEY WORDS

Flavonoid ● granulosa cells ● antioxidant ● steroid hormones ● transforming growth factor

Introduction

Flavonoid glucosides such as quercetin and isoquercitrin occur widely in the plant kingdom and are among the most common flavonoids in the human diet. Isoquercitrin (quercetin-3-O-β-D-glucopyranoside) is commonly found in fruits, vegetables, cereals, various plant-derived foods and beverages such as tea and wine, as well as in medicinal herbs such as St. John's wort (*Hypericum perforatum* L.) (Paulke et al., 2006; Hasumura et al., 2004; Valentová et al., 2014). It has been reported in comprehensive database Phenol-Explorer, which notes polyphenol content in foods, that content of isoquercitrin ranges between 0.0067 mg

isoquercitrin/100 g (kiwi juice) and 41.95 mg isoquercitrin/100 g (fresh black chokeberries – fruits of *Aronia melanocarpa*) (Neveu et al., 2010). Although this phytonutrient is widely distributed, it is very difficult to obtain a sufficient amount in a pure state for the food technology and pharmaceutical industry since isoquercitrin contents in plant materials are extremely low (Lu et al., 2013). In addition, considering the recommended daily dose of fruits and vegetables (5x100 g) average daily intake of isoquercitrin could be estimated at 3-12 mg (Valentová et al., 2014). Water soluble enzymatically modified isoquercitrin is generally regarded as safe for ingestion by the United States Food and Drug Administration (FDA 2007) and is also approved in Japan as a food additive (JFA 2007). On a daily basis, up to 4.9 $mg.kg^{-1}$ per day of enzymatically modified (α-glucosylated) isoquercitrin is acceptable (Valentová *et al.* 2014).

Dietary flavonoids, including isoquercitrin, possess neuroprotective, cardioprotective, chemopreventive, antiallergic, anti-inflammatory, and antioxidant properties (Appleton 2010). Its screening for clinical purposes has recently attracted a great deal of interest for a number of health issues including inflammation, atherosclerosis (Reuter *et al.* 2010) cancers of pancreas (Chen *et al.* 2015*)*, liver (Huang *et al.* 2014), kidney (Buonerba *et al.* 2018), colon (Amado *et al.* 2014), bladder (Wu *et al.* 2017), ovary (Michalcova *et al.* 2019) as well as ROS-induced diseases particularly for mesenchymal stem cell transplantation therapy (Li *et al.* 2016).

Granulosa cells involved in the process of ovarian steroidogenesis and folliculogenesis are of clinical importance during oocyte development, and mainly secrete progesterone and estradiol, among various other factors. During menstrual cycle they turn into granulosa lutein (~~h~~GL) cells at the time of the luteinizing hormone (LH) surge. The ~~h~~GL cells predominantly start secreting progesterone which is accompanied by a decline in estradiol production (Vander *et al.* 2001). HGL5 is an immortalized cell line derived from primary ~~h~~GL cells after

transformation with the E6 and E7 regions of human papillomavirus 16 (Rainey *et al.* 1994). They are capable of a quick growth and formation of large cultures apart from other qualities consistent with primary ovarian granulosa cells. HGL5 cell line forms an attractive model not only for investigating the mechanisms relating to steroid biosynthesis but also other pathways involved with ~~h~~GL function (Rainey *et al.* 1994; Havelock *et al.* 2004; Bouraki *et al.* 2012). The present study aimed at examining the viability, survival, apoptosis, release of 17β-estradiol, progesterone, human transforming growth factor-β2 (TGF-β2) and TGF-β2 receptor, and intracellular ROS generation by HGL5 cells after isoquercitrin treatment at the concentrations of 5, 10, 25, 50, and 100 $\mu g.ml^{-1}$.

Methods

Cell culture and treatment

Isoquercitrin (quercetin 3-O-β-D-glucopyranoside, purity 96.5 %) was prepared by selective enzymatic derhamnosylation of rutin using recombinant α-L-rhamnosidase from *Aspergillus terreus* (Weignerová *et al.* 2012). Purity of quercetin 3-O-β-D-glucopyranoside (isoquercitrin) was determined by HPLC by the method described in detail in the papers Weignerová *et al.* (2012) and Gerstorferová *et al.* (2012). Authenticity of this compound was determined by 1H and 13C NMR spectroscopy as detailed in the above papers. Immortalized human ovarian granulosa cells HGL5 (ABM®, BC, Canada) were cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotic solution (Invitrogen, CA, USA). Cells were cultured in plates without (control group) or with isoquercitrin at concentrations of 5, 10, 25, 50, and 100 $\mu g.ml^{-1}$ for 24 hours. As a positive control 0.1% DMSO (dimethyl sulfoxide, Sigma Aldrich, St. Louis, MO, USA, ≥99.5% purity) was used, as previously described (Baldovská *et al.* 2020). All the procedures followed were in accordance with institutional guidelines.

Cell viability

Cell viability was examined using AlamarBlue (BioSource International, Nivelles, Belgium) assay (Michalcova *et al.* 2019). Human ovarian granulosa cells were seeded into 96-well microplates (100µl well^{-1}) at a concentration of 1,5 x 10^4 cells.ml^{-1} at standard culture conditions of 5% CO_2 in air at 37°C. Cells were grown in culture for 24 hours without (control group) or with isoquercitrin (5, 10, 25, 50, and 100 µg.ml^{-1}), or with 0.1 % DMSO (as positive control). Resazurin reduction (oxidized indigo blue state into the reduced pink state) was measured by recording the absorbance at 560 nm using a microplate reader (Multiskan FC, ThermoFisher Scientific, Finland) and expressed as percentage.

Live, dead and apoptosis assay by flow cytometry

Numbers of live, apoptotic and dead cells were detected by the rate of uptake and retention of certain dyes as described previously (Michalcova *et al.* 2019). Cells were seeded in 6-well culture plates at a density of 0.5 × 10^6 cells per well in culture medium (control) and/or supplemented with isoquercitrin (at concentrations 5, 10, 25, 50, and 100 µg.ml^{-1}) for 24 hours, whereas positive control received 0.1% DMSO. Apoptotic cells were measured by staining with specific nuclear fluorochrome Yo-Pro-1 (Molecular Probes, Lucerne, Switzerland) and specific membrane marker Annexin V-FITC (AnV; Annexin V Apoptosis Detection Kit, Canvax, Cordoba, Spain). Dead cells were measured by staining with propidium iodide (PI; Molecular Probes, Lucerne, Switzerland). Briefly, after centrifugation (300× g for 5 min), cell pellets were adjusted to 1 × 10^6 cells per ml in PBS (without Ca and Mg) and stained with 1 µl of Yo-Pro-1 solution (100 µmol.l^{-1}) for 15 min in dark at room temperature. Annexin V staining was done according to manufacturer's instructions. Cells were stained with 4 µl of propidium iodide (50 µg.ml^{-1}) in each tube just prior to the analysis using flow cytometer (FACS Calibur, BD Biosciences, USA). At least 50 000 events (cells) were analyzed in each sample and data analysis was done using Cell Quest Pro software (BD Biosciences, USA). Three different populations were identified using this assay: live unstained cells (Yo-Pro-1$^-$

/PI^- and AnV^-/PI^-), apoptotic cells (Yo-Pro-$1^+/PI^-$ and AnV^+/PI^-), and dead cells (only PI^+) (Figure 1).

ELISA (enzyme-linked immunosorbent assay)

Concentrations of secreted 17β-estradiol, progesterone, TGF-β2 and TGF-β2 receptor were determined using ELISA kit (CUSABIO, Houston, USA) as described previously (Michalcova *et al.* 2019; Baldovská *et al.* 2020). Cells were re-seeded in 24-well culture plates at a density of 1 x 10^5 cells per well and then incubated in culture medium (control) and/or with isoquercitrin (at concentrations 5, 10, 25, 50, and 100 $\mu g.ml^{-1}$) for 24 hours and the release of 17β-estradiol, progesterone, TGF-β2 and TGF-β2 receptor was measured. Briefly, antibody specific for 17β-estradiol, progesterone, TGF-β2 and/or TGF-β2 receptor was pre-coated on a microplate. Standards and samples were pipetted into the wells and any TGF-β2 and/or TGF-β2 receptor present was bound by the immobilized antibody. After removal of any unbound substances, abiotin-conjugated antibody specific for 17β-estradiol, progesterone, TGF-β2 and TGF-β2 receptor was added to the wells. After washing, avidin conjugated horseradish peroxidase was added to the wells. Washing was done to remove any unbound avidin-enzyme reagent, and substrate solution was added to the wells and colour was developed in proportion to the amount of TGF-β2 bound in the initial step. Colour development was stopped and the intensity of the colour was measured spectrophotometrically.

ROS assay

Intracellular ROS generation was assessed by chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) as a probe (Michalcova *et al.* 2019; Sharma *et al.* 2017). Test samples consisted of 10 µl luminol each (5 mM) and 400 µl experimental sample or control. Negative controls were prepared by replacing the HGL5 cell suspension with 400 µl of culture medium each. Positive controls included 400 µl of each medium, 10 µl luminol and 50 µl hydrogen peroxide (30 %; 8.8 M; Sigma-Aldrich).

Chemiluminescence was measured on 48-well plates in 15 cycles of 1 minute using the Glomax Multi+ Combined Spectro-Fluoro Luminometer (Promega Corporation, WI, USA). Results were expressed as relative light units (RLU).$s^{-1}.10^{-6}$ cells (Michalcova *et al.* 2019; Tvrdá *et al.* 2016).

Statistical analysis

For statistical analysis, data were expressed as means with standard errors of means. All experiments were done in triplicate. One-way ANOVA along with Dunnett's tests were used to establish statistically significant differences at $p<0.05$.

Results

In culture, HGL5 cells did not lose viability after isoquercitrin supplementation. As examined by AlamarBlue assay, isoquercitrin treatment did not cause any significant change ($p>0.05$) in the viability of human ovarian granulosa cells HGL5 at all the concentrations used in the study, as compared with control (Figure 2). Similarly, as shown by flow cytometry analysis, isoquercitrin treatment did not cause any significant change ($p>0.05$) in the proportion of live, dead and apoptotic HGL5 cells at all the concentrations used in the study (Table 1).

As detected by ELISA, isoquercitrin treatment did not cause any significant change ($p>0.05$) ~~neither~~ in the release of steroid hormones 17β-estradiol and progesterone by HGL5 cells (Figure 3) as well as ~~nor~~ in the release of human TGF-β2 and binding TGF-β2 receptor (Figure 4) at all of the concentrations used in the study. On the other hand, isoquercitrin treatment was capable of reducing intracellular ROS generation at concentrations of 5, 10, 25 $\mu g.ml^{-1}$ ($p<0.001$) and 50 $\mu g.ml^{-1}$ ($p<0.01$) used in the study, as determined by chemiluminescence assay. However, the highest concentration of 100 $\mu g.ml^{-1}$ isoquercitrin led to oxidative stress resulting in an increase ($p<0.05$) in intracellular production of ROS (Figure 5).

Discussion

In the ovarian follicle, granulosa cells constitute the principal somatic cell type, which is involved in the process of steroidogenesis and folliculogenesis (Ai *et al.* 2019). Based on the phase of development, granulosa cells secrete a number of factors. These cells express follicle stimulating hormone (FSH) receptors during the first half of the menstrual cycle. Under the influence of FSH granulosa cells further express aromatase that converts androgens, produced in theca cells, to estradiol. Thereafter the granulosa cells start predominantly secreting progesterone during the LH surge as they turn into ~~h~~GL cells (Vander *et al.* 2001). In the present study, we used HGL5 cells a suitable cellular model to investigate the effect of dietary bioflavonoid isoquercitrin at the concentrations ranging from 5 - 100 $\mu g.ml^{-1}$ on cell viability, survival, apoptosis, release of 17β-estradiol, progesterone and human transforming growth factor-β2 (TGF-β2) and TGF-β2 receptor as well as the ROS production. To our knowledge, this is the first report which has looked into the influence of isoquercitrin on ovarian granulosa cells.

Similar to our findings, treatment with isoquercitrin did not affect the viability of human ovarian cancer cells OVCAR-3 as determined by AlamarBlue assay (Michalcova *et al*. 2019). Furthermore, concentrations of 25, 50, and 100 $\mu mol.l^{-1}$ isoquercitrin isolated from the aerial parts of *Hyptis fasciculata* did not cause any change in the viability of human brain cancer cells after 24, 48, and 72 hours (Amado *et al.* 2009). When cultured with isoquercitrin at 50, 100 and 200 μM concentrations for 72 hours, MTT assay also showed no change of viability in rat hepatoma cells H4IIE (Zhou *et al.* 2014). Isolated from *Acer okamotoanum*, isoquercitrin at the concentration range of 1-10 $\mu g.ml^{-1}$ did not affect the viability of SH-SY5Y human neuronal cells, too. On the other hand, isoquercitrin was capable of protecting the cells by increasing their viability against hydrogen peroxide (H_2O_2)-induced oxidative stress (Kim *et al.* 2019).

Similar to the findings of the present study, isoquercitrin, isolated from the aerial parts of *Hyptis fasciculata*, did not affect caspase-3 dependent apoptosis at concentrations up to 100 $mmol.l^{-1}$ in human brain cancer cells (Amado *et al.* 2009). In another previous study,

isoquercitrin was found to cause down-regulation of apoptotic protein expression such as cleaved caspase-9, -3, PARP, and p53. It also inhibited H_2O_2-induced apoptosis in the cellular system and the treatment further showed attenuation of apoptotic rate in the Hoechst 33342/PI double staining and AnnexinV-FITC/PI staining. Anti-apoptotic effect was further associated with the Akt/GSK3b signaling pathway, and isoquercitrin was recommended for clinical use owing to its capability to interfere with the progression of endothelial injury-associated cardiovascular disease (Zhu *et al.* 2016). On the other hand, potential clinical use of isoquercitrin in cancer cells is believed to be mediated by its pro-apoptotic property. Therapeutic doses of isoquercitrin extracted from *Bidens pilosa* L. retarded proliferation, induced apoptosis, and the cell cycle was arrested in the G1 phase in human bladder cancer cells 5637 and T24 (Chen *et al.* 2016). Isolated from *Bidens bipinnata* L. extract, it also promoted apoptosis, inhibited cell proliferation, and blocked the cell cycle via the mitogen-activated protein kinase (MAPK) signaling pathway in human liver cancer cells HepG2 and Hep3B (Huang *et al.* 2014).

Recently, our research group has reported the production of steroid hormones by HGL5 cells and that dry pomegranate extract affected the release of 17β-estradiol when cultured for 24 hours (Baldovska *et al.* 2019). Similarly, previous studies also showed secretion of estradiol and progesterone by HGL cells (Rainey *et al.* 1994; Havelock *et al.* 2004). However, it has been reported, that the HGL5 cell line is not responsive to FSH because of the lack of gonadotropin receptors (Rainey *et al.* 1994). As a potent luteinizing granulosa cell survival factor, progesterone was earlier found to promote the expression of epidermal growth factor family member amphiregulin and epiregulin thereby helping maintain the viability of luteinizing granulosa cells in primates (Puttabyatappa *et al.* 2013). Members of the TGF-β superfamily are expressed by ovarian somatic cells and oocytes are involved in folliculogenesis, including intraovarian control mechanisms, follicular assembly, growth, differentiation and progression, and thus fertility (Knight *et al.* 2006; Trombly *et al.* 2009). Another study conducted to explore the effects of the TGF-β superfamily members and their

receptors on human granulosa cells during folliculogenesis showed expression of TGF-β superfamily members and their receptors in a human nonluteinized granulosa cell line HGrC1, including the type I and II receptors (Iwase *et al.* 2012). Previous studies have reported the production of TGF-β1 and TGF-β2 by ovarian cells (Puttabyatappa *et al.* 2013; Roy *et al.* 1994; Bristol *et al*. 2004). In the present study, HGL5 cells have also been able to release both TGF-β2 and TGF-β2 receptor although isoquercitrin did not have any impact on their expression. Furthermore, Michalcova *et al.* (2019) has reported, that the release of human TGF-β1 and binding of TGF-β1 receptor by ovarian cancer cells was not affected by isoquercitrin.

Notably, the results of the present study on the effect of isoquercitrin on human granulosa cells indicate, that isoquercitrin could scavenge intracellular ROS production at lower concentrations and is able to decrease oxidative stress in HGL5 cells *in vitro*. Isoquercitrin isolated from *Thuja orientalis* was able to scavenge reactive oxygen species (H_2O_2, •OH and O2•-) as demonstrated by staining of cultures as well as the generation of individual radical species at 50 μM concentration (Jung *et al.* 2010). Isolated from *Acer okamotoanum*, isoquercitrin was able to ameliorate H_2O_2-induced oxidative stress by successfully inhibiting the production of ROS in human neuronal cells at a concentration of 10 $\mu g.ml^{-1}$. The antioxidant property of isoquercitrin molecule was believed to be attributed by its catechol moiety in B ring as the biological activity of flavonoids depend on the number and position of –OH as well as the presence of sugar. Based on the findings, isoquercitrin has been considered useful as a preventive and therapeutic agent for neurodegenerative diseases including Alzheimer's disease (Kim *et al.* 2019). On the other hand, in rat hippocampal neuronal cells pretreatment with isoquercitrin at 25, 50 and 100 $\mu g.ml^{-1}$ for 24 hours prior to 4 hours of oxygen glucose deprivation and 24 hours of normoxia was not sufficient to reduce the generation of ROS although a concentration-dependent trend of decline was noted (Chen *et al.* 2017). Antioxidant activity of isoquercitrin at 10–100 μM concentration range was also evident by the activation of glutathione peroxidase enzyme apart from the reduction of

malondiadehyde levels in 6-hydroxydopamine-induced PC-12 cells and the reduction of superoxide dismutase activity (Magalingam *et al*. 2016), which, in turn, is believed to catalyze the detoxification of detrimental superoxide radicals to less toxic molecules thus leading to attenuation of oxidative stress (Duong *et al.* 2008).

In the light of the previous findings on the role of isoquercitrin on various cells, tissues and cell lines of both healthy and disease models, the findings of the present study on human ovarian granulosa cells HGL5 indicate that isoquercitrin may be able to protect the ovarian functions from oxidative stress through a pathway involving the inhibition of intracellular ROS generation. Further investigations may prove very useful in confirming the hypothesis of the protective role of isoquercitrin at proper therapeutic concentrations against ovarian aging as well as other pathologies of the ovary whose etiology involves oxidative stress.

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Figure legends

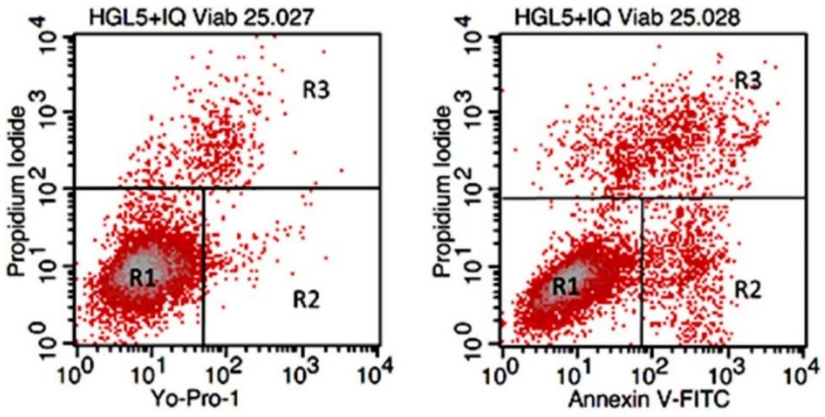

Figure 1. Flow cytometry dot plots used for measuring the live, dead and apoptotic cells: R1 – Live unstained cells (Yo-Pro-1^-/PI^- and AnV^-/PI^-), apoptotic cells (Yo-Pro-1^+/PI^- and AnV^+/PI^-), and dead cells (only PI^+).

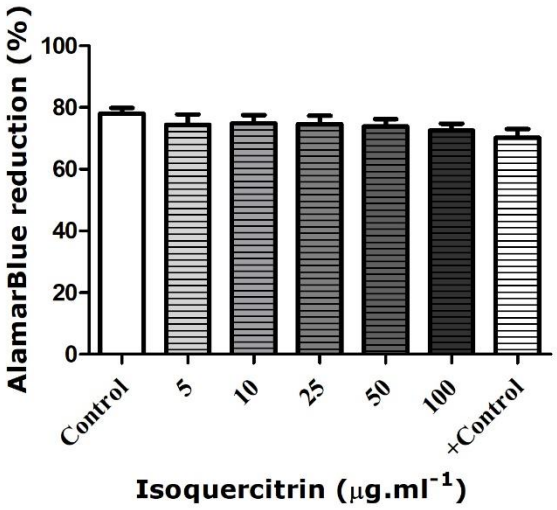

Figure 2. Viability of human ovarian granulosa cells HGL5 without (Control) or with isoquercitrin treatment (5, 10, 25, 50, 100 µg.ml^{-1}). Positive control (+Control) with 0.1 % DMSO. Significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett's multiple comparison tests. The data are expressed as means ± SEM. AlamarBlue assay.

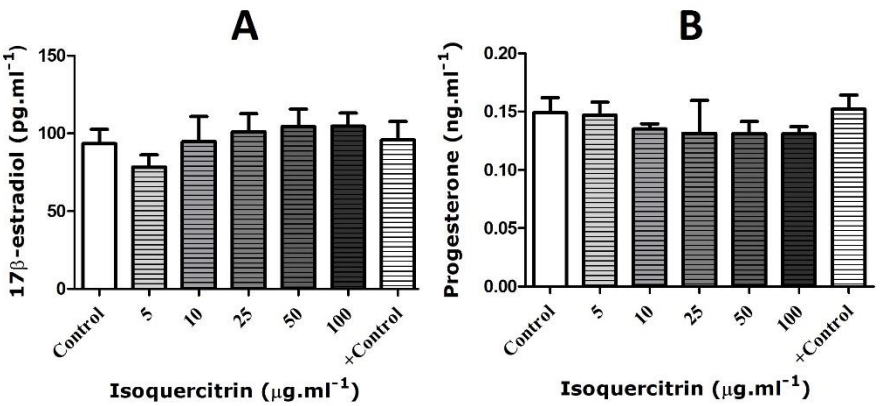

Figure 3. Release of 17β-estradiol (A) and progesterone (B) by human ovarian granulosa cells HGL5 after treatment with isoquercitrin (5, 10, 25, 50, 100 μg.ml^{-1}). Control represents culture medium without isoquercitrin; positive control (+Control) means culture medium enriched by 0.1% DMSO. Significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett's multiple comparison tests. The data are expressed as means ± SEM. ELISA.

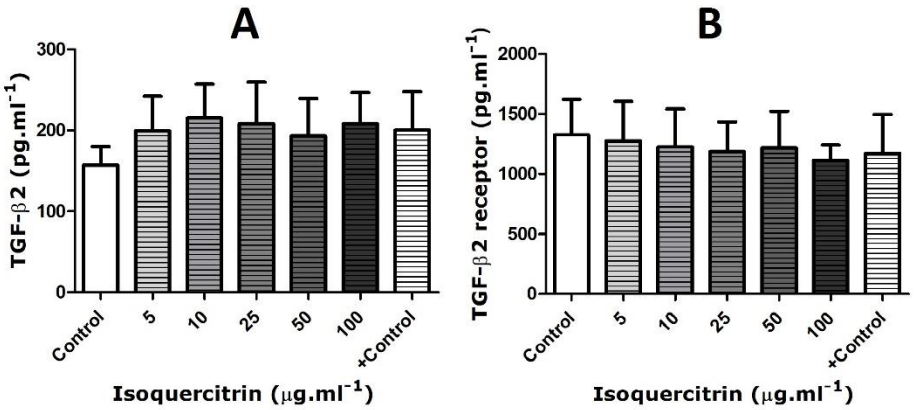

Figure 4. Release of TGF-β2 (A) and TGF-β2 receptor (B) by human ovarian granulosa cells HGL5 after treatment with isoquercitrin (5, 10, 25, 50, 100 μg.ml^{-1}). Control represents culture medium without isoquercitrin; positive control (+Control) means culture medium enriched by 0.1% DMSO. Significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett's multiple comparison tests. The data are expressed as means ± SEM. ELISA.

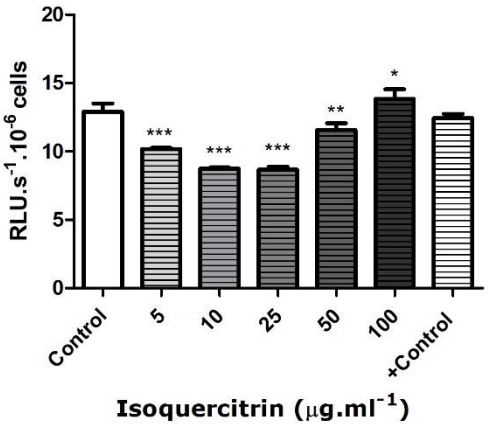

Figure 5. Intracellular ROS generation by human ovarian granulosa cells HGL5 after treatment with isoquercitrin (5, 10, 25, 50, 100 µg.ml^{-1}). Control represents culture medium without isoquercitrin; positive control (+Control) means culture medium enriched by 0.1% DMSO. Significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett's multiple comparison tests. The data are expressed as means ± SEM. Chemiluminescence assay. *p<0.05, **p<0.01, ***p<0.001.

Table 1. Proportion of live, dead and apoptotic HGL5 cells after isoquercitrin treatment at 5, 10, 25, 50, and 100 µg.ml^{-1} concentrations for 24 hours. Live cells remained unstained by specific nuclear fluorochrome Yo-Pro-1 and propidium iodide (PI) and specific membrane marker Annexin V-FITC (Yo-Pro-1$^-$/PI$^-$ and AnV$^-$/PI$^-$), whereas dead cells were stained by propidium iodide (only PI$^+$) and apoptotic cells were stained by specific nuclear fluorochrome Yo-Pro-1 and specific membrane marker Annexin V-FITC but unstained by propidium iodide (Yo-Pro-1$^+$/PI$^-$ and AnV$^+$/PI$^-$).

Parameter Yo-Pro-1/PI	**Control**	**5**	**10**	**25**	**50**	**100**	**Positive control**
		µg.ml^{-1}					
Live (%)	79.98 ± 5.82	80.94 ± 5.64	83.11 ± 5.22	82.63 ± 5.45	89.86 ± 2.92	84.96 ± 3.45	85.73 ± 4.10
Dead (%)	16.36 ± 1.21	15.48 ± 1.38	11.32 ± 3.95	12.45 ± 3.04	8.54 ± 2.69	10.49 ± 2.97	11.95 ± 3.91
Apoptotic (%)	5.73 ± 1.88	5.62 ± 1.80	5.57 ± 1.95	4.92 ± 1.92	3.98 ± 1.20	4.55 ± 1.48	4.88 ± 1.31
Parameter AnV/PI	**Control**	**5**	**10**	**25**	**50**	**100**	**Positive control**
		µg.ml^{-1}					
Live (%)	81.54 ± 7.65	79.16 ± 6.6	78.20 ± 9.80	79.58 ± 9.80	89.90 ± 2.81	87.80 ± 3.65	85.07 ± 3.75
Dead (%)	18.85 ± 6.77	19.75 ± 6.40	25.14 ± 5.86	24.12 ± 5.24	10.18 ± 1.08	10.61 ± 2.43	12.91 ± 3.47
Apoptotic (%)	1.75 ± 0.20	1.89 ± 0.25	2.04 ± 0.49	1.52 ± 0.36	1.55 ± 0.29	1.60 ± 0.33	2.02 ± 0.45