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Moringa oleifera extract modulates the expression of fertility related genes and elevation of calcium ions in sheep oocytes

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ABSTRACT

Success of *in vitro* maturation of mammalian oocytes and subsequently embryo production are normally affected by several factors such as hormonal alterations and culture conditions including nutrients or natural extracts. The leaves of *Moringa oleifera* are a good source for both macro- and micronutrients, and rich source of β-carotene, protein, vitamin C, calcium, and potassium. Gene expression techniques have become a powerful tool to analyze the relative abundance of transcripts related to quality of oocytes. In the present study, the relationship between mRNA expression profiles of maturation related genes [Cyclin B, cell division control (CDC2, CD44), extracellular signal-regulated kinases (ERK2) and c-mos genes], progression of meiosis and calcium concentration of sheep oocytes was evaluated. To carry out this study cumulus–oocyte complexes (COCs) aspirated from sheep ovaries were used. COCs were allocated in several groups cultured in TCM-199 medium without or with hormones plus different concentration of *M. oleifera* extract (20, 50 and 100 μg/ml). The results indicated that the relative abundance of the maturation related genes was affected by the concentration of *M. oleifera* extract and meiotic progression. Moreover, sheep oocytes cultured with hormones plus *M. oleifera* extract expressed high levels of the $[Ca^{2+}]$ concentration. The results suggest that *M. oleifera* combined with hormone supplementations improved the rate of maturation of sheep oocytes and could have acted as a promoter to induce mRNA expression and synthesis of essential protein, e.g. MPF, for the maturational processes.
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1. Introduction

In the Arabian economy, sheep play a role as suppliers of meat, milk and wool. Since sheep have their place in agriculture, ways have to be found to improve their level and efficiency of production and quality of their products. Efficient production depends among others, genetic improvement, and *in vitro* embryo production technology is a useful tool in this respect (Gilchrist and Thompson, 2007). Ovine *in vitro* embryo production is one of the future sheep breeding strategies for the development of biotechnologies. Since the maturation of oocytes is one of the prerequisites of successful IVF, many workers have studied the different aspects of *in vitro* maturation (IVM) of mammalian oocyles (Farag et al., 2009; Khalil et al., 2010).

In some studies, the supplementation of the IVM media with different combinations of hormones such as gonadotropins plus estradiol have been found to be essential for acquisition of developmental capacity of oocytes in cattle (Brackett et al., 1989) and buffaloes (Chauhan et al., 1997; Khalil et al., 2010). The addition of hormone combinations (gonadotropins plus estradiol) with a source of sera such as estrous goat serum (EGS) to TCM-199 medium has also been found to be necessary for achieving high maturation and cleavage rates of fertilized goat oocytes (Mogas et al., 1997a,b).

The developmental competence of oocytes may be a function of the presence or abundance of specific transcripts in their mRNA pools (Bachvarova, 1985). These pools of stored RNA are essential to ensure proper protein synthesis necessary for oocytes meiotic maturation, fertilization, and the first embryonic cell divisions. The main transcripts produced during maturation of the cumulus–oocyte complexes (COCs) encode regulators of the cell cycle such as maturation promoting factor (MPF), c-mos protooncogene (MOS) protein, and mitogen-activated protein kinase (MAPK) (Calder et al., 2003). MPF is a protein complex composed of two subunits including a catalytic subunit [p34cdc2 kinase] and a regulatory subunit [Cyclin B] (Taieb et al., 1997). The MPF activity in oocytes has been well characterized that active MPF from a

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competent oocyte can induce condensation of chromatin in the immature oocyte nucleus and reorganization of the cytoplasm (Yamashita et al., 2000).

The family of mitogen-activated protein (MAP) kinases, namely extracellular signal-regulated kinases (ERKs), has been shown to have an important role during the M-phase (Kubelka et al., 2002). The activation of MAP kinase is important for the transition of oocytes from Metaphase I (MI) to Metaphase II (MII) (Dedieu et al., 1996). Activation of MAP kinase may be regulated by a signaling pathway through synthesis of Mos kinase (proto-oncogene Mos kinase) (Peter et al., 2002; Verlhac et al., 2000). The mos protooncogene encodes the protein Mos (Maxwell and Arlinghaus, 1985), which is expressed at high levels in oocytes undergoing maturation (Gebauer and Richter, 1997).

A transient increase in the intracellular calcium concentration has long been known to be required for the induction of oocyte maturation, or GVBD, as it is blocked by injection of calcium chelators into the cell (Pesty et al., 1998). Mouse oocytes dissected from the follicles and spontaneously resuming meiosis *in vitro* display repetitive calcium transients prior to GVBD as well (Carroll et al., 1994).

Several plant species have proven to be important to both human and animal for nutrition and health maintenance (Ahmed et al., 2007). *M. oleifera* Lam. (syn. *Moringa pterygosperma*) is a rapidly growing tree that is native to Asia and Africa (Tsaknis et al., 1999; Fahey, 2005). This plant is extraordinary because all of its parts are edible and possess nutritional and medicinal values. Its leaves are an excellent source of vitamin A (four times the amount in carrots), vitamin C (seven times the amount in oranges), vitamin B, calcium (four times the amount in milk), protein (twice the amount in milk), and potassium (three times the amount in bananas) (Cajuday and Pocsidio, 2010). In addition, *Moringa* contains specific plant pigments with demonstrated potent antioxidant properties such as the carotenoids – lutein, alpha-carotene and beta-carotene, xanthins, and chlorophyll; other phytochemicals with known powerful antioxidant ability – kaempferol, quercetin, rutin and caffeoylquinic acids; powerful antioxidant vitamins – C, E, and A and essential micronutrients with antioxidant activity – selenium and zinc (Cajuday and Pocsidio, 2010).

In addition, *M. oleifera* is used in several tropical countries for medicinal purposes (Foidl et al., 1999). Extracts of various *Moringa* tissues have been used as anti-cancer, anti-trypanosomal, antimicrobial, anti-inflammatory and hepatoprotective agents (Cajuday and Pocsidio, 2010). In addition, leaf extracts have been shown to regulate thyroid status and cholesterol levels in rats (Ghasi et al., 2000; Tahiliani and Kar, 2000). To date, there is an ongoing scientific investigation on some of the reported therapeutic and medicinal properties of *Moringa* plant. Interestingly, the *Moringa* fruit is said to increase sexual libido in men in India (Cajuday and Pocsidio, 2010). Also, Cajuday and Pocsidio (2010) reported an increase in the sperm count in male mice when 1% concentration of *Moringa* ethanolic leaf extract was administered subcutaneously for two weeks.

To the best of our knowledge, no published data are available about the effect of *M. oleifera* on maturation of ruminant oocytes. Therefore, the role of the plant in improving *in vitro* maturation and consequently embryo production is our focus of interest. To perform the aim of this study assessment of meiotic progression, expression of maturation related genes and intracellular calcium concentration in sheep oocytes cultured *in vitro* were carried out.

2. Materials and methods

2.1. Plant material and extraction

The leaves of *M. oleifera* (MO) were collected from the farm of Egyptian Scientific Society of Moringa, Egypt. The collected material was air-dried, reduced to powder and kept for extraction. Dry powdered MO leaves (200 g) were extracted with 1000 ml of ethanol, shacked and mixed three times per day. First, ethanol 96% was used and after 24 h solutions were filtered and in the second step ethanol 70% was added to the remained dry materials. Afterwards, the hydro-alcoholic extract was filtered using a cotton funnel and repeat four times. The extract was concentrated using a rotator evaporator under pressure at 50 °C. Finally, the extract was dried and the remainder was later lyophilized. The resulting brownish and dark extract were filtered and then stored in fridge in dark place until use.

2.2. Experimental design

2.2.1. Oocytes collection

Sheep ovaries were collected at a local abattoir immediately after slaughter. The ovaries ($n = 536$) were transported to the laboratory in 0.9% saline supplemented with 50 μg/ml gentamycin sulfate at 30–35 °C within 2 h. Oocytes from all visible antral follicles (3–6 mm in a diameter) were aspirated with a 20-gauge hypodermic needle attached to a 5 ml disposable syringe containing 1 ml of aspiration medium. The aspiration medium consisted of Dulbecco's phosphate buffer saline (D-PBS) supplemented with 0.03 g/ml bovine serum albumin and 50 μg/ml gentamycin sulfate (Chauhan et al., 1997).

2.2.2. In vitro maturation

Cumulus–oocyte complexes (COCs) (with an unexpanded mass of cumulus cells and homogenous cytoplasm) were recovered under a stereomicroscope (Labomed, Labo America, Inc., USA). The COCs were washed once with aspiration medium and twice in basic culture medium TCM-199. This media (TCM-199, Sigma, St. Louis, MO, USA) was enriched with 50 μg/ml gentamycin sulfate without any serum or hormone supplementations to serve as a negative control. The same non-supplemented medium (TCM-199) was used as treatment groups with several supplements as follows: (1) TCM-199 medium supplemented with 20 IU/ml PMSG (GIBCO/BRL, Grand Island, N.Y, USA) + 10 IU/ml hCG + 1 μg/ml 17 β-estradiol (E2, Sigma, St. Louis, MO, USA) + 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) to act as a positive control; (2–4) same medium with the previous supplements in treatment (1) + 20 or 50 or 100 μg of *Moringa* extract/ml of medium.

In addition, the non-supplemented medium alone (negative control) and the different supplemented media without (positive control) or with *Moringa* extract were tested on sheep oocytes (COCs) to be serve as a maturation medium. Each treatment consisted of five replicates. The non-supplemented medium or the media enriched with hormone supplements with or without *Moringa* extract were sterilized using 0.22 μm Millipore filter.

For all experiments, 10–15 oocytes of COCs were transferred separately into a 50 μl drop of each type of culture media (control medium or medium plus hormone supplements with or without *Moringa* extract), covered with sterile mineral oil in a polystyrene culture dish (3.5 mm × 10 mm) which had been previously kept for about 2 h in a CO_2 incubator before the oocytes were added.

The oocytes (COCs) were cultured for 28–29 h at 39 °C in an atmosphere of 5% CO_2 in air with 95% humidity. Following the culture period, the degree of cumulus expansion of COCs was determined. The *in vitro* matured oocytes (COCs) were used for (1) accelerating the rate of nuclear maturation, irrespective of the degree of expansion (Bolamba et al., 2006); (2) determination of the expression of maturation related genes; and (3) Ca^{2+} measurements (Table 1).

2.3. Cytogenetic analysis for assessment of the nuclear maturation

To determine the rate of nuclear maturation (the proportion of oocytes whose nuclei reached metaphase II), the cumulus cells of COCs were removed by vortexing. The cumulus-free COCs with homogenous cytoplasm were then fixed in solutions of acetic acid:ethanol (1:3 v/v) in culture dishes (35 × 10 mm) for at least 48 h at 4 °C. Fixed oocytes were transferred to glass slides; silicon gel was used to maintain a coverslip in contact with the oocytes. The slides were immersed in 1% aceto-orcein stain for 30 min. Then, slides were washed three times in ascending concentrations of ethanol to remove the surplus orcein dye as follows: 5 s in 70% ethanol, then 1 and 3 min in absolute ethanol (Khalil et al., 2010). Oocytes were examined under a light microscope (1000× magnification) and classified as being at one of the following stages: germinal vesicle stage (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). Oocytes with no visible or abnormal chromatin configuration were classified as degenerate (Beker et al., 2000).

2.4. Gene expression analysis using quantitative real-time PCR

2.4.1. RNA extraction and cDNA synthesis

To determine the gene expression of selected genes, sheep oocytes of each treatment were used. Total RNA from pooled COCs were extracted using the Dynabeads mRNA DIRECT kit according to the manufacturer's instructions with minor modifications (Wrenzycki et al., 1999; Khalil et al., 2010). Briefly, pools of 10–15 frozen oocytes (Table 1) from each group were lysed by adding 30 Nl of lysis/binding buffer (cell lysis/mRNA binding buffer, 100 mM Tris–HCl, pH 8.0; 500 mM LiCl; 10 mM EDTA; 1% LiDS (SDS); 5 mM DTT) in 0.6 ml siliconized polypropyl tubes. After vortexing for 10 s, centrifugation for 30 s at 1000 *g* and incubation at room temperature for

Table 1
Experimental design clarifies replications and number of ovaries and oocytes used.

Groups	Meiotic progression analysis[a]		Gene expression analysis[b]		Ca^{2+} measurement[c]	
	No. of ovaries	No. of oocytes	No. of ovaries	No. of oocytes	No. of ovaries	No. of oocytes
TCM-199	35	135	34	137	35	140
TCM-199 + suppl	37	148	36	141	36	137
TCM-199 + suppl + MOE20	36	144	37	138	36	142
TCM-199 + suppl + MOE50	36	137	36	142	37	141
TCM-199 + suppl + MOE100	35	140	35	144	35	138
Total	179	704	178	702	179	698

[a,b,c] No of replications of analysis was "5" in which each replicate contained at least 7 ovaries and 25–30 oocytes; Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

10 min, 5 Nl of Dynabeads Oligo (dT) 25, prewashed twice with 60 Nl of lysis/binding buffer, were pipetted into the fluid. After 5 min of incubation at 20 °C under constant shaking to induce binding of poly(A)-RNAs to oligo (dT)25 Dynabeads, the beads were separated on ice by a Dynal MPC-E-1 magnetic separator (Dynal, Hamburg, Germany). After washing once with 40 Nl washing buffer 1 (10 mM Tris/HCl, pH 8.0; 0.15 M LiCl; 1 mM EDTA; 0.1% LiDS) and three times with 40 Nl washing buffer 2 (10 mM Tris/HCl, pH 8.0; 0.15 M LiCl; 1 mM EDTA), Poly(A)-RNAs were then eluted from the beads by incubation with 11 Nl sterile water at 65 °C for 2 min.

Aliquots were immediately used for reverse transcription (RT) using the PCR RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany), to obtain the widest array of cDNAs. To create a cDNA copy using 1 Nl oligo dT primer, the RT reaction was carried out in a final volume of 20 μl at 25 °C for 10 min, followed by 1 h at 42 °C, and finished with a denaturation step at 99 °C for 5 min and immediate cooling on ice. The cDNA then was used immediately in the following PCR reaction or stored at −20 °C until use.

2.4.2. Quantitative real-time PCR

The first strand cDNA (2 Ng) from different categories of sheep oocytes was used as templates for PCR with a pair of specific primers. The sequences of specific primers used are listed in Table 2.

QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the cortex cDNA copy number. PCR reactions were set up in 25 μl reaction mixtures containing 12.5 μl 1 × SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μl 0.2 μM sense and antisense primers, 6.5 μl distilled water, and 5 μl of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0 °C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0 °C for 15 s; (b) at 94 °C for 30 s, 60 °C for 30 s; and (c) at 72.0 °C for 30 s. The third step consisted of 71 cycles which started at 60.0 °C and then increased about 0.5 °C every 10 s up to 95.0 °C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

2.4.3. Calculation of gene expression

The amplification efficiency (*Ef*) was calculated from the slope of the standard curve using the following formula:

$$Ef = 10^{-1/\text{slope}}$$

$$\text{Efficiency}\,(\%) = (Ef - 1) \times 100$$

For the $2^{-\Delta\Delta CT}$ calculation to be valid, the efficiency (*Ef*) of the target amplification (CDC2, DC44, c-mos, Cyclin B and ERK2) and the efficiency of the reference amplification (β-actin) must be approximately the same. To calculate the relative efficiency of target and reference amplification the following formula was used:

$$\Delta C_{T(\text{test})} = C_{T(\text{target,test})} - C_{T(\text{reference, test})},$$

$$\Delta CT\,(\text{calibrator}) = C_{T(\text{target, calibrator})} - C_{T(\text{reference, calibrator})},$$

$$\Delta\Delta CT = \Delta C_{T(\text{Test})} - \Delta C_{T(\text{calibrator})}.$$

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

The β-actin gene was used as housekeeping gene due to that (a) the β-actin gene encodes a structural protein of cytoskeleton, and is the most widely used gene for normalization in the experiments of gene expression (Pohjanvirta et al., 2006). (b) β-Actin showed high stability in fresh and *in vitro* cultured goat preantral follicles (Frota et al., 2010).

2.5. Preparation of oocytes for Ca^{2+} measurement

Oocytes were denuded of their cumulus cells by placing them in hyaluronidase (Sigma, Poole, UK) (11 IU/ml) in HEPES-buffered Earle's balanced salt solution (EBSS; Life Technologies Ltd.) and aspirating them through fine bore (150–200 mm) pipettes for 1–3 min. Oocytes were then washed in HEPES-buffered EBSS supplemented with 0.5 mM pyruvate and 10% (v/v) bovine serum albumin. Cumulus-free oocytes were kept in the above medium and maintained at 37 °C for 0.5–6 h prior to measurement of $[Ca^{2+}]$i.

2.6. Measurement of $[Ca^{2+}]$i

Measurement of oocyte $[Ca^{2+}]$i was carried out according to Herbert et al. (1997). Briefly, Fura-2, a dual excitation wavelength Ca^{2+} indicator, was used to measure levels of $[Ca^{2+}]$i. Oocytes were incubated with 1.5 mM of the esterified form of the dye (Fura-2 AM; Molecular Probes Inc, OR, USA) at 37 °C for 15–20 min prior to the commencement of $[Ca^{2+}]$i measurement. A Nikon fluorescence microscope was used to measure $[Ca^{2+}]$i. Individual oocytes were illuminated alternately for 500 ms at 350 and 380 nm which are the respective excitation wavelengths of free and bound Fura-2.

2.7. Statistical analysis

Data of mRNAs abundance and levels of $[Ca^{2+}]$i were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS) (SAS, 1982). Scheffé-test was used to assess the significant differences between groups of the study including control and those supplemented with hormones and/or *M. oleifera* extract. The values were expressed as mean ± SEM. All statements of significant were based on probability of $P < 0.05$. On the other hand, data of meiotic progression of the cultured oocytes were expressed as percentage. Moreover, the significant differences for the data of nuclear maturation rates were analyzed using Chi-square test. Differences of the rates of nuclear maturation with a probability value less than 0.05 were considered significant.

3. Results

3.1. Effect of hormones and M. oleifera extract supplements on the meiotic progression

Table 3 shows the state of meiotic progression of the sheep oocytes *in vitro* cultured without or with hormones and *M. oleifera* extract supplements. The results indicated that most oocytes cultured in TCM-199 medium without supplements were arrested in the GV stage (37%); however, 33.3% and 29.6% of these oocytes had resumed GVBD and MI stages of meiosis, respectively. In contrary, sheep oocytes cultured with hormone supplements reached AI/TI and MII stages with 31.8% and 48%, respectively. Moreover, an improvement in progression of meiosis was shown in oocytes cultures with different concentration of *M. oleifera* extract, where, most oocytes reached AI/TI and MII stages. Sheep oocytes cultured with low, medium and high concentrations of *M. oleifera* extract reached AI/TI and MII stages with 28.8 + 53.8, 26.3 + 59.1 and 25 + 64.3%, respectively.

3.2. Effect of hormones and M. oleifera extract supplements on the gene expression

To assess the effect of the hormones and *M. oleifera* extract supplements on the expression levels of maturation related genes quantitative real-time PCR assay was used. Amplification of sheep oocyte cDNA with CD44, CDC2, c-mos, Cyclin B, and ERK2 specific primers resulted in quantitative PCR products (Figs. 1–5). These products of the studied genes were detected in all oocytes whether they cultured with or without hormones and *M. oleifera* extract supplements.

The relative abundance of CD44, CDC2, c-mos, Cyclin B and ERK2 mRNA was affected by the hormones and *M. oleifera* extract supplements ($P < 0.05$) (Figs. 1–5). The expression levels of CD44, CDC2,

Table 2
Primer sequences used for qPCR.

Gene	Primer sequence (5′–3′)	References
CD44	F: CAA CAC CTC CCA ATA TGA CAC R: TTC TTC TGC CCA CAC CTT CT	Aminafshar et al. (2014)
CDC2	F: ATT CTA TCC CTC CTG GTC AGT TCA T R: CAC TTC TGG CCA CAC TTC ATT ATT G	Khalil et al. (2010)
c-mos	F: CTT GGA CCT GAA GCC AGC GAA CAT T R: CGC CGA GAG GGA CGG ACG GAG ATT G	Khalil et al. (2010)
Cyclin B	F: GAG GGG ATC CAA ACC TTT GTA GTG A R: CAA TTT CTG GAG GGT ACA TTT CTT C	Khalil et al. (2010)
ERK2	F: CAC CGA CCA TCG AGC AGA TGA AAG A R: ACG GGG ATC CAA GAA TAC CCA GAA T	Khalil et al. (2010)
β-Actin	F: CAA CTG GGA CGA CAT GGA R: TGG TGG TGA AGC TGT AGC	Aminafshar et al. (2014)

F, forward primer; R, reverse primer.

Table 3
Effect of supplementation of hormones and *Moringa oleifera* extract on meiotic progression of the sheep oocytes.

Groups	Total of COCs	Mean of COCs per replicates	State of nucleus				
			GV M ± SE (%)	GVBD M ± SE (%)	MI M ± SE (%)	AI/TI M ± SE (%)	MII M ± SE (%)
TCM-199	135	27 ± 1.2	10 ± 0.06 (37.0)	9 ± 0.04[a] (33.3)	8 ± 0.05[a] (29.6)	–	–
TCM-199 + suppl	148	29.6 ± 2.3	00	2 ± 0.01[b] (6.8)	4 ± 0.02[b] (13.5)	9.4 ± 0.04[a] (31.8)	14.2 ± 0.08[b] (48.0)
TCM-199 + suppl + MOE20	144	28.8 ± 1.6	00	00	5 ± 0.06[b] (17.4)	8.3 ± 0.08[ab] (28.8)	15.5 ± 0.07[ab] (53.8)
TCM-199 + suppl + MOE50	137	27.4 ± 1.4	00	00	4 ± 0.05[b] (14.6)	7.2 ± 0.07[b] (26.3)	16.2 ± 0.10[ab] (59.1)
TCM-199 + suppl + MOE100	140	28 ± 2.2	00	00	3 ± 0.01[b] (10.7)	7 ± 0.06[b] (25.0)	18 ± 0.11[a] (64.3)

Suppl, hormones supplements; MOE, *Moringa oleifera* extract; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphaseI; AI/TI, anaphaseI/telophaseI; MII, metaphase II. [a,b] Mean values in the same column with different superscript differ significantly ($P < 0.05$).

c-mos, Cyclin B and ERK2 mRNA were higher in sheep oocytes cultured in TCM-199 medium without any supplementation of hormones or *M. oleifera* extract compared with those cultured with the supplements. However, the expression levels of these genes were gradually decreased with the supplementation of the hormones and increment of the *M. oleifera* extract doses, where, the decline of the expression levels of the tested genes was significantly different

There were no differences in the relative abundance of all tested genes between sheep oocytes cultured with hormones supplements alone or hormones supplements plus low dose of *M. oleifera*

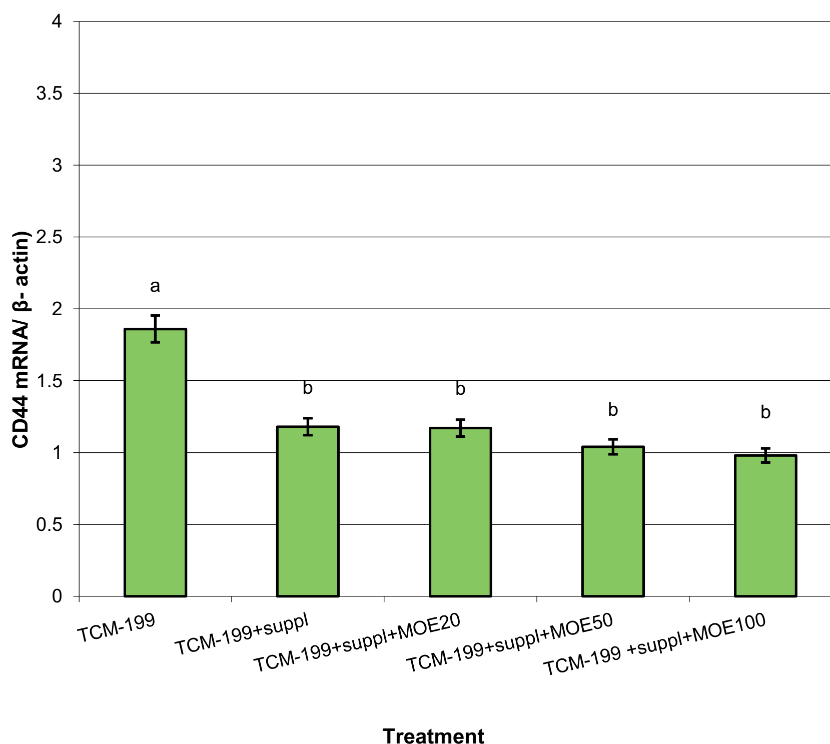

Fig. 1. The relative expression of CD44 gene in sheep oocytes without/or with supplements of hormones and *Moringa oleifera* extract. [a,b]: Mean values in the same column with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

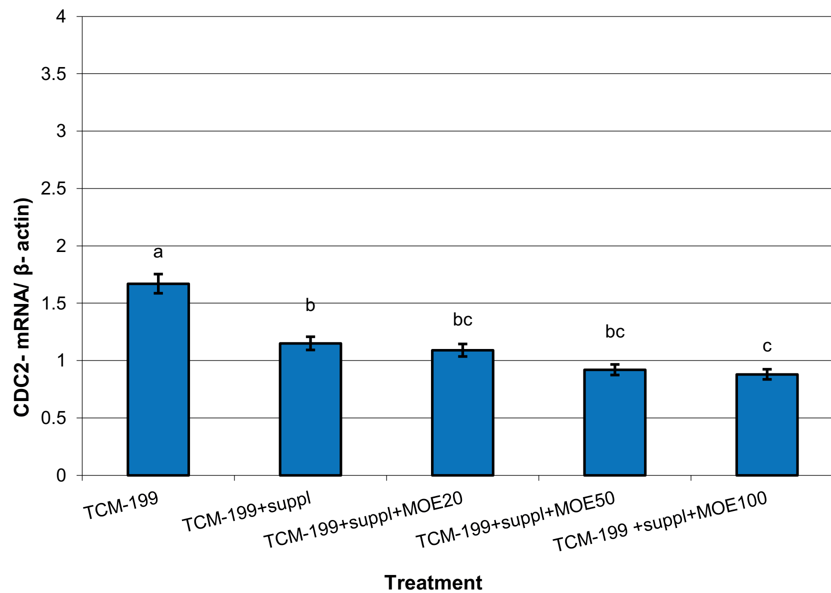

Fig. 2. The relative expression of CDC2 gene in sheep oocytes without/or with supplements of hormones and *Moringa oleifera* extract. [a,b,c]: Mean values in the same column with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

extract. While, there were significant differences in expression levels between the oocytes cultured with hormones supplements plus medium or high doses and other treatments, where, the expression values reached lowest levels in the oocytes cultures medium and high doses of the *M. oleifera* extract (Figs. 1–5).

3.3. *Effect of hormones and* M. oleifera *extract supplements on the Ca^{2+} concentration*

When *in vitro* cultured sheep oocytes were exposed to hormones and *M. oleifera* extract supplements, three intensity of intracellular calcium $[Ca^{2+}]i$ responses were seen: type 1, a large spike of $[Ca^{2+}]I$, type 2, a minor elevation; and type 3, a single baseline as no response of $[Ca^{2+}]i$ (Fig. 6).

The results revealed that most sheep oocytes (55%) cultured without any supplements indicated single baseline as no response of $[Ca^{2+}]i$, type 3. However, most the oocytes (50%) supplemented with hormones only revealed type 2 response of $[Ca^{2+}]I$. In contrary, the type 1, large spike of $[Ca^{2+}]I$, was the majority response in the sheep oocytes cultured with hormone plus *M. oleifera* extract supplements, where, the type 1 response of $[Ca^{2+}]I$ was observed in 45, 60 and 70% of the oocytes cultured with low, medium and high doses of *M. oleifera* extract (Fig. 6).

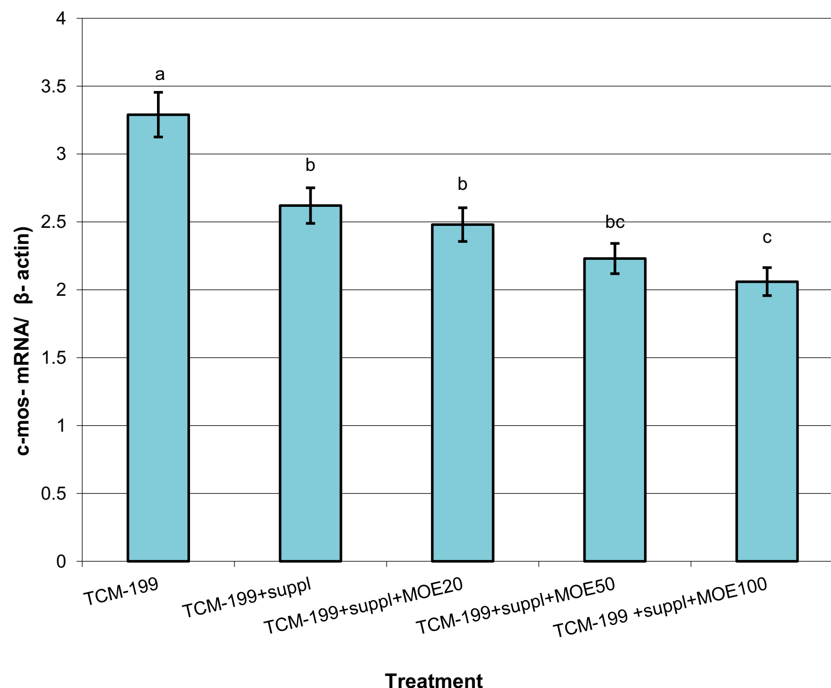

Fig. 3. The relative expression of c-mos gene in sheep oocytes without/or with supplements of hormones and *Moringa oleifera* extract. [a,b,c]: Mean values in the same column with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

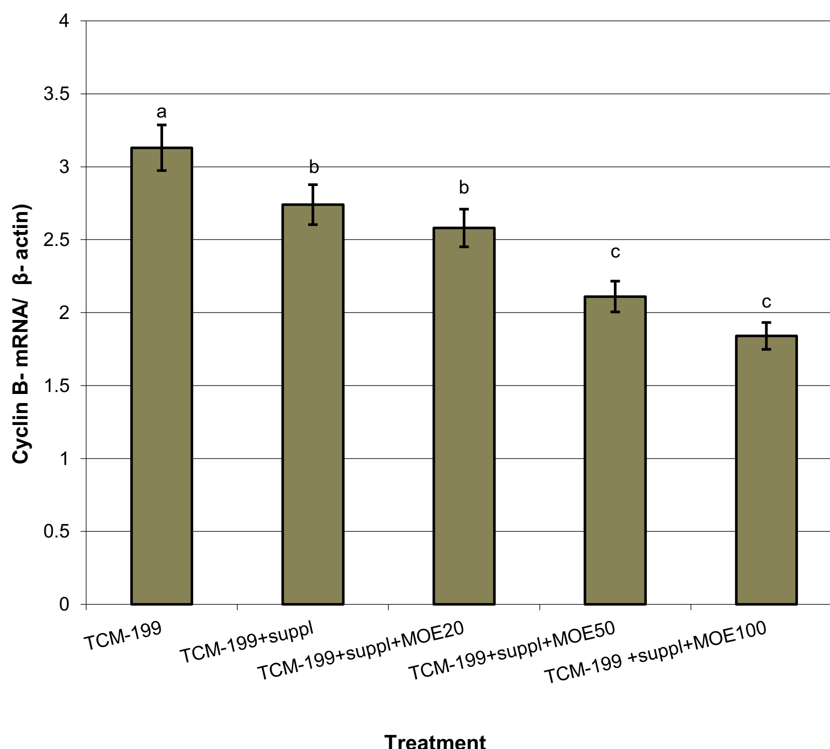

Fig. 4. The relative expression of Cyclin B gene in sheep oocytes without/or with supplements of hormones and *Moringa oleifera* extract. [a,b,c]: Mean values in the same column with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

In addition, Table 4 shows concentration of total calcium in sheep oocytes cultured *in vitro* in relation to the supplementation of hormones and *M. oleifera* extract. The results revealed that oocytes cultured without any supplements showed low concentration of $[Ca^{2+}]$. However, the $[Ca^{2+}]$ concentration increased to 2.6-fold in the oocytes cultured with the hormones supplements only compared with those cultured without supplements. Moreover, sheep oocytes cultured with hormones plus *M. oleifera* extract expressed

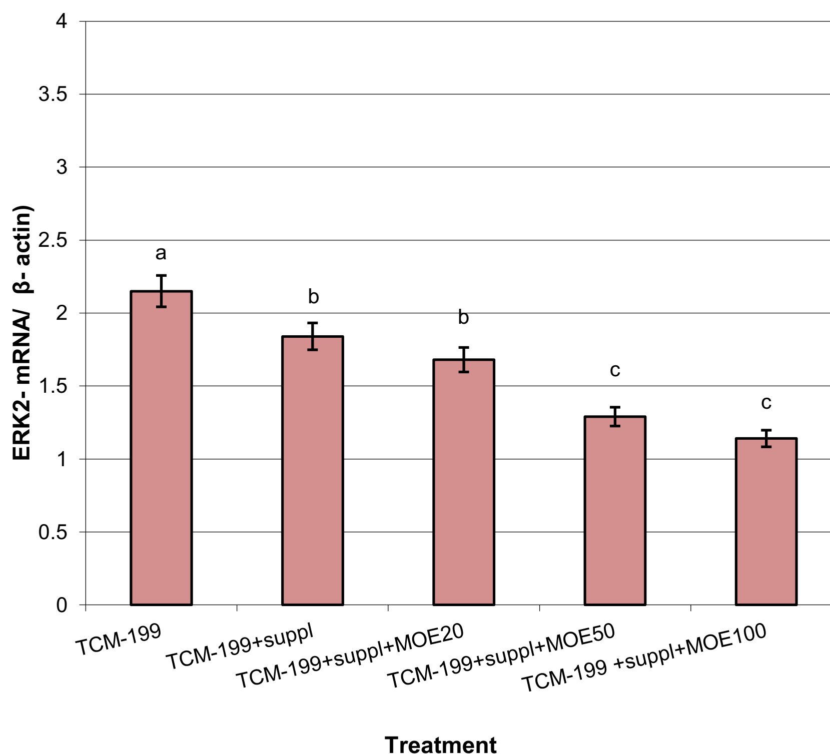

Fig. 5. The relative expression of ERK2 gene in sheep oocytes without/or with supplements of hormones and *Moringa oleifera* extract. [a,b,c]: Mean values in the same column with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

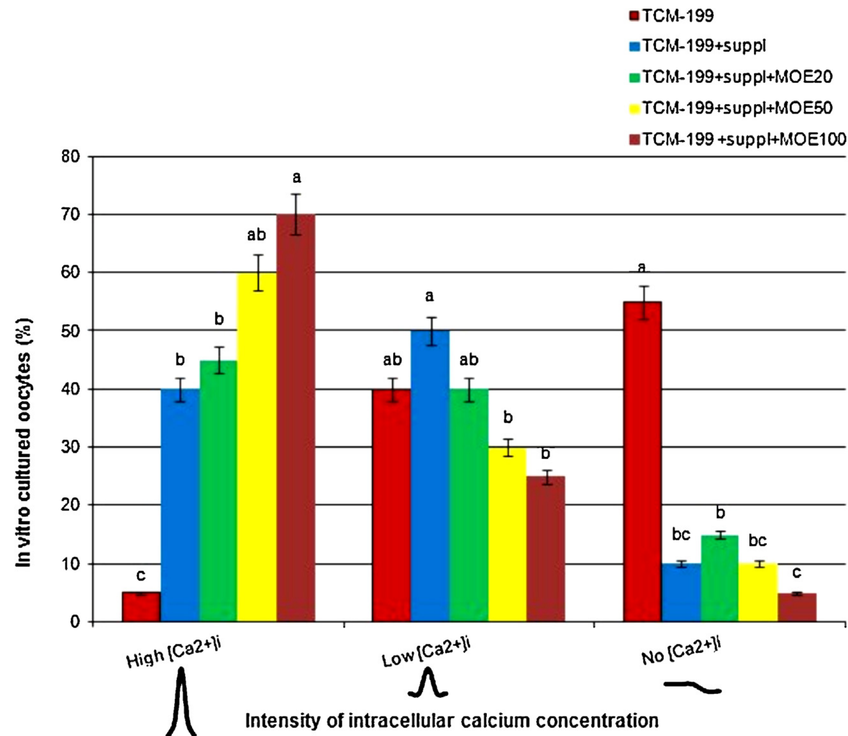

Fig. 6. Incidences of the different intensity of intracellular calcium concentration ($[Ca^{2+}]$i) response following supplementation of hormones and *Moringa oleifera* extract of *in vitro* matured sheep oocytes. [a,b,c]: Mean values of column between groups with different superscript differ significantly ($P < 0.05$). Suppl, hormones supplements; MOE, *Moringa oleifera* extract.

high levels of the $[Ca^{2+}]$ concentration, where, the concentration of the $[Ca^{2+}]$ increased to 2.8, 4 and 4.7-fold in oocytes cultured with low, medium and high doses of *M. oleifera*, respectively, compared with those cultured without supplements.

4. Discussion

The current study showed that supplementation of hormone combinations (PMSG + hCG + F2 + FBS) to the culture media (TCM-199) improved maturation rate of sheep COCs compared to the control media (without additives). Our findings were supported by the results of several studies on the *in vitro* maturation of goat oocytes. These studies have supplemented the maturation medium with FSH, LH and 17β-esradiol and FCS (Mogas et al., 1995) or FCS (Pawshe et al., 1996). In the experiment conducted by Mogas et al. (1995), supplementation of maturation medium with hormones + FCS enhanced the maturation rate to 55%. Pawshe et al. (1996) reported that 50–65% of oocytes became matured using media supplemented with hormones + FCS. Moreover, the present data are also consistent with those reported by Mogas et al. (1997a,b) who achieved a high *in vitro* maturation rate of goat oocytes in the presence of hormonal supplementation (FSH + LH + E2) plus estrous goat serum (EGS). In another study, Keskintepe et al. (1994) reported that the cleavage rate of caprine oocytes previously matured in TCM-199 medium enriched with FCS and hormonal supplements (FSH + LH + E2) was significantly higher than in hormone-free medium.

It has been reported that variations in the rate of the *in vitro* maturation the oocytes may be due to the composition of the medium (Krisher and Bavister, 1998). Maturation media supplemented with essential and non-essential amino acids supported maturation and development after fertilization more than that supplemented with essential amino acids or glutamine alone. Furthermore, in a previous study Bae and Foote (1975) found that the addition of glutamine to the media had a beneficial effect by providing both energy and ammonia nitrogen to the maturing oocytes of the rabbit. Therefore, the higher maturation rate *in vitro* of sheep oocytes which was achieved with TCM-199 medium supplemented with hormones in the present work might be attributed to some factors in its composition such as essential and non-essential amino acids, glutamine and insulin which stimulates DNA and RNA synthesis and enhances cell division in both media (Bilodeau-Goeseels, 2006, Gilchrist and Thompson, 2007). Moreover, gonadotropins are the primary regulators of nuclear maturation in mammalian oocytes *in vitro*, and one of the functions of its preovulatory surge is to suppress the granulose cell factors that inhibit meiosis (Pawshe et al., 1996). On the other hand, estradiol has been found to improve the completion of

Table 4
Concentration of total calcium in sheep oocytes cultured *in vitro* in relation to supplementation of hormones and *Moringa oleifera* extract.

Groups	Total of COCs	Mean of COCs per replicates	Total calcium (ng/COC; mean ± SEM)
TCM-199	140	28 ± 1.1	1.8 ± 0.11[c]
TCM-199 + suppl	137	27.4 ± 1.3	4.73 ± 0.36[b]
TCM-199 + suppl + MOE20	142	28.4 ± 2.1	5.12 ± 0.37[b]
TCM-199 + suppl + MOE50	141	28.2 ± 1.6	7.25 ± 2.13[a]
TCM-199 + suppl + MOE100	138	27.6 ± 1.4	8.53 ± 2.21[a]

Suppl, hormones supplements; MOE, *Moringa oleifera* extract. [a,b,c] Mean values in the same column with different superscript differ significantly ($P < 0.05$).

maturational changes (Farag et al., 2009) and also supported the synthesis of presumed male pronuclear growth factor (Fukui and Ono, 1989).

One of the main objectives of the current work is to evaluate the effect of *M. oleifera* extract as an additive in the culture media on the maturation rate of sheep oocytes. Up to date there are no published data concerning the effect of *M. oleifera* on oocyte maturation in animals. However, Odeyinka et al. (2008) discussed the effect of *M. oleifera* on reproductive performance of rabbits. They found that animals fed on 100% *Moringa* had higher values in litter size at birth, litter weight at birth and litter weight at weaning than those fed on 100% Centrosema.

Moreover, another study discussed the effect of *M. oleifera* leaf powder on fertility of male mice including sperm count and histology of testis and epididymis which found that administration of *M. oleifera* showed significantly higher testes and epididymis weight in comparison to control animals. They concluded that improvement of fertility by *M. oleifera* administration may be due to its chemical composition in leaves as they are excellent source of vitamin B, calcium, protein and potassium. In addition, its leaves contain also beta-carotene and other phytochemicals with known powerful antioxidant ability (kaempferol, quercetin, rutin and caffeoylquinic acids); powerful antioxidant vitamins (C, E, and A) and essential micronutrients with antioxidant activity (selenium and zinc as explained) which play an important role in fertility performance (Jaiswal et al., 2009; Vongsak et al., 2014). In agreement with the above findings, the current study showed an improvement in progression of meiosis of sheep oocytes cultured with different concentration of *M. oleifera* extract compared with those cultured without additives of *M. oleifera* extract.

Gaining knowledge about the variation/chronology of gene expression during oocyte maturation is crucial for optimization of IVF and other artificial reproduction technologies. Studies involving expression pattern of developmentally important genes in oocytes and embryos have not been widely investigated (Khalil et al., 2010; Lechniak, 2002). Also, study the expression of maturation related genes during IVM of the sheep oocytes has not been investigated yet. Based on the mRNA transcripts in sheep oocytes in our study, mRNAs for genes that encode components of MPF were higher in immature oocytes cultured in TCM-199 without supplements or hormones or *M. oleifera* extract than matured oocytes cultured in TCM-199 with hormones plus *M. oleifera* extract.

Wu et al. (1997) and Khalil et al. (2010) reported that levels of MPF (Cyclin B + CDC2) were high in immatured bovine and buffalo oocytes (GV stage) and became low in matured oocytes (MII stage). We also found in the current study that Cyclin B and CDC2 mRNA were high in the oocytes with low maturation rates and decreased gradually in oocytes with high rates of meiosis maturation. This result correlates the resumption of meiosis with the abundance of MPF component mRNAs. The depletion of these mRNAs could be associated with mRNA degradation or translation into MPF-component proteins. Furthermore, Cyclin B mRNA probably is in a translationally inactive state (masked) in immature GV-stage oocytes. Therefore, initiation of Cyclin B translation could be one of the early events leading to oocyte meiotic resumption. Tremblay et al. (2005) mentioned that oocytes can synthesize and store maternal mRNA in an inactive translational state until the start of IVM. Moreover, Anguita et al. (2007) reported that the amount of CDC2 protein was detected after *in vitro* maturation of goat oocytes which clearly shows that the transcript has been translated during the culture period and was associated with the ability to complete meiosis and to develop into embryos.

Extracellular signal-regulated kinase is required to convert the pre-MPF to the active form. In the current study it was found that the relative abundance of ERK2 mRNA was significantly high in immatured oocyted cultured in TCM-199 alone and then declined ($P < 0.01$) in matured oocytes cultured with hormones plus *M. oleifera* extract. In consistent with our results, ERK2-mRNA in the matured bovine oocytes was decreased so that the synthesis of the MAP protein required for the activation of the MPF (Krischek and Meinecke, 2002). Salamone et al. (2001) observed the kinetics of MPF and MAPK activity during oocyte maturation in calf and cow oocytes, concluding that kinase activity was low at the GV stage and increased several fold at MII stage.

On the other hand, MAP protein could be stimulated by c-MOS protein (Lee et al., 2000). The MOS protein is also coded by maternal RNA stored during oocyte growth. In the present study high-level accumulation of c-mos transcripts was observed in immatured oocyts compared with matured oocytes which are consistent with previous studies on bovine oocytes (Nganvongpanit et al., 2006). Hirao and Eppig (1997) reported that, the c-mos protein plays a main role as a cytostatic factor (CSF) to prevent the MII oocyte from developing parthenogenetically until the time of fertilization. This role of c-mos protein may explain why the level of c-mos-mRNA in the present study remained high until the end of oocyte maturation.

The capability of the *M. oleifera* extract to induce the oocyte maturation may be due to many of chemicals components existing in its leaves. It has been reported that the leaves of *M. oleifera* are valuable source of calcium and act as a good source of natural antioxidants (Siddhuraju and Becker, 2003). Therefore, *M. oleifera* extract can induce the elevation time of the intracellular calcium required for resumption of meiosis. Moreover, our results revealed also that sheep oocytes cultured with hormones plus *M. oleifera* extract expressed high levels of the [Ca^{2+}] concentration, where, the concentration of the [Ca^{2+}] increased to 2.8, 4 and 4.7-fold in oocytes cultured with low, medium and high doses of *M. oleifera.*

When meiosis begins, cumulus cells send Ca^{2+} ions to the oocytes *via* gap junctions between the cumulus cells and the oocyte (sheep: Mattioli et al., 1998; cattle: Tosti et al., 2000). The mechanism of how Ca^{2+} affects meiosis progression was reported by Levesque and Sirard (1996). Ca^{2+} acts as a promoter to induce mRNA expression and the essential protein synthesis for the maturational processes, e.g. MPF synthesis (Levesque and Sirard, 1996).

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