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Effects of co-incubation with conspecific ampulla oviductal epithelial cells and media composition on cryotolerance and developmental competence of *in vitro* matured sheep oocytes

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ABSTRACT

Developmental potential of cryopreserved *in vitro* matured oocytes is very low in nearly all mammalian species studied to date. Despite relatively high cleavage rates, the vitrified/warmed metaphase II oocytes have a decreased rate of blastocyst formation, which can be attributed to the elevated cytoplasmic lipid content and lipid droplet fragmentation. Secretory products of ampulla oviductal epithelial cells (AECs) at the periovulatory stage of the ovarian cycle enhance the viability of *in vitro* matured oocytes. The present study was undertaken to determine if co-culture of cumulus-oophorus complexes (COCs) with conspecific AECs or reducing the lipid content of *in vitro* matured ovine oocytes would improve their cryotolerance and ensuing developmental competence. Ovine COCs aspirated from the slaughterhouse ovaries were matured in the following media or culture conditions: TCM199 + FBS + AECs (T1); TCM199 + FBS (T2); TCM199 + BSA (T3); TCM199 + 0.6 mg/mL of L-carnitine (T4); TCM199+ L-carnitine + FBS (T5), or TCM199 only (Ctr). Subsequently, the oocytes were vitrified and used for *in vitro* fertilization (IVF). The lowest degree of zona pellucida (ZP) hardening following vitrification of *in vitro* matured sheep oocytes was observed in T1 and T5 ($P < 0.05$). Cleavage, blastocyst formation and ensuing development (i.e., total cell numbers) as well as blastocyst hatching rates were all greater ($P < 0.05$) in T1 compared with the remaining groups; *in vitro* matured COCs in T4 and Ctr did not develop beyond the cleavage stage. The inner cell mass: trophectoderm cell ratio in T1 (1:3.29) was significantly greater compared with T2 (1:3.39), T3 (1:3.40) and T5 (1:3.44). The present results indicate that the ovine COCs/AECs co-culture system had the most positive influence on cryotolerance, ZP hardening, and developmental competence of *in vitro* matured oocytes.

1. Introduction

The prospect of irreversibly losing ovarian function (women) and the genetic gain or salvage due to long-term storage and transfer (domestic livestock and endangered animal species) appear to be the most compelling reasons for oocyte freezing [1,2]. Even though a vast majority of women undergoing elective egg freezing do not use them later on, there appears to be a steady increase in the number of female millennials and younger generation X women using egg-freezing services [3]. Oocyte preservation in animals permits the indefinite storage of unfertilized eggs usually obtained from hormonally super stimulated donor females or *post mortem*, and the transfer or exchange of genetic material without the necessity of animal transportation. However, mammalian oocytes are highly sensitive to chilling and freezing. Despite numerous efforts to improve oocyte cryopreservation protocols, low survival rates of vitrified oocytes remain the major limitation in gamete cryobiology. Vitrification of both *in vitro*

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matured or ovulated oocytes is associated with abnormal spindle formation leading to chromosomal aberrations and/or mitotic arrest, altered distribution of cortical granules and increased polyspermy or, on the contrary, the premature cortical granule exocytosis resulting in zona pellucida (ZP) hardening [4].

Lipid accumulation and cytoplasmic fragmentation in oocytes during *in vitro* maturation (IVM) may exert adverse effects on later stages of *in vitro* embryo production (IVP) and vitrification/warming procedures [5]. Fetal calf serum (FBS) is commonly added to IVM media to provide COCs with growth factors and other bioactive molecules that are essential for proper and timely oocyte maturation; FBS is also the major source of fatty acids utilized by oocytes during the IVM procedure. Several studies have shown that using lipid reducing agents in IVP media may effectively prevent the formation of cytoplasmic lipid droplets [6,7]. The elimination of lipid droplets or reducing the lipid content of oocytes with various chemical reagents (e.g., L-carnitine promoting the transport of fatty acids from the cytosol to mitochondria for beta-oxidation [6–9]; can improve their cryotolerance [8].

One of the problems associated with oocyte freezing is the induction of a primary activation event, namely the zona pellucida (ZP) hardening, which significantly impedes ensuing fertilization and may affect implantation of resultant blastocysts [10,11]. The ZP hardening is evoked by a fusion of cortical granules with the plasma membrane, and the release of their content into the ZP layers. The membrane fusion event is Ca^{2+}-dependent and it is normally triggered by an increase in intracellular Ca^{2+}levels initiated by the sperm-egg fusion [10,11].

Cytokines secreted by epithelial cells of female tubular genitalia may also influence the viability of gametes and pre-implantation embryos [12]. There are two major classes of cytokines produced by the female reproductive tract: cytokines acting as survival agents [granulocyte macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), heparin-binding epidermal growth factor (HB-EGF), and insulin-like growth factor (IGFII)] and apoptosis-inducing cytokines [tumor necrosis factor alpha (TNFα), TNF-related apoptosis-inducing ligand (TRAIL) and interferon gamma (IFNg)]. The balance between these two types of cytokines regulates the competence of germ cells and progression of early embryonic development. However, the IVP media currently used do not contain cytokines in spite of numerous publications supporting their positive influence around fertilization and in early pregnancy [12]. Therefore, *in vitro* matured oocytes are unlikely to possess similar properties to those of ovulated oocytes [13,14].

Recently, we have developed and evaluated a new co-culture system of ovine cumulus-oophorus complexes (COCs) with ampulla oviductal epithelial cells (AECs) collected during the periovulatory stage of the interovulatory interval [15,16]. The results of those studies showed that oocytes matured in the co-culture system were significantly more competent compared with those incubated in a standard culture medium or the conventional (monolayer) co-culture system, wherein the epithelial cells are exposed to enzymatic detachment and the attainment of sufficient confluency prior to their use in co-culture systems takes several days; all those factors significantly reduce the biopotency of the incubated cells. These observations are indicative of the beneficial effects exerted by the secretory products of AECs on oocyte fertilizing ability and ensuing development of cultured embryos. Similar studies do not exist for vitrified oocytes used for *in vitro* fertilization.

Considering all the facts above, we hypothesized that the ovarian cycle-stage specific co-culture system (i.e., aspirated oocytes matured with conspecific AECs from cyclic females) and/or the reduction of lipid content of IVM media would improve cryotolerance of ovine oocytes. The present experiment set out to determine if: i. co-incubation of COCs with epithelial cells obtained from the ampullary segment of the oviduct of sexually mature females in metestrus and/or ii. the modification of IVM media composition aimed to reduce the amount of bioavailable fatty acids would affect cryotolerance of *in vitro* matured sheep oocytes. To evaluate the effects of modified IVM conditions, we followed the oocyte and early embryo development from warming to *in vitro* fertilization (IVF) and culture (IVC) procedures, and assessed oocyte nuclear maturation, ZP hardening and blastocyst differentiation.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Animals and oocyte recovery

The present study utilized clinically healthy cyclic ewes (September–February; longitude-35.69°N, latitude 51.39°E) of the Lori-Bakhtiary breed, aged 2–3-years. The ovaries (n = 730) were collected immediately after slaughter, placed in an insulated container filled with physiologic saline solution (0.9% NaCl) supplemented with penicillin-streptomycin (100 μg/mL; Gibco, Grand Island, NY, USA), and transported to the laboratory at 37 °C within 2 h of collection. COCs were aspirated from transparent antral follicles measuring 2–6 mm in diameter using an aspiration pump (MEDAP Sekretsauger P7040, Tilburg, NL), fitted with a disposable vacuum line (length: 35 cm, internal diameter: 3 mm) and set at the constant flow rate of 10 mL H_2O/min and a disposable 20-gauge needle [17,18]. The medium used for oocyte recovery was HEPES-TCM supplemented with 10% (FBS), 0.2 mM of sodium pyruvate, 5 μg/mL of gentamicin, 100 μl/mL heparin. The oocytes with at least three layers of cumulus granulosa cells and with uniform granulated cytoplasm were used for subsequent experimental procedures.

2.3. Preparation of ampulla oviductal epithelial cells (AECs)

Complete ovine reproductive tracts were collected immediately after slaughter and the oviducts ipsilateral to the ovaries with corpora heamorrhagica (i.e., ovarian antral follicles that ruptured within the last 24 h) were used for harvesting AECs. Such oviducts were used in this study because they contain epithelial cells that had been exposed to elevated(pre-ovulatory) estradiol concentrations and other secretory products from follicular fluid, which trigger the production of the trophic, oocyte-nourishing proteins [19]. Before washing the oviducts in phosphate buffered saline without Ca^{2+}/Mg^{2+} (PBS^{-}), both ends of the oviduct were tied up with sterile surgical sutures. The oviducts were then dipped in 70% ethanol and transported on ice to the laboratory in PBS solution supplemented with 2% penicillin/streptomycin. After the arrival to the laboratory, the oviducts were dipped again in 70% ethanol and washed in HEPES-buffered tissue culture medium (TCM-199), in a laminar flow hood (BioLAF, Milano, Italy). Identification of the ampulla and the isthmus was based primarily on a difference in their outer diameter and transparency [16]. The ampulla was then divided into two or three 3–4–cm segments that were gently squeezed, in a stripping motion, with forceps to recover epithelial cells [15,19,20]. A yellowish matter containing epithelial cells was collected into the culture medium TCM199, supplemented with 2% estrous cow serum and 0.25 mg/mL of gentamicin. The cell suspension was pipetted 10–15 times using a 1000-μL filtered tip (JET Biofil, Guangzhou, China) and then passed 5 times through a 1-mL