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From Cell Biology to Tissue Engineering

Effects of frutalin on early follicle morphology, ultrastructure and gene expression in cultured goat ovarian cortical tissue

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Summary. Frutalin is a galactose-binding lectin that has an irreversible cytotoxic effect on HeLa cervical cancer cells, by inducing apoptosis and inhibiting cell proliferation. It was previously shown that after *in vitro* incubation, frutalin is internalized into HeLa cells nucleus, which indicates that frutalin apoptosis-inducing activity might be linked with its nuclear localization. Considering that drugs commonly used for cancer treatment have a deleterious effect on germ cells, the aim of this study was to evaluate the effect of frutalin on the activation, survival, ultrastructure and gene expression in follicles cultured within ovarian tissue. Goat ovarian fragments were cultured for 6 days in α-MEM$^+$ alone or supplemented with frutalin (1, 10, 50, 100 or 200 μg/ml). Non-culturad and cultured tissues were processed for histological and ultrastructural analysis and they were also stored to evaluate the expression of anti- and pro-apoptotic genes by quantitative polymerase chain reaction (qPCR). The results showed that the frutalin, at all concentrations tested, reduced follicular survival when compared with control medium. Higher concentrations of frutalin (50, 100 or 200 μg/ml) also reduced follicular survival when compared with those tissues cultured with 1 or 10 μg/ml of frutalin. The ultrastructural analysis showed that atretic cultured follicles had retracted oocytes and a large number of vacuoles spread throughout the cytoplasm. In addition, signs of damage of mitochondrial membranes and cristae were observed. Moreover, although a dose-response effect on gene expression has not been observed, when compared with tissues culture in control medium, the presence of frutalin increased in mRNA expression pro-apoptotic genes. In conclusion, frutalin reduces follicular survival at all concentrations tested, its effects being more pronounced when high concentrations of this lectin (50, 100 and 200 μg/ml) are used. Gene expression profile and ultrastrutural features of cultured follicles suggest that follicular death in goat ovarian tissue cultured in presence of frutalin occurs via necrosis.

Key words: Frutalin, Ovary, Goat, Follicles

Introduction

Lectins are a well-known group of carbohydrate-binding proteins that mediate a variety of biological processes, especially the recognition of cancer-associated oligosaccharides (Kabir, 1998). Cancer cells exhibit cellular changes in glycosylation (Kim and Varki, 1997) and it has been reported that lectins are able to agglutinate malignantly transformed cells, but not their normal parental cells (Inbar and Sachs, 1969; Fang et al.,

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Abbreviations. TNFA, tumor necrosis factor-[alpha]; TNFRI, Tumor necrosis factor receptor 1; TNFRII: Tumor necrosis factor receptor 2; BAX, BCL2-Associated X Protein; BCL2, B cell lymphoma gene 2; MEM, Minimum essential medium.

2011). In an attempt to develop alternative treatments for cancer diseases, frutalin (a galactose-binding jacalin-related lectin) has received considerable attention because of its biological properties (Brando-Lima et al., 2005; Oliveira et al., 2009, 2011). This lectin belongs to the family derived from *Artocarpus incisa* and specifically binds α-D-galactose (Moreira et al., 1998; Nobre et al., 2010). Previous *in vitro* studies have shown that frutalin is internalized into HeLa cells nucleus and has an irreversible cytotoxic effect on these cells, by inducing apoptosis and inhibiting cell proliferation (Oliveira et al., 2011). However, it is well known that drugs commonly used for cancer treatment have a deleterious effect on germ cells (Arnon et al., 2001; Akdemir et al., 2014), which results in primordial follicle depletion and, as a consequence, premature menopause, subfertility, infertility and associated symptoms (Jeruss and Woodruff, 2009; Schmidt et al., 2010). Considering that frutalin is capable of inducing apoptosis in cancer cells (Oliveira et al., 2011) and, thus, may be potentially used in the future as an anticancer agent (Brando-Lima et al., 2005; Oliveira et al., 2009), it is very important to evaluate whether this lectin has deleterious effects on female gametes, e.g., on the oocytes enclosed in primordial follicles.

Primordial follicles are very important for fertility preservation, as they are the most abundant follicle class (Hornick et al., 2012). In primate species, the primordial follicle pool is established shortly before birth (Gougeon, 1996; Bristol-Gould et al., 2006). Females are born with 1 million primordial follicles, and this number continuously declines with age, as dormant primordial follicles are either lost by degeneration or activated to proceed through folliculogenesis (Faddy et al., 1992; Adhikari and Liu, 2009). Ovarian follicular death, also known as follicular atresia, is an apoptotic process controlled by hormones, and may occur at any stage of development (Carroll et al., 1990; Kaipia and Hsueh, 1997). The balance between several pro-apoptotic (BAX, BCL2-associated X protein) and pro-survival molecules (BCL2, MCL1 and BCLXL) regulates the process of apoptosis in ovarian follicles (Hussein, 2005; Matsuda et al., 2012). Tumor necrosis factor-α (TNFA) and its receptors (TNFRI and TNFRII) also have critical roles in the induction of apoptosis. The action of this factor is through interaction with its receptors on the cell surface, resulting in the aggregation of receptors and recruitment of signaling proteins. The TNFRI acts through its death domain in its cytoplasmic region, which interacts with the intracellular transducer and results in aggregation and activation molecules, such as caspases, leading to cell death (Hussein, 2005). However, it is unknown whether frutalin regulates the expression of mRNAs for factors involved in apoptosis, nor if it induces morphological or ultrastructural changes in primordial follicles cultured within goat ovarian cortical tissue.

The aim of this study was to evaluate the effect of different concentrations of frutalin on follicular activation, survival, ultrastructure, and expression of mRNA for *TNFA* and its receptor (*TNFRI*), as well as that for *BCL2, BAX* and the *CASPASES 3* and *6*, after 6-days culture of caprine ovarian cortical tissue. This species was chosen because it has been successfully used as an experimental model for studying the causes, mechanisms and therapy of human diseases (Haldar and Ghosh, 2014).

Materials and methods

Chemicals

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO).

Isolation and purification of frutalin

Mature seeds from *Artocarpus incisa* L. were cut into small fragments, dried in acetone (P.A.) and ground into a fine powder using a coffee mill. The powder was incubated at a 1:10 ratio (w/v) in phosphate-buffered saline (PBS, pH 7.4) at room temperature with continuous stirring for 2 h before centrifugation at 10,000 x g for 20 min at 5°C. The supernatant (crude extract) was precipitated with ammonium sulfate (0-90% of saturation, $F_{0/90}$) and centrifuged at 12 000 x g for 30 min at 5°C. The pellet was suspended and dialyzed in PBS pH 7.4. The protein solution ($F_{0/90}$) was applied to an agarose-D-galactose column (8x1 cm) equilibrated with the same buffer. After removing the unbound material, frutalin was eluted with 100 mM D-galactose in an equilibrium solution. The presence and purity of the lectin in the pooled fractions were checked by SDS-PAGE 15%. Fractions containing pure frutalin were dialyzed against PBS pH 7.4 and concentrated in 10 KDa Amicon® (Millipore) tubes (Moreira et al., 1998).

Effect of different concentrations of frutalin on survival and growth of primordial follicles

Ovaries (n=12) were obtained from six cross-breed goats (*Capra hircus*) collected at a slaughterhouse. Immediately postmortem, ovaries were washed in 70% alcohol for 10 sec followed by two times in saline solution (0.9% NaCl) containing antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Ovaries were transported within 1 h to the laboratory in a saline solution containing antibiotics at 4°C.

The culture system was earlier described in detail by Ribeiro et al. (2015). Briefly, ovarian cortical tissue from the same ovarian pair was cut in 21 slices (3x3x1 mm) using scissors and scalpel under sterile conditions. The tissue pieces were distributed for the following analyses: transmission electron microscopy (one fragment), classical histology (one fragment) and polymerase chain reaction (PCR) (one fragment) in uncultured control. The remaining fragments were cultured for 6 days (three

fragments per treatment). Cortical tissues were transferred to 24-well culture dishes containing 1 mL of culture media. Culture was performed at 39°C in 5% CO_2 in a humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2-7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, antibiotics 100 IU/mL penicillin and 100 mg/mL streptomycin, 50 μg/mL ascorbic acid, 3.0 mg/mL of bovine serum albumin (α-MEM$^+$). The ovarian cortical fragments were cultured in control medium (α-MEM$^+$) alone or supplemented with different concentrations of frutalin (1, 10, 50, 100 or 200 μg/mL). Every 2 days, the culture medium was replaced with fresh medium. After culture in each treatment, fragments were fixed for classical histology, transmission electron microscopy or stored at -80°C for PCR. This procedure was repeated six times.

For histological studies, after six days of culture, the pieces of ovarian tissue were fixed overnight at room temperature in 4% paraformaldehyde in PBS pH 7.4. After fixation, the ovarian fragments were dehydrated in a graded series of ethanol, clarified with xylene, and embedded in paraffin. For each piece of ovarian cortex, 7 μm sections were mounted on slides and stained with eosin and hematoxylin. Coded anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at x100 and x400 magnification. The developmental stages of follicles were classified as primordial follicles (one layer of flattened or flattened and cuboidal granulosa cells around the oocyte) or growing follicles (primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte) (Silva et al., 2004).

These follicles were further classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that were well organized in one or more layers, and had no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte with a pyknotic nucleus and/or surrounded by disorganized granulosa cells detached from the basement membrane. Overall, a number ranging from 247 to 398 follicles were evaluated for each treatment. The percentages of healthy primordial and developing follicles were calculated before (fresh control) and after culture in a particular medium.

Ultrastructural analysis of cultured ovarian tissue

To better examine follicular morphology, transmission electron microscopy (TEM) was performed to analyze the ultrastructure of normal follicles from uncultured and cultured tissues, as well as from follicles with signs of atresia after 6 days of culture. Experiments and analyses involving electron microscopy were performed in the Center of Microscopy at the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (http://www.microscopia.ufmg.br). A portion with a maximum dimension of 1 mm^3 was cut from each fragment of ovarian tissue and fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer, pH 7.2) for 3 h at room temperature. Specimens were 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 at room temperature, washed in sodium cacodylate buffer and counterstained with 5% uranyl acetate. The samples were then dehydrated through a gradient of acetone solutions and thereafter embedded in epoxy resin (Epoxy-Embedding Kit, Fluka Chemika-BioChemika). Afterwards, semi-thin sections (2 μm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400x magnification. Subsequently, ultra-thin sections were counterstained with uranyl acetate and lead citrate and examined under a transmission electron microscope (FEI, Tecnai G2 Spirit, Oregon, USA). The density and integrity of ooplasmic and granulosa cell organelles, as well as vacuolization, and basement membrane integrity were evaluated.

Expression of mRNA for TNFA, TNFRI, CASPASE 3, CASPASE 6, BAX and BCL2 in cultured cortical tissue

To evaluate the effects of frutalin on the mRNA expression of *TNFA, TNF* receptor I *(TNFRI),* TNF receptor II *(TNFRII), CASPASE 3, CASPASE 6, BAX* and *BCL2*, for each treatment and for uncultured control, two fragments were collected and then stored at -80°C until extraction of total RNA, being in total 14 pieces of ovarian cortex per animal. 6-day cultured 2 fragments from each treatment. Total RNA extraction was performed using a Trizol® purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer instructions, 800μl of Trizol solution were added to each frozen sample and the lysate was aspirated through a 20-gauge needle before centrifugation at 10 000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNase-free DNase (340 Kunitz units/ mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 μl RNase-free water.

The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham Biosciences, Cambridge, UK) and 2 μg of total RNA was used for reverse transcription. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled in ice. Reverse transcription was performed in a total volume of 20 μl, which was comprised of 10 μL of sample RNA, 4 µL 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNase out, 150 units Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C, and then stored at 20°C.

Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase. Quantification of mRNA was performed using SYBR Green. PCR reactions were composed of 1 μL cDNA as a template in 7.5 μL of SYBR GreenMaster Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μL of ultra-pure water, and 0.5 M of each primer. The primers were designed by using the Primer QuestSM program (http://www.idtdna.com) to perform amplification of *TNFA, TNFRI, TNFRII, CASPASE 3, CASPASE 6, BAX* and *BCL2*, and housekeeping gene *BACTINA* (Table 1). The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Realplex (Eppendorf, Germany). The ΔΔCt method was used to transform the Ct values into normalized relative expression levels (Livak and Schmittgen, 2001).

Statistical analysis

The percentages of primordial and developing follicles, as well as of those classified as morphologically normal after 6 days of culture in medium supplemented with different concentrations of frutalin were compared by Fisher's exact test (Graph pad Instat). Levels of mRNA for *TNFA, TNFRI, TNFRII, CASPASE 3, CASPASE 6, BAX* and *BCL2*, in cultured fragments were analyzed by using the non-parametric KruskalWallis test ($P<0.05$), and the values were expressed as mean ± stand and error of the mean (SEM). The differences were considered significant when $P<0.05$.

Table 1. Primer pairs used in real-time PCR.

Target gene	Primer sequence (5'→3')	Sense (s) anti-sense(s)	Position	GenBank accession no.
TNFα	CCACGTTGTAGCCGACATCA	s	466-485	GI:402693442
	ATGAGGTAAAGCCCGTCAGC	as	578-597	
TNFRI	CTGGTGATTGTCTTCGGGCT	s	936-955	GI:2290397
	TGCCCGCAAATGATGGAGTA	as	1020-1039	
TNFRII	GTAGCTCAGAGGCGTCTTCC	s	102-121	GI:2613148
	GCCGCTGCAAACATTGACA	as	157-175	
CASPASE 3	CACGGAAGCAAATCAGTGGAC	s	73-93	GI:379067369
	CGACAGGCCATGCCAGTATT	as	181-200	
CASPASE 6	ACTCCGTGGCAGAAGGTTATTA	s	589-610	GI:548469419
	TGAGAAACCTTCCTGTTCACCA	as	712-733	
BAX	GCCCTTTTCTACTTTGCCAGC	s	334-354	GI:41386763
	GGCCGTCCCAACCACCC	as	465-481	
BCL2	GGTAGGTGCTCGTCTGGATG	s	2317-2336	GI:22652876
	GGCCACACACGTGGTTTTAC	as	2421-2440	
BACTIN	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	

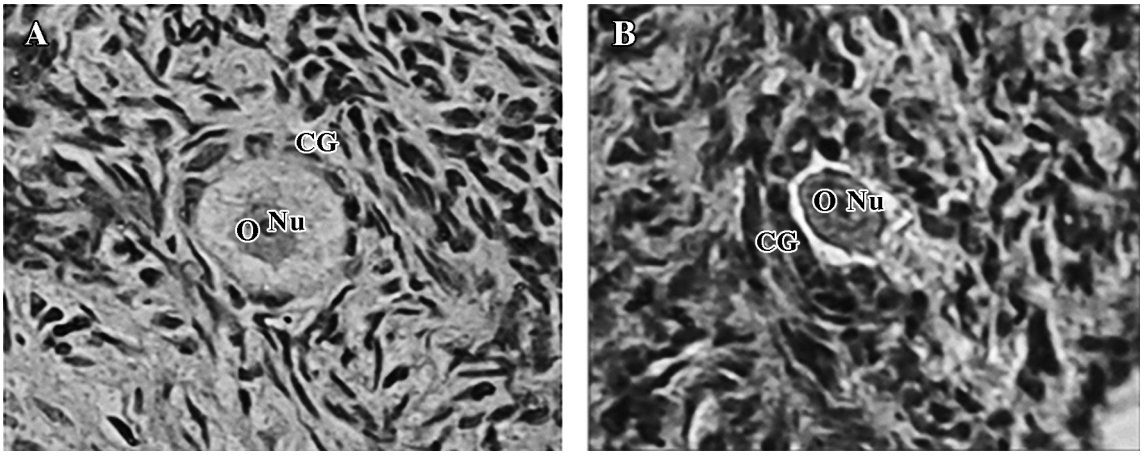

Fig. 1. Histological section of uncultured tissue showing a normal primordial follicle **(A)** and a degenerated primary follicle **(B).** Degenerated follicle had a shrunken oocyte, with pyknotic nucleus or disorganized granulosa cells. Symbols: O, oocyte; Nu, nucleus; GC, granulosa cells. × 400.

Results

Effect of different concentrations of frutalin on survival and activation of primordial follicles

Histological analysis showed the presence of normal (Fig. 1A) and degenerated (Fig. 1B) follicles in cultured ovarian cortical fragments. Degenerated follicles had a shrunken oocyte, with pyknotic nucleus or disorganized granulosa cells. After 6 days, the percentage of normal follicles after culture of ovarian tissue in α-MEM$^+$ alone or supplemented with different concentrations of frutalin (0, 1, 10, 50, 100 or 200 µg/ml) decreased significantly when compared with that of uncultured follicles from fresh control. In addition, the percentage of normal follicles after culture in medium with 1, 10, 50, 100 or 200 μg/ mL of frutalin was significantly lower than that of tissues cultured in α-MEM$^+$ alone. Moreover, the percentage of normal follicles after culture in medium with 50, 100 or 200 μg/mL of frutalin was significantly reduced when compared with that of tissues cultured in medium with 1 or 10 μg/mL of frutalin (Fig. 2).

After 6 days culture of ovarian fragments in the different media, a significant reduction in the percentage of primordial follicles (Fig. 3A) and an increase in the percentage of developing follicles were observed, when compared with the respective percentages within fresh uncultured tissues ($P<0.05$). At all concentrations tested, the presence of frutalin in culture media did not influence the percentage of developing follicles *in vitro* compared to what was found in tissues cultured in α-MEM$^+$ alone (Fig. 3B).

Ultrastructural aspects of follicles before and after culture

Normal primordial follicles from uncultured tissues exhibited sparse vesicles spread throughout the cytoplasm in all the oocytes and regular nuclear membrane. Oocyte and granulosa cells were well connected. Granulosa cells had irregularly-shaped nuclei, with a high nuclear-to-cytoplasm ratio. The cytoplasm contained a great number of elongated mitochondria with lamellar cristae, as well as endoplasmic reticulum. The oocyte cytoplasm also contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes, although there were occasional elongated forms with parallel cristae. Golgi complexes were rarely observed. Both, smooth and rough endoplasmic reticula were present, either as isolated aggregations or as complex associations with mitochondria and vesicles (Figs. 4, 5). Normal follicles from cultured tissues had similar characteristics (Fig. 6), except for a discreet increase in the number of vacuoles and a higher number of elongated mitochondria (Fig. 7). Connections between oocyte and granulosa cells were also seen (Fig. 8).

Degenerated follicles from cultured tissues had a

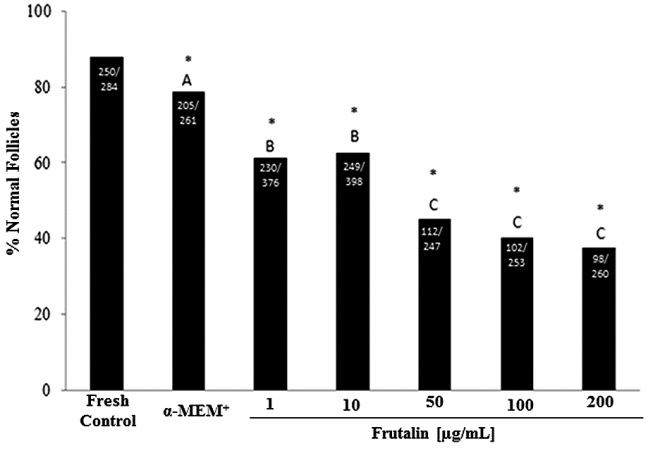

Fig. 2. Percentages of normal follicles in fresh control and after culture in α-MEM$^+$ supplemented with frutalin (1, 10, 50, 100 or 200 μg/mL). Follicle counts from a total of six goats were pooled. *: Differs significantly from fresh control; A,B,C: Differences between treatments after 6 days.

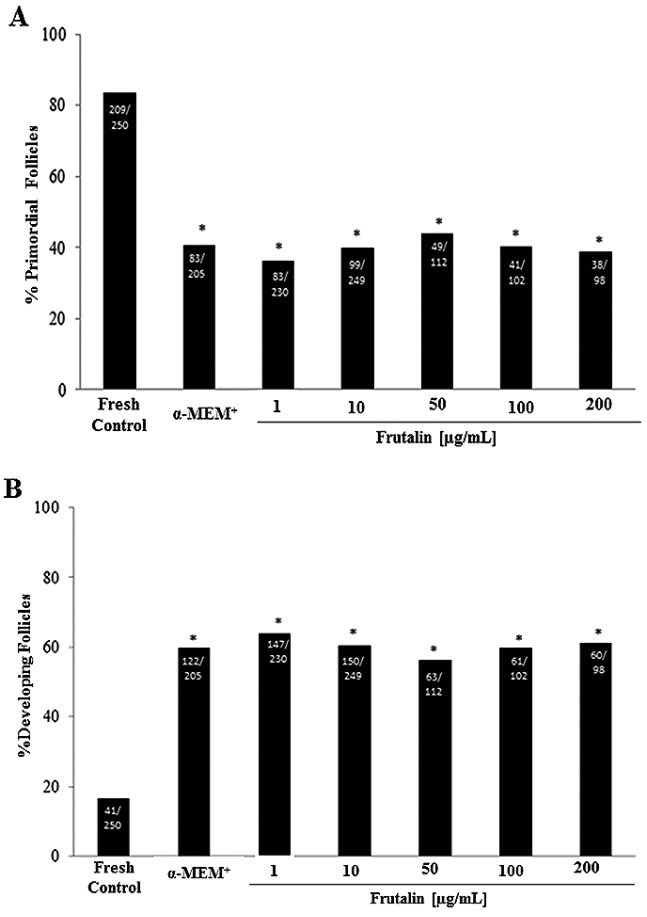

Fig. 3. Percentages of follicles in fresh control and after culture in α-MEM$^+$ supplemented with frutalin (1, 10, 50, 100 or 200 μg/mL). Follicle counts from a total of six goats were pooled. **A.** Primordial follicles. **B.** Developing follicles. *: Differs significantly from fresh control.

retracted oocyte and large irregularity of the follicular, oocyte and nuclear outlines. In the oocyte, a large number of vacuoles spread throughout the cytoplasm were seen (Fig. 9A). In addition, signs of damage to mitochondrial membranes and cristae were observed. Granulosa cells had a swollen aspect, with a low density of organelles in their cytoplasm (Fig. 9B). Granulosa cells had lost their connections with the oocyte basement membrane while a rupture in their cytoplasmic membrane was observed. Generally, organelles were unrecognizable in granulosa cells (Fig. 9B). In tissues cultured in the presence of frutalin, fragmented follicles

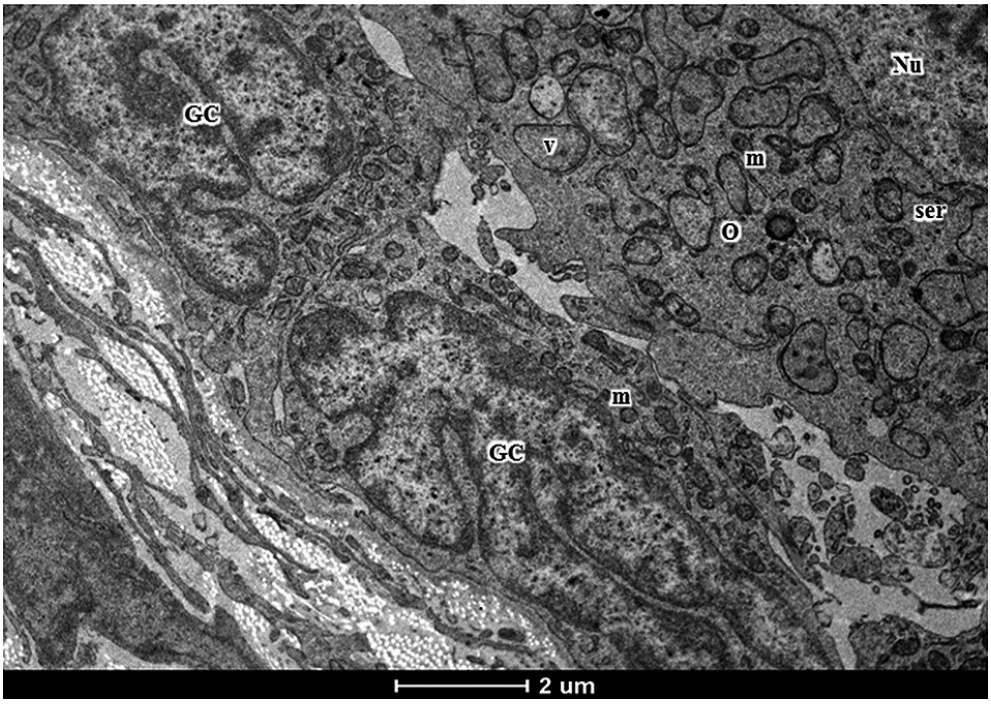

Fig. 4. Electron micrograph of a normal primordial follicle from uncultured tissue. O: oocyte; Nu: nucleus of oocyte; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles.

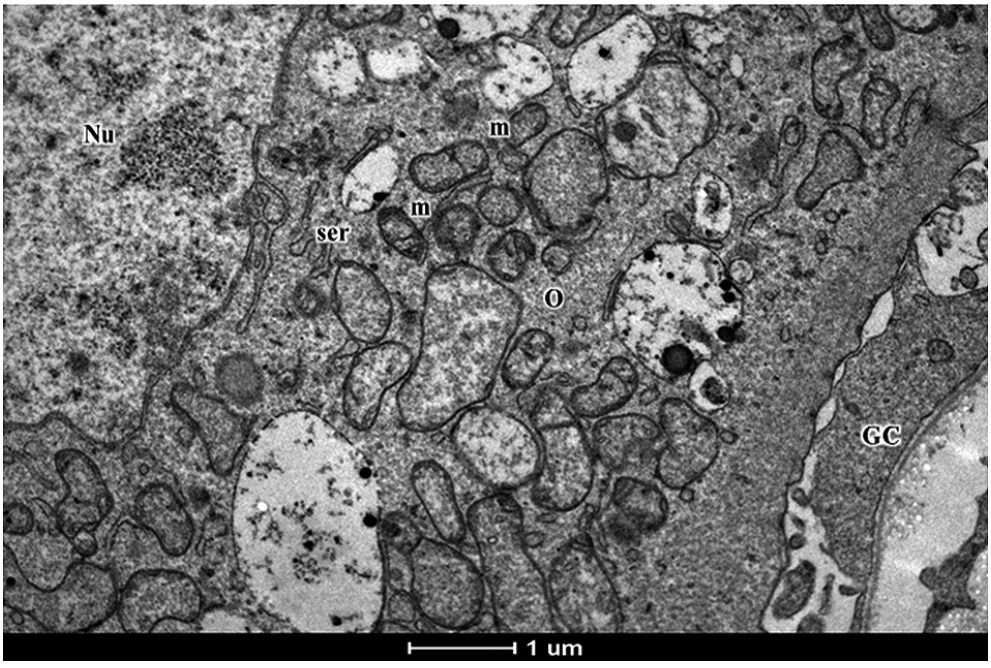

Fig. 5. Detail of an oocyte from uncultured tissue with nucleus and cytoplasmic organelles. O: oocyte; Nu: nucleus of oocyte; GC, granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles.

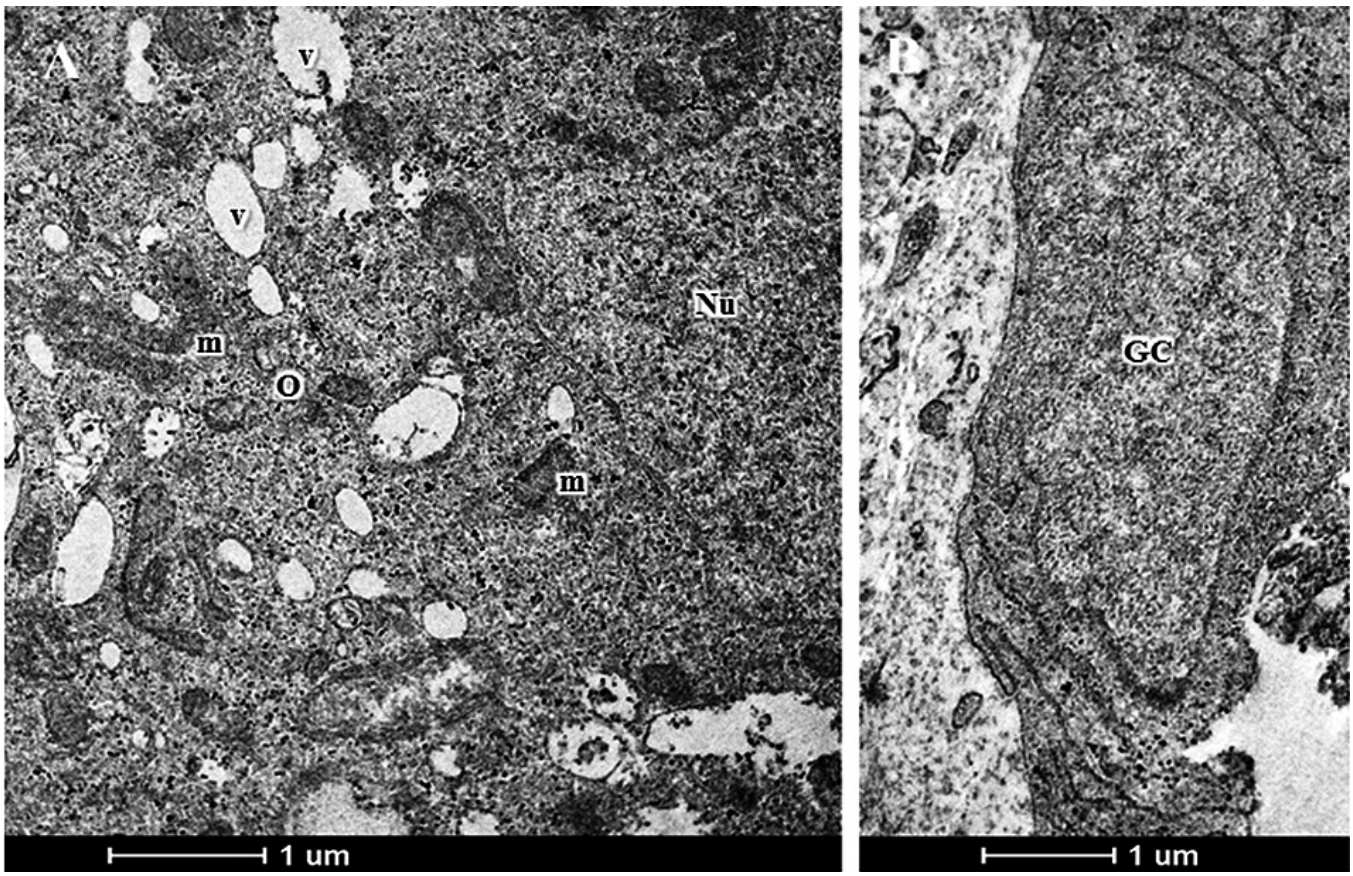

Fig. 6. Electron micrograph of a normal primordial follicle from cultured tissue in α-MEM$^+$ only. **A.** Oocyte. **B.** Granulosa cell. O: oocyte; Nu: nucleus; m: mitochondria; v: vesicles.

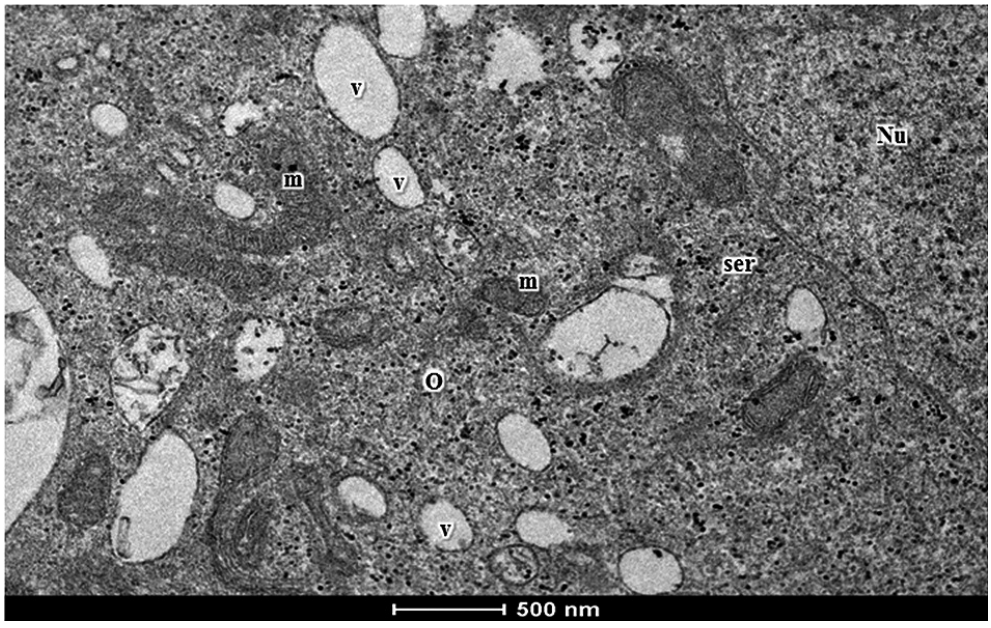

Fig. 7. Detail of an oocyte from primordial follicle from cultured tissue in α-MEM$^+$ only with nucleus and cytoplasmic organelles. O: oocyte; Nu: nucleus of oocyte; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles.

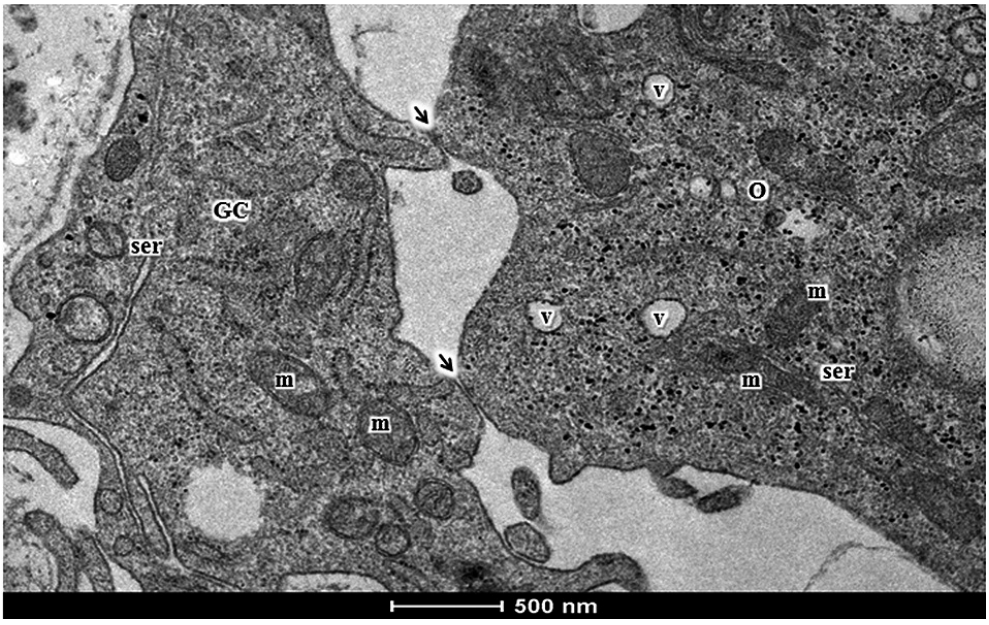

Fig. 8. Connections (arrows) between oocyte and granulosa from primordial follicles present in cultured tissue in α-MEM+ only. m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles; O: oocyte; GC: granulosa cell.

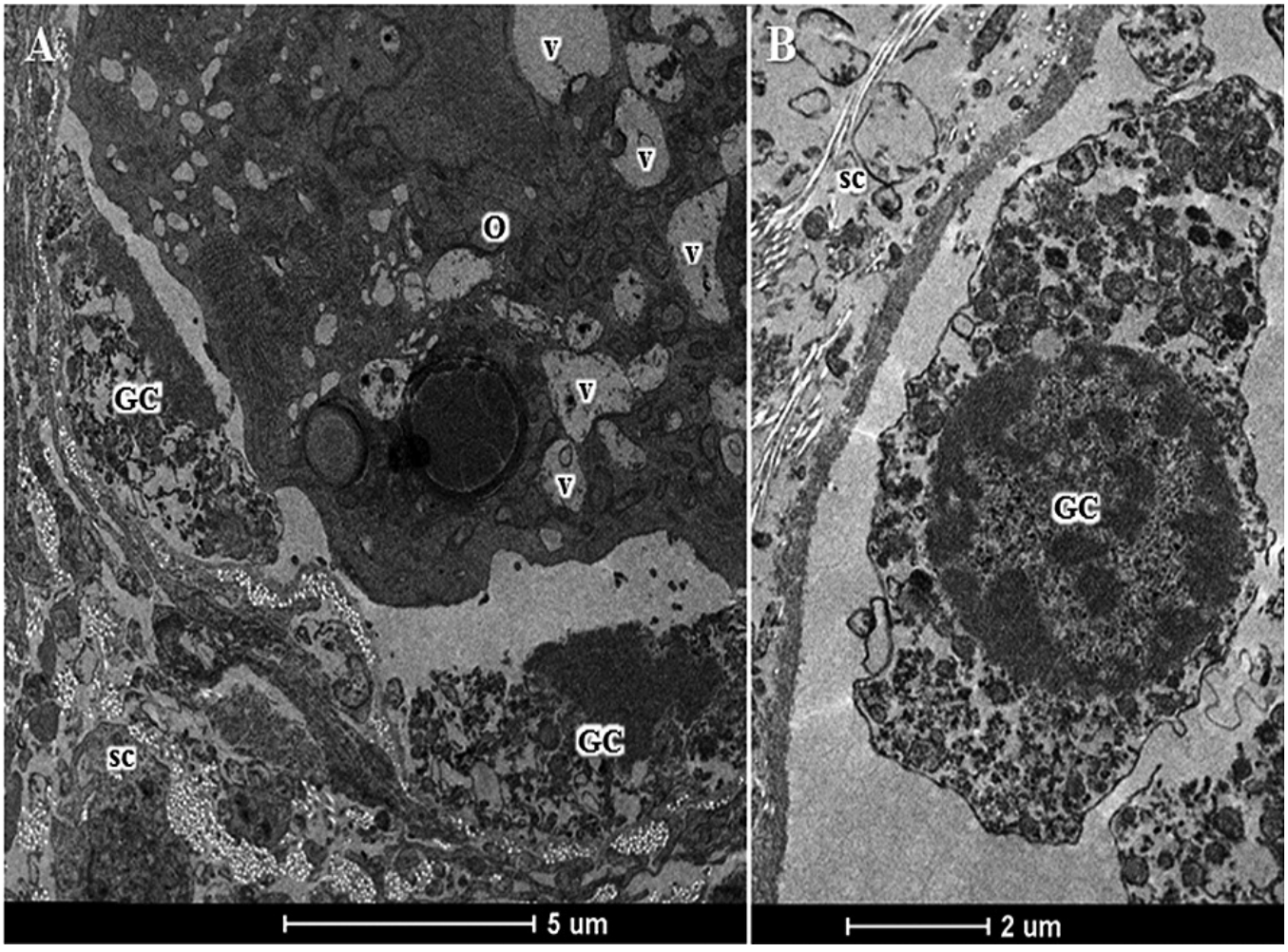

Fig. 9. Electron micrograph of a degenerated primordial follicle from tissue cultured in presence of frutalin (100 μg/mL). **A.** Retracted oocyte and large irregularity of the follicular oocyte and its nuclear outlines. **B.** Cytoplasmic low density of granulosa cells. O: oocyte; GC: granulosa cell; v: vesicles; sc: stromal cells.

and stromal cells were commonly observed (Fig. 9A,B).

Effects of frutalin on gene expression in cultured cortical tissue

After comparing the levels of mRNA between uncultured tisseues and tissues cultured in control medium, most of the mRNAs had their levels reduced after culture. However, only the expression of mRNAs for TNFRI was significantly lower than those seen in uncultured control. In contrast, cultured tissues had higher levels of mRNA for BAX than those seen in uncultured control (Fig. 10).

When compared with tissues cultured only in control medium (α-MEM$^+$), the presence of 10 μg/ml frutalin significantly increased the levels of mRNA for *TNFA* (Fig. 11A), *CASPASE 6* (Fig. 11E) and *BAX* (Fig. 11F). The levels of mRNA for *BAX* (Fig. 11F) were also higher in ovarian tissues cultured in medium containing 100 μg/mL of frutalin than those cultured in control medium. In addition, the levels of mRNA of *CASPASE 3* (Fig. 11D) were significantly increased in ovarian tissues cultured in medium containing 50 and 200 μg/ml frutalin compared to α-MEM$^+$. The presence of 200 μg/mL frutalin also increased the levels of mRNA of *TNFRI* (Fig. 11B), *TNFRII* (Fig. 11C) and *BCL2* (Fig. 11G) when compared to tissues cultured in α-MEM$^+$ alone.

Discussion

Frutalin is a tetrameric lectin expressed in breadfruit plant seeds (Monteiro-Moreira et al., 2015) that, similarly to other galactose-binding lectins [e.g. jacalin (Kabir, 1998), has successfully been used in immunobiological research on cell recognition (Brando-Lima et al., 2005; Oliveira et al., 2009). This study shows for the first time that frutalin does not influence caprine ovarian-cortical-tissue-enclosed primordial follicle activation *in vitro*, most of the follicles spontaneously started growth in all cultures including the control medium. Similar results have been described for bovine cortical tissue pieces cultured in serum-free control medium (Wandji et al., 1996; Braw-Tal and Yossef, 1997) and those from sheep (Santos et al., 2014). To explain the massive growth of primordial follicles during *in vitro* culture, Cushman et al. (2002) suggested that an inhibitor of medullary origin regulates activation *in vivo* and that separation of the cortex from medulla causes primordial follicles to activate *in vitro*. However, more recently, Kawamura et al. (2013) demonstrated that ovarian fragmentation increased actin polymerization and disrupted the Hippo signaling pathway, leading to increased expression of growth factors. Secreted connective tissue growth factor and related factors promoted primordial follicle growth *in vitro* (Hsueh et al., 2015). Our current data furthermore show decreased follicular survival in all media containing frutalin, the effect being stronger (±50% decrease) in the presence of relatively high concentrations (50-200 μg/mL) of this compound, than in the media with lower concentrations (1 and 10 μg/mL; ±30% decrease). Thus, frutalin has a deleterious effect on caprine primordial follicles *in vitro*. Despite this, the still relatively high survival rate of ovarian follicles after culture in the presence of low concentrations of frutalin strengthens our hope for the development of an anticancer therapy that results in less severe damage to the ovarian follicle population. Previous *in vitro* studies have shown concentrations higher than 18 μg/mL have a toxic effect on Hela cells, but, probably, an *in vivo* effect on cancer cells will require higher concentrations. It is clear that, apart from the concentration of the drug in the culture medium, other aspects, such as its half-life and metabolism, should be taken into account in future studies, but such studies open up numerous prospects for progress in this area. It is important to note that frutalin was previously used as a histochemical biomarker for prostate cancer (Oliveira et al., 2009).

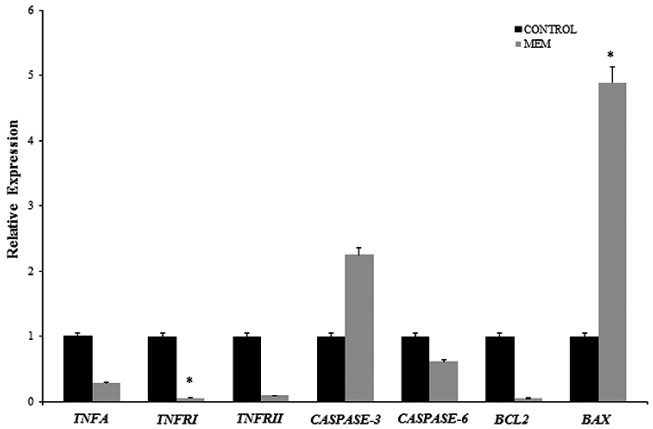

Fig. 10. Levels of *mRNA for TNFA, TNFRI, TNFRII, CASPASE 3, CASPASE 6, BAX and BCL2* in tissues cultured in α-MEM$^+$ when compared with fresh uncultured tissues. Symbols: *: Differs significantly from fresh control.

The present study shows morphological and ultrastructural changes that occur during degeneration of caprine preantral follicles enclosed in ovarian cortical tissues that have been cultured in the presence of frutalin. Ultrastructural analysis shows that healthy follicles have oocyte and granulosa cells well connected while the oocyte contains sparse vesicles spread throughout the cytoplasm, a great number of mitochondria with lamellar cristae and well-developed rough endoplasmic reticulum. In contrast, cultured follicles that became atretic have retracted oocytes, large numbers of vacuoles throughout the cytoplasm, and swollen granulosa cells, with a low density of organelles. In addition, signs of damage to mitochondrial membranes and cristae were observed. Similar changes were previously described in goat ovaries stored *in vitro* (Silva et al., 2002). The observed morphological and ultrastructural changes indicate that the concerned atretic

follicular cells undergo cell death by necrosis, a process different from apoptosis (Ziegler and Groscurth, 2004; Celestino et al., 2009). The main features of cells dying by necrosis are increased cell volume, chromatin aggregation, large vacuolization in the cytoplasm, disruption of cytoplasm, loss of plasma membrane integrity and subsequent cell disruption (Fink and Cookson, 2005). Cellular necrosis has long been regarded as an incidental and uncontrolled form of cell death. In contrast, the morphology associated with apoptosis is characterized by nuclear and cytoplasmic condensation and cellular fragmentation into membrane-bound fragments. These fragments or apoptotic bodies are taken up by other cells and degraded within phagosomes (Fink and Cookson, 2005). A new regulated form of cell death, termed necroptosis, has been identified recently. Necroptosis is a form of caspase-independent programmed cell death associated with morphological changes similar to those that occur in necrosis (Preyat et al., 2016). Necroptosis can be induced by extracellular cytokines, pathogens, and several pharmacological compounds. Interestingly, most ligands known to induce necroptosis, including, notably, *TNF-α*, can also promote apoptosis (Preyat et al., 2016).

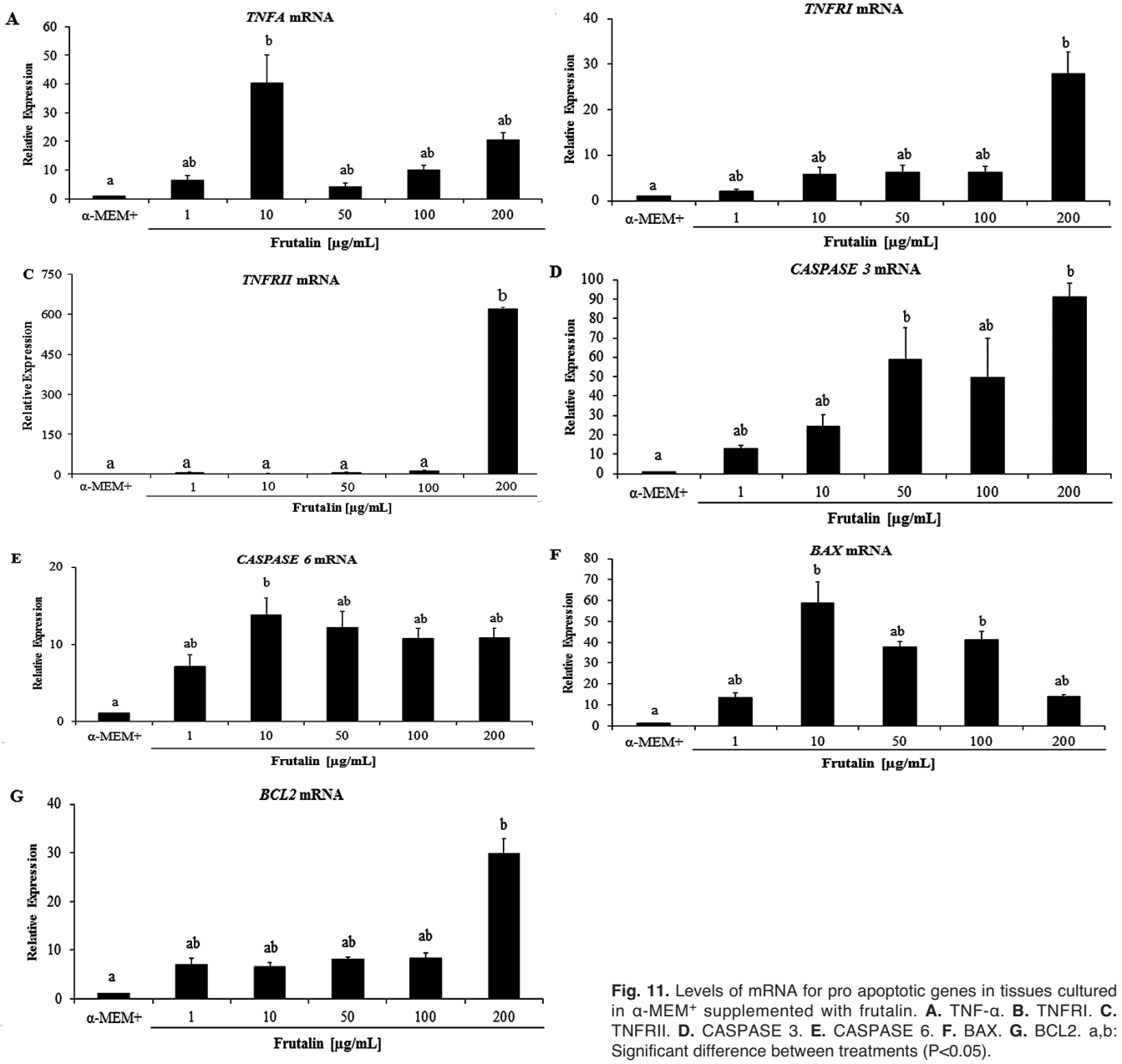

Fig. 11. Levels of mRNA for pro apoptotic genes in tissues cultured in α-MEM^{+} supplemented with frutalin. **A.** TNF-α. **B.** TNFRI. **C.** TNFRII. **D.** CASPASE 3. **E.** CASPASE 6. **F.** BAX. **G.** BCL2. a,b: Significant difference between treatments (P<0.05).

Autophagy, another form of cell death, features degradation of cellular components within the dying cell in autophagic vacuoles (Clarke, 1990). The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation (Fink and Cookson, 2005). Since some of these features were also seen in degenerated follicles, the possibility that autophagy can also be involved cannot be excluded.

Real time PCR has shown that tissues cultured in control medium had reduced levels of mRNA for *TNFRI*, but increased expression of BAX, showing that the cultute system itself influences gene expression. Despite the absence of a dose-response effect of frutalin on the expression of genes for *TNFA, TNFRI, TNFRII, CASPASE 3* and *6, BAX* and *BCL2*, this lectin increased the mRNA expression of pro-apoptotic genes (*TNFα* [frutalin 10 μg/mL], *TNFRI* [frutalin 200 μg/mL], *CASPASE 3* [frutalin 50 and 200 μg/mL], *CASPASE 6* [frutalin 10 μg/mL], and *BAX* [frutalin 10 μg/mL]). It is well known that TNFα is able to elicit pro-apoptotic signals in various cellular types (Sethu and Melendez, 2011) and that TNFRI contains an intracellular death domain that is necessary for signaling pathways associated with apoptosis (Hsu et al., 1995). After binding to its receptor, TNFα triggers pro-apoptotic pathways, such as the loss of mitochondrial membrane potential and activation of terminal proteases, such as the caspase-3 family of proteases (Miyashita et al., 1998). TNFRII does not contain a death motif but still recruits adaptor proteins including TRAF2, and thus, it is thought to be able to signal apoptosis (reviwed by MacEwan, 2002). After receiving the death signals, the pro-apoptotic BAX protein allows cytochrome c to leak out of the mitochondria, which then activates the caspase cascade, leading to cell death (Hussein, 2005). CASPASE 3 activation has been associated with the execution phase of apoptosis and has previously been reported to mediate proteolysis of numerous cellular substrates, including the nuclease DFF40/CAD, which cleaves DNA into oligonucleosomes, poly(ADP ribose)polymerase, protein kinase Cδ, and U1-70 kDa (Lazebnik et al., 1994; Ghayur et al., 1996; Rosen and Casciola-Rosen, 1997). In addition, caspase 6 cleaves lamins during apoptotic cell death (Orth et al., 1996).

Considering that the population of normal follicles has significantly decreased and both ultrastructural changes indicative of necrosis and a transitorily increased expression of pro-apoptotic genes has been detected after culture of ovarian tissue in the presence of frutalin, it is concluded that under these circumstances frutalin is able to trigger follicular cell death mainly via necrotic or necroptotic pathway. Several lectins possess proapoptotic and proautophagic properties in cancer cell lines (Fu et al., 2011; Li et al., 2011) and Oliveira et al. (2011) reported that frutalin induces apoptosis of HeLa cells. In contrast to apoptosis, necroptosis is largely independent of executioner caspases, such as CASPASE 3, 6 and 7 (Preyat et al., 2016). Although necroptosis often appears to occur when apoptosis is abortive, such as in situations of caspase inhibition (Preyat et al., 2016), the cellular factors regulating the choice between these two forms of regulated cell death have not been fully uncovered. To reinforce the possibility that the apoptotic pathway may have been interrupted, an unexpected increase in the expression of *BCL2* mRNA was seen after culture of ovarian tissue in the presence of frutalin (200 μg/mL). The expressed factor is a membrane-associated protein that resides in the nuclear envelope and mitochondria and exerts its pro-survival functions by modulating the mitochondrial the release of cytochrome c (Hussein, 2005).

Conclusions

The putative anticancer lectin frutalin does not influence *in vitro* caprine primordial follicle activation. Frutalin, however, reduces *in vitro* follicular survival, although at low concentrations (1 and 10 μg/mL) the percentage of healthy follicles is still high (>60%). Ultrastructural features of cultured follicles demonstrate that necrosis is the main type of cell death in follicles cultured in the presence of frutalin. Since this lectin, depending on the concentration, has low to moderate toxic effect on early follicles, future studies to evaluate the effects of frutalin on ovarian, oocyte and embryo function *in vivo* are needed to determine the consequences of this treatment far patients.

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