Gene expression and maturation evaluation of sheep oocytes cultured in medium supplemented with natural antioxidant source

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Abstract

Any improvements in assisted reproduction techniques at experimental level are important in *in vitro* production of large animal and human embryos. The *in vitro* embryo production system includes three major steps, namely *in vitro* maturation of the primary oocytes, *in vitro* fertilization of the matured oocytes, and *in vitro* culture of presumptive embryos. The success of *in vitro* fertilization and embryo culture depends on the success of *in vitro* maturation. Fenugreek seed extract (FSE) is an important source of steroid hormones, antioxidants, and anti-inflammatories. This study aims to investigate the effect of FSE on the maturation progression, glutathione (GSH) concentration, and expression of certain genes controlling maturation such as growth differentiation factor-9 (GDF-9), transforming growth factor beta receptor 1 (TGFβR1), activin A receptor type II-like 1 (ACVRL1), hyaluronan synthase 2 (HAS2), C-Myc, B-cell leukaemia/lymphoma gene-2 (BCL-2), and BCL2-associated X protein (Bax) of matured sheep oocytes. To carry out this study, cumulus-oocyte complexes (COCs) aspirated from sheep ovaries were cultured in TCM-199 medium supplemented with various concentrations of FSE (0, 1 and 10 μg/mL). The results indicated that the mean values of matured oocytes were 65.2 compared with 47.8 in the control group. Concerning the candidate genes, expression was improved in oocytes matured with FSE when compared with oocytes cultured without extract. In conclusion, the addition of FSE to the maturation medium at 1 or 10 μg/mL concentrations improved the maturation of oocytes.

Keywords: Fenugreek seed extract, *in vitro* maturation, maturation genes, oocytes, sheep
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Introduction

In vitro maturation (IVM) is one of the essential steps in the *in vitro* fertilization (IVF) process (Mahdi *et al.*, 2008). *In vivo* conditions cannot be mimicked totally under *in vitro* situations, but developmental capabilities of oocytes can be improved by adding various substances to the maturation media (Choi *et al.,* 2001).

Fenugreek (*Trigonella foenum graecum* L.) is an annual plant from the family Papilionaceae–Leguminosae and is cultivated extensively in India, the Mediterranean region, North Africa and Yemen (Kassem *et al.*, 2006). Studies have shown that fenugreek seed has antioxidant properties (Hibasami *et al.*, 2003). In addition, fenugreek seed polyphenols have been shown to prevent oxidative haemolysis and lipid peroxidation induced by hydrogen peroxide (H_2O_2) *in vitro* in human erythrocytes (Kaviarasan *et al.*, 2004). In the authors' previous study (Barakat *et al.*, 2010), the addition of fenugreek extract to a maturation medium was found to enhance the quality of a less effective medium (CR1aa), and to optimize the effect of TCM-199 on *in vitro* maturation of Egyptian buffalo oocytes. Moreover, oral administration of fenugreek extract increased the total number and quality of collected mice oocytes significantly, as mentioned by Hassan & Abdel-Wahhab (2006).

Spices and herbs possess antioxidant activity and can be applied to preserve lipid peroxidation in biological systems (Mohsen *et al.*, 2012). Fenugreek *(Trigonella foenum graecum)* is an important spice. Its dried seeds have wide applications in food and beverages as flavouring additives and in medicines. The antioxidant property of the plant material is due to the presence of many active phytochemicals, including

vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, and sterols. Currently, research is directed towards finding naturally occurring antioxidants of plant origin (Mohsen *et al.*, 2012). Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species by inhibiting enzymes or by chelating trace elements (Young & Woodside, 2001). Therefore, it has been used for liver therapy (Yoo *et al.*, 2008).

A major problem in IVF is that oocytes and embryo culture are affected by increased oxidative stress (Gaspmini *et al.*, 2000; Guerin*et al.*, 2001; Sadeesh *et al.*, 2014). Oxidative stress damages gametes and embryos owing to free radicals that are generated by endogenous processes such as normal cellular metabolism and exogenous factors such as chemicals that are added to the culture media, hyperoxia, and exposure to light (Ileana *et al.*, 2012). During *in vivo* oocyte maturation, fertilization and embryonic development, enzymatic and non-enzymatic antioxidants are present in follicular fluid (Catherine *et al.*, 2009).

Cumulus-oocyte complexes (COCs) are present in the reproductive tract secretions that aid in controlling the propagation of free radical reactions, thus providing precise regulation of the redox state of the embryo. The innate antioxidant defences in embryos are not sufficient to counter the oxidative stress encountered during *in vitro* culture (Dröge, 2002; Oyawoye *et al.*, 2003; Tracy *et al.*, 2009; Rajesh *et al.*, 2010). Numerous antioxidant chemicals have been added as supplements to culture media in mammalian *in vitro* embryo culture (Rajesh *et al.*, 2010). These include proteins, vitamins, antioxidant enzymes, metal chelators, thiol compounds, vitamin E, α-tocopherol, and its derivatives, ascorbic acid and cysteamine (Sies & Stahl, 1995; Olson & Seidel; 2000; Guerin *et al.*, 2001; Rajesh *et al.*, 2010; Beheshti *et al.*, 2011; Ileana *et al.*, 2012). In addition, Barakat *et al.* (2014) found that green tea extract used as an antioxidant in culture media of sheep oocytes resulted in an improvement of embryo development to blastocyst stage.

The molecular modifications of COCs during maturation include accumulation of mRNA and proteins, which originate from long stages of development during oogenesis and folliculogenesis (Kempisty *et al.*, 2011b). In metaphase II (MII) oocytes, these accumulated molecules become a template for the synthesis of proteins during the consecutive steps of embryo growth. During the long period of oocyte maturation and differentiation (folliculo- and oogenesis), large amounts of mRNA and proteins become synthesized and accumulated (Kempisty *et al.*, 2011a). These oocyte-specific molecules, also called 'maternal factors', are involved in sequential steps of embryogenesis. Li *et al.* (2010) described maternal-effect genes as mRNA templates, which appear between the last stages of oocyte maturation and embryonic genome activation, and are necessary for early embryo development. Molecular changes in COCs include the accumulation of appropriate amounts of several substrates, enzymes and other molecules involved in biochemical reactions inside the cytoplasm. Biochemical changes have pronounced effects on the cell, which may be defined as the ability of the oocyte to change the cell organellum activity in relation to modified culture conditions (for example using transcription or translation activators or inhibitors) (Kempisty *et al.*, 2011a). Bone morphogenetic protein 15 (BMP15) and growth differentiation factor-9 (GDF-9) are oocyte-specific growth factors that appear to play key roles in granulosa cell development and fertility in most mammalian species (Eppig, 2001). GDF-9 and bone morphogenetic protein 15 (BMP15) are two members of the transforming growth factor receptor superfamily (Chang, 2002) that are expressed by oocytes and play key roles in granulosa cell development and fertility in animal models (Dong *et al.*, 1996; Yan *et al.*, 2001). Moreover, the actions of recombinant GDF-9 resemble those of native factors secreted by growing oocytes isolated from secondary follicles in promoting the synthesis of proteins by granulosa cells associated with the secondary to tertiary transition stage during follicular development (Latham *et al.*, 2004). Oocytes are essential for follicular formation and development (Eppig, 2001; Matzuk *et al.*, 2002), because oocyte-derived factors such as GDF-9 and BMP15 are involved in regulation of follicle formation, growth and granulocyte differentiation (Peng *et al.*, 2013; Baerwald *et al.*, 2012). Genes that encode steroidogenic enzymes for androgen production are expressed to a certain extent in stromal cells (SCs). These results confirm that granulosa cells (GCs) stimulate ovarian stromal cells differentiation into theca cells (TCs). After that, they lose the capacity to stimulate the steroidogenic function of TCs and retain only the capacity of maintaining their steroidogenic function (Spaczynski *et al.*, 2005). In addition, and according to Hengde *et al.* (2013), supplementing a-lipoic acid significantly reduced the cellular apoptosis rate of nucleus transfer blastocysts by inhibiting the expression of apoptotic activators such as Bax, Bad, Caspase-3, and CytC genes and promoting cumulus-oocyte complexes to synthesize GSH and express antioxidant enzymes such as GPX4 and SOD genes. During embryonic development, cell apoptosis is a major phenomenon that eliminates abnormal cells through embryo internal cell selection (Shimizu*et al.*, 2012).

Owing to the persisting low efficiency of IVM of some mammalian oocytes, the aim of this study was to examine the role of FSE as a natural source for antioxidants during IVM of Neaimi sheep oocytes, and its effect on gene expression and the maturation of oocytes.

Materials and Methods

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

The FSE was extracted once at the beginning of the experiments. Fenugreek seeds were heated in water (10 g/100 mL) until boiling, and then evaporated to 25% of its original volume. Afterwards, the suspension was spread and left to dry completely (Maghsoud *et al.*, 2013).

To evaluate the role of FSE as a natural source for antioxidants and estradiol 17β, IVM was carried out under these culture conditions: i) defined maturation medium (GI); ii) defined maturation medium + 1 µgFSE/mL (GII); and iii) defined maturation medium + 10 µgFSE/mL (GIII). The defined maturation medium consisted of Tissue culture medium-199 with earl's + 10% foetal bovine serum (Gibco, USA) + 0.023 IU FSH/ml + 0.23 IU LH/ml + 1 µg estradiol 17 β/ml + 50 µg/ml kanamycin. Group I served as the control group and Groups II and III were considered to evaluate the effect of FSE on the IVM outcome.

Ovaries of adult sheep were obtained from a local slaughterhouse, placed in isotonic sodium chloride solution (0.9% NaCl), and supplemented with penicillin (200 IU/mL) and streptomycin (200 µg/mL) at 30–35 °C. Collected ovaries were transported to the laboratory within one to three hours of collection. Ovaries were washed three times with pre-warmed fresh saline (37 °C), and all visible follicles with a diameter from 2 to 8 mm were aspirated with a 20-gauge needle. After aspiration, COCs were selected (Figure1) for IVM. They were washed three times with maturation medium and were cultivated for 22–24 hours at 38.5 °C at 5% CO_2 and high humidity conditions.
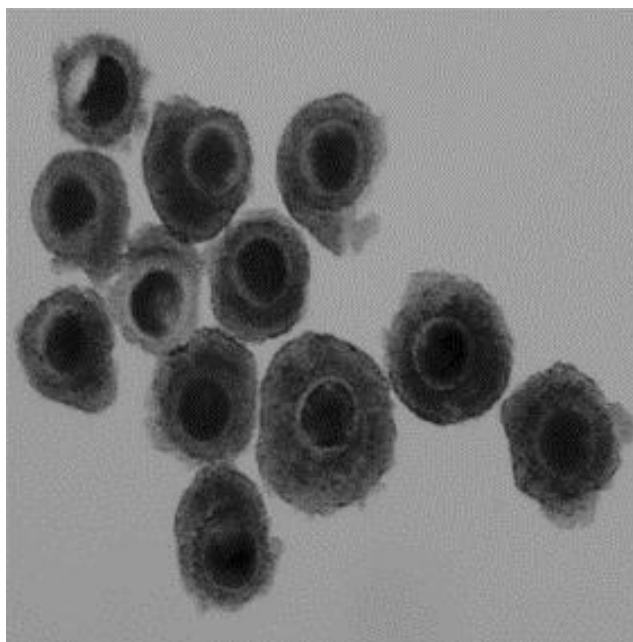

Figure 1 Different grades of cumulus oocyte complexes before *in vitro* maturation

Estimation of nuclear maturation was carried out with the classical method. After 22–24 hours, matured oocytes were denuded with 0.1% hyaluronidase and gentle mouth pipetting. Denuded oocytes were then fixed in acetic acid : ethanol (1 : 3, v/v) for 24–48 hours, and were stained with 1% aceto-orcein in 45% (v/v) acetic acid (Rao *et al.*, 2002). The stages of oocytes were classified according to Santos *et al.* (2006): intact germinal vesicle (GV), namely unidentifiable nucleolus and very fine filaments of chromatin; germinal vesicle breakdown (GVBD), presence of different patterns of chromatin condensation, chromosomes coiled up and no visible individual chromosomes; metaphase I (MI); anaphase I (AI); and metaphase II (MII).

The intracellular content of GSH was measured as described by Funahashi *et al.* (1994). The frozen samples were thawed at room temperature, then frozen again. This procedure was repeated three times. Five microlitres of 0.2 M sodium phosphate containing 10 mM EDTA (Sigma, assay buffer) and 5 mL of 1.25 M phosphoric acid (Fluka, Buchs, Switzerland) were added to a 1.5 mL microfuge tube containing 30 oocytes. Samples were stored at -80 °C until use. Concentrations of GSH in the oocytes were determined with the dithionitrobenzonic acid-glutathione disulphide (DTNB-GSSG) reductase-recycling assay (Anderson, 1985). Briefly, 700 µL of 0.33 mg NADPH/mL in stock buffer, 100 µL of 6 mM DTNB in stock buffer and 190

µL water were added with mixing into the microfuge tube. To initiate the reaction, 10 µL glutathione reductase was added with mixing. The formation of 5-thio-2-nitrobenzoic acid was followed continuously and the absorbance was monitored at 412 nm with a spectrophotometer (Ultraspec-2000, Pharmacia Biotech Ltd., Cambridge, UK) and recorded at 30 seconds and 5 minutes. The reagent blank and GSH standards (0.1–1.0 nmol) were assayed under the same conditions. The amount of GSH in each sample was determined by comparing it with a standard curve prepared at the same time.

The oocytes were washed three additional times in the phosphate buffer solution provided with a Cells-to-cDNA II kit (Ambion, Texas, USA), then transferred in a minimal volume of medium to 1.5 mL tubes and centrifuged at 17000 g for 10 min at 4 °C to recover the cell pellet. All samples were stored at -80 °C until assayed. cDNA was synthesized according to the manufacturer's instructions.

To prepare a cell lysate, 50 µL of the provided cell lysis buffer was added to a 0.5 mL tube containing the pelleted cells and mixed using a vortex for 5 min. The suspension was then heated using a block-type thermal cycler system according to the manufacturer's instructions.

Cell lysates were supplemented with RNase-free certified DNase I (10 IU/µL) provided in the Cells-to-cDNA II kit (0.16 IU/µL final concentration) and incubated at 37 °C for 5 minutes. Immediately after the end of the incubation, each tube was heated for 15 min at 75 °C to inactivate the DNase. To synthesize the first strand of cDNA according to the Cells-to-cDNA II kit protocol, 5 µL cell lysate, 4 µL dNTP mix, 2 µL oligo (dT) and 5 µL RNase-free water were assembled in an RNase-free 0.5 mL tube, then heated for 3 min at 70 °C. Afterwards, this mixture was cooled on ice, then 2 µL 10 × RT buffer, 1 µL M-MLV reverse transcriptase and 1 µL RNase inhibitor were added to the reaction tubes. Reverse transcription was carried out for 30 min at 42 °C, followed by incubation at 95 °C for 10 min. RT-minus products were produced as controls for each sample to demonstrate that the template for the PCR product was cDNA, not genomic DNA contamination.

Genes encoding activin like kinase5 (ALK5), GDF-9, transforming growth factor-receptor 1: (TGF-R1), HAS2, BCL-2, C-Myc, Bax and P54 transcript quantity were measured by Real Time-Polymerase change reaction (RT-PCR). The sequences of primers were designed using the GenBank genes sequences and synthesized by Applied Biosystems UK, Ltd. (Table 1). The RT-PCR cycling conditions consisted of initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation step at 94 °C for 30 sec., annealing step at 60 °C for 30 sec., and extension step at 72 °C for 45 seconds. This cycle was followed by final extension at 72 °C for 5 min. The data were presented as the fold change in gene expression normalized to the endogenous reference gene (β- actin) and relative to a calibrator. The RT-PCR data were analysed using the relative gene expression (that is, $2^{-\triangle\triangle C_T}$) method, as described in Applied Biosystems User Bulletin No. 2. The RT-PCR machine used was that of Applied Biosystems®. The expression was normalized to the endogenous reference gene (β- actin) and relative to a calibrator.

Table 1 Primer sequences and GenBank accession numbers of genes under study and housekeeping gene

Gene	Forward Primer	Reverse primer	Annealing temperature	Accession numbers
GDF-9	AGCTGAAGTGGGACAACTGG	ACACAGGATGGTCTTGGCAC	60 °C	NM_001142888.2
TGFβR1	ATGCCAATGGAGCAGCTAGG	ATGGCAGTTTCCTGGGTCTG	60 °C	XM_004004226.1
ACVRL1	GACCCCTTGAAACCCTCTCG	GAGGCGACGTACCTTCTAGC	60 °C	XM_004007400.1
HAS2	GTCTGTTTGCCTTGGCAGTG	TGTTGGTGAGAGGACCATGC	60 °C	XM_004015096.1
C-Myc	ACACACAACGTTTTGGAGCG	GGGATCTGGTCACGAAGAGC	60 °C	NM_001009426.1
BCL-2	ATGACTTCTCTCGGCGCTAC	CTCCACACACATGACCCCTC	60 °C	HM630309.1
Bax	TGCATCCACCAAGAAGCTGAG	AGGAAGTCCAATGTCCAGCC	60 °C	XM_004015363.1
B- Actin	AGGCCAACCGTGAGAAGATG	AATCGCACGAGGCCAATCTC	60 °C	NM_001009784.1

GDF-9: growth differentiation factor-9
TGFβR1: transforming growth factor beta receptor 1
ACVRL1: activin A receptor type II-like 1
HAS2: hyaluronan synthase 2

Statistical analyses for all data were carried out using one-way analysis of variance (ANOVA) followed by Duncan's test using the SPSS program. Results were expressed as mean ± SE. A probability of $P \leq 0.05$ was considered statistically significant.

Results

In terms of the effect of adding FSE to the maturation medium on the nuclear maturation of sheep oocytes, the results in Table 2 show no significant differences between Groups II and III for all nuclear stages (GV, GVBD, MI, AI, and MII), while there were significant differences between Groups II, III and control in relation to GVBD, MI, and Anaph.I stages. On the other hand, there was no significant difference in GV stage among groups. As for the MII stage, the mean value of Group III was significantly higher when compared with Group I (control), while it was not significantly different from Group II. At the same time, the mean values of Groups I and II were not significantly different (Table 2).

Table 2 Effect of fenugreek seed extract on *in vitro* nuclear maturation (mean ± SEM) of Saudi Arabian sheep oocytes

Traits Groups		Nuclear maturation				
		GV	GVBD	MI	AI	MII
Estradiol 17β (Control) (Group I)		$15.6^a \pm 0.7$	$11.4^a \pm 1.0$	$12.4^a \pm 1.1$	$8.2^a \pm 0.7$	$47.8^a \pm 6.3$
	1 µg/ml (Group II)	$13.2^a \pm 0.7$	$16.2^b \pm 1.3$	$16.4^b \pm 1.0$	$11.0^b \pm 0.7$	$51.4^{ab} \pm 2.7$
FSE	10 µg/ml (Group III)	$15.2^a \pm 0.9$	$15.2^b \pm 0.7$	$15.8^b \pm 1.1$	$10.8^b \pm 0.9$	$65.2^b \pm 3.8$

[ab] Mean values in the same columns with different superscripts differ significantly ($P \leq 0.05$)
GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; AI: anaphase I; MII: metaphase II, FSE: fenugreek seed extract

Results of GSH concentrations, as shown in Figure 2, reveal a significant increase in GSH levels in oocytes matured in a maturation medium supplemented with 10 µg/mL(Group III) (0.33 ± 0.1 nmol/oocytes) compared with Group I and II (0.15 ± 0.03 and 0.18 ± 0.7 nmol/oocytes, respectively).
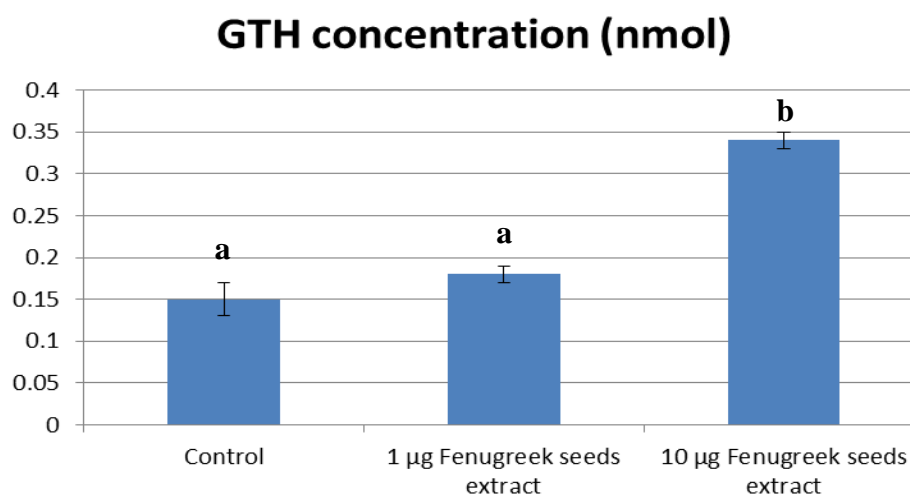

Figure 2 Effect of fenugreek seed extract on the concentration of the intracellular glutathione in sheep oocytes during *in vitro* maturation

The RT-PCR analyses (Table 3) showed that the mRNA expression of genes encoding ACVRL1, GDF-9, TGFβR1, HAS2, and C-Myc had a significant increase in Groups II and III compared with Group I (control) ($P \leq 0.05$). Results of mRNA RT-PCR showed that expression of both BCL-2 (pro-survival) and Bax (pro-apoptosis) were significantly decreased in Group II and III if compared with Group I (control group) ($P \leq 0.05$). In addition, there were no significant differences on the expression of these all genes between Group II and III ($P \leq 0.05$).

Table 3 Effect of administrations of fenugreek seed extract on the expression of activin A receptor type II-like 1, growth differentiation factor-9, transforming growth factor beta receptor 1, hyaluronan synthase 2, C-Myc, BCL2-associated X protein and B-cell leukaemia/lymphoma gene-2 genes in sheep oocytes matured *in vitro* (mean ± SEM)

Traits groups		The fold change in gene expression						
		ACVRL1	GDF-9	TGFβR1	HAS2	C-Myc	Bax	BCL-2
Control (Group I)		$1^a \pm 0.07$	$1.0^a \pm 0.05$	$1.0^a \pm 0.05$	$1.0^a \pm 0.06$	$1.0^a \pm 0.07$	$1.00^b \pm 0.07$	$1.00^b \pm 0.05$
FSE	1 µg/ml (Group II)	$1.45^b \pm 06$	$1.51^b \pm 0.03$	$1.41^b \pm 0.06$	$1.23^b \pm 0.04$	$1.95^b \pm 0.08$	$0.60^a \pm 0.03$	$0.61^a \pm 0.04$
	10 µg/ml (Group III)	$1.54^b \pm 0.9$	$1.47^b \pm 0.04$	$1.48^b \pm 0.04$	$1.34^b \pm 0.03$	$2.01^b \pm 0.06$	$0.52^a \pm 0.04$	$0.57^a \pm 0.03$

[ab] Mean values in the same columns with different superscripts differ significantly ($P \leq 0.05$)
FSE: fenugreek seed extract
GDF-9: Growth differentiation factor-9
TGFβR1: Transforming growth factor beta receptor 1
ACVRL1: Activin A receptor type II-like 1
HAS2: Hyaluronan synthase 2
BCL-2: B-cell leukaemia/lymphoma gene-2
Bax: BCL2-associated X protein

Discussion

This study is the first to report the effect of FSE as a natural antioxidant source on gene expression and *in vitro* maturation of sheep oocytes. The effect of FSE on the *in vitro* maturation of oocytes and thus the development of embryos was reported recently (Barakat & Ahmed, 2013). Previous studies have shown that increasing the oxidative stress in the maturation medium affects the success of maturation and thus the production of embryos in different animals (Geshi *et al.*, 2000; Harvey *et al.*, 2002; Watson *et al.*, 1994). Oxidative stress results from the increase of reactive oxygen species, that is, free radicals (Miesel *et al.*, 1993). Moreover, free radicals have been found in the cultivation media for aerobic organisms, oocytes and embryos (Guerin *et al.*, 2001; Martin-Romero *et al.*, 2008). Additionally, *in vitro* maturation of oocytes requires the presence of estradiol in the medium to complete the meiotic division. COCs secrete estradiol during cultivation in a steroid-free medium (Pellicer; 1997; Dode & Graves, 2002; Mingoti *et al.*, 2002). The current study agreed with previous studies concerning the importance of supplementing the maturation medium with antioxidants and estradiol to improve *in vitro* oocyte maturation. The results of the current study showed that the mean of maturation, intracellular GSH concentration and gene expression increased when using FSE compared with the control group. In addition, these findings agree with the results reported by Sur *et al.* (2001), Hibasami *et al.* (2003), Barakat & Ahmed (2013) and Barakat *et al.* (2014), who found that the addition of a source of antioxidants in the maturation medium enhanced the *in vitro* maturation rate and embryonic development. Previous studies showed that there is a relationship between the maturation rate and the concentration of intracellular GSH (Eppig, 1996; Abeydeera *et al.*, 2000; Luberda, 2005). This is illustrated by the current results, in which the authors found a significant increase in the *in vitro* maturation rate of sheep oocytes with an increased concentration of GSH. In contrast, there is no relationship between GSH concentration and the blastocyst formation rate (Viet *et al.*, 2009).

According to the current results, GDF-9 was upregulated in Groups I and II compared with control. Furthermore, the levels of other related genes to DD9 as HAS2, TGFβR1, transforming growth factor receptor 1, and C-Myc were increased. In this case, GDF-9 promoted granulosa cell proliferation, suppressed the expression of LH/CG receptor (LHCGR) in granulosa cells, and stimulated the expression of genes in cumulus cells, HAS2, and prostaglandin synthetase 2 (Ptgs2) (also known as cyclooxygenase 2 (Cox2)), which are required for the expansion of the cumulus oophorus and ovulation, thus mimicking the actions of native oocyte-derived factors observed in oocyte cumulus cell co-culture experiments (Elvin *et al.*, 1999; Elvin *et al.*, 2000). GDF-9, zygote arrest 1 (ZAR1) and BMP15, which are maternal effect genes, are expressed preferentially in the oocyte and are usually present in early embryos and then degraded at the time of maternal-to-embryonic transition, in which there is a shift from mRNAs and proteins stored in the oocyte to those transcribed and synthesized by the embryo (Yadav *et al.*, 2013). GDF-9 is an oocyte-specific paracrine factor, which is expressed throughout most stages of folliculogenesis and persists after fertilization

through pre-implantation embryo development (Pennetier *et al.*, 2004). GDF-9 and BMP15, both of which are members of the transforming growth factor-b superfamily, are expressed in oocytes and are secreted to act on cumulus cells (McNatty *et al.*, 2005). BMP15 transcript has been shown to be present in oocytes and embryos in cattle (Pennetier *et al.*, 2004), sheep (Bebbere *et al.*, 2008) and pigs (Li *et al.*, 2008) because apoptosis is believed to play an important role in protecting the cells from cytotoxic conditions by removing damaged cells. The current data showed that Bax and BCL-2 (apoptotic or bad genes) were significantly down-regulated in Groups I and II compared with control, where the inhibition of the levels of expression of these two genes occurred in the oocytes treated with FSE in the maturation medium. In contrast with the current results, Yadav *et al.* (2013) demonstrated that exposure of buffalo oocytes and embryos to elevated temperatures for a duration of time that is physiologically relevant severely compromises their developmental competence, increases apoptosis and affects stress-, apoptosis- and development-related genes (GDF-9, caspase-3, BID and Bax, BCL-XL and MCL-1, GLUT1, ZAR1, and BMP). The current results found that the levels of expression of both -2 and apoptotic Bax were significantly decreased in mature oocyte cells that were treated with FSE, as in Groups I and II, compared with control group. Mingning *et al.* (2013) concluded that the interaction of granulosa cells with ovarian cells could reduce the frequency of apoptotic cells of ovarian cortical stromal cells by decreasing the expression of pro-apoptotic genes Bax and bad mRNA in ovarian cortical stromal cells, ovarian medullary stromal cells and theca cells, and increasing the expression of pro-survival gene BCL-2 mRNA in ovarian stromal cells to promote ovarian cell proliferation. These findings suggested that granulosa cells prevented cell apoptosis of ovarian cortical stromal cells, and enhanced the capacity of anti-apoptosis of ovarian cortical stromal cells, ovarian medullary stromal cells and theca cells, but had no detrimental effect on cell proliferation of ovarian stromal cells. The present results indicated that adding FSE to the maturation medium significantly reduced BCL-2 and Bax genes expression in embryos, thus improving oocyte quality by reducing BCL-2 and Bax gene expression and inhibiting cell apoptosis. In this study, the authors also observed that treatment of cells with FSE resulted in decreased levels of both BCL-2 and Bax mRNA. According to its structure and function, BCL-2 is classified as a pro-apoptotic gene, anti-apoptotic gene, and regulated gene. Sun *et al.*, (2012) concluded that Bax initiate apoptosis by forming a pore in the mitochondrial outer membrane, which allows cytochrome C to escape into the cytoplasm and activates the pro-apoptotic caspase cascade. In addition, the BCL-2–associated death promoter (Bad) protein is a pro-apoptotic member of the BCL-2 gene family, which is involved in initiating apoptosis. According to the current data, FSE downregulated Bax and BCL-2 with high significance so it prevented cell apoptosis and enhanced the anti-apoptotic machinery. Thus, BCL-2, Bad, and Bax measurements in cells may reflect cell apoptotic degree. Studies in mice and cows have reported that granulosa cells are metabolically coupled to oocytes via gap junctions throughout their growth and during initial stages of maturation. This metabolic coupling is responsible for protein and phosphorylation changes associated with the resumption of meiosis (Spaczynski *et al.*, 2005). During oocyte maturation from GV to MII stage, oocytes generally cannot express genes, but need to synthesize some proteins and their phosphorylation changes for cytoplasmic maturation to promote nuclear changes. In the current study, that authors detected ACVRL1, GDF-9, transforming growth factor beta receptor 1 (TGFβR1), HAS2, β-cell lymphoma protein 2 (BCL-2), C-Myc, Bax and P54 gene expressions in MII oocytes. Although the oocyte itself does not express some genes during maturation, this gap junction of oocyte with cumulus cells may result in expressing some genes in cumulus cells to be transported into oocytes during IVM. This inference needs to be confirmed in future studies.

Conclusion

The addition of FSE to oocyte IVM medium at 10 μg/mL improved the maturation rate and increased the intracellular GSH concentration and gene expression of the studied genes. This improvement is attributed to the antioxidant effect regulating growth factor genes, anti-apoptotic genes and apoptotic genes.

Authors' Contributions

IAHB and RAA were in charge of the experimental design and implementation. IAHB and KMAZ wrote the manuscript. ARAH and RAA participated in correcting the manuscript. IAHB carried out statistical analysis.

Conflict of Interest Declaration

The authors wish to confirm that there are no conflicts of interest associated with the publication of this manuscript, and that it has been read and approved by all authors.

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