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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