Research in Veterinary Science 120 (2018) 33–40

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effects of frutalin and doxorubicin on growth, ultrastructure and gene expression in goat secondary follicles cultured *in vitro*

M.A.A. Soares-Costa[a], J.J.N. Costa[a], A.W.B. Silva[a], E.V. Cunha[a], L.R.F.M. Paulino[a], B.R. Silva[a], A.L.C. Silva[b], R. van den Hurk[c], J.R.V. Silva[a,*]

[a] Biotechnology Nucleus of Sobral (NUBIS), Federal University of Ceará, Sobral, Ceará, Brazil
[b] Molecular Biotechnology Laboratory (LabBMol), Federal University of Ceará, Fortaleza, Ceará, Brazil
[c] Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

ARTICLE INFO

Keywords:
Frutalin
Doxorubicin
Goat
Secondary follicles
in-vitro culture
Gene expression

ABSTRACT

This study evaluated the effects of frutalin (0.6, 6.0 or 60.0 μg/mL) and doxorubicin (0.3 μg/mL) on survival, growth and ultrastructure of *in-vitro* cultured goat secondary follicles. The effects of these substances on the levels of mRNA for *Casp3*, *Casp6, Bax*, and *Bcl2* were also investigated. Results showed that, after 6 days of culture, frutalin or doxorubicin reduced the percentage of normal follicles ($P < 0.05$), but doxorubicin had higher toxicity than frutalin. Except for follicles cultured with 0.6 μg/mL frutalin, follicular growth rate was reduced after culture with doxorubicin or frutalin ($P < 0.05$). The presence doxorubicin or 60.0 μg/mL frutalin increased the levels of mRNA for *Casp3*, *Casp6, Bax*, and *Bcl2* ($P < 0.05$). Higher mRNA levels for *Casp3*, *Casp6 and Bax* were found in follicles cultured with doxorubicin, but higher levels of *Bcl2* mRNA were found in follicles cultured with frutalin ($P < 0.05$). In conclusion, frutalin has lower toxic effects than doxorubicin on secondary follicles cultured *in vitro*.

1. Introduction

Lectins are proteins that recognize carbohydrates and glycoconjugates in cells, tissue sections, and biological fluids, having large potential for biomedical applications (Santos et al., 2013; Silva et al., 2014). Frutalin, an α-D-galactose-specific carbohydrate-binding lectin, induces *in vivo* and *in vitro* migration of neutrophils, chemotaxis, reorganization of cytoskeleton, and interferes with cellular motility and oxidative response (Brando-Lima et al., 2005; Oliveira et al., 2009; Oliveira et al., 2011). Previous *in-vitro* studies have shown that frutalin, at concentration of 60 μg/mL, induces apoptosis and inhibits proliferation of HeLa cells (Oliveira et al., 2011). However, it is well known that chemical agents used to destroy cancer cells also damage ovarian function and female fertility (Morgan et al., 2012; De Vos et al., 2014). For example, doxorubicin, which is widely used, at the concentration of 0.3 μg/mL for the treatment of different cancers, including sarcomas (Maruzzo et al., 2013) and ovarian tumors (Nicoletta et al., 2014). Doxorubicin has deleterious effects on female fertility even at low doses (Nishi et al., 2017). The adverse effects of this substance includes ovarian toxicity in mice by reducing ovulation rate and ovary size (Ben-Aharon et al., 2010), as well as increasing infertility and precocious menopause rate in women with ovarian activity (Letourneau et al., 2012). Doxorubicin intercalates into DNA, and disrupts the action of topoisomerase-II-mediated DNA repair and promotes generation of free radicals and damages to cellular membranes, DNA and proteins (Gewirtz, 1999). Doxorubicin induces apoptosis or programmed cell death in mice ovarian follicles (Ben-Aharon et al., 2010). Apoptosis is regulated by balance between proapoptotic and antiapoptotic molecules, such as *Bax* and *Bcl2*, respectively. Caspases are essential proteins in the process of apoptosis, caspase 3 and 6 both acting as effectors (Kaipia and Hsueh, 1997; Hussein, 2005; Matsuda et al., 2012).

Oliveira et al. (2011) reported that frutalin induces apoptosis in Hella cells and thus, it is relevant to investigate the effects of this lectin on early follicles, since they represent > 90% of the follicular population. Recently, an *in-vitro* study showed that frutalin did not influence caprine primordial follicle activation, but increased mRNA expression of pro-apoptotic genes and induced changes in the structural characteristics of primordial follicles that were cultured within ovarian tissue fragments (Soares et al., 2018). However, it is still unknown whether frutalin has toxic effects on secondary follicles cultured *in vitro*. Additionally, the effects of frutalin on gene expression and ultrastructural features of secondary follicles cultured *in vitro* were still not

* Corresponding author: Biotechnology Nucleus of Sobral - NUBIS, Federal University of Ceará, Av. Comandante Maurocélio Rocha Ponte 100, CEP 62041-040 Sobral, CE, Brazil.
E-mail address: jrvsilva@ufc.br (J.R.V. Silva).

https://doi.org/10.1016/j.rvsc.2018.08.006
Received 23 December 2017; Received in revised form 24 August 2018; Accepted 29 August 2018

reported.

The aim of the current study is to investigate and to compare the effects of frutalin and doxorubicin on *in-vitro* survival and ultrastruture of 6-days cultured goat secondary follicles, and on mRNA expression of *Casp3*, *Casp6, Bax*, and *Bcl2* therein.

2. Materials and methods

2.1. Chemicals

The culture media and most of the chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO). Substances purchased from other companies were mentioned in the text.

2.2. Isolation and purification of frutalin

Frutalin was isolated from mature seeds of *Artocarpus incisa* L. as described previously (Moreira et al., 1998; Soares et al., 2018). In short, after recovering the seed extract, the protein solution was applied to an agarose–D-galactose column and frutalin was eluted in phosphate-buffered saline (PBS, pH 7.4). The presence and purity of the frutalin were checked by SDS-PAGE.

2.3. Source of ovaries

Goat ovaries (*Capra hircus*) (n = 50) were collected in a local abattoir and washed in 70% alcohol for 10 s, as well as in saline solution (0.9% NaCl) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovaries were transported to the laboratory in a thermal container at 4 °C within 1 h, as described by Chaves et al. (2008).

2.4. Isolation and culture of goat secondary follicles

To isolate secondary follicles, ovarian cortical fragments (1 to 2 mm thick) were recovered from the ovarian surface. Then, the ovarian tissue was placed in minimum essential medium (MEM) plus HEPES. Secondary follicles (~200 µm in size) were visualized under a stereo microscope (SMZ 645 Nikon, Tokyo, Japan) and dissected from slices of ovarian cortex using 26 gauge needles. After isolation, secondary follicles were put 100 µL drops of fresh medium to evaluate the follicular integrity. Follicles that had a visible oocyte, well-organized granulosa cells, intact basement membrane and absence of antral cavity were selected for culture.

Secondary follicles were cultured individually in 100 µL drops of medium in petri dishes (60x15mm, Corning, USA). The control medium was α-MEM supplemented with bovine serum albumin (3 mg/mL), 2 mM hypoxanthine, 2 mM glutamine, 10 ng/mL insulin, 5.5 µg/mL transferring, 5 ng/mL selenium, 50 µg/mL ascorbic acid and 100 ng/mL FSH (α-MEM$^+$). The secondary follicles were randomly cultured in α-MEM$^+$ (control medium) alone or supplemented with 0.3 µg/mL doxorubicin, and 0.6, 6.0 or 60.0 µg/mL frutalin. Concentrations of doxorubicin and frutalin were chosen according to results of previous studies (Oliveira et al., 2011; Maruzzo et al., 2013). Around 40 follicles were cultured in each treatment, and the experiment was repeated four times. The follicles were cultured at 39 °C, in 5% CO_2 in air for 6 days. Every other day, 60 µL of fresh medium were replaced in each drop. After culture, follicle growth, the presence of antral cavity and the percentages of normal follicles were evaluated. In addition, some follicles were stained to evaluate their viability by fluorescence microscopy or stored at −80 °C for PCR.

2.5. Morphological evaluation of cultured follicles

Follicles were evaluated with stereomicroscope (SMZ 645 Nikon, Tokyo, Japan), ant those that had a spherical oocyte surrounded by well-organized granulosa cells, as well as intact basement membrane and stromal-thecal outer layer were classified as morphologically normal. Follicles with dark oocyte and opaque granulosa cells were considered degenerated. To evaluate follicle diameters, two perpendicular measurements were made in the normal follicles using an inverted microscope with NIS Elements 2.4 software (Nikon, Nikon Instruments Inc., Americas). Moreover, the percentages of secondary follicles that reached antrum formation *in vitro* were determined. Once a translucent cavity was visible between the granulosa cells, the antrum was considered to be formed.

2.6. Assessment of secondary follicle viability by fluorescence microscopy

Follicles cultured in all treatments were evaluated by using fluorescent probes to determine their viability. To this end, follicles were incubated in 100 µL droplets of medium (α-MEM$^+$) that contained 4 mM calcein-AM and 2 mM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37 °C for 15 min. Then, the follicles were washed in α-MEM$^+$ and evaluated under fluorescence microscope (Nikon, Eclipse, TS 100). Oocytes and granulosa cells were classified as viable when the cytoplasm was stained positively with calcein-AM (green) and when the chromatin was not labeled with ethidium homodimer-1 (red) (Schotanus et al., 1995; Van Den Hurk et al., 1998).

2.7. Ultrastructural analysis

Ultrastructural evaluation of secondary follicles from fresh control (n = 5), as well as those cultured in control medium alone (n = 5) or supplemented with frutalin [60 µg/mL] (n = 5) or doxorubicin (n = 5) was performed as described previously (Soares et al., 2018). The follicles were fixed in Karnovsky solution (2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2), and post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h. After washing in sodium cacodylate buffer, the follicles were counterstained with 5% uranyl acetate. The samples were then dehydrated in acetone and embedded in epoxy resin (Epoxy-Embedding Kit, Fluka Chemika-BioChemika). Semi-thin sections (2 µm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400× magnification. Subsequently, ultra-thin sections (70 nm) were obtained and counterstained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Fei Tecnai Spirit).

2.8. Quantification of mRNA for Casp3, Casp6, Bax, and Bcl2 in cultured follicles

From four samples of five follicles cultured in each treatment, total RNA extraction and real time PCR were performed as described previously (Soares et al., 2018). Quantification of mRNA was performed using SYBR Green in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The primers were designed to amplify mRNA for *Casp3*, *Casp6, Bax*, and *Bcl2* and *B-actin* (housekeeping gene) (Table 1). Primer efficiency was determined by using serial dilutions of the target cDNA and the specificity of each primer pair was confirmed by melting curve analysis of the PCR products. The reactions were performed in triplicate in a StepOnePlus™ Real Time System (Applied Biosystems, Foster City, California, USA). Negative controls consisted of reactions without cDNA. The relative quantification of gene expression was evaluated by delta-delta-CT (*dd*CT) method (Livak and Schmittgen, 2001). The data were calculated from three independent replicates.

2.9. Statistical analysis

Data of follicular diameter were subjected to logarithmic transformation (log10 (x)) and comparisons among the different treatments