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Caffeine and oocyte vitrification: Sheep as an animal model

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ABSTRACT

Oocyte cryopreservation is valuable way of preserving the female germ line. Vitrification of immature ovine oocytes decreased the levels of both maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) in metaphase II (MII) oocytes after IVM. Our aims were 1) to evaluate the effects of vitrification of ovine GV-oocytes on spindle assembly, MPF/MAP kinases activities, and preimplantation development following IVM and IVF, 2) to elucidate the impact of caffeine supplementation during IVM on the quality and development of vitrified/warmed ovine GV-oocytes. Cumulus-oocyte complexes (COCs) from mature ewes were divided into vitrified, toxicity and control groups. Oocytes from each group were matured *in vitro* for 18 h in caffeine free IVM medium and denuded oocytes were incubated in maturation medium supplemented with 10 mM (+) or without (−) caffeine for another 6 h. At 24 h.p.m., oocytes were evaluated for spindle configuration, MPF/MAP kinases activities or fertilized and cultured *in vitro* for 7 days. Caffeine supplementation did not significantly affect the percentages of oocytes with normal spindle assembly in all the groups. Caffeine supplementation during IVM did not increase the activities of both kinases in vitrified groups. Cleavage and blastocyst development were significantly lower in vitrified groups than in control. Caffeine supplementation during the last 6 h of IVM did not significantly improve the cleavage and blastocyst rates in vitrified group. In conclusion, caffeine treatment during *in vitro* maturation has no positive impact on the quality and development of vitrified/warmed ovine GV-oocytes after IVM/IVF and embryo culture.

1. Introduction

Cryopreservation of gametes and embryos has become an integral part of assisted reproduction technologies (ART). In particular, sperm cryopreservation has been the most widely and successfully used in humans as well as in variety of mammalian species [1]. Embryo cryopreservation has also become a routine practise in clinical ART and has resulted in delivery of many healthy babies [2]. However, embryo cryopreservation has some drawbacks; it requires the availability of male partner to produce those embryos, in addition, embryo cryopreservation is prohibited due to ethical, legal and religious implications in some countries [3]. Cryopreservation of unfertilized oocytes is an alternative option, giving flexibility in the time of *in vitro* fertilization (IVF) and a potential to establish oocyte banking with oocyte donation [4–8]. There are two techniques applied to the cryopreservation of gametes and embryos: controlled slow freezing, which was favoured in early procedures, and ultrarapid cooling by vitrification, which is now widely used as it produces less damage to the oocytes and embryos than slow freezing [9].

Although extensive research have been conducted on the cryopreservation of metaphase II (MII) oocytes, vitrification of oocytes at this stage can disrupt the meiotic spindle [10], which could be avoided by freezing of oocytes at the germinal vesicle (GV) stages. Freezing of oocytes at this stage has many clinical advantages in human and animal reproduction. [11–15]. Though, numerous studies have been conducted to cryopreserve GV-oocytes in many mammalian species [12,13,15–27], and reportedly produced live births in humans and animals [17,28,29], the blastocyst development rate remains low

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[23,24,30]. One major obstacle is the requirement of the frozen GV-oocytes to be matured *in vitro* (IVM) prior to fertilization [19]. No standard IVM protocol for GV-oocytes has yet been established in humans and in some animal species [31–33].

It is well known that, oocyte meiotic maturation is controlled by the levels of two cytoplasmic protein kinases; maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK). High activities of both kinases are responsible for the onset of germinal vesicle breakdown and are also essential for the maintenance of the oocytes at MII-stage [34]. Previous studies showed that vitrification of immature ovine oocytes decreased the levels of both kinases after IVM [13].

Caffeine (1,3,7-trimethylxanthine), a phosphodiesterase inhibitor, has been reported to induce dephosphorylation of Y-15 and T14 of p34cdc2, which may have been occurred by inhibition of Myt1/Wee1 kinase resulting in an increase in the activity of MPF in cultured mammalian cells, Xenopus, and porcine oocytes [35]. In sheep, previous studies showed that treatment of *in vitro* matured oocytes with caffeine increased the activities of both MPF and MAPK kinases, improved frequencies of nuclear envelope breakdown and chromosome condensation of transferred nuclei, increased total cell numbers, and reduced the frequency of apoptotic nuclei in blastocyst embryos produced by somatic cell nuclear transfer (SCNT) [36–38]. Moreover, in aged denuded ovine oocytes, caffeine treatment increased the blastocyst development rates and decreased the frequency of polyspermy following IVF [39]. However, little is known about its role on the development of vitrified/warmed oocytes. Our aims were 1) to evaluate the effects of vitrification of ovine GV-oocytes on spindle assembly, MPF/MAP kinases activities, and preimplantation development following IVM and IVF, 2) to elucidate the impact of caffeine supplementation during IVM on the quality and development of vitrified/warmed ovine GV-oocytes.

2. Materials and methods

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK).

2.1. Oocyte collection

Ovine ovaries were collected from a local slaughterhouse (Nottingham, UK) and kept in a thermos flask filled with pre-warmed phosphate-buffered saline (PBS) at 25 °C during transportation to the laboratory. Cumulus–oocyte complexes (COCs) were aspirated using a 21-gauge needle attached to a 10-mL syringe from 2 to 3-mm follicles. The follicular fluid and COCs were placed into 50-mL conical tubes and kept in a warming box at 39 °C for 15 min so that COCs sank to the bottom of the tubes. The upper follicular fluid was removed and 5 mL of follicular fluid containing COCs was poured into a 90-mm Petri dish containing oocyte washing medium consisting of HEPES-buffered TCM 199 (H-TCM 199; Gibco BRL BRL/Life Technologies, Paisley, Renfrewshire, UK) supplemented with 10% v/v fetal bovine serum (FBS; Gibco). The COCs, with at least two to three compact layers of cumulus cells and a homogeneous cytoplasm, were selected under a light microscope (Leica, Wetzlar, Germany). The selected oocytes were randomly divided into three groups; vitrified, toxicity (exposed only to vitrification and warming solutions without freezing), and control (directly subjected to IVM).

2.2. Vitrification and warming of COCs

COCs were vitrified according to the method described by Moawad *et al.* [13]. Briefly, COCs were rinsed three times in base medium (BM; H-TCM 199 supplemented with 10% FBS), and then transferred into 500 µL equilibration solution (10% v/v ethylene glycol (EG) plus 0.25 M trehalose in BM) for 3 min on a warm stage at 39 °C. 3–5 oocytes were then transferred into a small droplet of 20 µL vitrification solution (20% v/v EG and 20% v/v dimethylsulfoxide (DMSO)) before being immediately treated in warming solution as toxicity or vitrified. For vitrification, the cryoloop (Hampton Research, Aliso Viejo, CA, USA) was dipped in the vitrification solution so that a thin film was created by surface tension. Three to five oocytes were gently placed on the film using a glass-mouth pipette and the cryoloop device containing the oocytes was plunged directly into a cryovial that had been submerged and filled with liquid nitrogen (LN_2). The cap of the cryovial was then tightened and the vial was returned to LN_2. The whole process was performed in 1 min. For thawing, the cap of the cryovial submerged in LN_2 was carefully unscrewed and opened and the cryoloop containing the vitrified oocytes was transferred immediately into warming solution, which consisted of 500 mL BM plus 10% v/v EG and 1 M trehalose. The oocytes came off the cryoloop into the solution and were kept in this solution for 3 min before being transferred into 500 µL BM plus 0.5 M trehalose and then to BM (3 min in each solution). All solutions were kept at 39 °C. Finally, the COCs were examined morphologically to assess their viability under a stereomicroscope (MZ 12.5; Leica Microsystems, Wetzlar, Germany). Oocytes with a spherical and symmetrical shape and evenly granulated cytoplasm were regarded as viable, whereas oocytes exhibiting membrane damage, a swollen or ruptured zona pellucida and/or degenerated cytoplasm were considered as non-viable. Only morphologically viable COCs were selected for further experiments.

2.3. In vitro maturation

IVM was performed as described previously [15]. Briefly, COCs from vitrified, toxicity and control groups were washed twice in maturation medium (bicarbonate-buffered TCM 199 with Earle salts; Gibco) supplemented with 10% FBS, 5 µg/mL FSH (Vetropharm, Belleville, ON, Canada), 5 µg/mL LH (Vetropharm), 1 µg/mL 17β-oestradiol, 0.3 mM sodium pyruvate, 100 µM cysteamine and 50 µg/mL gentamicin. Groups of 40–45 COCs were then transferred into four-well dishes (Nunc, Roskilde, Denmark) containing 500 µL maturation medium covered with mineral oil. All dishes were pre-warmed in an incubator at 39 °C under 5% CO_2 in air before use. All COCs were cultured in the medium for 18 h under the same conditions.

2.4. Caffeine treatment

At 18 h post IVM, cumulus cells were removed from all the groups by repeated pipetting of COCs in H-TCM 199 supplemented with 4 mg/mL polyvinylpyrrolidone (PVP) and 300 IU/mL hyaluronidase. After washing in maturation medium, oocytes from each group were incubated in maturation medium supplemented either with 10 mM (+) or without (−) caffeine for another 6 h. IVM was then continued till 24 h.

2.5. Spindle and chromosome configuration

IVM oocytes were immunostained for tubulin and counterstained to assess chromosomes as previously described [13]. Briefly, oocytes (at least 44 oocytes/group) were fixed in 4% (w/v) paraformaldehyde (PFA) for 30 min at room temperature and then washed three times (10 min each) in PBS supplemented with 20% FBS (PBS-FBS). All oocytes were then transferred to PBS-FBS containing 0.5% Triton X-100 (permeabilization medium) and kept in this medium for 30 min at room temperature. Subsequently, the primary antibody (mouse monoclonal anti-α-tubulin antibody; 1:200 dilution) was added and oocytes were further cultured at 4 °C overnight. Then, oocytes were rinsed three times in PBS-FBS as described above before being incubated with a fluorescein isothiocyanate (FITC)-labelled goat secondary antibody (1:200 dilution) at room temperature for 60 min. Finally, oocytes were stained with 10 µg/mL Hoechst 33342 and then rinsed three times in PBS-FBS. Oocytes were then transferred to a small drop of Vectashield