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

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ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 17 June 2015
Accepted 19 June 2015
Available online 29 June 2015

Keywords:
Sheep
IVM
Oocytes
Maturation related genes
qRT-PCR
Ca²⁺

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 [Ca²⁺] 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 maturation 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 oocytes (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...
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 protoonco- gene 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; Falkey, 2003). 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, shaken 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. After 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 Dulbeco’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 maturation medium (TCM-199). This medium (TCM-199, Sigma, 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 a positive control with groups of solutions as follows: (1) TCM-199 medium supplemented with 20 μl/ml PMSG (GBICO/BRL, Grand Island, NY, USA) + 10 IU/ml hCG + 1 μg/ml 17 β-estradiol (EZ, 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 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 droplet 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 CO₂ incubator before the oocytes were added. The oocytes (COCs) were cultured for 28–29 h at 39 °C in an atmosphere of 5% CO₂ 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) determining 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) Ca²⁺ 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 (A1), telophase I (T1) 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

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 μl 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 10 000 g and incubation at room temperature for
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 NI washing buffer (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 NI 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 cDNA copy number. PCR reactions were set up in 20 Nl reaction mixtures containing 12.5 lM sense and antisense primers, 6.5 lM of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0 °C for 1 min, second step was at 95.0 °C for 30 s, 60 °C for 30 s, the third 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; and (c) at 72.0 °C for 30 s. The amplification efficiency (EF) was calculated from the slope of the standard curve using the following formula:

\[ EF = 10^{\frac{1}{\text{slope}}} \]

Efficiency (%) = \( EF - 1 \times 100 \)

For the \( 2^{-\Delta\Delta Ct} \) calculation to be valid, the efficiency (EF) of the target amplification (CD2C, DC4C, 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:

\[ \Delta Ct (\text{target}) = Ct (\text{target}) - Ct (\text{reference}) \]

\[ \Delta Ct (\text{calibrator}) = Ct (\text{target, calibrator}) - Ct (\text{reference, calibrator}) \]

\[ \Delta\Delta Ct = \Delta Ct (\text{target}) - \Delta Ct (\text{calibrator}) \]

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 vitre cultured goat preantral follicles (Frota et al., 2010).

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, CD2C, 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 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 MI stages with 31.8% and 48%, respectively. Moreover, an improvement in progression of meiosis was shown in oocytes cultured without extract supplements. The results indicated that most oocytes reached AI/TI and MI stages. Sheep oocytes cultured with \( M. \) oleifera extract reached AI/TI and MI stages with 28.8 + 35.8. 26.3 + 59.1 and 25 + 64.3%, respectively.

To measure [Ca\(^{2+}\)]i, individual oocytes were illuminated alternately for 500 ms at 37 °C to check the quality of the used primers. 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\(^{2+}\)]. 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 monitor [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.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of ovaries</th>
<th>No. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>35</td>
<td>135</td>
</tr>
<tr>
<td>TCM-199 + suppl</td>
<td>37</td>
<td>148</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE20</td>
<td>36</td>
<td>144</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE30</td>
<td>36</td>
<td>137</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE100</td>
<td>35</td>
<td>140</td>
</tr>
<tr>
<td>Total</td>
<td>179</td>
<td>704</td>
</tr>
</tbody>
</table>

Data of mRNAs abundance and levels of [Ca\(^{2+}\)]i were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS, 1982). Scheffe-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 MI 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 MI stages. Sheep oocytes cultured with low, medium and high concentrations of \( M. \) oleifera extract reached AI/TI and MI stages with 28.8 + 35.8, 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, CD2C, 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, CD2C, c-mos, Cyclin B and ERK2 specific mRNAs was affected by the hormones and \( M. \) oleifera extract supplements (P < 0.05) (Figs. 1–5). The expression levels of CD44, CD2C,
Table 2
Primer sequences used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–3′)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>F: CAA CAC CTC CCA ATA TGA CAC</td>
<td>Aminafshar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>R: TIC TIC TGC CCA CAC CTT CT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: ATT CTA TCC CTC CTG GTC AGT TCA T</td>
<td>Khalil et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R: CAC TCG CCA CAC TIC ATT ATT G</td>
<td></td>
</tr>
<tr>
<td>c-mos</td>
<td>F: CTT GGA CCT GAA GCC AGC TAA T</td>
<td>Khalil et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R: CAG GGC ATC CAA ACC TTT GTA GTC A</td>
<td>Khalil et al. (2010)</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>F: GAG GGG ATC CAA ACC TTT GTA GTG T</td>
<td>Khalil et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R: CAA TTT CTG GAG GGT ACA TTT CT T</td>
<td></td>
</tr>
<tr>
<td>ERK2</td>
<td>F: CAC CGA CCA TCG AGC AGA TGA AAG A</td>
<td>Khalil et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R: ACG GGG ATC CAA GAA TAC CCA GAA T</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: CAA CTG GGA CGA CAT GGA</td>
<td>Aminafshar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>R: TGG TGG TGA AGC TGT AGC</td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total of COCs</th>
<th>Mean of COCs per replicates</th>
<th>State of nucleus</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
<td>GVBD</td>
<td>MI</td>
<td>AI/TI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>TCM-199</td>
<td>135</td>
<td>27 ± 1.2</td>
<td>10 ± 0.06 (37.0)</td>
<td>9 ± 0.04 (33.3)</td>
<td>8 ± 0.05 (29.6)</td>
<td>–</td>
</tr>
<tr>
<td>TCM-199 + suppl</td>
<td>148</td>
<td>29.6 ± 2.3</td>
<td>00</td>
<td>2 ± 0.01 (6.8)</td>
<td>4 ± 0.02 (13.5)</td>
<td>9.4 ± 0.04 (31.8)</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE20</td>
<td>144</td>
<td>28.8 ± 1.6</td>
<td>00</td>
<td>00</td>
<td>5 ± 0.06 (17.4)</td>
<td>8.3 ± 0.08 (28.8)</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE50</td>
<td>137</td>
<td>27.4 ± 1.4</td>
<td>00</td>
<td>00</td>
<td>4 ± 0.05 (14.6)</td>
<td>7.2 ± 0.07 (26.3)</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE100</td>
<td>140</td>
<td>28 ± 2.2</td>
<td>00</td>
<td>00</td>
<td>3 ± 0.07 (10.7)</td>
<td>7 ± 0.06 (25.0)</td>
</tr>
</tbody>
</table>

Suppl, hormones supplements; MOE, Moringa oleifera extract; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI/TI, anaphase I/telophase I; 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 extract.

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.
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$^{2+}$] responses were seen: type 1, a large spike of [Ca$^{2+}$], type 2, a minor elevation; and type 3, a single baseline as no response of [Ca$^{2+}$] (*Fig*. 6).

The results revealed that most sheep oocytes (55%) cultured without any supplements indicated single baseline as no response of [Ca$^{2+}$], type 3. However, most the oocytes (50%) supplemented with hormones only revealed type 2 response of [Ca$^{2+}$]. In contrary, the type 1, large spike of [Ca$^{2+}$], was the majority response in the sheep oocytes cultured with hormone plus *M. oleifera* extract supplements, where, the type 1 response of [Ca$^{2+}$] was observed in 45, 60 and 70% of the oocytes cultured with low, medium and high doses of *M. oleifera* extract (*Fig*. 6).
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 \([\text{Ca}^{2+}]\). However, the \([\text{Ca}^{2+}]\) 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...
high levels of the [Ca\textsuperscript{2+}] concentration, where, the concentration of the [Ca\textsuperscript{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 + E2 + 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 17\textbeta-estradiol 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, Kesektepe 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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total of COCs</th>
<th>Mean of COCs per replicates</th>
<th>Total calcium (ng/COC; mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>140</td>
<td>28 ± 1.1</td>
<td>1.8 ± 0.11*</td>
</tr>
<tr>
<td>TCM-199 + suppl</td>
<td>137</td>
<td>27.4 ± 1.3</td>
<td>4.73 ± 0.36*</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE20</td>
<td>142</td>
<td>28.4 ± 2.1</td>
<td>5.12 ± 0.37*</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE50</td>
<td>141</td>
<td>28.2 ± 1.6</td>
<td>7.25 ± 2.13*</td>
</tr>
<tr>
<td>TCM-199 + suppl + MOE100</td>
<td>138</td>
<td>27.6 ± 1.4</td>
<td>8.53 ± 2.21*</td>
</tr>
</tbody>
</table>

Suppl, hormones supplements; MOE, *Moringa oleifera* extract. *abc* 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% Centrosoma.

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 phytocompounds 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 (Jaishwal 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 immature 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 absence 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 oocytes 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 oocytes 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 cytokinetic factor (CSF) to prevent the MI 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 that sheep oocytes cultured with hormones plus M. oleifera extract expressed high levels of the [Ca2+] concentration, where, the concentration of the [Ca2+] 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 Ca2+ 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 Ca2+ affects meiosis progression was reported by Levesque and Sirard (1996). Ca2+ 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).

Acknowledgement

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding this Research group no. (RG-1435-058).

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