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Curcacycline A and B modulate apoptosis induced by heat stress in sheep oocytes during *in vitro* maturation

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ABSTRACT

The main objective of the current study was to investigate the effect of Curcacycline A and B against heat stress induced apoptosis. Cumulus-oocyte complexes (COCs) were allocated in several groups were cultured at 39 °C and 42 °C in TCM-199 supplemented either with hormones alone, or with two cyclic peptides isolated from the latex of *Jatropha curcas*, namely Curcacycline A and B. In general, the results revealed a concrete reduction in the maturation rate of sheep oocytes cultured in TCM-199 medium at 42 °C. At the same temperature, higher percentages of sheep oocytes cultured with Curcacycline A or A + B were matured compared with TCM-199 medium alone. Both the expression of apoptotic related-genes (Bax, Bcl-2, Caspase 3, P53 and C-myc) and the generation of reactive oxygen species (ROS) increased significantly in oocytes cultured in TCM-199 medium at 42 °C. The addition of Curcacycline A and B supplements to the culture media at 42 °C suppressed the effect of heat stress on the expression of apoptotic genes and on the generation of ROS. In conclusion, results indicated that the two cyclic peptides inhibited the negative effect of heat stress on meiotic maturation, expression of apoptotic genes and ROS generation in oocytes.

1. Introduction

Sheep rearing plays an important role in the Arabian economy as a major source of meat, milk and wool. Climate changes, however, have the potential to impact the economic viability of livestock production systems not only in Arabian countries but worldwide. Higher temperatures are among the major factors responsible for reduced fertility of farm animals (Hansen, 2009), and it has been reported that the viability of bovine oocytes and embryos is lower during the warm seasons than in the cold seasons (Roth, 2008; Edwards et al., 1997). This seasonal depression of reproductive performance can be determined by several factors, including management, an inappropriate environment, age and species-specific sensitivity to these factors (Badinga et al., 1985). *In vitro* studies have also demonstrated that heat stress has a negative effect on the viability and development capacity of mammalian embryos (Tseng et al., 2004; Roth, 2008; Hansen, 2009). Additionally, several studies have shown that heat stress caused infertility, not only by affecting hormonal secretion (Wolfenson et al., 2000) and embryo development (Edwards and Hansen, 1997; Rivera and Hansen, 2001) but also by damaging the oocyte. Oocytes harvested from cows during the summer showed a reduced ability to develop into blastocysts after fertilization *in vitro* (Rutledge et al., 1999; Al-Katanani et al., 2002). Heifers exposed to heat stress between the onset of estrus and insemination had an increased proportion of abnormal and retarded embryos (Putney et al., 1989). This suggests that the process of oocyte maturation is susceptible to heat stress. In fact, it has been shown that exposure of bovine oocytes to elevated temperature during *in vitro* maturation decreased their subsequent cleavage and blastocyst rates (Roth and Hansen, 2004). Therefore, evaluation of the effect of two temperature degrees on the oocyte maturation was examined, in which one of them was 39 °C (as normal temperature used in routine *in vitro* maturation work) and the another one was 42 °C (as heat stress inducer, Santos Junior et al., 2013).

It is likely that inhibition of apoptosis by means of anti-apoptotic factors might be a useful tool for decreasing the deterioration in oocyte developmental competence due to heat shock (Jousan and Hansen, 2004). Apoptosis is known to play a critical role in the effects of thermal stress, both at the early stage of follicular development and during the maturation of the oocytes (Ju et al., 1999; Paula-Lopes and Hansen, 2002). In this regard, Zhandi et al. (2009) revealed that HS during *in vitro* oocyte maturation can profoundly induce apoptosis in blastocysts. Additionally, fractions (approx.

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15–30%) of oocytes exposed to elevated temperature undergo apoptosis, as determined by TUNEL labelling of the pronucleus (Roth and Hansen, 2004, 2005; Soto and Smith, 2009). Inhibition of heat-shock-induced apoptosis with a caspase inhibitor (Roth and Hansen 2004), sphingosine 1-phosphate (Roth and Hansen, 2004, 2005) or a BH4 peptide (Soto and Smith, 2009) reduced the effect of elevated culture temperature on oocyte competence for fertilization and subsequent development.

Cyclic peptides are peptides formed into a ring via amide ester or disulphide bonds (Insanu et al., 2012) and are quite stable in terms of enzymatic degradation (Wu et al., 2007). Cyclic peptides are able to cross membranes more readily than linear peptides because they have less zwitterionic characteristics, and their rigid structure leads them to exhibit higher affinity and selectivity for binding with protein (Sakai et al., 1996). Cyclic peptides also exhibit a wide range of activities such as antibiotic (gramicidin), anthelmintic (yunnanin F), antineoplastic (dolastatin 3), phytotoxic (tentoxin, HC-toxin) and insecticidal (destruxin family), and also serve as cytostatic (chlamydocin) or antiviral (sansalvamide A) agents (Hwang et al., 1999; Poojary and Belagali, 2005; Insanu et al., 2012). In nature, cyclic peptides are non-ribosomely biosynthesized by complex multi-enzymes in the cytosol. A rich source of cyclic peptides is the family of Euphorbiaceae, especially the genus *Jatropha*, from which a number of cyclic peptides have been characterized. Originally, curcacycline A and B were isolated from *Jatropha curcas* latex. Curcacycline A is an octapeptide with moderate dose-dependent inhibitory activity on human T-cell proliferation and the classical pathway complement system. Curcacycline B, a cyclic nonapeptide, enhances rotamase activity of cyclophillin (Baraguey et al., 2000; Mongkolvisut et al., 2006). It has been shown that both curcacycline A and B have anti-apoptotic activity. Mongkolvisut et al. (2006) observed that cell debris and apoptotic cell nuclei were absent in treated cell cultures, suggesting that both peptides have anti-proliferative but not cytotoxic activity. Both peptides (A and B at 50 μM) exhibited cell migration activity in confluent human Capan II pancreatic carcinoma cells by 30 and 20%, respectively. These peptides, therefore, have the potential to inhibit basic cytoskeleton-dependent cellular processes such as neurite outgrowth, cell proliferation, and cell migration.

Although the structure of curcacycline A and B was established more than twenty years ago, there is still a lack of information regarding their anti-degradation effects in oocytes. The objectives of this research are to evaluate the anti-apoptotic effect of both curcacyclines against heat stress on *in vitro* maturation of sheep oocytes.

2. Materials and methods

2.1. Chemicals and plastics

TCM-199 medium (M-4530), foetal bovine serum (F- 7524), 178-estradiol (E-2758), and mineral oil (M-8410) were purchased from Sigma Chemicals Co. (St. Louis, Mo., USA). hCG (Pregnyl®, Nile Co. for Pharmaceutical and Chemical Industries A.R.E). PMSG (Folligon®, Intervet International B.V., GIBCO/BRL, Grand Island, N.Y, USA). Polystyrene plastic culture dishes (35 × 10 mm, 60 × 10 mm) and 0.22 μm millipore membrane filters were purchased from Nunclon, Nalge Nunc International, Roskide, Denmark.

2.2. Plant material

Jatropha curcas L. was grown in Luxor governorate (in a man-made plantation located in the Hebail region), Egypt. It was collected and identified in the Botany Department of the National Research Center, Egypt. Crude latex was collected early in the morning by cutting off leaf stalks and adding a few drops of MeOH to prevent the latex from extra foaming. The latex was kept at −20 °C before use.

2.3. Plant extraction

The two cyclic peptides, namely curcacycline A and B, were isolated from the latex of *Jatropha curcas* L. according to Insanu et al. (2012).

2.4. Oocyte collection and maturation

Sheep ovaries were collected at a local abattoir immediately after slaughter. The ovaries (n = 819) were transported to the laboratory in 0.9% saline supplemented with 50 μg/mL gentamycin sulphate at between 30 and 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 sulphate (Chauhan et al., 1997). 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 the aspiration medium, twice in the basic culture medium TCM-199 and enriched with 50 μg/ml gentamycin sulphate. This medium (TCM-199) was used in five different treatment groups, as follows: (Group 1) sheep oocytes were cultured at 39 °C using TCM-199 medium supplemented with 20 iu/ml PMSG + 10 iu/mL hCG + 1 μg/mL 17 β −estradiol (E2) + 10% foetal bovine serum (FBS). This group served as the control; (Groups 2–4) sheep oocytes were again cultured at 39 °C using the same supplemented medium as in group (1) but this time with the addition of 50 μg/mL of Curcacycline A, Curcacycline B and Curcacycline A + B, respectively; (Group 5) sheep oocytes were cultured at 42 °C using the same supplemented medium as used in group 1; (Groups 6–8) sheep oocytes were cultured at 42 °C using the same supplemented medium as in group (1) with the addition of 50 μg/mL of Curcacycline A, Curcacycline B and Curcacycline A + B, respectively. Groups 5–8 represent the heat stress treatment groups.

Five replicates were performed for each treatment. The supplemented medium, with or without Curcacycline A and Curcacycline B, was sterilized using a 0.22 μm Millipore filter. The dose of Curcacycline A and Curcacycline B used in this study was selected according to Insanu et al. (2012). In all the experiments, 10–15 oocytes of COCs were transferred separately into a 50 μL drop of each type of culture medium (with or without Curcacycline A and Curcacycline B), 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. The oocytes (COCs) were cultured for 28 h at either 39 °C or 42 °C in an atmosphere of 5% CO_2 in air with 95% humidity. The *in vitro* cultured oocytes (COCs) were used for examination of the nuclear maturation rate (Bolamba et al., 2006); determination of the expression of apoptotic related genes; and measurement of the reactive oxygen species (Dalvit et al., 2005).

2.5. Determination of the nuclear maturation by cytogenetic analysis

To examine the nuclear maturation rate (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; silicone gel

was used to maintain a coverslip in contact with the oocytes. The slides were immersed in 1% aceto-orcein stain for 30 min before being washed three times in ascending concentrations of ethanol in order to remove the surplus orcein dye as follows: 5 sec. 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).

2.6. Gene expression analysis using quantitative real-time PCR

RNA extraction and cDNA synthesis: To determine the gene expression of selected genes, sheep oocytes from each treatment were used. Total RNA from pooled COCs was 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 sec, centrifugation for 30 sec at 1000 g and incubation at room temperature for 10 min, 5 μL of Dynabeads Oligo (dT) 25, prewashed twice with 60 μL of lysis/binding buffer, was 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 using a Dynal MPC-E-1 magnetic separator (Dynal, Hamburg, Germany). After washing once with 40 μL 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 μL 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 μL 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), in order to obtain the widest array of cDNAs. To create a cDNA copy using 1 μL 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 was then used immediately in the following PCR reaction or stored at −20 °C until use.

Quantitative Real Time PCR: The first strand cDNA (2 Ng) from different categories of sheep oocytes were 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 primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The reaction programme was allocated to 3 steps. The first step was at 95 °C for 3 min. The second step consisted of 40 cycles in which each cycle was divided into three further steps: (a) at 95 °C for 15 sec; (b) at 94 °C for 30 sec, 60 °C for 30 sec; and (c) at 72 °C for 30 sec. The third step consisted of 71 cycles which started at 60 °C and then increased about 0.5 °C every 10 sec up to 95 °C. At the end of each qRT-PCR a melting curve analysis was performed at 95 °C to check the quality of the used primers. Each qRT-PCR was repeated for each treatment, generating at least five new cDNA products per group. Additionally, each of the samples was analyzed in duplicates. The average of the coefficient of variation (CV) for each primer pair was low (e.g. Bax CV = 0.044; Bcl2 = 0.055; p53CV = 0.057; Caspase3CV = 0.064 and; C-myc CV = 0.68).

Calculation of gene expression: the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet:

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

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

The relative quantification of the target to the reference was determined by using the

$2^{-\mathrm{D}\Delta\mathrm{CT}}$ method if Ef for the target (Bax, Bcl2, Caspase 3, P53 and C-myc) and the reference primers (GAPDH) are as follows:

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

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

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

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

2.7. Assessment of ROS production

To measure the production of ROS, cultured oocytes were completely denuded by vortex agitation in physiological saline solution for 1 min and then washed to eliminate cumulus cells. Samples of 15 denuded oocytes from the different groups were incubated in 40 mmol/l Tris-HCl buffer pH 7.0 at 37 °C for 30 min in the presence of 5 μmol/l 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA), washed, sonicated at 50 W for 1 min and centrifuged at 4 °C and 10,000 × *g* for 20 min. Fluorescence was monitored in the supernatant using a spectrofluorometer at 488-nm excitation and at 525-nm emission (Cetica et al., 2001). Corrections for autofluorescence were made by inclusion of parallel blanks in each experiment. Data was expressed as Arbitrary ROS Units/denuded oocyte (means ± SEM) × 10^{-3}. The accuracy and precision of the method were evaluated by analyzing five replicates for each group. In addition, the samples were analyzed in duplicates. The coefficients of variation for each treatment are all <0.1 and the average coefficient of variation for all assays was equal 0.06.

3. Statistical analysis

Data of gene expression and ROS generation 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 two temperatures and four treatments of the study including control and those supplemented with 50 μg/mL of Curcacycline A, Curcacycline B and Curcacycline A + B, respectively. 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.

4. Results

4.1. Effect of Curcacycline A and Curcacycline B supplements against heat stress on the meiotic progression

Table 3 summarises the state of meiotic progression in sheep oocytes (Fig. 1) cultured *in vitro* at 39 °C and 42 °C with Curcacycline A and Curcacycline B supplements. The results show that most

Table 1
Experimental design clarifies the number of ovaries and oocytes used in each group for each test.

Groups	Meiotic progression		Gene expression		ROS production	
	No. of ovaries	No. of oocytes	No. of ovaries	No. of oocytes	No. of ovaries	No. of oocytes
TCM-199 (39 °C)	33	138	34	138	35	143
TCM-199 + C-CyclineA (39 °C)	34	139	33	140	34	139
TCM-199 + C-CyclineB (39 °C)	35	143	35	144	34	140
TCM-199 + C-CyclineA + B (39 °C)	33	138	34	138	33	137
TCM-199 (42 °C)	35	144	35	143	35	142
TCM-199 + C-CyclineA (42 °C)	33	138	34	141	34	140
TCM-199 + C-CyclineB (42 °C)	34	139	33	138	34	141
TCM-199 + C-CyclineA + B (42 °C)	35	144	35	145	35	143
Total	272	1123	273	1127	274	1125

Table 2
Primer sequences, annealing temperature and expected fragment sizes of apoptotic and housekeeping genes.

Gene	Primer sequence and direction (5'–3')	GenBank accession ID/References	Annealing temperature (°C)	Amplicon size (bp)
Bax	F: TGGAGATGAATTGGACAGTAAC R: GCCTTGAGCACCAGTTTG	XM_004015363.1	61	174
Bcl-2	F: CAGGAGAAATCAAACAGGG R: GTGTGTGGAGAGCGTCAAC	XM_004020687.1	59	171
Caspase3	F: TAGCAAGTTTCTTCAGAGGG R: GTCTCAATACCACAGTCCAG	Ebrahimi et al. (2010)	60	105
P53	F: GGAAGAATCGCAGGCAGAACT R: GGAGAGCTCGGAGGACAGAA	Ebrahimi et al. (2010)	60	109
C-myc	F: GGATAGTGGAAATACGGGCT R: GTGGTAGAAGTTCTCCTCCT	Ebrahimi et al. (2010)	60	125
GAPDH	F: AGTGTCGCTGTTGAAGTCG R: GAAACCTGCCAAGTATGATG	NM_001190390.1	60	121

F: Forward primer; R: Reverse primer.

Table 3
Effect of Curcacycline A and Curcacycline against heat stress on meiotic progression of the sheep oocytes.

Groups	Total of COCs	Mean of COCs per replicates M ± SD	State of nucleus				
			GV M ± SD (%)	GVBD M ± SD (%)	MI M ± SD (%)	AI/TI M ± SD (%)	MII M ± SD (%)
TCM-199 (39 °C)	138	27.6 ± 0.9	0.0 ± 0.0[c] (0.0)	2.6 ± 0.5[b,c] (9.4)	4.8 ± 0.4[b] (17.4)	7.6 ± 0.9[a,b] (27.5)	12.6 ± 1.1[a] (45.7)
TCM-199+ C-CyclineA (39 °C)	139	27.8 ± 1.3	0.0 ± 0.0[c] (0.0)	2.4 ± 0.9[b,c] (8.6)	4.4 ± 1.3[b] (15.8)	8.2 ± 1.6[a] (29.5)	12.8 ± 1.5[a] (46.0)
TCM-199+ C-CyclineB (39 °C)	143	28.6 ± 1.1	2.4 ± 0.5[b] (8.4)	3.2 ± 0.8[b] (11.2)	6.8 ± 1.3[a,b] (23.8)	7.4 ± 1.8[a,b] (25.9)	8.8 ± 1.5[b] (30.8)
TCM-199+ C-CyclineA + B (39 °C)	138	27.6 ± 1.5	0.0 ± 0.0[c] (0.0)	1.8 ± 0.8[c] (6.5)	5.8 ± 1.4[b] (21.0)	8.2 ± 0.8[a] (29.7)	11.8 ± 1.3[a] (42.8)
TCM-199 (42 °C)	144	28.8 ± 2.2	4.6 ± 1.1[a] (16.0)	6.2 ± 1.3[a] (21.5)	8.0 ± 0.9[a] (27.8)	5.6 ± 0.9[b] (19.4)	4.4 ± 1.1[d] (15.3)
TCM-199+ C-CyclineA (42 °C)	138	27.6 ± 1.1	3.6 ± 1.1[a,b] (13.0)	5.8 ± 1.5[a] (21.0)	5.2 ± 0.8[b] (18.8)	5.4 ± 1.3[b] (19.6)	7.6 ± 0.9[c] (27.5)
TCM-199+ C-CyclineB (42 °C)	139	27.8 ± 1.8	4.8 ± 1.3[a] (17.3)	6.6 ± 1.1[a] (23.7)	7.8 ± 0.8[a] (28.1)	6.2 ± 1.3[b] (22.3)	2.4 ± 0.9[e] (8.6)
TCM-199+ C-CyclineA + B (42 °C)	144	28.8 ± 1.5	3.8 ± 1.3[a,b] (13.2)	6.2 ± 0.8[a] (21.5)	6.8 ± 1.1[a,b] (23.6)	5.6 ± 0.9[b] (19.4)	6.4 ± 1.5[c] (22.2)

C-Cycline: Curcacycline; GV = Germinal vesicle, GV = Germinal vesicle breakdown, MI = MetaphaseI, AI/TI = AnaphaseI/TelophaseI, MII = Metaphase II.
[a] With different superscripts are significantly different ($P \leq 0.05$).
[b] With different superscripts are significantly different ($P \leq 0.05$).
[c] With different superscripts are significantly different ($P \leq 0.05$).
[d] With different superscripts are significantly different ($P \leq 0.05$).
[e] With different superscripts are significantly different ($P \leq 0.05$).

oocytes cultured in TCM-199 medium at 39 °C without Curcacycline A and Curcacycline B supplements attained AI/TI + MII stages (73.2%). Similarly, most oocytes cultured in TCM-199 medium at 39 °C with Curcacycline A alone or with a mixture of Curcacycline A + B supplements reached AI/TI + MII stages (75.5 and 72.5%, respectively). In contrast, only 56.7% of sheep oocytes attained AI/TI + MII stages when cultured in TCM-199 medium at 39 °C with Curcacycline B alone (Table 3). Furthermore, a concrete reduction was observed in the maturation rate of sheep oocytes cultured in TCM-199 medium at 42 °C, with only 34.7% of oocytes reaching the AI/TI + MII stages (Table 3). Following the same trend, low percentages of sheep oocytes attained the AI/TI + MII stages when they were cultured at 42 °C in TCM-199 medium with Curcacycline A (47.1%), Curcacycline B (30.9%) or Curcacycline A + B (41.6%), respectively (Table 3).

4.2. The effect of Curcacycline A and Curcacycline B supplements against heat stress induced expression of apoptotic genes

Quantitative real time-PCR assay was used to assess the effect of Curcacycline A and Curcacycline B supplements on heat stress induced expression apoptotic genes in sheep oocytes. Amplification of sheep oocyte cDNA with Bax, Bcl2, Caspase 3, P53 and C-myc specific primers resulted in quantitative PCR products (Figs. 2–6). These products of the studied genes were detected in all oocytes regardless of whether they were cultured with or without Curcacycline A and Curcacycline B supplements.

The results revealed that expression levels of Bax, Caspase 3, P53 and C-myc mRNA were lower in sheep oocytes cultured at 39 °C in TCM-199 medium alone or with Curcacycline A and Curcacycline B supplements than those cultured at 42 °C in TCM-199 medium alone (Figs. 2, 4–6). The addition of Curcacycline A and

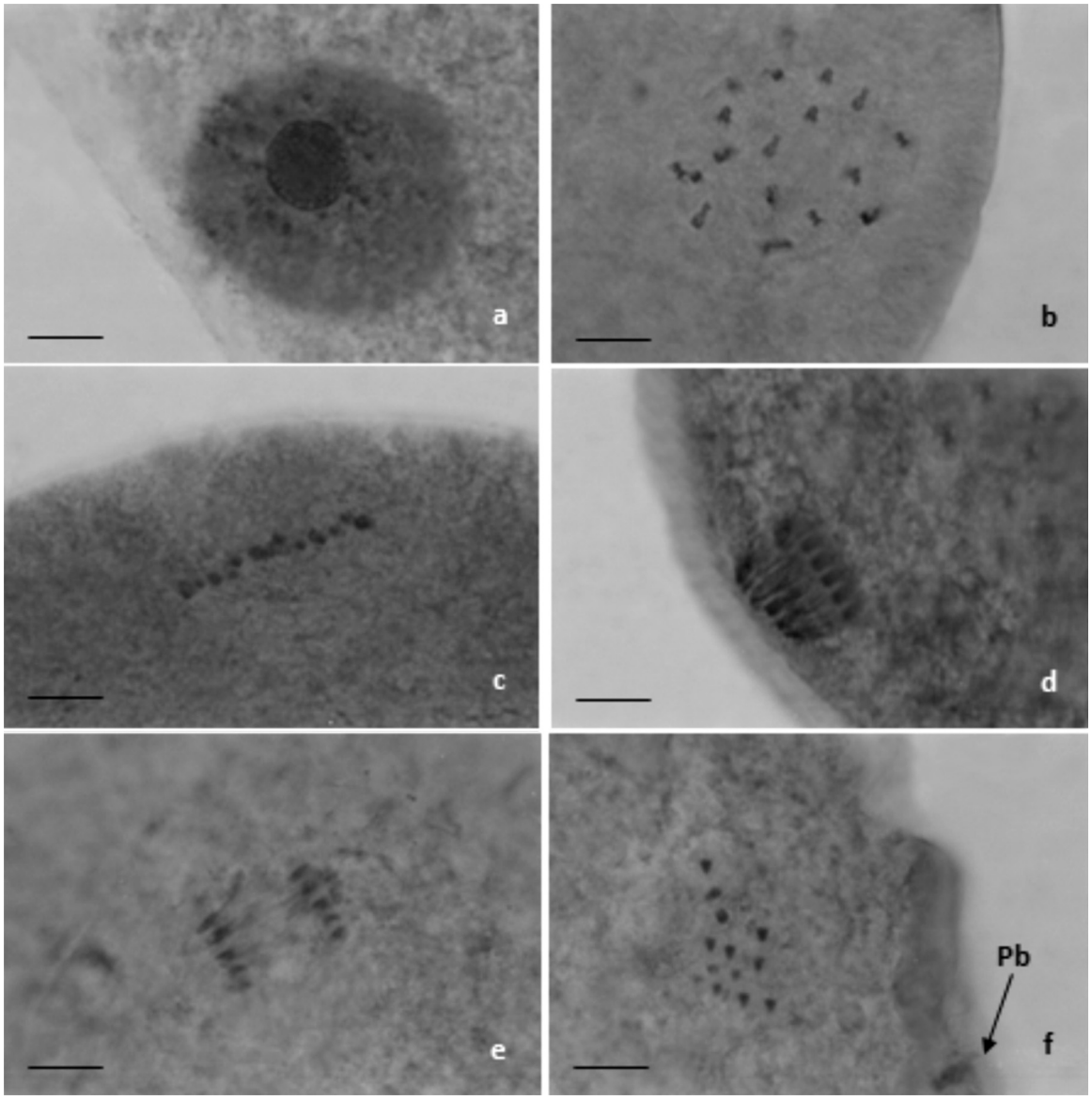

Fig. 1. Photomicrographs of stained whole mounted sheep oocytes representing various nuclear stages during maturation. (a) Germinal vesicle stage (GV) including a nucleus: (Nu) (bar = 10 μm). (b) Diakinesis of prophase I (bar = 15 μm). (c) Metaphase I (bar = 15 μm). (d) Anaphase I (bar = 15 μm). (e) Telophase I (bar = 15 μm). (f) Oocyte at metaphase II and extrusion of the first polar body (PB) (bar = 20 μm).

Curcacycline B supplements to the culture media at 42 °C served, however, to suppress the effect of heat stress on the expression of Bax, Bcl2, Caspase 3, P53 and C-myc genes. Regarding the expression of the Bcl-2 gene, the results revealed that its expression levels were higher in sheep oocytes cultured at 39 °C in TCM-199 medium alone or with Curcacycline A and Curcacycline B supplements than those cultured at 42 °C (Fig. 3). Moreover, the addition of Curcacycline A and Curcacycline B supplements to the culture media at 42 °C significantly increased the expression levels of Bcl-2 mRNA compared with those cultured at 42 °C in TCM-199 medium alone (Fig. 3).

4.3. *Effect of Curcacycline A and Curcacycline B supplements against heat stress induced ROS production*

Assessment of ROS generation by spectrofluorometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA), revealed that ROS levels were consistently low in the sheep oocytes cultured at 39 °C in the TCM-199 medium alone or with Curcacycline A and Curcacycline B supplements (Table 4). A significant increase in ROS levels was observed, however, in the sheep oocytes cultured at 42 °C in TCM-199 medium alone. This increase was significantly moderated ($p < 0.05$) by the addition of Curcacycline A and Curcacycline B supplements to the culture media at 42 °C (Table 4).

5. Discussion

This study aimed to investigate the effect of heat stress on the rate of maturation, expression of apoptotic related genes and ROS generation in sheep oocytes during *in vitro* maturation. Comparison between sheep oocytes cultured with the same culture media (TCM-199) containing hormone supplementation combinations (PMSG + hCG + F2 + FBS) but at two different temperature conditions (39 and 42 °C) revealed that the maturation rate was higher at 39 °C than at 42 °C. Our findings support the results of several other studies on the *in vitro* maturation of sheep and bovine oocytes (dos Santos Junior et al., 2013; Payton et al., 2004). In addition, Hansen (2009) reported that heat stress can disrupt

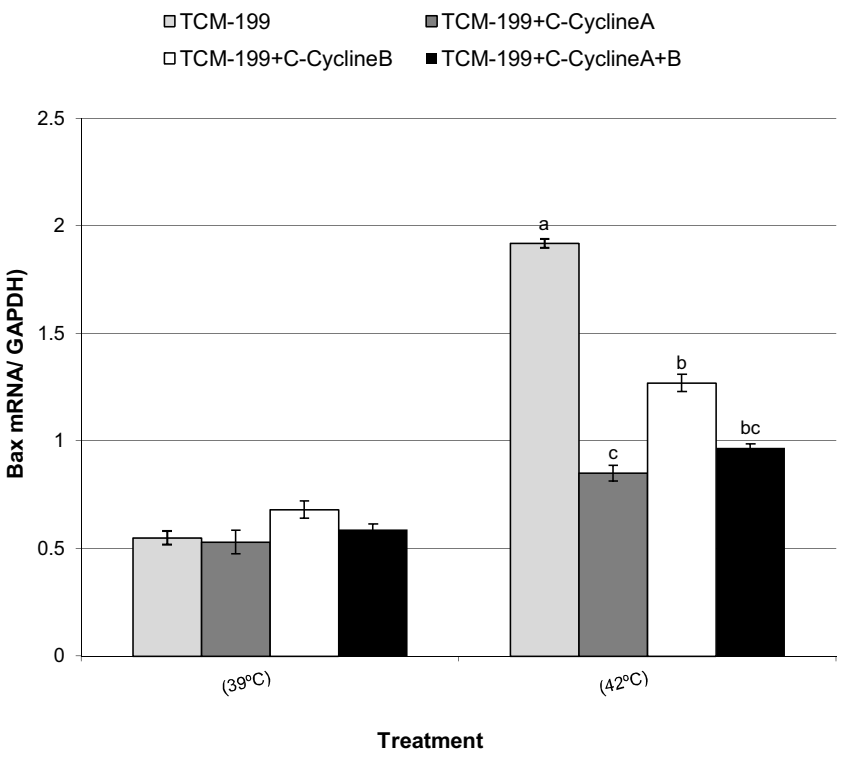

Fig. 2. The relative expression of Bax gene in sheep oocytes exposed to heat stress and treated with Curcacycline A and Curcacycline B. Mean values within tissues with unlike lowercase letters (a–c) were significantly different ($P < 0.05$), while mean values within tissues with similar lowercase letters (c, b, and bc) or without lowercase letters were not significantly different ($P > 0.05$). C-Cycline: Curcacycline.

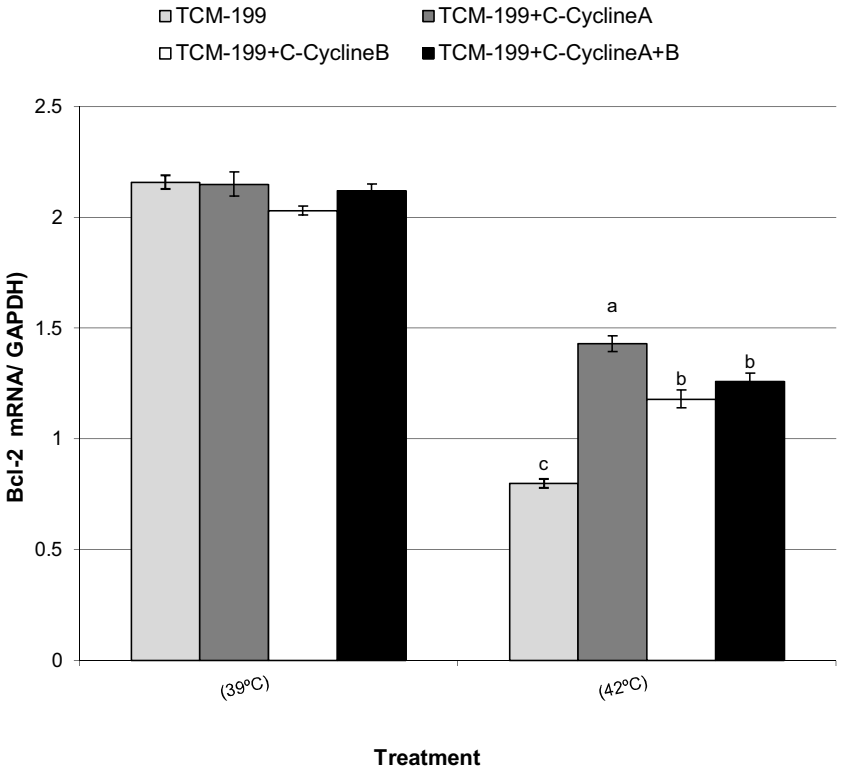

Fig. 3. The relative expression of Bcl-2 gene in sheep oocytes exposed to heat stress and treated with Curcacycline A and Curcacycline B. Mean values within tissues with unlike lowercase letters (a–c) were significantly different ($P < 0.05$), while mean values within tissues with similar lowercase letters (c, b, and bc) or without lowercase letters were not significantly different ($P > 0.05$). C-Cycline: Curcacycline.

the development and function of the oocyte, and several studies have also reported that the competence of oocytes for fertilization and subsequent development is reduced during times of the year associated with heat stress (Zeron et al., 2001; Al-Katanani et al.,

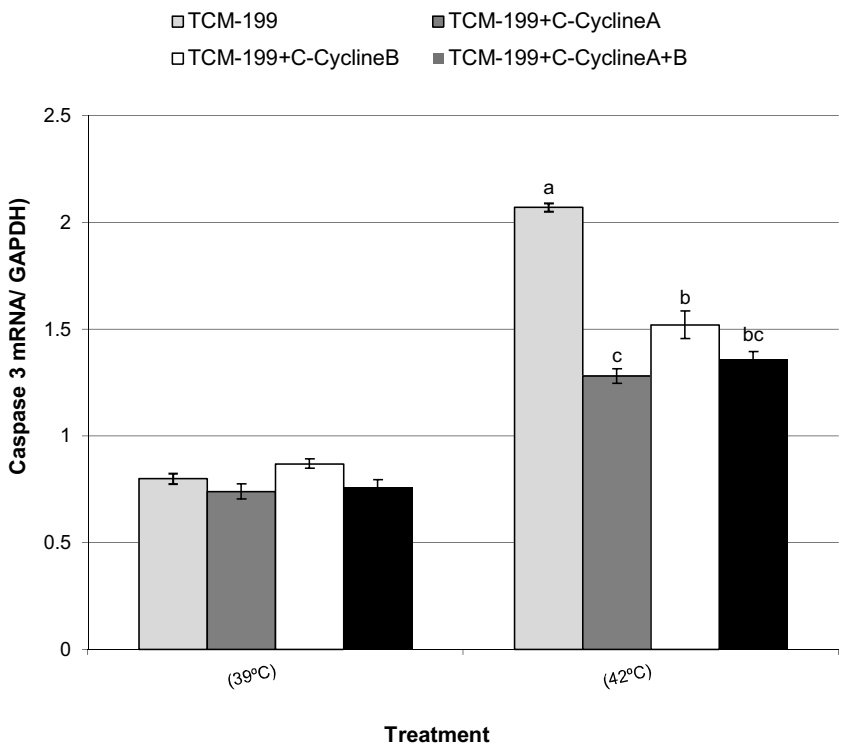

Fig. 4. The relative expression of Caspase 3 gene in sheep oocytes exposed to heat stress and treated with Curcacycline A and Curcacycline B. Mean values within tissues with unlike lowercase letters (a–c) were significantly different ($P < 0.05$), while mean values within tissues with similar lowercase letters (c, b, and bc) or without lowercase letters were not significantly different ($P > 0.05$). C-Cycline: Curcacycline.

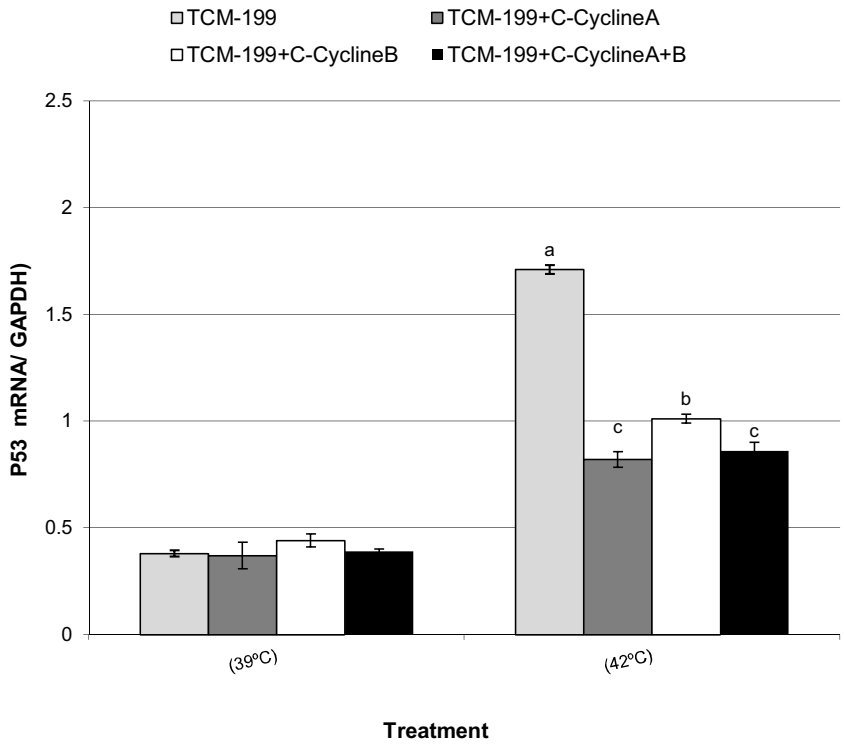

Fig. 5. The relative expression of P53 gene in sheep oocytes exposed to heat stress and treated with Curcacycline A and Curcacycline B. Mean values within tissues with unlike lowercase letters (a–c) were significantly different ($P < 0.05$), while mean values within tissues with similar lowercase letters (c, b, and bc) or without lowercase letters were not significantly different ($P > 0.05$). C-Cycline: Curcacycline.

2002; Sartori et al., 2002). The depression of nuclear maturation is one of the changes caused by heat shock in bovine oocytes. Exposure of GV-stage (Payton et al., 2004) or maturing oocytes (Roth and Hansen, 2005; Paula-Lopes et al., 2008) to 41 °C heat shock decreased the proportion of oocytes that reached MII stage following IVM. These findings agreed closely with our results, where

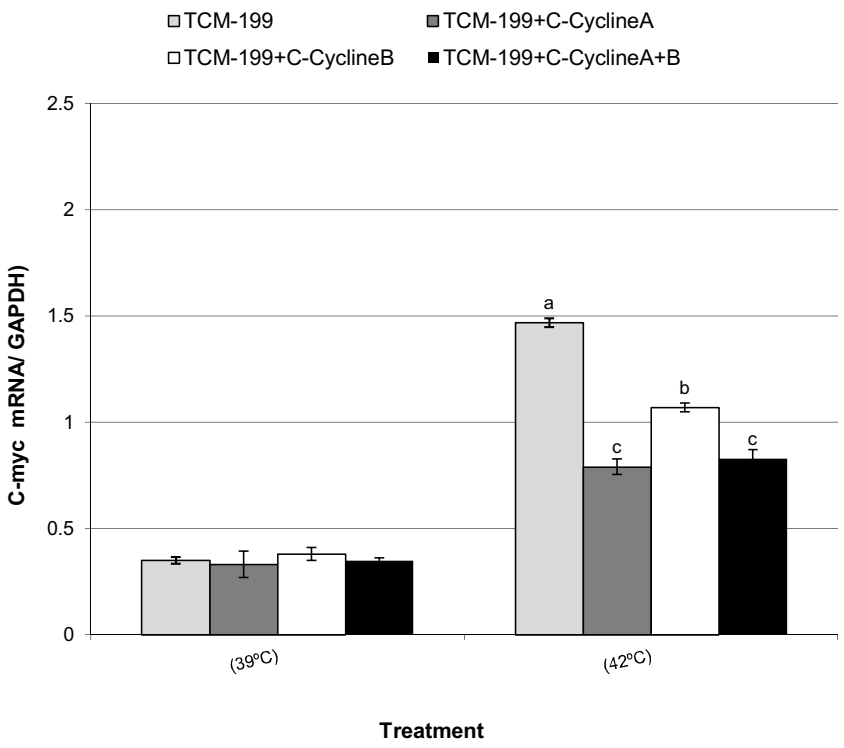

Fig. 6. The relative expression of C-myc gene in sheep oocytes exposed to heat stress and treated with Curcacycline A and Curcacycline B. Mean values within tissues with unlike lowercase letters (a–c) were significantly different ($P < 0.05$), while mean values within tissues with similar lowercase letters (c, b, and bc) or without lowercase letters were not significantly different ($P > 0.05$). C-Cycline: Curcacycline.

Table 4
Effect of Curcacycline A and Curcacycline against heat stress induced ROS production in sheep oocytes.

Groups	Total of COCs	Mean of COCs per replicates (M ± SD)	ROS levels (ROS units/Oocyte.min 10^{-3}) (Mean ± SD)
TCM-199 (39 °C)	143	28.6 ± 1.8	4.4 ± 0.89[c]
TCM-199+ C-CyclineA (39 °C)	139	27.8 ± 1.5	4.6 ± 0.55[c]
TCM-199+ C-CyclineB (39 °C)	140	28.0 ± 1.2	5.2 ± 0.84[c]
TCM-199+ C-CyclineA + B (39 °C)	137	27.4 ± 1.5	6.2 ± 1.48[c]
TCM-199 (42 °C)	142	28.4 ± 2.3	20.6 ± 2.41[a]
TCM-199+ C-CyclineA (42 °C)	140	28.0 ± 1.0	11.6 ± 1.14[b]
TCM-199+ C-CyclineB (42 °C)	141	28.0 ± 1.6	13.8 ± 1.92[b]
TCM-199+ C-CyclineA + B (42 °C)	143	28.6 ± 1.7	12.6 ± 1.82[b]

ROS levels determined by spectrofluorometry using 2′,7′- dichlorodihydrofluorescein diacetate assay; C-Cycline: Curcacycline.
[a] With different superscripts are significantly different ($P < 0.05$).
[b] With different superscripts are significantly different ($P < 0.05$).
[c] With different superscripts are significantly different ($P < 0.05$).

the maturation rate in sheep oocytes cultured in TCM-199 medium decreased from 73.2% at 39 °C to 34.7% at 42 °C. The mechanism by which heat stress reduces the maturation rate of oocytes probably involves cytoplasmic and nuclear alterations. Several studies have indicated that damage to oocytes during the maturation process by heat shock seems to involve the generation of reactive oxygen species, since both the effects of heat stress *in vivo* (Roth et al., 2008) and heat shock *in vitro* (Lawrence et al., 2004) were reduced by administration of antioxidants. These observations also concur with our results since we show that ROS generation increased significantly in the sheep oocytes cultured at 42 °C compared with those cultured at 39 °C.

Apoptosis also plays a critical role in the effects of thermal stress on the maturing oocyte in cattle. A fraction (approx. 15–30%) of oocytes exposed to elevated temperature undergoes apoptosis (Roth and Hansen 2004, 2005; Soto and Smith, 2009). Inhibition of heat-shock-induced apoptosis with a caspase inhibitor (Roth and Hansen, 2004), sphingosine 1-phosphate (Roth and Hansen, 2004, 2005) or a BH4 peptide (Soto and Smith, 2009) reduced the effect of elevated culture temperature on oocyte competence for fertilization and subsequent development. Our results also found that the expression of apoptotic related genes was significantly higher in the sheep oocytes cultured at 42 °C compared to those cultured at 39 °C, indicating that heat stress reduces the rate of maturation by enhancing the apoptosis pathway. There are two important regulatory families involved in the apoptosis pathway: the Bcl-2 and Caspase families. The Bcl-2 family consists of two types: anti-apoptotic such as Bcl-2 and Bcl-XL, and pro-apoptotic such as Bax and Bid (Antonsson and Martinou, 2000; Kim and Tilly, 2004). Bcl-2 is a membrane-associated protein present in the nuclear envelope and mitochondria and is fully capable of promoting germ cell survival in females, irrespective of the developmental status of the oocyte (Flaws et al., 2001). The pro survival function of Bcl-2 is exerted by first modulating the mitochondrial release

of cytochrome c, causing the interaction of Apaf-1 with caspase 9, and, secondly, through binding to Bax, and finally blocking apoptosis induced by C-myc (Hussein, 2005; Kim and Tilly, 2004). Bax is a pro-apoptotic gene that resides either in the cytoplasm or cell membrane and is a Bcl-2 antagonist whose cytoplasmic elevation is sufficient to cause induction of oocyte apoptosis (Morita and Tilly, 1999). Consistent with these findings, the present study found that heat stress induced apoptosis in sheep oocytes cultured at 42 °C led to increased expression values of Bax and C-myc and decreased expression of Bcl-2.

Caspases, including caspase 1–3, also exist in cells as inactive proenzymes that become activated upon initiation of apoptosis. A number of proteins, such as PARP (poly ADP ribose polymerase), histone H1 and lamins are destroyed by the action of caspases which lead to the morphological changes that occur in apoptotic cells (Mockridge et al., 2000). The existence of intracellular caspase activity is definitive confirmation of cellular degeneration, since they are apoptosis-specific proteases; particularly caspase 3 (Hussein, 2005). In addition, P53, which is a stress response gene, is induced by DNA damage leading to the activation of downstream effector genes (include Bax). During this procedure, the release of mitochondrial cytochrome is promoted while anti-apoptotic genes continue to function normally (Hussein, 2005). In the same way, our results also found that heat shock induced apoptosis in sheep oocytes cultured at 42 °C led to increased expression levels of Caspase and P53.

In the present study Curcacycline A and B, as two cyclic peptides isolated from the latex of *Jatropha curcas* L., were used to modulate the apoptosis process induced by heat shock. The results revealed that the cyclic peptide, Curcacycline A, protected sheep oocytes against heat stress induced suppression of maturation rate, alteration in the expression of apoptotic genes and ROS generation in oocytes cultured at 42 °C compared to those cultured only with TCM-199 medium. Moreover, Curcacycline B has also similar effect on the sheep oocytes against heat stress, where it suppressed the alterations in the expression of apoptotic genes and ROS generation in oocytes cultured at 42 °C. However, Curcacycline B has low protective action against inhibition of oocyte maturation induced by heat stress, where it expressed similar maturation rate compared with that in oocytes cultured at 42 °C. It is known that the transcription process during gene expression is taking time span shorter than that required to translate the mRNA molecules to protein needed for stimulating oocyte maturation (Dekel, 1999). Therefore, the anti-apoptotic action of Curcacycline B occurs earlier on the gene expression level than that required for protein synthesis. So, it seems likely that Curcacycline B needs more time to express its anti-apoptotic action on the oocyte maturation.

To date, no other published data is available regarding the effects of Curcacycline A and B on oocytes, either *in vivo* or *in vitro*. Although several compounds isolated from *Jatropha curcas* L such as Curcin (a ribosome-inactivating protein, Zhaoa et al., 2012) and Phorbol Esters (Oskoueian et al., 2012) have apoptotic properties, Curcacycline A and B do not have this action (Devappa et al., 2010). Devappa et al. (2010) reported that cell debris and apoptotic cell nuclei were absent in the treated cell cultures of human melanoma, suggesting both peptides have an anti-proliferation but no cytotoxic activity. In this regard, our results may suggest that Curcacycline A and B protect sheep oocytes against heat stress through the modulation of the apoptosis pathway by altering the expression of apoptotic genes and suppressing ROS generation.

In conclusion, the current study is the first to examine the effect of Curcacycline A and B against heat stress during *in vitro* oocyte maturation. The results indicated that the two cyclic peptides inhibited the negative effect of heat stress on the meiotic maturation, provoked an alteration in the expression of apoptotic genes and suppressed ROS generation in sheep oocytes.

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