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Developmental competence of mature yak vitrified–warmed oocytes is enhanced by IGF-I via modulation of CIRP during in vitro maturation

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ABSTRACT

The objective of this study was to investigate whether developmental competence of mature vitrified–warmed yak (*Bos grunniens*) oocytes can be enhanced by supplemented insulin-like growth factor I (IGF-1) during in vitro maturation (IVM), and its relationship with the expression of cold-inducible RNA-binding protein (CIRP). In experiment 1, immature yak oocytes were divided into four groups, and IVM supplemented with 0, 50, 100 and 200 ng/mL IGF-1 was evaluated; the mRNA and protein expression levels of CIRP in mature oocytes in the four groups were evaluated using quantitative real-time PCR and western blotting analyses. In experiment 2, the mature yak oocytes in the four groups were cryopreserved using the Cryotop (CT) method, followed by chemical activation and in vitro culture for two days and eight days to determine cleavage, blastocyst rates, and total cell number in the blastocysts. Mature yak oocytes without vitrification served as a control group. The outcomes were as following: (1) the expression of CIRP in the matured oocytes was up-regulated in the IGF-1 groups and was highest expression was observed in the 100 ng/mL IGF-1 treatment group. (2) In the vitrified–warmed groups, the rates of cleavage and blastocyst were also highest in the 100 ng/mL IGF-1 treatment group (81.04 ± 1.06%% and 32.16 ± 1.01%), which were close to the rates observed in groups without vitrification (83.25 ± 0.85% and 32.54 ± 0.34%). The rates of cleavage and blastocyst in the other vitrified–warmed groups were 70.92 ± 1.32% and 27.33 ± 1.31% (0 ng/mL); 72.73 ± 0.74% and 29.41 ± 0.84% (50 ng/mL); 72.43 ± 0.61% and 27.61 ± 0.59% (200 ng/mL), respectively. There was no significant difference in the total cell number per blastocysts between the vitrified–warmed groups and group without vitrification. Thus, we conclude that the enhancement in developmental competence of mature yak vitrified–warmed oocytes after the addition of IGF-1 during IVM might result from the regulation of CIRP expression in mature yak oocytes prior to vitrification.

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1. Introduction

The breeding of yak (*Bos grunniens*) is one of the main sources of income for humans communities living in cold highlands surrounding the Qinghai-Tibet Plateau. This species is considered to have a low reproductive efficiency because it is a seasonally polyoestrous animal; the breeding season of this animal ranges from July to October, and the calving season occurs from April to July [31,34]. Thus, technologies, such as in vitro fertilization (IVF) and nuclear transfer are important to enhance the reproductive efficiency of yaks. Nevertheless, the birth of live offspring using these technologies has not been reported. One of the main reasons for this is the limited availability of oocytes as the optimal time for embryo transplantation in yaks is from July to October; however, yaks are slaughtered for meat from September to December, particularly the female yak, whose ovaries are mainly collected for recovered oocytes to produce embryos in vitro. Oocyte cryopreservation may serve as a valid alternative to overcome the limited availability of oocytes, allowing the enhancement of in vitro embryo production.

The development of cryopreservation techniques for mature

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metaphase II stage (MII) oocytes has provided many benefits for subsequent development [1,2]. These techniques can be applied not only to the breeding of livestock animals but also to the clinical practice of reproductive medicine [4]. However, there has been no publication in this field in yaks, and subsequent developmental competence of cryopreserved oocytes still needs enhancement.

Some proteins in oocytes and embryos play important roles in maintaining the developmental competence of oocytes or embryos in vitrification [28]. Cells can respond to cold or other stress by increasing the synthesis of a group of specific proteins, such as cold-inducible RNA-binding protein (CIRP) [29]. The precise functions of CIRP are still unclear; however, one of their roles is to protect cells against adverse environmental stress [28,29]. In neural stem cells, moderately low temperature stimulates the expression of CIRP, which protects neural stem cell from apoptosis [23]. Thus, an analysis of CIRP levels in mature oocytes prior to vitrification will be helpful for exploring the role of CIRP in the protection of oocytes from damage caused by the cryopreservation procedure. Numerous studies have reported that insulin-like growth factor I (IGF-1) enhanced the development and cryotolerance of bovine blastocysts [11,22]. The motility of yak frozen semen could also be enhanced by IGF-1 [18]. However, no detailed research study has been performed on yak mature oocyte cryopreservation to determine the correlation between the CIRP expression level and developmental competence of mature oocyte. In addition, whether there is some cryotolerance of IGF-1 on yak oocytes during in vitro maturation also requires verification.

2. Materials and methods

2.1. Chemical agents

All chemicals and reagents were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA) unless otherwise indicated. All of the media used in the present study were supplemented with penicillin (100 IU/mL) and streptomycin (0.1 mg/mL) and filter-sterilized (0.22 μm) prior to use.

2.2. Preparation of mature oocytes

Yak ovaries were derived from a local abattoir, transported to the laboratory in 30–35 °C saline within 4 h after slaughter. Cumulus–oocyte complexes (COCs) were recovered from follicles that were 2–8 mm in diameter using a 18-gauge needle connected to a 10-mL syringe. Oocytes surrounded by at least two layers of intact, compact cumulus cells and with a homogenous cytoplasm were selected for in vitro maturation (IVM) [6,20]. COCs were washed three times in IVM medium on a heating stage at 37 °C in a sterile room. The IVM medium consisted of TCM-199 with 25 mM HEPES, 2 mM $NaHCO_3$, 10% FBS, 50 μg/mL follicle-stimulating hormone (FSH), 50 μg/mL luteinizing hormone (LH), and 1 μg/mL 17β-oestradiol. Approximately 40–45 COCs were placed in 500-μL of IVM medium in four-well dishes (Nunc, Roskilde, Denmark) and cultured with oil overlay for 24 h at 37 °C in 5% CO_2 in ambient humidified air [32]. Depending on the experimental series, the culture medium was supplemented with 0, 50, 100, and 200 ng/mL IGF-1. After 24 h of IVM, the COCs were denuded of cumulus cells by treatment with COCs with hyaluronidase (100 IU/mL). Oocytes with an extruded first polar body were defined as matured (Fig. 1).

2.3. Quantitative RT-PCR analysis of CIRP mRNA levels in yak mature oocytes

Briefly, 24 h after IVM, fifteen matured oocytes without cumulus cells in four groups, were collected after being washed in D-PBS (without Ca^{2+}, Mg^{2+}) three times and used for total RNA extraction. Total RNA was extracted from matured oocytes using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration of the extracts was determined from the absorbance at 260 nm. All of the samples had a 260/280 nm absorbance ratio of approximately 1.8–2.0. The first strand was synthesized using SuperScript™ III First Strand Synthesis kit (Invitrogen, Chicago, IL, USA). The samples were stored in the freezer (−20 °C) until further analysis.

Quantitative RT-PCR (qRT-PCR) was performed using the Real Time PCR Detection System (ABI ViiA™ 7; Applied Biosystems, Foster City, CA, USA). The reactions consisted of 2 μL of cDNA, 0.5 μL of forward and reverse specific gene primers, 10 μL of SYBR Green II master mix, with 0.4 μL of ROX (Takara, Dalian, China), and 6.6 μL of water in a total volume of 20 μL. The optimal PCR conditions were as follows: one cycle of denaturation at 95 °C for 10 s; 40 cycles of denaturation at 95 °C for 10 s, annealing at the appropriate temperature for 15 s, and extension fluorescence acquisition at 72 °C for 10 s. For the PCR studies, the gene expression levels were quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene for cDNA normalization. The oocytes were used as control samples in the unfrozen group. The primer sequences and annealing temperature are shown in Table 1.

2.4. Analysis of CIRP protein levels in yak mature oocytes with western blotting analyses

Briefly, thirty matured oocytes without cumulus cells in the four groups were washed three times in ice-cold PBS, and the total proteins were extracted in extraction buffer (Beyotime, China). The proteins was separated on a 10% SDS-PAGE gel using a Bio-Rad apparatus and then transferred electrophoretically onto enhanced chemiluminescence (ECL) polyvinylidene fluoride (PVDF) membranes (Amersham, USA) using a Bio-Rad Mini Trans-Blot Cell (Bio-Rad, USA). The membranes were then blocked with 5% non-fat dry milk and 0.1% Tween 20 in Tris-buffered saline and subsequently incubated with primary antibody goat anti-CIRP antibody (ab106230, Abcam, USA) at 37 °C for 2 h, which was diluted to 1:500 in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween 20. Next, the membrane was washed three times in Tris-buffered saline, each time for 10 min. Horseradish peroxidase conjugated rabbit anti-goat IgG (sc-2768; Santa Cruz Biotechnology; dilution 1:6000 in PBS containing 1% BSA) was used to detect the conjugates and antibody–antigen complexes. The membrane was then washed three times in Tris-buffered saline, each time for 10 min, and was visualized using ECL detection kit (Beyotime, China). The intensity of the blots was measured using a densitometric analysis system (Bio-Rad). The intensity of the β-actin bands was used for normalization.

2.5. Vitrification and warming

Vitrification was performed using the CT (Kitazato BioPharma, Shizuoka, Japan) method according to previously described procedures [10]. Briefly, the remaining yak mature oocytes in each group were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries, Osaka, Japan) and 7.5% dimethyl sulfoxide (DMSO) in HEPES-buffered TCM-199 with 20% FBS base medium for 3 min at room temperature (23–28 °C) and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO, and 0.5 M sucrose in the base medium for ~60 s at room temperature. Within this 60-s period, up to 15 oocytes were loaded onto the polypropylene strip of a Cryotop device with a minimal amount of vitrification solution (<0.1 μL), and CT was directly immersed in liquid nitrogen (LN), and was protected with a cap. For warming, a

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