Application of intra- and extracellular sugars and dimethylsulfoxide to human oocyte cryopreservation

Abdelmoneim Younis · David Carnovale ·
William Butler · Ali Eroglu

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Abstract

Purpose Oocyte cryopreservation may avoid many complications of human embryo freezing and provide future fertility for women undergoing cancer therapy. The objective of this study was to explore the application of intra- and extracellular sugars in combination with small amounts of dimethylsulfoxide (DMSO) to human oocyte cryopreservation as an alternative approach.

Methods Discarded human oocytes that were obtained from IVF patients under informed consent and IRB approval, were cryopreserved by slow cooling to -196°C after being randomly distributed into three groups: (i) DMSO control without intra- and extracellular sugar; (ii) extracellular sugar (raffinose) + DMSO; and (iii) intra- and extracellular sugar (trehalose and raffinose, respectively) + DMSO. Subsequently, all cryopreserved oocytes were thawed rapidly, and their survival was assessed by morphological criteria after 24 h of culture.

Results A total of 71 oocytes were evaluated in three groups with survival rates of 88.5% (23/26), 68.2% (15/22), and 52.2% (12/23) for intra- and extracellular sugar

+DMSO, extracellular sugar+DMSO, and DMSO control groups, respectively.

Conclusion These results support the use of intra- and extracellular sugars as an alternative approach for cryopreservation of human oocytes.

Keywords Cryopreservation · Human oocyte · Trehalose · Raffinose · DMSO

Introduction

Although a gradual progress in slow-cooling and vitrification protocols and the use of ICSI for fertilization have led to an increasing number of live births of frozen thawed human oocytes [1–9], ASRM guidelines still consider cryopreservation of human oocytes as an experimental procedure [10]. Nonetheless, many women understandably have interest in this emerging technology and view oocytes cryopreservation as an elective fertility preservation strategy that may help them to realize their longer-term reproductive goals [11]. Alternative novel approaches such as the use of intra- and extracellular sugars [12–14] may improve the outcome of human oocyte cryopreservation. We report here our initial results of a pre-clinical cryopreservation protocol using failed-to-fertilized (FTF) metaphase II (MII) oocytes.

Materials and methods

This prospective study investigated the survival of human FTF oocytes after cryopreservation in LN_2 as described subsequently. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Institutional

A. Younis (✉) · D. Carnovale · W. Butler
Central Georgia Fertility Institute,
Department of Obstetrics and Gynecology,
Mercer University School of Medicine,
4075 Elnora Dr.,
Macon, GA 31210, USA
e-mail: Younis.Abdelmoneim@mccg.org

A. Eroglu (✉)
Institute of Molecular Medicine and Genetics,
Department of Medicine and Department of Obstetrics
and Gynecology, Medical College of Georgia,
1120 15th Street, CB-2803,
Augusta, GA 30912, USA
e-mail: aeroglu@mcg.edu

review board (IRB) approval was obtained from the Medical Center of Central Georgia-Mercer University School of Medicine to use FTF discarded human oocytes in the present study. All women participating in this study signed an IRB-approved consent form describing the investigational nature of oocyte freezing. The discarded human oocytes were randomized to undergo freezing and thawing in three different groups: (i) DMSO control [0.5 M DMSO in the absence of intra- extracellular sugars]; (ii) extracellular sugar [0.3 M raffinose] + 0.5 M DMSO; and (iii) intra- and extracellular sugar [0.1 M trehalose and 0.3 M raffinose, respectively] + 0.5 M DMSO. Raffinose (Fluka, Germany) was used as extracellular sugar in the cryopreservation medium based on its slightly higher glass transition temperature.

Trehalose microinjection

Microinjection of trehalose (Fluka) into oocytes was performed in a 80- μ L drop of Hepes-buffered Hypermedium [14, 15] supplemented with 3 mg/mL human serum albumin (HSA *InVitro* Care Inc) and 0.15 M trehalose. The microinjection dish also had a 40- μ L drop of 0.8 M trehalose solution prepared in 15 mM Hepes [15]. The concentrated trehalose solution (i.e., 0.8 M) was microinjected into oocytes to achieve an intracellular trehalose concentration of $\sim$ 0.15 M. Both drops were covered with sterile mineral oil (Humco, Clinton, SC). Approximately 10 min before microinjection, oocytes were transferred to the microinjection drop to allow equilibration with the hypertonic extracellular osmolarity. During this period, small amounts of 0.8 M trehalose solution were aspirated into an ICSI needle (MIC-CUST-30; Humagen Fertility Diagnostics, Charlottesville, VA). Next, the injection needle loaded with 0.8 M trehalose was moved to the microinjection drop containing the equilibrated oocytes. The injection needle and holding pipette were gently lowered and focused in accordance with the outer right border of the oolemma on the equatorial plane at 3 o'clock. The trehalose solution was injected slowly in the center of

the oocytes. The amount of intracellular trehalose was determined by the osmometric behavior of the oocytes in response to the microinjection (See Fig. 1). The re-expansion of the microinjected oocytes in the hypertonic medium (i.e., Hypermedium containing 0.15 M trehalose) to their isotonic volume indicated introduction of 0.15 M trehalose into oocytes [15].

Freezing procedure

Hepes-buffered Hypermedium containing 10% FBS was used to prepare freezing solutions containing only DMSO (0.5 M) or both DMSO (0.5 M) and raffinose (0.3 M). Before cryopreservation, oocytes were transferred to 1:1 dilution of the freezing solutions at ambient temperature, where they were held for 5 min, and then transferred to the final freezing solutions for additional 10 min of equilibration with the extracellular osmolarity. During the final equilibration period, the oocytes, along with 0.20 mL of freezing medium, were aspirated into 0.25-mL plastic straws (TS Scientific, Perkasio, PA). The straws were then placed vertically in a programmable freezer (Planer Kryo 10-II, TS Scientific) at 0°C and cooled to -6°C at a rate of 2°C/min. After manual seeding of extracellular ice using a pre-chilled metal forceps and holding at -6°C for 10 min, the straws were cooled first to -60°C at 1°C/min and then -135°C at 5°C/min followed by plunging into LN₂.

Thawing procedure

Thawing for all groups was carried out by holding the straws in ambient air for 30 s then by immersing in a water bath at 31°C until the ice has fully melted, about 10–30 s. Approximately 2–3 min after thawing, the contents of the straws (0.20 mL) were gently released into 1 mL of Hepes-buffered Hypermedium containing 0.1 M galactose and held therein for 5 min. Galactose was selected for cryoprotectant removal based on an earlier published study [16]. Next, the oocytes were transferred into fresh Hepes-buffered Hypermedium containing 0.1 M galactose for

Fig. 1 Trehalose injection into human oocytes. **a** A shrunken oocyte equilibrated in the hypertonic medium before microinjection. **b** Microinjection the trehalose solution using an ICSI needle. **c** Re-expansion of the microinjected oocyte in the hypertonic medium

another 5 min. Subsequently, the oocytes were washed in a new drop of the same medium before transferring to culture drops covered with sterile mineral oil. The trehalose-injected oocytes were cultured in bicarbonate-buffered Hypermedium containing 0.1 M galactose while non-injected oocytes were cultured in the same medium without addition of 0.1 M galactose. All cultures were carried out at 37°C in an atmosphere of 5% CO₂ in air. The cryosurvival was assessed after a culture period of 24 h. Oocytes were designated as surviving based on the morphological criteria when they had an intact zona pellucida, intact oolemma, and a refractive cytoplasm after 24 h of culture.

Statistical analysis

Data were analyzed by Fisher's exact test using GraphPad Prism (GraphPad Software Inc., SanDiego, CA). Differences among the groups considered statistically significant when the *p*-value was less than 0.05.

Results

Results are summarized in Fig. 2. In the DMSO control group, 52.2% of the cryopreserved oocytes survived after thawing and overnight culture. Addition of extracellular sugar (i.e., 0.3 M raffinose) improved the cryosurvival to 68.2%; however, this was not statistically significant. In contrast, the presence of both intra- and extracellular sugars significantly improved the cryosurvival to 88.5% (*p*=0.009) indicating beneficial effect of sugars as intra- and extracellular cryoprotectants.

Discussion

This study using a small but sufficient number of human oocytes demonstrates efficacy of intra- and extracellular

sugars along with small amounts of DMSO in cryopreservation of human oocytes. The results of the present study are in accord with the survival schemes in nature where a variety of organisms such as certain frogs, tardigrades, insects, and brine shrimp survive freezing and extreme drying by accumulating intra- and extracellular sugars [17, 18]. Our results also support findings of previous studies showing the beneficial effect of intra- and extracellular sugars in cryoprotection of mammalian cells [12, 19] and oocytes [13, 14].

The protective actions of sugars such as trehalose can be attributed to their high glass transition temperature compared to conventional penetrating cryoprotectants and their stabilizing effect on lipid membranes as a result of direct interaction with polar head groups [20–22]. Furthermore, findings of several studies show that sugars afford remarkable protection against osmotic, chemical, and hypoxic stresses [23–26], which cells may experience during cryopreservation procedures. Experimental findings also indicate non-toxicity of sugars compared with well-known toxicity of conventional cryoprotectants such as DMSO, propanediol, and glycerol [12, 27–31]. Consequently, intra- and extracellular sugars can be used with small amounts of conventional cryoprotectants to minimize the cryoprotectant toxicity while maximizing overall cryoprotection. In fact, this has recently been demonstrated by obtaining healthy offspring from cryopreserved mouse oocytes [14].

It should also be noted that mammalian cell membranes practically impermeable to sugars, which represents a disadvantage for the application of sugars to oocyte cryopreservation. However, sugars such as trehalose are very effective at quite low concentrations and can be introduced into oocytes using a regular ICSI setup as demonstrated in the present study. Earlier studies using mouse oocytes and zygotes also showed that microinjected trehalose at its effective concentrations was non-toxic and quickly eliminated during embryonic development [15, 29].

Fig. 2 Survival of human oocytes cryopreserved using intra- and extracellular sugars along with small amounts of DMSO. [Raff]ex: extracellular raffinose, [Tre]in: intracellular trehalose. (please enter the group names under the bars as follows: DMSO control (0.5 M); [Raff]ex = 0.3 M +DMSO = 0.5 M; [Tre]in = 0.1 M+[Raff]ex = 0.3 M +DMSO = 0.5 M)

In recent years, several studies reported excellent survival rates after vitrification of human oocytes [6, 9, 32–34]. These results are encouraging; yet the vitrification procedure used in these studies requires direct contact with LN₂, and thus is of concern due to contamination risk. In the present study, we were able to obtain comparable survival rates in a closed system. However, it remains to be determined to what extent the surviving oocytes are functional. Therefore, studies involving nonhuman primate oocytes have been planned to address this issue.

In conclusion, the findings of this study support the use of intra- and extracellular sugars as an alternative approach for cryopreservation of human oocytes. Further clinical studies are needed to demonstrate fertilization and developmental capacity of human oocytes cryopreserved using intra- and extracellular sugars along with small amounts of a conventional cryoprotectant such as DMSO.

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