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Genistein affects proliferation and migration of bovine oviductal epithelial cells

Daniela C. García, Pablo A. Valdecantos, Dora C. Miceli, Mariela Roldán-Olarte *

Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI San Miguel de Tucumán, Argentina

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ABSTRACT

Genistein is one of the most abundant isoflavones in soybean. This molecule induces cell cycle arrest and apoptosis in different normal and cancer cells. Genistein has been of considerable interest due to its adverse effects on bovine reproduction, altering estrous cycle, implantation and fetal development and producing subfertility or infertility. The objective of this work was to study the effects of genistein on the expression of selected genes involved in the regulation of cell cycle and apoptosis. Primary cultures of bovine oviductal epithelial cells (BOEC) were treated with different genistein concentrations (0.2, 2 and 10 μM) to analyze *CYCLIN B1*, *BCL-2* and *BAX* gene expression by Real-time RT-PCR. Results showed that genistein down-regulated *CYCLIN B1* expression, affecting cell cycle progression, and caused a decrease in the *BCL-2*/*BAX* ratio starting at 2 μM of genistein. In addition, in order to determine if genistein affects BOEC migration, *in vitro* wound healing assays were performed. A significant reduction in cell migration after 12 h of culture was observed at both 0.2 and 10 μM genistein concentrations. Also, in the presence of genistein the percentage of mitotic cells decreased, although apoptotic cells percentages were not affected. These findings indicate that genistein has an inhibitory effect on BOEC proliferation and migration, suggesting that it could influence the normal physiology of the oviductal epithelium.

1. Introduction

Human and animal diet includes isoflavones derived from different plants, in particular those derived from soybean, which has become an important source of protein in animal fodder (Woclawek-Potocka et al., 2013). Genistein is the major isoflavone constituent of soybeans (Allred et al., 2001; Dixon and Ferreira, 2002). It has been described in different cellular models that genistein inhibits cell cycle at G2/M and induces apoptosis (Yan et al., 2010); genistein is also known as an inhibitor of tyrosine kinase proteins (Banerjee et al., 2008) that regulate the expression of transcription factors (Akiyama et al., 1987; Li et al., 2008). Several proteins that control the cell cycle have been described as affected by genistein, including the CDK1/*CYCLIN B1* complex, the anti-apoptotic *BCL-2* and the pro-apoptotic *BAX* (Han et al., 2013; Moskot et al., 2015). At physiological concentrations, genistein exerts an inhibitory effect on the migration of normal and human tumor cells (Miekus and Madeja, 2007).

Genistein is of significant interest because of its adverse effect on bovine reproduction, altering the estrous cycle and ovulation and producing subfertility/infertility as well as implantation and fetal development defects (Woclawek-Potocka et al., 2013). The mammalian oviduct plays an important role in gamete transport, fertilization and early embryo development (Hunter, 2012). Oviductal epithelial cells synthesize and secrete macromolecules to the lumen and, with other molecules derived from plasma and follicular fluid, provide an optimal microenvironment for reproductive functions (Killian, 2004). Various components of the animal diet could affect the composition of the oviductal fluid (Leroy et al., 2010) and generate changes in oviductal morphology and physiology (Jefferson et al., 2012). Taking into account that genistein affects cellular processes by altering signaling pathways and the expression of cell cycle- and apoptosis-regulating genes (Liu et al., 2013; Zhang et al., 2015) and that the oviductal epithelium has an important role in the maintenance of an optimal microenvironment for successful reproduction, the aim of this work was to determine if genistein affects apoptosis and/or proliferation and to evaluate BOEC migration in *in vitro* conditions.

2. Materials and methods

2.1 Expression of CYCLIN B1, BCL-2 and BAX genes in cell suspension cultures

2.1.1 Bovine oviductal epithelial cell suspension cultures

Genital tracts from young beef cows (*Bos taurus*) were collected at the local abattoir within 20 min of death and transported to the

* Corresponding author.
E-mail address: emroldanolarte@fbqf.unt.edu.ar (M. Roldán-Olarte).

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laboratory in sodium phosphate buffer (PBS), pH 7.4, at 4 °C for processing within 3 h after collection. Ipsilateral oviducts obtained from seven animals corresponding to the mid-luteal stage (days 6–12) were used in each experiment, as described previously (García et al., 2014). Oviducts were ligated, dissected, and washed in fresh PBS and then dipped in 70% ethanol. After discarding infundibulum and uterotubal junction, oviducts were gently squeezed in a stripping motion with forceps to obtain epithelial cells (Rottmayer et al., 2006). Biological material was collected in culture medium TCM-199 (Gibco, Life Technology, Burlington, ON, Canada) supplemented with 10% bovine fetal serum (Internegocios, Buenos Aires, Argentina), 25 mM Hepes (Gibco, Life Technology, Burlington, ON, Canada), 0.2 mM sodium pyruvate, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of fungizone (Gibco, Life Technology, Burlington, ON, Canada). The cell suspension was pipetted 15 times through a 21-gauge syringe needle. Three steps of washing were performed, each followed by 25 min sedimentation in culture medium in a cell culture incubator. Cell viability at seeding was analyzed by trypan blue (Sigma) staining and microscopic observation of beating cilia. Cells were grown in 25 mm^2 bottle cultures (Nunclon, Roskilde, Denmark) in 5 ml TCM-199 supplemented as described earlier. Suspension cultures were performed at 38.5 °C in a humidified atmosphere and 5% CO_2. After 48 h, cell cultures were washed twice with PBS. The epithelial nature of the cultured cells was confirmed by immunocytochemical analysis, using monoclonal Anti Pan Cytokeratine clone PCK-26 antibody (Sigma, C1801); more than 90% of the cells were cytokeratin positive (García et al., 2014).

20 mg of cell suspension culture was placed in each well of a 24-well culture plate (Nunclon, Roskilde, Denmark) in 500 μl of serum-free medium containing 1% bovine serum albumin (Sigma) for 2 h. Oviductal cells culture media were supplemented with genistein (Sigma, G6649) at different concentrations: 0.2, 2 and 10 μM or supplemented with 1 μl/ml DMSO, used as a vehicle of genistein in control cultures. Genistein concentrations were selected based on the findings of other studies (Reinhart et al., 1999; Woclawek-Potocka et al., 2013). Cells were incubated for 24 h at 38.5 °C in a humidified atmosphere and 5% CO_2.

2.1.2 Total RNA isolation

Bovine oviductal epithelial cell cultures ($n = 3$ per treatment) were isolated using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). The yield of total RNA was determined spectroscopically at 260 nm. RNA quantity and quality were verified after electrophoresis on 1% (*w*/*v*) formaldehyde agarose gel containing 0.06 μg/ml Sybr ® Safe DNA Gel Stain (Invitrogen, Burlington, ONT, Canada).

2.1.3 Reverse Transcription

Total RNA (1 μg) from each cell culture was reverse transcribed using Moloney murine leukaemia virus enzyme (Promega, Madison, WI, USA) and oligo(dT)17 (García et al., 2014). A 25 μl final volume of the reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pM oligo(dT), 10 mM dithiothreitol and 200 units of reverse transcriptase was incubated at 42 °C for 1 h followed by a reverse transcriptase inactivation at 94 °C for 5 min.

2.1.4 Real Time RT-PCR

In order to determine *CYCLIN B1*, *BCL-2* and *BAX* expression levels, real time RT-PCR assays were carried out in a BioRad CFX96 system. Real-time PCR was performed with a total volume of 20 μl, each reaction containing 5 μl of 1/20 dilution of cDNA determined by a standard curve, 5 μl of a 2 μM of primer pairs stock solution (forward and reverse), 1 μl of EvaGreen® qPCR (Biotium), 2.0 U of Taq DNA polymerase (Invitrogen, Burlington, ONT, Canada), 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$ and 0.2 mM of each dNTP. All primers were designed to amplify a product with a size of 117–171 bp (Table 1), and the specificity of each primer set was confirmed by running the PCR products on a 1.5% agarose gel. PCR conditions were as follows: 94 °C for 2 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s using a melting-curve program (increasing the temperature from 65 °C to 95 °C at a rate of 0.5 °C every 10 s) and continuous fluorescence measurement. Sequence-specific products were identified by generating a melting curve, the Ct value represented the number of cycles at which a fluorescent signal rose statistically above background and relative gene expression was quantified. Standard curves were performed using different concentrations of the cDNA samples to determine the efficiency of primers. The calculation used was previously detailed by other authors (Livak and Schmittgen, 2001). The relative ratios of mRNA levels were normalized. Genes were first normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) Ct value. The ratio of the relative values of *BCL-2* and *BAX* was also determined (*BCL-2*/*BAX*). Each gene was analyzed in triplicate.

2.2 Wound healing assay in monolayer cell cultures

2.2.1 Oviductal epithelial monolayer cell cultures

For each experiment, four ipsilateral oviducts at the mid-luteal stage were used to obtain oviductal epithelial cells for primary cultures. The collected cells were incubated at 38.5 °C, 5% CO_2 and 100% humidity for 48 h to obtain cell suspension cultures. Later incubation of these cells cultures for 5 days allowed to obtain monolayer cell cultures. Then, cultures were washed twice with PBS, resuspended in 2 ml of trypsin-EDTA (Gibco BRL) and incubated for 3 min at 37 °C. Trypsin action was blocked with 2 ml of culture medium (Lopera-Vasquez et al., 2016). The cells were recovered in a sterile Petri dish and disaggregated mechanically with a pipette. Cells were collected in a 50 ml sterile conical tube with 15 ml medium TCM-199 and washed twice at room temperature. Material was collected in culture medium TCM-199 supplemented with 10% bovine fetal serum, 25 mM Hepes 0.2 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. The cells (2×10^5/ml) were incubated at 38.5 °C, 5% CO_2 and 100% humidity in 24-well culture plates until confluence. After that, monolayer cell cultures were incubated with 500 μl of serum-free medium containing 1% bovine serum albumin for 2 h.

2.2.2 Wound healing assay

Wound healing assays were performed to study cell migration capacity. Artificial gaps were made in each monolayer using a 200 μl pipette tip. Immediately after creating a scratch of cultures, cells were washed and incubated in medium supplemented with genistein at 0.2

Table 1
Primers used to amplify specific bovine transcripts from oviductal epithelial cells.

Gene symbol	Gene name		Primer sequence (5′ - 3′)	Fragment size (bp)	GenBank access No.
BAX	BCL2-Associated X Protein	Forward	GACTCTCCCCGAGAGGTCTT	171	NM_173894.1
		Reverse	GTCCAATGTCCAGCCCATGA		
BCL2	B-Cell CLL/Lymphoma 2	Forward	GAGTTCGGAGGGGTCATGTG	158	NM_001166486.1
		Reverse	GGGCCATACAGCTCCACAAA		
CYCLIN B1	Cyclin B1	Forward	AAGACGGAGCGGATCCAAAC	121	NM_001045872.1
		Reverse	CCAGTGACTTCACGACCCAT		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward	AGATGGTGAAGGTCGGAGTG	117	NM_001034034
		Reverse	GAAGGTCAATGAAGGGGTCA		

and 10 μM ($n = 3$). Control cells were incubated in medium plus 1 μl/ml DMSO. Images were captured at different intervals (0, 6, 12 and 24 h) using inverted light microscopy (Zeiss Axiovert 25) and photographed ($n = 3$) with a digital camera (Arcano). In order to obtain the same field of interest during the observations, etches were made on the bottom of each well and used as microscopic reference points to make sure that measurements were always carried out at the exact same spot (Jordaens et al., 2015). Measurements of each scratch area were obtained using the ImageJ software at every time point and the percentage of wound region (% WR) was determined (wound region/total size of image per 100) (Cardona, 2015).

2.2.3 Staining procedure of BOEC cultures with Hoëchst

Monolayer cells with genistein 0.2 and 10 μM, and controls ($n = 3$) were fixed at 24 h of culture in 4% formaldehyde in PBS, pH 7.4, for 1 h at room temperature and stored in ethanol 70% at 4 °C, for no more than 7 days. Samples were washed five times with PBS. Cells were incubated in 1 μg/ml Hoëchst 33342 solution for 15 min in the dark and washed three times with PBS, mounted in 5 μl of mountain medium and covered with a coverslip. Samples were examined immediately at 400× in an epifluorescence microscope (Olympus BX40). Staining culture cells were photographed at different fields ($n = 5$) of each well culture plate. Microphotographs were analyzed using the ImageJ software. The total number of cells was determined by counting nuclei dye. Also, mitotic and apoptotic cells were recorded. Apoptosis was determined by examining nuclear morphology, detecting the presence of chromatin condensation or nuclear fragmentation (Syed Abdul Rahman et al., 2013; Ulukaya et al., 2011). Percentages of apoptotic and mitotic cells were determined (number of apoptotic or mitotic cells/total cells per 100) for each image analyzed.

2.2.4 Statistical studies

All the experiments were carried out in triplicate and the means of the data were calculated. Real Time RT-PCR, wound closure and percentage of apoptotic and mitotic cells data are expressed as mean ± standard error of the mean (SEM). The statistical analysis of the data was performed using the Minitab 15 and the InfoStat (InfoStat 2015, http://www.infostat.com.ar) statistical softwares. ANOVA was applied for residues analysis. When they showed normality, significant differences between the mean values were determined (LSD Fisher's test, $p < 0.05$).

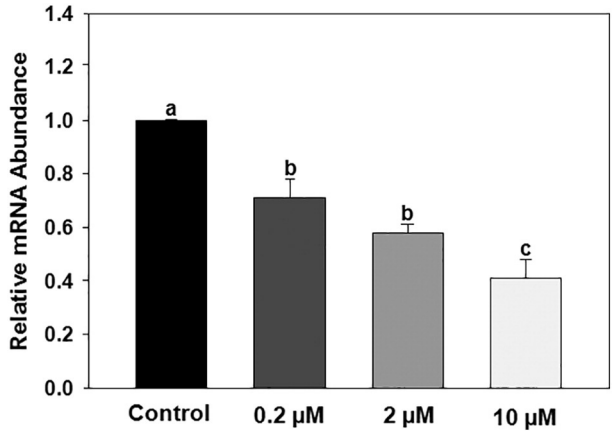

Fig. 1. *CYCLIN B1* expression in bovine oviductal epithelial cell cultures. Bars represent relative mRNA levels at different genistein concentrations in the culture medium. Values are the mean ± SEM ($n = 3$). a, b and c indicate significant differences between treatments ($p < 0.05$).

3. Results

3.1 CYCLIN B1 expression in oviductal epithelial cells

Real Time RT-PCR analysis was used to evaluate *CYCLIN B1* expression in BOEC cultures treated with increasing genistein concentrations: 0.2 μM, 2 μM and 10 μM. As shown in Fig. 1, genistein induced a concentration-dependent decrease in *CYCLIN B1* mRNA expression. Significant differences were found when comparing treatments *versus* control. The lowest expression level was observed at 10 μM of genistein.

3.2 BCL-2/BAX ratio in bovine oviductal epithelial culture cells

mRNA expression of *BCL-2* and *BAX* was analyzed by Real Time RT-PCR in BOEC cultures at 24 h with different genistein concentrations (0.2 μM, 2 μM and 10 μM); then, the *BCL-2/BAX* ratio was calculated (Table 2). No significant variations were observed at genistein 0.2 μM, but higher genistein concentrations (2 and 10 μM) significantly reduced this ratio (Fig. 2).

3.3 Wound healing assay

Monolayer cultures were incubated with 0.2 and 10 μM of genistein and wound healing assays were performed. In the control group, the scratched areas showed a progressively decrease from 0 to 24 h. As shown in Fig. 3, since 12 h, genistein significantly affected cell ability to close the scratched area at both assayed concentrations (Fig. 3). In order to analyze if genistein affected proliferation and/or apoptosis of BOEC in these experiments, a nuclear Hoëchst staining was performed on monolayer cultures with and without genistein. Total cells per field were counted and the percentages of mitosis and apoptosis were determined. Mitotic cell percentage was significantly reduced in cultures treated with 10 μM of genistein compared with the control; genistein at 0.2 μM did not affect the mitotic index. No significant differences were observed in the percentage of apoptotic cells between treated and control cells (Fig. 4).

4. Discussion

The oviduct plays a pivotal role in mammalian reproduction by providing an optimal environment for sperm capacitation, fertilization and transport of gametes and embryos (Gonella-Diaza et al., 2015; Killian, 2004; Lopera-Vasquez et al., 2016; Maillo et al., 2016a; Maillo et al., 2016b). Genistein is an isoflavone that affects important process in the female reproductive tract. A previous report claims that morphology, histology and the expression of mucosal immune response genes on the oviduct were affected by genistein (Jefferson et al., 2012). Genistein affects the synthesis of leukaemia inhibitory factor in human and bovine oviductal cells in a concentration-dependent manner. This factor plays a key role in fertilization and implantation (Aghajanova, 2010; Reinhart et al., 1999). It is known that this isoflavone also induces endothelin-1, a vasoconstrictor that plays an important role within the endocrine/reproductive system and regulates tubal contractility (Reinhart et al., 2003). Genistein has deleterious effects and may affect the biology and physiology of the oviduct (Jefferson et al., 2012). Alterations in the epithelial oviductal cells are not completely understood; this is

Table 2
BCL-2, *BAX* and *BCL-2/BAX* ratio expression in bovine oviductal epithelial culture cells.

Genistein	BCL-2	BAX	BCL-2/BAX
Control	1.00 ± 0.001	1.00 ± 0.002	1.00 ± 0.001[a]
0.2 μM	1.25 ± 0.12	0.73 ± 0.12	1.22 ± 0.25[a]
2 μM	0.88 ± 0.03	1.64 ± 0.28	0.27 ± 0.03[b]
10 μM	0.70 ± 0.17	1.19 ± 0.22	0.32 ± 0.02[b]

a, b indicate differences between samples ($p < 0.05$).

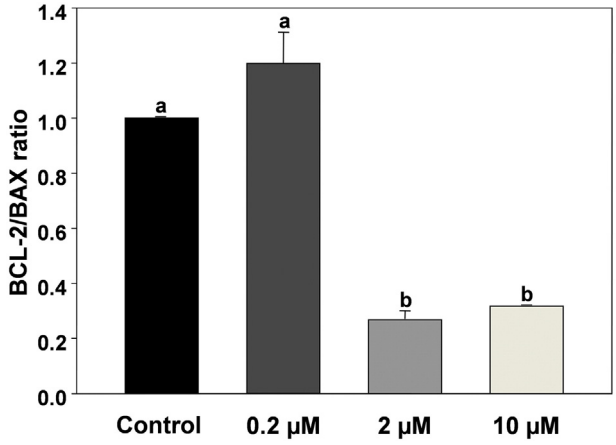

Fig. 2. Relationship between *BCL-2/BAX* expression in bovine oviductal epithelial cell cultures. Bars represent the ratio of *BCL-2/BAX* expression levels at different genistein concentrations in the culture medium. Values are the mean ± SEM ($n = 3$). a and b indicate significant differences between treatments ($p < 0.05$).

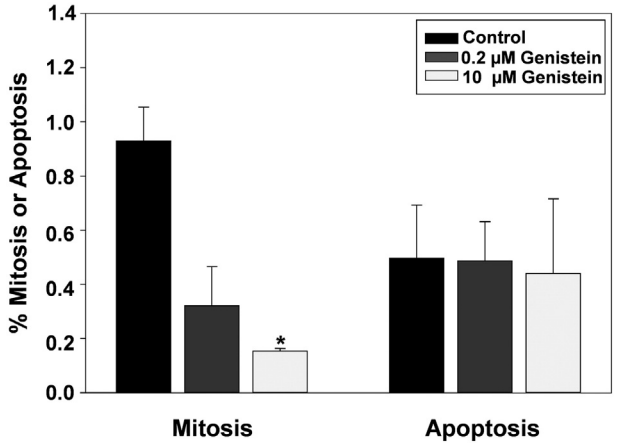

Fig. 4. Percentage of mitotic and apoptotic cells of bovine oviductal epithelial cell cultures stained with Hoëchst. Mitosis or apoptosis were represented as percentages of cells incubated with genistein at different concentrations after 24 h. Values are the mean ± SEM ($n = 3$). * indicates differences between control and treated samples ($p < 0.05$).

why in this work the action of genistein over oviductal epithelial cells was analyzed in order to determine how apoptosis, proliferation and migration are affected.

In the present work, mid-luteal oviducts were used. In this stage, we previously studied the expression of genes involved in oviduct extracellular matrix remodeling, which could be relevant to understand the changes in oviductal physiology (García et al., 2014; Roldán-Olarte et al., 2012) since matrix reorganization is closely associated with tissue morphogenesis and cell proliferation (Gonella-Diaza et al., 2015). Bovine oviductal epithelial cell cultures in suspension were used in this research because this model retains their ciliary and secretory activity (Walter, 1995) and maintains functional morphology. Ultrastructural features and the epithelial cell structure are similar to *in vivo* conditions (Rottmayer et al., 2006).

CYCLIN B1 is a key molecule for G2/M transition during the cell cycle; it has been found that genistein decreases its expression in different cellular types (Moskot et al., 2015). In this work the results indicate that genistein induces a down-regulation of the *CYCLIN B1* mRNA expression in the BOEC. The inhibitory effect on *CYCLIN B1* expression was observed at different concentrations in a dose-dependent manner. Also, the percentages of mitotic cells were affected with the addition of 10 μM of genistein when cultures were stained with a Hoëchst dye. This is the first evidence of the effect of genistein on bovine oviduct studying cell proliferation by expression of *CYCLIN B1*. Nevertheless, other cell cycle-related genes/proteins such as *CYCLIN A1*, *CYCLIN D1*, proliferating cell nuclear antigen (PCNA), MET proto-oncogene receptor tyrosine kinase (C-MET), Ki-67 as well cell cycle affecting factors like connective tissue growth factor (CTGF), platelet derived growth factor D (PDGFD) and transforming growth factor-beta (TGF-β) family should also be useful to confirm genistein effect on BOEC.

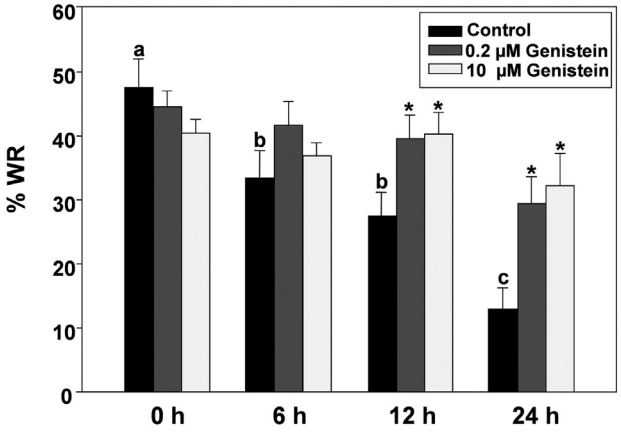

Fig. 3. Quantification of *in vitro* wound region (WR) in bovine oviductal epithelial cell cultures. Bars represent the percentage (% WR) in cultures with genistein at different times. Values are the mean ± SEM ($n = 3$). Different letters represent significant differences between samples ($p < 0.05$). * indicates differences between control and treated samples at the same time point.

The ratio between the anti-apoptotic *BCL-2* and the pro-apoptotic *BAX* predicts cell response to the apoptotic pathways (Gazzaniga et al., 1996). In the present study, the mRNA expression of both *BCL-2* and *BAX* were evaluated in BOEC cultures exposed to different genistein concentrations. Results suggest that the reduction in *BCL-2/BAX* ratio observed at 2 μM and 10 μM of genistein and those obtained by others authors (Choi et al., 1998) would be related to the decrease in cell survival. Interestingly, as judged by the Hoëchst staining, genistein did not induce an increase in apoptosis in BOEC cultures, suggesting that the reduction in the *BCL-2/BAX* ratio may be due to the cell cycle arrest produced by genistein and indicates that genistein would not have a cytotoxic effect at the assayed concentrations. However, in order to confirm if genistein induces apoptosis, it would be necessary to study the expression levels of other genes like p53, caspases and apoptotic protease activating factor 1 (APAF-1) as well as cells with DNA fragmentation and phosphatidylserine translocation.

The wound healing assay is a well-known methodology for the *in vitro* study of cell proliferation and migration (Jordaens et al., 2015; Latifi-Pupovci et al., 2015). This method is performed after confluent cell monolayer disruption. Cell monolayer reconstruction during *in vitro* wound healing assay is the result of cell proliferation and migration from wound edges toward the open area once new cell-cell contacts are established (Cardona, 2015). Oviductal epithelial cell cultures were treated with 0.2 and 10 μM of genistein and wound healing was evaluated for 0, 6, 12 and 24 h. The effect of genistein showed that the percentage of wound edge region at 12 and 24 h did not decrease at the same rate as its respective controls.

These observations demonstrated that genistein has a significant action on the migration and proliferation of BOEC primary cultures from the mid-luteal stage. In this research BOECs from the follicular phase were not analyzed. Considering that high estradiol concentrations have an effect on the regulation of BOEC proliferation (Kamwanja and Hansen, 1993), further studies should be carried out to confirm if the action of genistein has a similar effect on cells under different hormonal conditions in the evaluated processes.

In conclusion, genistein has an inhibitory action on proliferation and migration on bovine oviductal epithelial cells in *in vitro* conditions. *In vivo* studies should be carried out to evaluate the effect of genistein

incorporated through diets rich in soybean used to feed farm animals on the above processes.

These results contribute to the knowledge of how genistein could affect the oviductal microenvironment, modifying molecular mechanisms involved in the maintenance of the normal physiology of the oviductal epithelium, necessary for successful reproductive events. Further studies would be necessary to clarify the effect of genistein on fertilization and embryo development.

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