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## Original article

Using RT-PCR and glutathione level to study the effect of follicular fluid on *in vitro* maturation and gene expression of sheep oocytesM. Al-Mutary<sup>b</sup>, M. Al-Ghadi<sup>a,\*</sup>, A. Al-himaidi<sup>a</sup>, D. Iwamoto<sup>a,c</sup>, Y. Al-anazi<sup>a</sup>, A. Ammari<sup>a</sup>, J. Ahmad<sup>a,1</sup>, A. Al-Khedhairi<sup>a,1</sup><sup>a</sup> Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia<sup>b</sup> Basic Sciences Department, College of Education, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia<sup>c</sup> Kinki University, Faculty of Biological-Oriented Sci. and Technology, Dept. of Genetic Engineering, Wakayama, Japan

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## ABSTRACT

The aim of this study is to evaluate the effect of sheep follicular fluid (SFF) supplementation of the *in vitro* maturation (IVM) media of sheep oocytes on the resumption of meiosis, glutathione (GSH) level, and expression of apoptosis (Bax, Bcl-2) as well as heat shock protein beta-1 (HSPB1) genes. Sheep ovaries were collected from the central slaughterhouse of Riyadh city, KSA. Oocytes were aspirated from 3 to 8 mm follicles. Sheep oocytes were cultured in maturation medium with different concentrations of sheep follicular fluid: 0% (control), 10%, 20% and 40% for 24 h. The results indicated that the maturation rate of oocytes was significantly ( $p \leq .05$ ) decreased in 40% SFF (36.87%) versus the control (61.3%), 10% SFF (63.95%) and 20% SFF (64.08%). The supplementation of the IVM medium with 10% SFF induced an intra-oocyte GSH concentration that was significantly higher than in sheep oocytes cultured with 20% and 40% SFF and similar to the GSH content in oocytes cultured without SFF. Real-time polymerase chain reaction analysis of gene expression revealed no significant differences in the Bax and HSPB1 genes between the control and 10% SFF, whereas they were significantly higher in 40% FF ( $p \leq .05$ ) compared to the control. The expression of Bax:Bcl-2 was significantly higher in 20% and 40% SFF compared to the control group. In conclusion, the addition of SFF to the IVM culture of sheep oocytes is recommended to support nuclear maturation and increase oocyte competence.

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## 1. Introduction

Oocytes must progress during folliculogenesis from the diplotene to the MII stage to become mature at the nuclear level. However, completing nuclear maturation does not ensure embryonic development. Oocyte maturation also includes cytoplasmic transformations to support fertilization and subsequent embryo development (Atef and Marc-Andre, 2002; Trounson et al., 2001; Yang et al., 1998).

The *in vitro* maturation (IVM) of oocytes has become a crucial step in the *in vitro* production (IVP) of sheep embryos. Although

oocytes matured *in vitro* and *in vivo* have similar rates of nuclear maturation, fertilization and cleavage, they clearly differ in their developmental potential (Blondin and Sirard, 1995; Dieleman et al., 2002; Dunning et al., 2007; Kyasari et al., 2012; Rizos et al., 2002). Thus, the blastocyst rate after *in vitro* oocyte maturation, fertilization and embryo culture is 30–40%, whereas 70% of *in vivo* mature oocytes develop into blastocysts (Dieleman et al., 2002; Humblot et al., 2005; Rizos et al., 2002; Romar et al., 2011; Smiljakovic and Tomek, 2006; van de Leemput et al., 1999). Thus, several substances have been added to test different culture conditions for IVM. One possible way to improve IVM efficiency is to supplement IVM medium with follicular fluid.

The components of follicular fluid are mainly derived from the serum and synthesized products of follicular cells and oocytes (Avery et al., 2003; Li et al., 2000). Follicular fluid contains an abundance of steroid hormones, FSH, LH, growth factors, cytokines, glucose, cholesterol, triglycerides, albumin, globulin, electrolytes, enzymes and nutrients (Angelucci et al., 2006; Edwards, 1974; Kor and Moradi, 2013; Tabatabaei and Mamoei, 2011; Tabatabaei et al., 2011). These factors may affect oocyte quality, fertilization

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and embryonic development. Follicular fluid is a natural medium for oocyte maturation during folliculogenesis and may be suitable as a supplement to allow the *in vitro* culture medium to mimic the *in vivo* microenvironment and promote development (Coleman et al., 2007; Driancourt and Thuel, 1998; Knight et al., 1996).

Glutathione (GSH) is the major non-protein sulfhydryl compound found in mammalian cells that protects the cells from oxidative stress (Droge, 2002; Pastore et al., 2003). Many factors, when added to IVM medium, promote cysteine uptake in oocytes and enhance glutathione synthesis in the ooplasm (de Matos and Furnus, 2000; Gasparini et al., 2000). Thus, the cytoplasmic glutathione concentration after IVM is a good marker of cytoplasmic maturation (Livingston et al., 2009; Lojic et al., 2012).

On the other hand, gene expression can be altered by the culture conditions not only in the oocyte but also in the blastocyst stages (Lonergan et al., 2003; Nemcova et al., 2006; Rizos et al., 2002). Knowledge regarding gene expression during oocyte maturation is crucial for the optimization of *in vitro* embryo production (Aswal et al., 2008). Some conditions for *in vitro* maturation can induce the expression of apoptotic genes (e.g., Bcl-2: anti-apoptotic and Bax: pro-apoptotic) in oocytes. The sensitivity of cells to apoptotic stimuli may depend on the balance of pro- and anti-apoptotic genes (Ebrahimi et al., 2010; Kim and Tilly, 2004; Yang and Rajamahendran, 2002). Bax gene expression was increased when serum was added to the culture medium (Rizos et al., 2002; Sanna, 2009) and in degenerated embryos compared to good quality embryos (Yang et al., 1998).

Heat shock protein (HSPB1) is induced by heat shock, freezing and slight changes in pH (Chernik et al., 2004). This protein plays an important role not only in various stress conditions but also in certain biological events, including gene activation, cell cycle arrest, differentiation and apoptosis (Palasz et al., 2008). These genes (Bax, Bcl-2 and HSPB1) could be considered good markers of oocyte maturation.

Some studies have shown that the use of supplementary follicular fluid plays a key role in the ability of oocytes to undergo nuclear and cytoplasmic maturation, fertilization and embryonic development in cattle (Ali et al., 2004; Kim et al., 1996; Larocca et al., 1993), humans (Chi et al., 1998), sheep (Guler et al., 2000; Shabankareh and Sarsaifi, 2008; Sun et al., 1994; Thompson, 2000), pigs (Huang et al., 2002; Ito et al., 2008), buffalo (Gupta et al., 2005; Nandi et al., 2004), horses (Bogh et al., 2002), and goats (Cognie et al., 2003; Masudul et al., 2012). However, the effect of follicular fluid on the glutathione level and gene expression of apoptotic genes has not been tested in sheep oocytes after IVM. The objectives of the current study were to investigate the effects of sheep follicular fluid on *in vitro* maturation, glutathione concentration and expression of apoptosis (Bax, Bcl-2) and heat shock protein (HSPB1) genes in sheep oocytes.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals and media used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

### 2.2. Preparation of follicular fluid

The follicular fluid was obtained by aspiration from normal follicles located on sheep ovaries collected from fresh slaughtered animals in abattoir. The fluid was pooled in 15-ml centrifuge tubes and centrifuged twice at 4000 rpm for 20 min to remove cellular debris. The supernatant was collected, filtered on a 0.45- $\mu$ m filter,

and then heat inactivated at 56 °C for 30 min and stored as aliquots in 1.5-ml Eppendorf tubes at –20 °C until use (Sun et al., 1994).

### 2.3. Experimental design

After oocyte collection from the ovaries, the cumulus oocyte complexes (COCs) were divided into four groups depending on the concentration of sheep follicular fluid (SFF) added to the *in vitro* maturation medium: 0% SFF (control); 10% SFF; 20% SFF; and 40% SFF. After maturation, all oocytes were subjected to 199 Hank's medium supplemented with 100 IU/ml hyaluronidase enzyme to remove the surrounding cumulus cells by repeat pipetting, and then, the denuded oocytes in each group were divided into 3 lines: first line, evaluation of nuclear maturation using aceto-orcein stain; second line, measurement of the intracellular glutathione concentration; and last line, detection of the expression of selected genes (Bax, Bcl-2, and HSPB1).

### 2.4. *In vitro* maturation

Sheep ovaries were collected from the central slaughterhouse in Riyadh, Saudi Arabia, and transported to the laboratory in normal saline at 30–35 °C in a thermos flask within 1–3 h after collection. The extra-ovarian tissues were removed, and the ovaries were washed three times with warm fresh normal saline (37°C). Oocytes were obtained by the aspiration of follicles (3–6 mm in diameter) using a 10-ml sterile syringe with a 19 G needle containing 0.5 ml of handling medium {TCM-199 (Hank's salts), supplemented with 10% FCS, 0.5 mM sodium pyruvate, 140  $\mu$ g/ml heparin and 50  $\mu$ g/ml gentamycin}. The aspirated follicular fluid containing the oocytes was placed in 15-ml tubes in a water bath (37 °C) for approximately 10 min to allow settling in the bottom of the tube. The COCs with more than three layers of intact cumulus cells and uniform cytoplasm were collected under a stereomicroscope by mouth pipette and washed three times in maturation medium {TCM-199 (Earle's salts) with 10% FCS, 0.5 mM sodium pyruvate, 0.02 AU/ml FSH, 0.023 AU/ml LH, 1  $\mu$ g/ml E2, 100  $\mu$ M cystamine, 50  $\mu$ g/ml gentamycin and 0% SFF for group 1, 10% for group 2, 20% for group 3, and 40% for group 4}. Groups of ten COCs were placed in 50- $\mu$ l maturation drops, which were covered with mineral oil and incubated in a humidified atmosphere and 5% CO<sub>2</sub> for 24 h at 38.5 °C.

### 2.5. Evaluation of oocyte meiotic maturation

Samples of denuded oocytes from each group were mounted on glass slides, covered and fixed on slides with 3 volumes of acetic acid to one volume of ethanol (v/v) for 24 h and stained with 1% aceto-orcein stain. The oocytes were examined using a phase contrast microscope (400x Olympus, CKX41 Japan) and assessed for oocyte nuclear status, including Germinal vesicle, Germinal vesicle Breakdown, Metaphase I and Metaphase II (Table 2).

### 2.6. Glutathione assay

Samples of 20 denuded oocytes from each groups (A, B, C and D) were washed three times in PBS, then transferred into a 1.5-ml microfuge tube containing 5  $\mu$ l of PBS and stored at –80 °C until use. The experiments were performed in four replicates in each group. The frozen samples were thawed at room temperature and centrifuged. The quantity of glutathione (GSH) in the oocytes was assayed according to the Glutathione kit instructions (Cat. No. CS0260). The amount of GSH in each sample was determined based on the standards and calculated for 20 oocytes.

### 2.7. Sample collection, RNA isolation and reverse transcription

Samples of 20 denuded oocytes from each group (0% SFF (control); 10% SFF; 20% SFF; and 40% SFF) were washed in 0.1% PBS-PVA, then transferred into an Eppendorf tube containing 5  $\mu$ L of 0.1% PBS-PVA and stored at  $-80^{\circ}\text{C}$  until RNA extraction. The experiments were performed in three replicates for each group. RNA was isolated from the oocytes, and complementary DNA (cDNA) was synthesized using a Cells-to-cDNA<sup>TM</sup>II Kit (Cat. No. AM1722) as described by the manufacturer's instructions. Briefly, pools of oocytes from each group were lysed by adding 100  $\mu$ L of ice-cold Cell Lysis II Buffer to each sample in 1.5-ml Eppendorf tubes. After vortexing and centrifugation, the samples were incubated at  $75^{\circ}\text{C}$  for 10 min. For genomic DNA removal, RNA samples were treated with 2  $\mu$ L of DNase I per 100  $\mu$ L of Cell Lysis II Buffer for 30 min at  $37^{\circ}\text{C}$ . To inactivate DNase I, the samples were heated at  $75^{\circ}\text{C}$  for 5 min. Reverse-transcription reactions were performed with 3  $\mu$ g of total RNA, dNTP Mix, Oligo(dT)18 Primer, 10X RT Buffer, and RNase Inhibitor in a total reaction volume of 20  $\mu$ L for 60 minutes at  $42^{\circ}\text{C}$ , followed by 10 minutes at  $95^{\circ}\text{C}$  to inactivate the reverse transcriptase. The integrity of the RNA was visualized on a 1% agarose gel using a gel documentation system (Universal Hood II, BioRad). Then, the RNA concentration was determined by spectrophotometry (NanoDrop, Wilmington, DE, USA) using 1- $\mu$ L samples.

### 2.8. Real-time polymerase chain reaction (RT-PCR)

Real-time PCR was performed in 50  $\mu$ L of reaction buffer containing 25  $\mu$ L of SYBR<sup>®</sup> Green Master Mix (Applied Biosystems, 4367659), 2  $\mu$ L of each of the forward and reverse primer pairs for each gene (10 pM), 2.5  $\mu$ L of cDNA, and 18.5  $\mu$ L of nuclease-free water. The primer sequences, annealing temperature, approximate sizes of the amplified fragments of all transcripts and GenBank<sup>®</sup> accession number are listed in Table 1. Histone H2AFZ (Histone 2A, member Z) mRNA was used as an internal standard. The program used for the amplification of the genes consisted of denaturing at  $95^{\circ}\text{C}$  for 10 min followed by 45 cycles

of PCR (denaturation at  $95^{\circ}\text{C}$  for 15 s, annealing at  $60^{\circ}\text{C}$  for one minute, and extension at  $72^{\circ}\text{C}$  for one minute). All reactions were conducted using the LightCycler<sup>®</sup> 480 real-time PCR machine (Cat. No. 04 640 268 001). The expression of each gene was calculated by  $2^{-\Delta\Delta\text{Ct}}$  (Schmittgen and Livak, 2008).

### 2.9. Statistical analysis

Statistical analyses of data from at least three replicate trials for each treatment comparison were performed using the SPSS version 20 software package (SPSS Inc., Chicago, IL, USA). Data were first evaluated using the Kolmogorov-Smirnov normalization test. The means of GV, GVBD, MI, MII and degenerated oocytes after *in vitro* maturation, glutathione concentration and relative gene expression in all groups were compared by one-way analysis of variance (ANOVA) and post-LSDDunnett's test.

## 3. Results

The overall percentages of mature oocytes in the different groups are reported in Table 2. The results show that the different concentrations of follicular fluid in the first three groups does not affect the maturation rate significantly, whereas the maturation rate is significantly ( $p \leq .05$ ) decreased in 40% SFF (36.87%). Simultaneously, the proportions of GV, GVBD, and MI oocytes were found to be higher in 40% SFF than the other groups (Table 2).

The glutathione levels measured in sheep oocytes after maturation using IVM media supplemented with different concentrations of follicular fluid are shown in Fig. 1. Our results indicate that the level of glutathione was significantly ( $p \leq .05$ ) lower in 20% SFF and 40% SFF compared to the control group. A decrease was also observed in the level of glutathione in 10% SFF compared to the control, but the difference was not significant. The maximum depletion of the glutathione level was observed in 40% SFF (Fig. 1).

The expression levels of apoptosis marker genes and heat shock protein genes are summarized in Figs. 2 and 3. The expression levels of the Bax and HSPB1 genes were significantly up-regulated in 40% SFF compared to the control group (Fig. 2), and

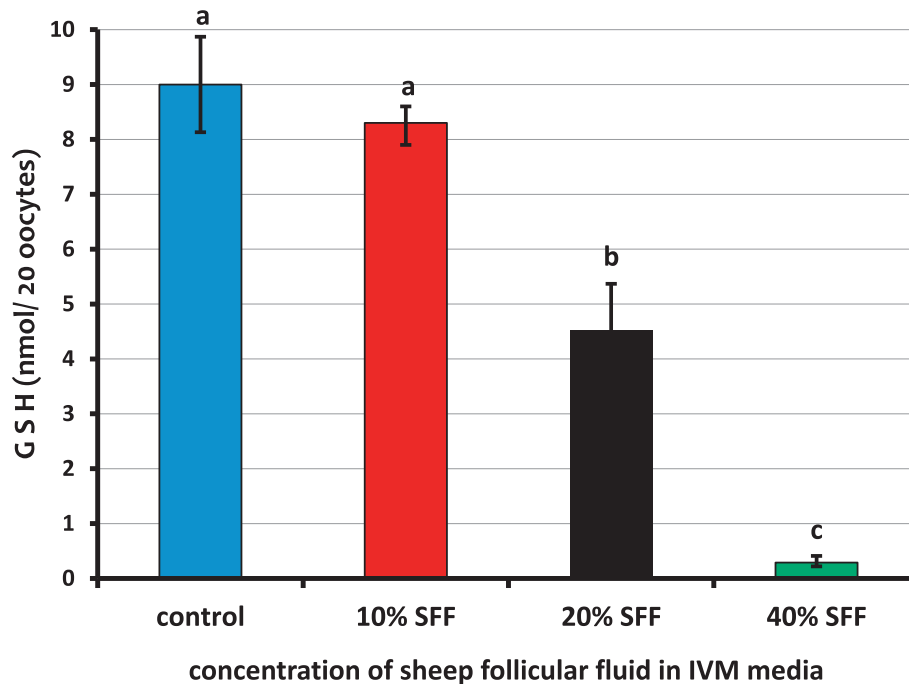
**Table 1**  
The sequence of primers for quantitative Real-time PCR.

Functions	Genes	Primer sequences (5'-3')	Size (bp)	Ref.
Endogenous control	H2AFZ	(F) AGGACGACTAGCCATGGACGTGTG(R) CCACCACCAGCAATTGTAGCCTTG	212	Sanna (2009)
Apoptosis genes	BAX	(F) CTACTTTGCCAGCAAACCTGG(R) TCCCAAAGTAGGAGAGGA	158	Sanna (2009)
	Bcl-2	(F): GCCGAGATGTCCAGTCAGC(R) :GACGCTCTCCACACATGAC	150	Ebrahimi et al. (2010)
Stress gene	HSPB1	(F) TCCCTGGACGTCAACCACTTCG(R) AGGTTTGCCGGGTGAGGATGTC	391	Sanna (2009)

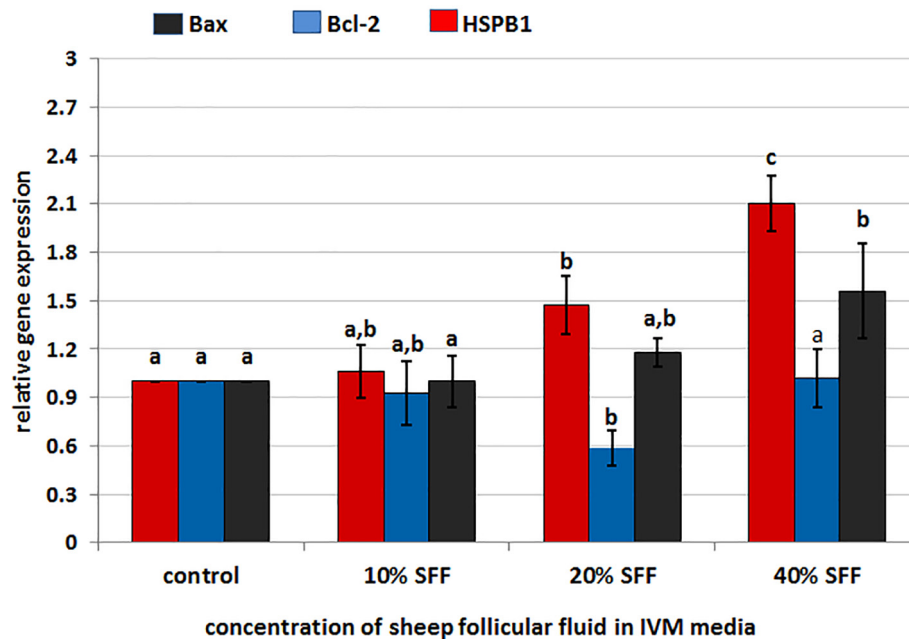
**Table 2**  
Effect of the supplementation *in vitro* maturation medium with SFF on maturation rates of sheep oocytes.

Nuclear status (Mean $\pm$ SEM)				Deg. oocytes (%)	Total oocytes	Concentration of SFF
M II (%)	M I (%)	GVBD (%)	GV (%)			
276 (61.39 $\pm$ 3.50) <sup>a</sup>	64 (14.23 $\pm$ 2.82) <sup>a</sup>	44 (9.7 $\pm$ 1.4) <sup>a</sup>	16 (3.55 $\pm$ 0.76) <sup>a,b</sup>	49 (11.15 $\pm$ 2.1)	449	0% SFF (control)
301 (63.95 $\pm$ 1.84) <sup>a</sup>	41 (6.99 $\pm$ 1.92) <sup>a</sup>	51 (11.09 $\pm$ 1.64) <sup>a,b</sup>	8 (1.58 $\pm$ 0.85) <sup>a</sup>	69 (14.6 $\pm$ 1.1)	470	10% SFF
273 (64.08 $\pm$ 6.47) <sup>a</sup>	78 (18.76 $\pm$ 2.31) <sup>b</sup>	41 (9.92 $\pm$ 1.51) <sup>a,b</sup>	8 (1.73 $\pm$ 0.78) <sup>a</sup>	46 (10.92 $\pm$ 1.1)	426	20% SFF
170 (36.87 $\pm$ 2.14) <sup>b</sup>	130 (28.32 $\pm$ 1.35) <sup>c</sup>	73 (15.89 $\pm$ 1.4) <sup>b</sup>	27 (5.86 $\pm$ 0.47) <sup>b</sup>	59 (13.04 $\pm$ 1.6)	459	40% SFF

Data are presented as the Mean  $\pm$  S.E.M. GV: germinal vesicle; GVBD: germinal vesicle break down; MI: metaphase-I; MII: metaphase-II; SFF sheep follicular fluid. Five replicates in each treatment. (a,b,c) letters with different superscript in the same column differ significantly ( $p \leq .05$ ).



**Fig. 1.** Glutathione levels in sheep oocytes with different concentrations of sheep follicular fluid during IVM. Columns with different superscript (a, b, c) differ significantly ( $p \leq .05$ ).



**Fig. 2.** Fold differences in relative gene expression for three different genes; Bax, Bcl-2 and HSPB1 genes in sheep oocytes matured in different concentrations of sheep follicular fluid during IVM. Columns with different superscript (a, b, c) for the same gene columns differ significantly ( $p \leq .05$ ).

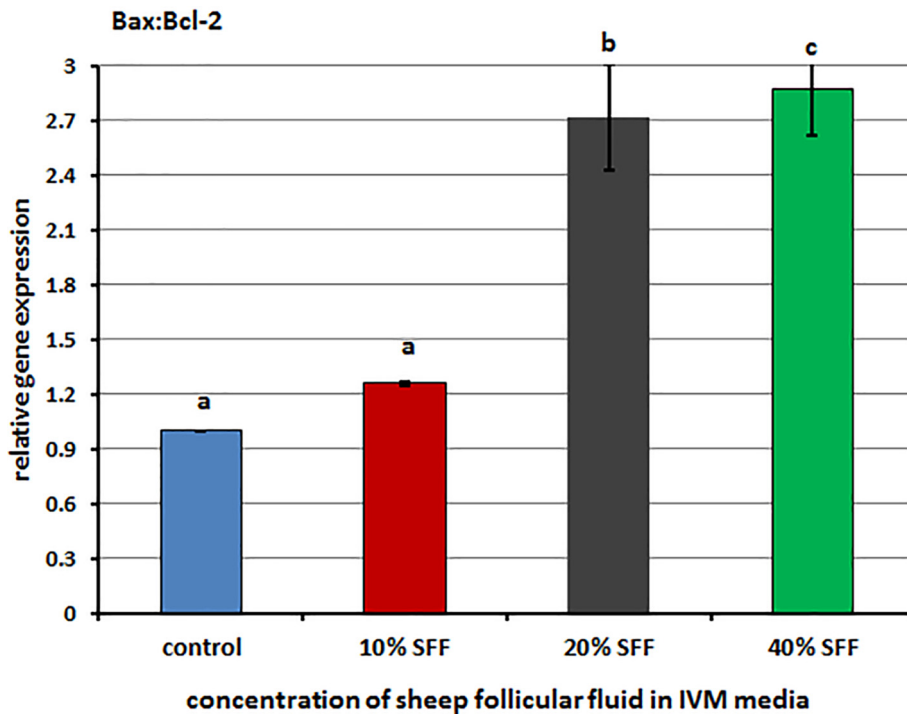
the level of expression of the Bcl-2 gene was down-regulated in 20% SFF compared to the control group (Fig. 2). The Bax:Bcl-2 ratio was up-regulated in all groups and significantly up-regulated in 20% SFF and 40% SFF compared with the control group (Fig. 3).

#### 4. Discussion

Follicular fluid contains many important hormones and growth factors that support the *in vivo* maturation of oocytes. The addition

of follicular fluid to the *in vitro* maturation medium of sheep oocytes may increase oocyte competence. Here, for the first time, we report the use of real-time RT-PCR to characterize the changes in the relative gene expression of apoptosis genes (Bax, Bcl-2) and heat shock protein (HSPB1) and the estimation of the glutathione concentration in sheep oocytes cultured at different concentrations of follicular fluid in IVM media.

It was observed that the addition of SFF to IVM media at 10% and 20% concentrations could support the *in vitro* maturation of



**Fig. 3.** Relative Quantitative RT-PCR representing the Bax/Bcl-2 ratio in sheep oocytes matured in different concentrations of sheep follicular fluid during IVF. Columns with different superscript (a, b, c) differ significantly ( $p \leq .05$ ).

sheep oocytes. The maturation rates of oocytes in 10% SFF (63.95%) and 20% SFF (64.08%) were similar to the maturation rates of the oocytes in the control group (61.39%), with no significant difference among the groups. These results were in good agreement with observations in cattle (Coleman et al., 2007) and in horses (Caillaud et al., 2008). In another study, (Sun et al., 1994) observed that the addition of SFF to IVF at a concentration of 20% significantly increased the rate of matured oocytes. Concurrently, an inhibitory effect of FF on the maturation rate was observed in 40% SFF at a 40% concentration, which is consistent with the findings of Kim et al. (1993). They reported that adding 60% follicular fluid to the IVF medium inhibits bovine oocyte maturation, probably due to coagulating the cumulus cell mass with a fibrin-like substance in the follicular fluid, whereas 10% follicular fluid stimulated both the maturation and developmental capacity of bovine oocytes (Kim et al., 1993). Supporting these results, we found that the glutathione levels of oocytes in the first three groups, 0% SFF, 10% SFF and 20% SFF, were significantly higher than in the last group, 40% SFF. It is known that the intracellular GSH level increases during maturation and peaks when the oocytes reach metaphase II (de Matos and Furnus, 2000; Perreault et al., 1988; Yoshida et al., 1993).

The *in vitro* maturation conditions can alter the acquisition of oocyte developmental competence and could influence the levels of certain oocyte transcripts (Nemcova et al., 2006; Watson et al., 2000). Many studies have used the expression of apoptotic genes as a quality marker for oocyte viability (Tatone et al., 2006; Yang and Rajamahendran, 2002). We used the sheep histone H2AFZ gene as an endogenous control for quantifying the expression of other genes, as described in (Nemcova et al., 2006; Sanna, 2009). The expression levels of Bax and HSPB1 showed no significant differences between the control and 10% SFF, but were significantly higher ( $p \leq .05$ ) in 40% SFF compared to the control group. Lonergan et al. (2003) reported the Bax and Bcl-2 genes to be significantly altered by modification of the culture conditions. Furthermore, the ratio of Bax:Bcl-2 could potentially be used as a

tool to compare various treatments (Rao et al., 2012). These results indicated the significantly heightened expression of Bax:Bcl-2 in 20% SFF and 40% SFF compared to the control group. As the expression of heat shock protein genes may be altered during oocyte culture, the expression of these genes can be used as an indicator of culture conditions (Warzych et al., 2007; Wrenzycki et al., 2005). It has been suggested that interaction in the expression levels of the Hsp-70 and Bax genes may occur under stress conditions, with Bax activation suppressed in cells with high Hsp-70 levels (Stankiewicz et al., 2005). Furthermore, oocytes undergoing apoptosis have poor developmental competence (Li et al., 2009). HSPB1 enhances the resistance of cells to the deleterious effects induced by heat shock, oxidative stress or conditions that trigger apoptosis (Paul et al., 2010). These data are similar to the findings of Avery et al. (2003), who reported that the maturation of cattle oocytes cultured in large quantities of bovine follicular fluid was inhibited compared with the maturation of cattle oocytes cultured in small amounts of follicular fluid. The sudden change of media in the *in vitro* environment probably caused sharp changes in oocyte metabolic pathways, negatively interfering with the maturation process (Cruz et al., 2014).

**In conclusion**, this study reports for the first time the use of real-time RT-PCR to characterize the changes in the relative gene expression of apoptosis genes (Bax, Bcl-2) and heat shock protein (HSPB1) and to estimate the glutathione concentration in sheep oocytes cultured at different concentrations of follicular fluid in IVF media. The data also suggest that the addition of a small amount of SFF to the IVF media of sheep oocytes may improve the progression of nuclear maturation and the developmental competence of sheep oocytes compared to the addition of large amount.

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