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Effects of trehalose vitrification and artificial oocyte activation on the development competence of human immature oocytes

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ABSTRACT

Sucrose and trehalose are conventional cryoprotectant additives for oocytes and embryos. Ethanol can artificially enhance activation of inseminated mature oocytes. This study aims to investigate whether artificial oocyte activation (AOA) with ethanol can promote the development competence of in vitro matured oocytes. A total of 810 human immature oocytes, obtained from 325 patients undergoing normal stimulated oocyte retrieval cycles, were in vitro maturated (IVM) either immediately after collection (Fresh group n = 291)) or after being vitrified as immature oocytes (Vitrified group n = 519). These groups were arbitrarily assigned. All fresh and vitrified oocytes which matured after a period of IVM then underwent intra-cytoplasmic sperm injection (ICSI). Half an hour following ICSI, they were either activated by 7% ethanol (AOA group) or left untreated (Non-AOA group). Fertilization, cleavage rate, blastocyst quality and aneuploidy rate were then evaluated. High-quality blastocysts were only obtained in both the fresh and vitrified groups which had undergone AOA after ICSI. Trehalose vitrification slightly, but not significantly, increased the formation rates of high-quality embryos (21.7% VS 15.4%, P > 0.05) and blastocysts (15.7% VS 7.69%, P > 0.05)) when compared with sucrose vitrification. Aneuploidy was observed in 12 of 24 (50%) of the AOA derived high quality blastocysts. High-quality blastocysts only developed from fresh or vitrified immature oocytes if the ICSI was followed by AOA. This information may be important for human immature oocytes commonly retrieved in normal stimulation cycles and may be particularly important for certain patient groups, such as cancer patients. AOA with an appropriate concentration of ethanol can enhance the developmental competence of embryos.

1. Introduction

Oocyte cryopreservation is an important tool in assisted reproductive technologies and in particular for cancer patients before chemotherapy [1,2]. However, it is unfeasible and risky for some cancer patients to retrieve mature oocytes (Metaphase II, MII) after hormone stimulation. Cryopreservation of immature oocytes (CIO) followed by in-vitro maturation (IVM) is an option for fertility preservation for normal in-vitro fertilization (IVF) patients as well as for cancer patients [3]. Unlike MII oocytes, immature oocytes, those at the germinal vesicle stage (GV oocytes) and at the metaphase I stage (MI oocytes), do not have chromosomes arranged on a spindle. Therefore, CIO can circumvent spindle damage [4,5]. However, only few cases of successful pregnancies have been reported for cryopreserved GV stage oocytes (CIO) which may be due to problems associated with IVM after cryopreservation [6–8]. In bovine embryos, cryopreservation causes alterations in the zona pellucida, reduces the development competence [9]. Cryopreservation of oocytes may also affect Ca^{2+} signaling, mitochondrial membrane potential and membrane proteins [10,11]. Artificial

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oocyte activation (AOA) can raise the fertilization and the cleavage rates [12–14], as well as reproductive outcomes in women with previous fertilization failure [15,16]. There are a variety of AOA methods for oocytes, including mechanical, electrical [17], and chemical activation. Chemical activation is the most commonly used method for AOA [18], chemical activating agents include calcium ionophore A23187 [19–21], strontium chloride (SrCl2) [22], ionomycin [21,23,24] and ethanol [25,26]. In 2009, Hou et al. used bovine oocytes to show that AOA with ethanol plus 6-DMAP achieved higher rates of embryo development than other activation agents (calcium ionophore A23187, or ionomycin alone, or in combination with DMAP or cycloheximide) [25]. Ethanol activation of fresh and cryopreserved human oocytes after ICSI can improve their subsequent developmental competence [26]. Therefore, in the current experiment, we chose ethanol as the activation agent for human in-vitro-matured oocyte, and monitored the effects of ethanol on their subsequent activation and developmental competence. Vitrified and fresh mature oocytes currently result in comparable pregnancy outcomes and are therefore suitable for a wide range of applications [27,28]. The vitrification solutions for human mature oocytes most commonly include sucrose as an additive as it acts as a non-penetrating cryoprotectant to aid dehydration and vitrification [29]. Trehalose is another sugar that can protect the membrane integrity of dried mammalian cells and can-if injected intracellularly improve the survival rate of mammalian cells and oocytes [30–34].

In this study, we cryopreserved immature oocytes in vitrification solutions supplemented with one of two non-penetrating cryoprotectant additives (trehalose or sucrose) and then evaluated their survival, in vivo maturation, fertilization and the development competence of embryos. We investigated the influence of AOA after ICSI on the fertilization outcome of the in-vitro-matured oocytes and the development competence of the resulting embryos.

2. Materials and methods

2.1. Reagents

The culture media (gamete, fertilization, cleavage and blastocyst) were purchased from Cook Australia Pty Ltd. (Brisbane, Australia). Recombinant human follicle stimulating hormone (r-hFSH) was purchased from Merck KGaA (Darmstadt, Germany). Human chorionic gonadotropin (hCG) was purchased from Livzon Pharmaceutical Group Inc (Zhuhai, China). MultiVysion Polybrene (PB) multi-color probe kit was purchased from Abbott Molecular (Abbott Park, IL, USA). HTF 1024 and serum protein substrate (SPS) were purchased from SAGE (Irvine, CA, USA). Other reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.2. Collection of immature oocytes

This study included a total of 810 oocytes obtained from 325 patients who underwent intra-cytoplasmic sperm injection (ICSI) at the First Affiliated Hospital of Anhui Medical University between February 2009 and December 2012. All patients were under the age of 35, and received a long or short regime of ovarian stimulation (GnRH analogue and r-hFSH) for superovulation. The patients received an intramuscular injection of 10,000 hCG when at least 2–3 oocytes reached the diameter of 18 mm or above, At 36 h after hCG injection, transvaginal ultrasound-guided oocyte retrieval was conducted. The cumulus-oocyte complexes (COCs) were isolated and cultured in fertilization medium at 37 °C with 6% CO2. After ovum pick-up, COCs were denuded enzymatically by 60–80 mU/ml hyaluronidase solution to remove the cumulus cells to assess nuclear maturity. All retrieved metaphase II (MII) oocytes were used for patients' treatment, whereas immature oocytes (GV or MI) were used for this study. This study was approved by the Ethical Committee at the First Affiliated Hospital of Anhui Medical University. All participants were prerequisite to sign a written informed consent.

2.3. Preparation of sperm and patient's serum

For sperm for ICSI, on the second day of oocyte retrieval, the paternt's semen was collected once again. One part of the semen was for the in-vitro-matured fresh oocyte, whereas the other part was frozen and stored in −196 °C liquid N_2 for the subsequent in-vitro-matured thawed oocyte. For patient's serum for IVM medium, one day before oocyte retrieval, the patient's serum was collected and one part was directly used to prepared IVM medium, while the other part was frozen and stored in −22 °C condition for the subsequent preparation of IVM medium. In this experiment, all of the patients used their own sperm and serum.

2.4. Grouping of immature oocytes

The Immature oocytes were arbitrarily assigned to one of 6 groups as shown in Fig. 1. The oocytes in the fresh group (n = 291) were placed in maturation medium immediately, while the remaining (n = 519) were vitrified, and stored in liquid nitrogen for one month before being warmed and placed in maturation medium. In the vitrified oocytes, a total of 274 immature oocytes underwent sucrose vitrification, and the data had been reported before [26]. Fresh and vitrified oocytes, which reached the MII phase after the maturation step underwent ICSI and were then either activated by 7% ethanol (Fresh AOA group; Vitrification AOA group) or left untreated (Fresh Non-AOA group; Vitrification Non-AOA group). Numbers in each group are shown in Fig. 1.

2.5. Immature oocyte vitrification

The vitrification of the oocytes was performed mainly as described in Ref. [35]. The minor modifications and variations in process is explained briefly here, the oocytes without cumulus cells were incubated in equilibration medium (EM) [30% SPS, HTF1024, and 7.5% ethylene glycol (EG, v/v), 7.5% 1.2-propanediol (PROH, v/v) with no sugar] at room temperature for 15 min, and then incubated in medium [HTF1024, 15% (v/v) PROH, 30% SPS, 15% (v/v) EG] and either 0.5 M sucrose or 0.5 M trehalose, at room temperature for 1 min. The oocytes were then put onto Cryotops (Kitazato, Japan) and plunged into liquid nitrogen. The Cryotops were removed from liquid nitrogen one month later, and placed in dishes with medium (HTF1024, 30% SPS, and 1.0 M sucrose or trehalose) at room temperature for 1 min, followed by diluent medium I and diluent medium II for almost 3 min each at room temperature. Diluent medium I contained (30% SPS, HTF1024, and 0.5 M sucrose or trehalose) and diluent medium II consisted of (HTF1024, 30% SPS, 0.25 M trehalose or sucrose), then all oocytes were immersed into washing medium (30% SPS and HTF1024) twice for almost 3 min each. All of the warmed oocytes were then cultured in maturation media [35]. An oocyte with irregular rim and diffuse cytoplasm was defined as non-viable.

2.6. IVM, ICSI and oocyte activation

The fresh and warmed immature oocytes with normal morphology were cultured in IVM medium prepared in our laboratory (80% (v/v) TCM199 medium, 0.22 mM pyruvic acid, 0.075 IU/