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Effects of Follicular Fluid on Developmental Competence and Gene Expression of *in vitro* Fertilized Sheep Embryos

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Authors' Contribution
MOG, AA-K and MGA-M designed the study. MOG, ARA-H, MGA-G, DI and JA performed experiments and did sampling. MOG, YA-A, AA and AA-K drafted the manuscript.

Key words
Embryonic development, Follicular fluid, Apoptosis genes, HSPB1.

ABSTRACT

This study was undertaken to evaluate the effect of follicular fluid supplementation on the oocyte *in vitro* maturation and early embryonic development after *in vitro* fertilization. Sheep follicular fluid (SFF) was collected from different ovaries and supplemented to the *in vitro* maturation (IVM) media with proportions 0%, 10%, 20% and 40%. Oocyte maturation was assessed in different treatments and then cumulus-oocyte complexes were used for *in vitro* fertilization. Relative quantitative expression of mRNA transcripts related to apoptosis (Bax and Bcl-2), embryo development (LAMA1, IL-6 and FGF4) and stress (HSPB1) were examined. The results showed that 10% and 20% SFF supplementation exerted no effect on maturation and cleavage percentage whereas 40% SFF supplementation significantly decreased maturation and cleavage. SFF supplemented at 10% concentration showed a significant increase in blastocyst development when compared with other groups. Gene expression analysis revealed a significant increase in BAX expression in groups supplemented with SFF 20% and 40%, whereas the expression of BCL-2 was significantly increased only in the 40% SFF supplemented group. The ratio of Bax to Bcl-2 was significantly increased in the embryos derived from oocytes matured in 20% and 40% SFF supplemented media. HSPB1 expression was significantly increased in group matured in 20% SFF. The relative expression of IL-6, LAMA1 and FGF4 genes did not show any difference between groups. In conclusion, IVM medium supplemented with 10% SFF showed the best rate of blastocyst development.

INTRODUCTION

The modification of *in vitro* maturation (IVM) media to imitate the *in vivo* and to support the maturation of oocytes is important for *in vitro* embryo production (IVEP) in animals (Lanzendorf *et al*., 1996; Dell'Aquila *et al*., 1997; Coleman *et al*., 2007). Thus, several substances have been supplemented to improve the culture conditions of the IVM. One of these substances is the follicular fluid (FF) that has various nutrients that may affect oocyte growth, fertilization, and embryo development (Tabatabaei and Mamoei, 2011; Duarte *et al*., 2012).

FF was used during IVM in cattle (Larocca *et al*., 1993; Kim *et al*., 1996; Ali *et al*., 2004), human (Chi *et al*., 1998), goat (Cognie *et al*., 2003; Masudul *et al*., 2012), pig (Huang *et al*., 2002; Ito *et al*., 2008), buffalo (Nandi *et al*., 2004; Gupta *et al*., 2005), horse (Bogh *et al*., 2002), and sheep (Sun *et al*., 1994). However, supplementation of the FF to the *in vitro* maturation media can lead to changes in mRNA abundance, influencing gene expression not only in the oocytes but also in the blastocyst stages (Watson *et al*., 2000; Young *et al*., 2001; Lonergan *et al*., 2003; Wrenzycki *et al*., 2005; Cruz *et al*., 2014). In general, the competence of oocyte is evaluated by maturation

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rate, fertilization rate, cleavage rate and blastulation ratio (Iwata *et al*., 2004). In addition, analysis of expression patterns of developmentally important genes can be used as good markers of the quality of embryos (Li *et al*., 2009). To date, the effect of sheep follicular fluid (SFF) supplementation during the *in vitro* maturation of sheep oocytes on gene expression and embryo development has not been investigated. In the present study, we evaluated the effect of SFF on oocyte maturation, pointing to changes in the expression of genes related to apoptosis (Bax and Bcl-2), embryo development (LAMA1, IL-6 and FGF4) and stress (HSPB1).

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and media used in the present study were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Collection and preparation of SFF

Mixed SFF was collected from ovaries that were brought from local slaughter house. The follicular fluid was aspirated by an 18 gauge needle attached to a 10 ml syringe and was pooled in a 15 ml tubes and centrifuged twice at 4000 rpm for 20 min to remove granulosa cells, blood cells and oocytes. The supernatants were collected, filtered by 0.45 µm filter. The SFF was heat-inactivated at 56°C for 30 min, aliquoted in 1.5 ml tube and stored at -20°C until use. All experiments were performed with the same batch.

Oocyte collection and IVM

Ovaries were collected from central slaughterhouse in Riyadh (Saudi Arabia) and transported within 2 h to the laboratory in 0.9% NaCl. The cumulus oocyte complexes (COCs) were obtained by aspiration using a sterile syringe onto 0.5 ml handling medium TCM-199 (Hank's salts) supplemented with 10% FCS, 0.5 mM Na–Pyruvate, 140 µg/ml heparin and 50 µg/ml gentamycin. The oocytes with or more than three layers of intact cumulus cells and uniform cytoplasm were collected under a stereomicroscope by mouth pipette. The COCs were then washed three times in 100 µl of maturation medium TCM-199 (Earle's salts) with 10% FCS, 0.5 mM Na–Pyruvate, 0.02 IU/ml follicle-stimulating hormone (FSH), 0.023 IU/ml luteinizing hormone (LH), 1 µg/ml E2, 100 µM cystamine, 50 µg/ml gentamycin and 0% SFF (Control: Group A), 10% SFF (Group B), 20% SFF (Group C) or 40% SFF(Group D). Then, groups of ten COCs were placed in 50 µl maturation drops which was covered with mineral oil and incubated in a 100% humidified atmosphere and 5% CO_2 for 24 h at 38.5ºC. Following incubation, samples of oocytes from each group were denuded from cumulus cells and mounted on slides, fixed in acetic acid:ethanol 3:1 (v/v) for 24 h, then stained with 1% aceto-orcein. The oocytes were examined using a phase contrast microscope (400x Olympus, CKX41 Japan) and assessed for oocyte nuclear status.

In vitro *fertilization of oocytes*

Fresh semen from ram was collected and washed in capacitation medium HEPES-Tyrode's Albumin Lactate Pyruvate (Caisson Labs, IVL02, USA) containing 6 mg/ml bovine serum albumin (BSA), 50 µg/ml gentamicin and 0.5 mM Na-pyruvate, centrifuged twice at 1800 rpm for 5 min. For swim up, 0.5 ml of semen was kept under 1 ml of capacitation medium in a 15 ml conical Falcon tube at 38.5 ºC for one hour. Before transfer to fertilization drops, the oocytes were washed four times in IVF-Tyrode's Albumin Lactate Pyruvate (Caisson Labs, IVL01, USA) with 6 mg/ml BSA, 140 µg/ml heparin and 50 µg/ml gentamycin. Insemination was carried out by adding 2.0×10^6 into fertilization medium that included 10 oocytes per 50 µl fertilization drop in an atmosphere of 5% CO_2 and humidified air at 38.5ºC for 24 h.

In vitro *culture of embryos*

After IVF, zygotes were denuded from cumulus cells by pipetting and washed in SOF medium (Caisson Labs, IVL03, USA) supplemented with 1% (v/v) BME-essential amino acids, 1% (v/v) MEM non-essential amino acids, 50 µg/ml gentamycin and 6 mg/ml BSA. They were then allocated to 50 µl drop of SOF medium (25 embryos/drop). The incubation conditions were humidified by 5%O_2, 5%CO_2, and 90% N_2 at 38.5 ºC. The culture was continued until 7-8 days post-fertilization.

Blastocyst staining

Blastocysts were transferred from culture medium and wash two times in 100 µl drop of 1% PBS-polyvinylpyrrolidone (PVP), fixed in alcohol, washed three times in 1% PBS-PVP, stained in Hoechst 33342 solution for 10 minutes, washed three times in 1% PBS-PVP. The blastocysts were mounted on the slides and examined them by fluorescent microscope.

RNA isolation and reverse transcription

All embryos were washed in 0.1% PBS-PVA. Then, the embryos (5 blastocyst: day 7) were placed in an Eppendorf tube containing 5 µl of 0.1% PBS-PVA and stored at -80°C (three replicates in each group) until RNA extraction. mRNA was extracted from pooled blastocysts from embryos and cDNA was synthesized using the Cells-

to-cDNA™ II Kit (Thermo Fisher Scientific, USA; Cat. No. AM1722). Briefly, frozen samples were lysed in 100 µl ice-cold Cell Lysis II Buffer. After vortexing and centrifugation, the samples were incubated at 75°C for 10 min. For genomic DNA removal, RNA samples were treated with 2 µl DNase I per 100 µl Cell Lysis II Buffer for 30 min at 37°C. To inactivate DNase I, the samples were heated at 75°C for 5 min. Reverse-transcription reactions were performed with 3 µg total RNA, dNTP Mix, Oligo(dT)18 Primer, 10X RT Buffer, RNase Inhibitor in total reaction volume of 20 µl for 60 min at 42°C, followed by 10 min at 95°C to inactivate the reverse transcriptase. The RNA concentration was determined by spectrophotometry (NanoDrop, Wilmington, DE, USA) using a 1 µl sample.

Real-time polymerase chain reaction

Real-time PCR was performed in 50 µl of reaction buffer containing 25 µl of SYBR® Green Master Mix (Applied Biosystems, USA), 2 µl each of forward and reverse primer pairs for each gene (400 nM), 2.5 µl of cDNA (ng/µl), 18.5 µl of nuclease-free water. Primer sequences and the approximate sizes of the amplified fragment of all transcripts are listed in Table I. The program used for the amplification of the genes consisted of denaturing at 95°C for 10 min and followed by 45 cycles of PCR (denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds). All reactions were carried out using the LightCycler® 480 real time PCR machine (Roche Life Science, USA). The expression of each gene was calculated by following formula according to Schmittgen and Livak (2008):

$2^{-\Delta\Delta CT}$={(CT gene of interest-CT internal control) treated sample - (CT gene of interest - CT internal control) control sample}

Statistical analysis

Statistical analysis of data from at least seven replicates for each treatment comparison was carried out using the SPSS ver. 21 software package (SPSS Inc., Chicago, IL, USA). Data were first evaluated using the Kolmogrov-Smirnov normalization test. The means of maturation and cleavage rates, different stages of in vitro development and relative gene expression in all groups were compared by one-way analysis of variance (ANOVA) and post-LSD Dunnett's test. Three replicates of gene expression of all genes were used.

Table I.- Details of primer sequences and fragment sizes of genes used for RT-PCR.

Functions	Symbol gene	Gene	Primer sequence (5-3)	Fragment size (bp)
Endogenous control	H2AFZ	H2A histone family, member Z	(F) AGGACGACTAGCCATGGACGTGTG (R) CCACCACCAGCAATTGTAGCCTTG	212
Embryo development	LAMA1	Laminin, Alpha 1	(F) CCCTGCCAGCAATGCACACATC (R) TCGGATGCCGTTCTGTTGAAGG	341
	IL6	Interleukin-6	(F) CGCCTTCACTCCATTCGCTGTC (R) CGCCTGATTGAACCCAGATTGG	307
	FGF4	Fibroblast Growth Factor 4	(F) AACGTGAGCATCGGCTTCCACC (R) TTGCTCAGGGCGATGAACATGC	284
Apoptosis	BAX	BCL2-associated X protein	(F) CTACTTTGCCAGCAAACTGG (R) TCCCAAAGTAGGAGAGGA	158
	Bcl-2	B-cell CLL/lymphoma 2	(F) GCCGAGATGTCCAGTCAGC (R) GACGCTCTCCACACACATGAC	150
Stress	HSPB1	Heat shock protein beta-1	(F) TCCCTGGACGTCAACCACTTCG (R) AGGTTTGGCGGGTGAGGATGTC	391

The primers were reported by Sanna (2009) and Ebrahimi *et al.* (2010).

Table II.- Effect of sheep follicular fluid on maturation rate, cleavage rate and blastocyst rate of sheep embryos.

Conc. of follicular fluid	No. of cultured oocytes (n)*	Maturation rate (%)	Cleavage rate (%)	Blastocyst rate (%)	Total cells of blastocyst
0% (Group A)**	626 (15)	(61.39± 3.50) a	(64.53 ± 3.42) a	(21.03 ± 1.85) a	(129.6 ± 8.08) a
10% (Group B)	605 (13)	(63.95± 1.84) a	(66.77 ± 2.68) a	(36.13 ± 3.41) b	(125 ± 8.05) a
20% (Group C)	279 (7)	(64.08± 6.47) a	(64.51 ± 3.89) a	(22.77 ± 4.79) a	(112.3 ± 13.1) b
40% (Group D)	299 (7)	(36.87± 2.14) b	(51.17 ± 3.14) b	(11.77 ± 0.51) a	None #

Data expressed as Mean ± SEM of the replicates. ** control group; * (n) number of replicates in each group. [a, b, c], significantly different ($P < 0.05$).

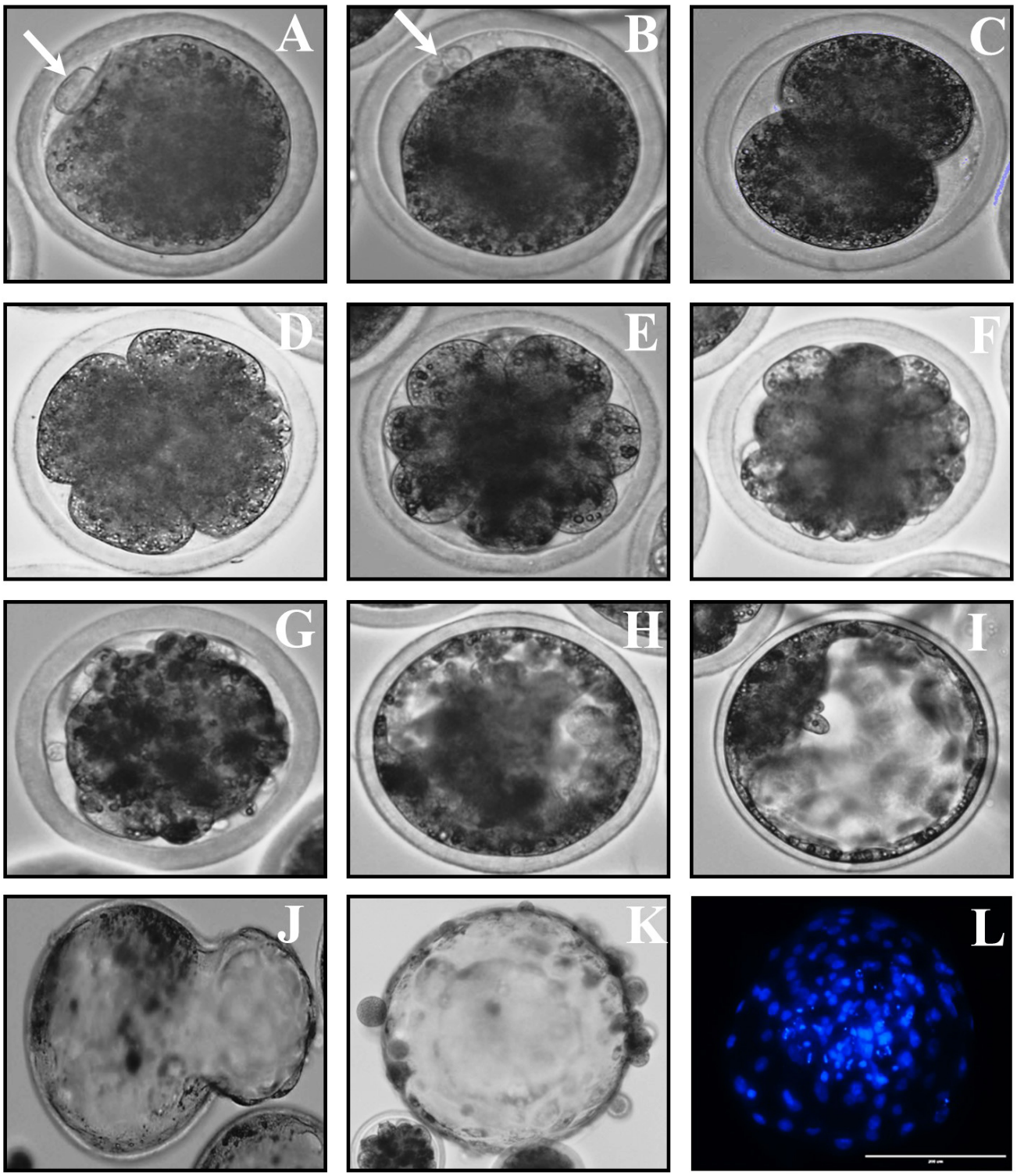

Fig. 1. The different stages of embryonic development of sheep embryos produced *in vitro*. A, Matured oocyte (the arrow = 1st polar body); B, Zygote (the arrow = 2nd polar body); C, 2 cells stage; D, 4 cells stage, € 8 cells stage; F, 16 cells stage; G, Morula stage; H, early blastocyst I, expanded blastocyst; J, hatching blastocyst; K, hatched blastocyst; L, blastocyst on day 7 stained with Hoechst.

RESULTS

Effect of SFF on maturation, cleavage and hatching rate of blastocyst

The different stages of sheep embryos produced *in vitro* are summarized in Figure 1. In this experiment, maturation rate, cleavage rate, blastocyst rate and total cells of blastocyst were observed for oocytes that matured in different concentrations of SFF. There were non-significant (P>0.05) differences in maturation and cleavage rates in groups supplemented with 20% and 40% SFF when compared with the control group. However, the maturation and cleavage rates were significantly (P<0.05) declined in group D when compered with all other groups. On the other hand, the blastocyst rate was significantly higher (P<0.05) in embryos that obtained from oocytes matured in 10% of SFF when compared with the control (Group A; 0% SFF), 20% SFF (Group C) and 40% SFF (Group D) supplemented groups. In addition, there was a significant decrease in the total cells of blastocyst in group C when compared with other groups (Table II). Furthermore, there was a significant increase in blastocyst hatching rate in group B when compared with the other groups as depicted in Figure 2.

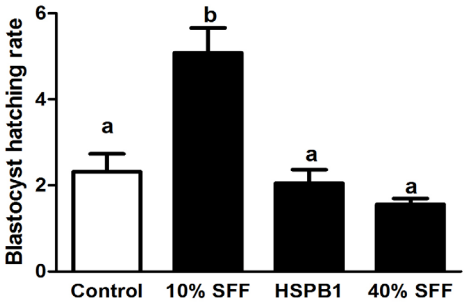

Fig. 2. Effect of sheep follicular fluid on hatching rate of blastocyst. Data are expressed as Mean ± SEM. Different superscript (a, b, c) within columns are significantly different, (P < 0.05).

Effect of SFF on the expression levels of genes related to apoptosis

The transcripts of Bax and Bcl-2 genes were detected in day7 blastocyst originating from oocytes matured in different treated groups. As depicted in Figure 3, the real time PCR results for the expression of pro-apoptotic gene Bax was highest in the groups supplemented with 20 and 40% SFF, whereas the expression of anti-apoptotic gene Bcl-2 was only highest in the 40% SFF supplemented group. In addition, the ratio of Bax to Bcl-2 was significantly higher (P<0.05) in the embryos that derived from oocytes matured in 20% and 40% of follicular fluid.

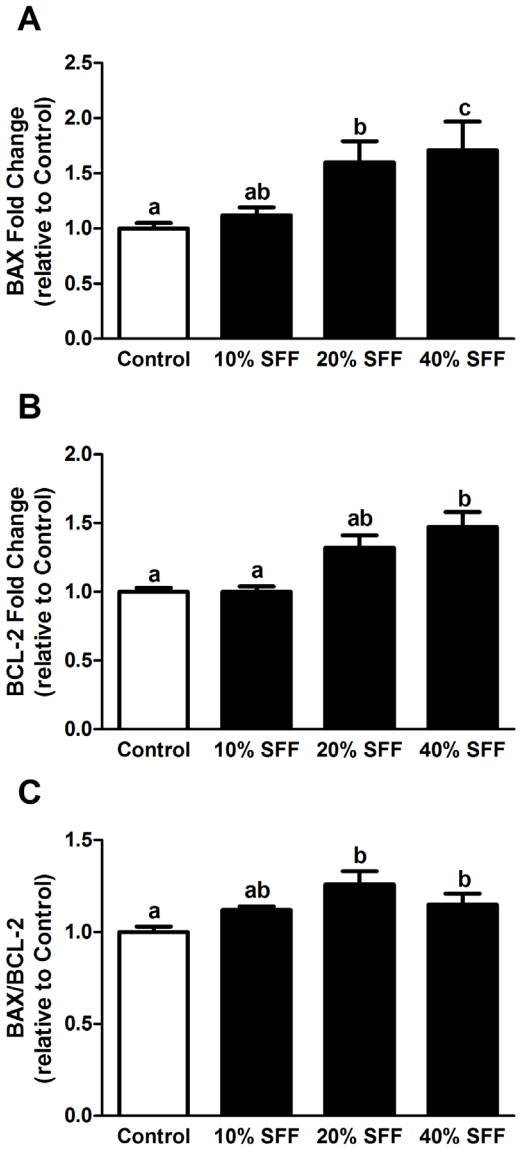

Fig. 3. Effect of sheep follicular fluid on BAX (A), BCL-2 gene expression(B) and BAX/BCL-2 (C) ratio of sheep embryos. Data are expressed as Mean ± SEM. Different superscript (a, b, c) within columns are significantly different, (P < 0.05).

Effect of SFF on the expression levels of genes related to stress (HSPB1)

The gene expression of HSBP1 was determined in day7 blastocyst originating from oocytes matured in different treatments (Fig. 4). HSPB1 expression was a significantly higher in the group supplemented with 20% SFF while showed non-significant changes in the groups supplemented with either 10 or 40% SFF when compared with the control group.

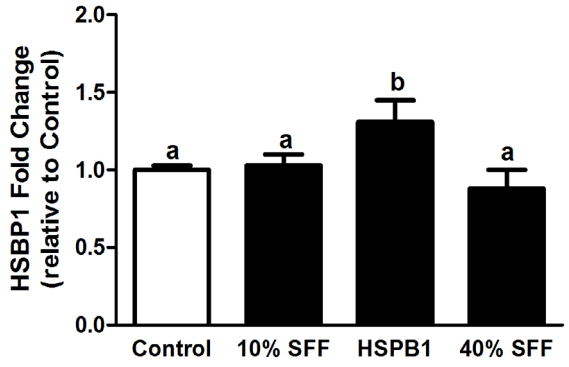

Fig. 4. Effect of sheep follicular fluid on HSBP1gene expression of sheep embryos. Data are expressed as Mean ± SEM. Different superscript (a, b, c) within columns are significantly different, (P < 0.05).

Effect of SFF on the expression levels of genes related to embryo development (FGF-4, IL-6 and LAMA1)

The transcripts of FGF-4, IL-6 and LAMA1 genes were detected in day 7 blastocyst originating from oocytes matured in different groups. Relative expression of analyzed genes are represented in Figure 5. The relative expression of mRNA FGF-4, IL-6 and LAMA1 genes did not differ between groups.

The findings of the study are summarized in Table III.

Table III.- A summary of the effect of follicular fluid on all parameters in this study.

Parameters	A	B	C	D
Maturation rate	---	---	---	LS
Cleavage rate	---	---	---	LS
Blastocyst rate	---	HS	---	---
Hatching rate of Blastocyst	---	HS	---	---
Total cells of Blastocyst	---	---	---	---
Bax gene expression	---	---	HS	HS
Bcl-2 gene expression	---	---	---	HS
Bax Bcl-2 ratio	---	---	HS	HS
HSPB1 gene expression	---	---	---	HS
LAMA1 gene expression	---	---	---	---
IL-6 gene expression	---	---	---	---
FGF4 gene expression	---	---	---	---

*High or low significant compare to control. SFF, sheep follicular fluid; A, conrol; B, 10% SFF; C, 20% SFF; D, 40% SFF; HS, high significant; LS, low significant.

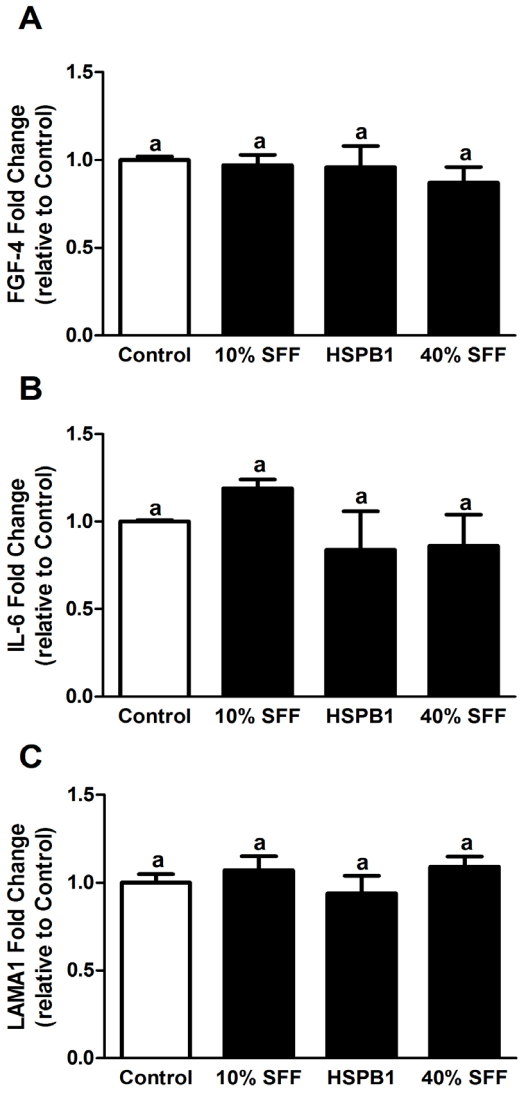

Fig. 5. Effect of sheep follicular fluid on FGFBP-4 (A), IL-6 (B) and LAMA1 (C) gene expression of sheep embryos. Data are expressed as Mean ± SEM. Different superscript (a, b, c) within columns are significantly different, (P < 0.05).

DISCUSSION

It has been observed that the embryos produced *in vivo* are of high quality and less apoptosis than those produced *in vitro* (Pomar *et al*., 2005; Melka *et al*., 2010), and the major reason that might contribute to depress oocyte and embryo competence is the *in vitro* conditions (Wohlres-Viana *et al*., 2011). In this study, we have used different concentrations of SFF in an attempt to optimize the *in vitro* conditions of oocyte maturation to be closer to the *in vivo* status. Some reports have shown high maturation rate in horse oocytes

that matured in pure follicular fluid (Dell'Aquila *et al*., 1997; Bogh *et al*., 2002). On the contrary, in our study, the lowest maturation and cleavage rate was shown in the group of maturation medium supplemented with 40% SFF. This result in sheep is similar to the report of Cruz *et al*. (2014) in bovine. They as well as others have observed that high concentrations of FF in the maturation medium reduce maturation rate, cleavage rate, blastocyst rate and cell number of ICM (Avery *et al*., 2003; Cruz *et al*., 2014). The low blastocyst rate obtained using high concentrations of FF might be affected by dilution and sudden change of the *in vitro* media that may negatively interfere with the maturation pathways (Cruz *et al*., 2014). Furthermore, we found that the hatching rate of blastocyst was significantly higher in the group that has less amount of follicular fluid than other groups. It is well established that the hatching of blastocyst is the most important factor for embryo to implant in uterus (Huisman *et al*., 2000; Hammadeh *et al*., 2011; Thompson *et al*., 2013). And the main factor affecting on the blastocyst yield and implantation rate is the oocyte competence (Lonergan *et al*., 2003). The composition of maturation media may improve blastocyst yield without increasing the total cells of embryo (Warzych *et al*., 2007), as observed in group B compare to control in this study.

Additionally, several studies have suggested that modification of the *in vitro* maturation media can be associated with changes in gene expression not only in oocyte but also in blastocyst (Rizos *et al*., 2002; Lonergan *et al*., 2003; Wrenzycki *et al*., 2005; Nemcova *et al*., 2006; Warzych *et al*., 2007; Heinzmann *et al*., 2011; Diederich *et al*., 2012; Boruszewska *et al*., 2015). To characterize the quality of embryos developed from oocytes matured in different media, we compared the relative expression of six genes in sheep blastocyst. Obviously, apoptosis detection is a useful tool to find out the viability of early embryonic development and to define consequences of stress impacts on *in vitro* maturation of oocytes (Melka *et al*., 2010; Bakri *et al*., 2016). Hence, BCL2-associated X protein (Bax) and B-cell CLL/lymphoma 2 (Bcl-2) were chosen in order to study suitable amount of FF for oocyte maturation. Based on our analysis of relative expression of apoptotic genes (Bax and Bcl-2), the embryos that obtained from IVM with large amount of follicular fluid (20% SFF and 40% SFF) have lower viability than other embryos. It is well known that suboptimal conditions during *in vitro* culture may impair embryo quality (Pomar *et al*., 2005). However, using the ratio of Bcl-2/Bax alone cannot predict the potential of *in vitro* embryo development (Praveen-Chakravarthi *et al*., 2015). Thus, the heat shock protein beta 1 (HSPB1) gene was chosen to determine this potential. This study found that HSPB1 high expression is located in blastocysts derived from oocytes matured in medium with 20% SFF. This observations agree with our findings in the Bcl-2 gene and with decreasing the total cells of blastocyst in same group. Mishra *et al*. (2017) reported that sheep embryos produced *in vitro* are highly sensitive to culture condition, which alters the expression level of apoptotic and antioxidant enzyme genes. Moreover, It was documented that HSPB1 and Bcl-2 genes protect the cell from apoptosis during stress conditions by using different pathways (Paul and Arrigo, 2000; Fulda *et al*., 2010; Banerjee *et al*., 2011; Acunzo *et al*., 2012; Liu *et al*., 2013).

Fibroblast growth factor-4 (FGF4), Laminin α1 (LAMA1) and Interleukin-6 (IL6) genes are known to have specific functions through early embryo development (Desai *et al*., 1999; Hallmann *et al*., 2005; Sanna, 2009; Valdez-Magana *et al*., 2014). No significant differences in relative expression of FGF4, LAMA1 and IL6 genes were found between groups treated with different concentrations of SFF. Previous studies revealed the role of paracrine signals that trigger positive feed-back loops promoting cellular specification (Arnold and Robertson, 2009). FGF4 has been reported to play a central role in promoting trophoblast proliferation (Chen *et al*., 2009) and in the development and maintenance of the trophoblast stem cell (Guzman-Ayala *et al*., 2004). Although the stress and apoptosis related genes were affected by SFF supplementation, FGF4, LAMA1 and IL-6 showed non-significant changes.

CONCLUSION

In conclusion, the present study showed that supplementation of the *in vitro* maturation (IVM) media with 10% sheep follicular fluid produced the best rate of blastocyst development. At this concentration, the sheep follicular fluid modulated genes related to apoptosis and stress whereas exerted no effect on the genes of FGF-4, IL-6 and LAMA1.

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Statement of conflict of interest

Authors have declared no conflicts of interest.

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