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GAMETE BIOLOGY

Oocyte morphology does not affect post-warming survival rate in an egg-cryobanking donation program

Amanda Souza Setti · Rita de Cássia Savio Figueira · Daniela Paes de Almeida Ferreira Braga · Renata Cristina Ferreira · Assumpto Iaconelli Jr. · Edson Borges Jr.

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Abstract
Purpose To evaluate whether oocyte dysmorphisms affect oocyte survival rates in an egg-cryobanking donation program.
Methods This study included 54 patients undergoing intracytoplasmic sperm injection. A total of 415 metaphase II oocytes were vitrified using the Cryotop method. Oocyte morphology was assessed immediately prior to oocyte vitrification under 400× magnification. The influence of dysmorphisms on post-thaw survival rates was assessed using regression analysis. Results were considered to be significant at the 5% critical level.
Results Oocyte survival rate was not affected by the presence of the following analysed oocyte abnormalities: increased cytoplasmic granularity, vacuoles in the ooplasm, aggregates of smooth endoplasmic reticulum in the ooplasm, large perivitelline space size, perivitelline space granularity, fragmented first polar body and zona pellucida abnormalities.
Conclusions Oocyte morphology, observed prior to vitrification, does not predict post-warming survival. The non-invasive identification of predictive markers for oocyte survival potential remains a difficult task.

Keywords Egg-cryobanking · Oocyte dysmorphism · Oocyte morphology · Oocyte quality · Oocyte vitrification

Capsule Oocyte morphology, observed prior to vitrification, does not predict post-warming survival.

A. S. Setti · D. P. d. A. F. Braga · A. Iaconelli Jr. · E. Borges Jr.
Sapientiae Institute – Educational and Research Centre in Assisted Reproduction,
Rua Vieira Maciel, 62,
04203-040 São Paulo, SP, Brazil

R. d. C. S. Figueira · D. P. d. A. F. Braga · R. C. Ferreira · A. Iaconelli Jr. · E. Borges Jr. (✉)
Fertility – Assisted Fertilisation Centre,
Av. Brigadeiro Luis Antonio, 4545,
01401-002 São Paulo, SP, Brazil
e-mail: science@sapientiae.org.br

Introduction

Oocyte cryopreservation has been widely used in assisted reproductive technologies (ART) as a main strategy for fertility preservation, oocyte donation programmes and storage of supernumerary oocytes for women requiring repeated in vitro fertilization (IVF) procedures [1].

Several strategies are available for oocyte cryopreservation. Slow freezing was firstly introduced and is still being used worldwide. More recently, vitrification, which uses highly concentrated cryoprotectants and an extremely rapid cooling rate, has been the method of choice for oocyte cryopreservation [2].

During cryopreservation, oocytes are exposed to mechanical, thermal and chemical stresses, which can result in compromised cell function and death [3]. Oocyte cryopreservation has been associated with intracellular organelles and cytoskeleton injuries, such as alteration in mitochondrial function [4], hardening [5] and fracturing [6] of the zona pellucida, microtubules damage [7, 8], ultrastructural alterations in microdomains and meiotic spindle damage [9–12], which are presumably linked to the reduced developmental potential of cryopreserved oocytes [13]. However, protection of the meiotic spindle has been inferred by the addition of cryoprotectants [14] and by de novo spindle reconstruction following warming [15].

It is well known that the majority of the oocytes retrieved from stimulated cycles (60–70%) exhibit one or

more abnormal morphological characteristics [16]. In order to increase post-warming survival rates, some authors have omitted oocytes with poor morphology [17, 18]. Therefore, the potential effects, if any, of these dysmorphisms on post-warming oocyte reformation is not known. The present study aimed to evaluate whether oocyte dysmorphisms affect oocyte survival rates in an egg-cryobanking donation program using the Cryotop vitrification method.

Materials and methods

Study design

A total of 415 metaphase II (MII) oocytes obtained from 54 patients undergoing intracytoplasmic sperm injection (ICSI) who decided to donate their surplus oocytes were vitrified using the Cryotop method. Oocyte morphology was assessed immediately prior to vitrification (2–3 h after retrieval) using an inverted Nikon Diaphot microscope (Eclipse TE 300, Nikon®, Tokyo, Japan) with a Hoffmann modulation contrast system under 400× magnification. The influence of dysmorphisms on survival rates after vitrification was assessed.

Controlled ovarian stimulation

Controlled ovarian stimulation was achieved by long pituitary down-regulation using a GnRH agonist (GnRH agonist, Lupron Kit, Abbott S.A., Sociète Française des Laboratoires, Paris). This step was followed by ovarian stimulation with recombinant FSH (Gonal-F, Serono, Geneva). Oocyte retrieval was performed using transvaginal ultrasound 35 h after the administration of recombinant hCG (rhCG; Ovidrel, Serono).

Preparation of oocytes

Retrieved oocytes were maintained in culture media (global® for fertilisation, LifeGlobal, Connecticut, USA) supplemented with 10% protein supplement (LGPS, LifeGlobal, Connecticut, USA) and covered with paraffin oil (Paraffin oil P.G., LifeGlobal, Connecticut, USA) for 2 to 3 h prior to cumulus cell removal. Surrounding cumulus cells were removed after exposure to a HEPES buffered medium containing hyaluronidase (80 IU/mL, LifeGlobal, Connecticut, USA). The remaining cumulus cells were then mechanically removed by gently pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, USA).

Denuded oocytes were assessed for nuclear status, and those observed to have released the first polar body were considered mature. The following intracytoplasmic morphological abnormalities were recorded: (a) increased cytoplasmic granularity (homogeneous), (b) vacuoles in the ooplasm and (c) aggregates of smooth endoplasmic reticulum in the ooplasm. The following extracytoplasmic morphological abnormalities were also recorded: (d) large perivitelline space size (e) perivitelline space granularity, (f) fragmented first polar body and (g) zona pellucida abnormalities.

Vitrification/warming procedure

The Cryotop method for oocyte vitrification was used as previously described by Kuwayama et al. [19]. Oocytes were equilibrated in 7.5% (v/v) ethylene glycol (EG) + 7.5% dimethylsulfoxide (DMSO) in TCM199 medium + 20% synthetic serum substitute (SSS) referred to as equilibrium solution-ES at room temperature for 15 min. This prepares the oocytes to be exposed to the vitrification solution. They were then placed into “vitrification solution-VS” that was the same as “ES” except that the concentrations were 15% ethylene glycol + 15% DMSO + 0.5 M sucrose. After 1 min in this solution, oocytes were aspirated into the pipette and deposited onto the end of the cryotop. Excess vitrification solution was then aspirated and the cryotop was immediately plunged into liquid nitrogen. For warming, the Cryotop was taken out of liquid nitrogen and instantly placed in 1.0 M sucrose in TCM199 + 20% SSS at 37°C. After 1 min oocytes were placed in 0.5 M sucrose in TCM199 + 20% SSS at room temperature for 3 min. Finally, two 5-minute washes were performed with TCM199 + 20% SSS at room temperature before incubating the oocytes in HTF media for 2 h before ICSI.

Intracytoplasmic sperm injection

Two hours after warming, ICSI was performed in a microinjection dish that was prepared with 4 μL droplets of buffered medium (Global® w/HEPES, LifeGlobal, Connecticut, USA) and covered with paraffin oil on an inverted microscope stage heated to 37.0±0.5°C. Approximately 16 h after ICSI, fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Embryos were kept in a 50 μL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10% protein supplement, covered with paraffin oil and stored in a humidified atmosphere under 6% CO2 at 37°C until transfer on day 3 of development.

Good quality embryos were defined as those having all of the following characteristics: 7 to 10 cells, less than 15% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation that has no inclusions, absence of perivitelline space granularity and absence of zona pellucida dysmorphism.

Statistical analysis

Linear regression models were used to assess the influence of oocyte dysmorphism's incidence on post thaw survival, fertilisation, embryo quality and implantation rates. To evaluate the predictive value of oocyte dysmorphisms on pregnancy and embryo transfer rates binary logistic models were used. Data from the linear regressions are presented as the regression coefficient (RC), R^2 and p values. The results of the logistic regression are presented as the odds ratio (OR), 95% confidence interval (CI) and p values.

The results were considered to be significant at the 5% critical level ($p<0.05$). Data analysis was performed using the Minitab (version 14) Statistical Program.

Results

The general characteristics of the donor cycles and ICSI outcomes are shown in Table 1. Of the 415 vitrified oocytes, 358 (86.3%) survived. The distribution of oocyte morphological abnormalities is shown in Table 2. Oocyte survival rate was not affected by the presence of the analysed oocyte abnormalities (Table 2).

Neither the incidence of intracytoplasmic nor the incidence of extracytoplasmic morphological abnormalities influenced oocyte post-thaw survival rate (RC: 0.1322, R^2: 2.6%, $P=0.597$ and RC: 0.2168, R^2: 1.8%, $P=0.788$, respectively). The total number of oocyte dysmorphisms within a cohort did not influence oocyte post-thaw survival rate (RC: 0.3318, R^2: 0.9%, $P=0.487$). Furthermore, oocyte survival rate was not affected by donor age (RC: −0.122, R^2: 2.5%, $P=0.254$), oestradiol level on the day of hCG administration (RC: −0.0005, R^2: 0.5%, $P=0.630$) or total dose of FSH administered (RC: 0.013, R^2: 5.8%, $P=0.079$).

Table 1 General characteristics of donor cycles and ICSI outcomes

Variable	Value
Female age	29.4±3.8
Dose of FSH administered (IU)	1250.5±415.3
Number of follicles	32.4±13.5
Number of retrieved oocytes	23.1±10.2
MII oocyte (%)	80.1
Fertilisation (%)	79.0
Good quality embryos (%)	56.1
Number of embryos transferred	2.0±0.5
Pregnancy rate (%)	20/50 (40%)
Implantation (%)	26/105 (24.8%)
Miscarriage (%)	2/20 (10%)

IU international units

None of the analysed oocyte dysmorphisms influenced ICSI outcomes (Table 3).

Discussion

Oocyte vitrification allowed the establishment of egg banks for donation. The main concern of egg banking is differential oocyte survival rates, although they are usually over 90% [20]. In this study, we aimed to predict survival rates by analysing the morphology of the donor oocyte prior to vitrification. We have failed to demonstrate any association between oocyte morphological dysmorphisms and oocyte post-thaw survival rate.

A crucial prerequisite for a successful egg cryobanking program is having efficient oocyte freezing/thawing technology. Furthermore, oocytes must overcome all of the effects of the physicochemical changes that occur during equilibrium and vitrification, successfully withstand the storage period and finally return to physiological temperatures. There are several factors that influence this process.

The human MII oocyte reveals particular susceptibility to freeze–thaw damage affecting mainly the meiotic spindle [21], the microfilaments [22], the zona pellucida and the cortical granules [21, 22]. It has been suggested that cryoinjury of the cytoskeletal components is responsible for the relative lack of success in preserving human oocytes [23].

Balaban and Urman [24] summarized the data in the literature that studied the effect of oocyte morphology on in vitro fertilisation (IVF). The authors suggested the extracytoplasmic dysmorphisms should be considered only a phenotypic deviation resulting from the heterogeneity of the oocytes retrieved and the severe cytoplasmic dysmorphisms, such as aggregates of smooth endoplasmic reticulum (SER), centrally severe granulation and excessive vacuolization, should be considered as abnormal and taken into consideration for the selection of a viable oocyte.

Because SER and excessive vacuolization are rarely observed dysmorphisms (in the present study they were observed in 1.9% and 4.4% of oocytes, respectively), it is difficult to evaluate their impact on post-warming oocyte survival and/or development. Despite the fact that a higher proportion of oocytes presenting cytoplasmic granulation were identified, we have failed to demonstrate any correlation between the dysmorphism and post-warming survival rates.

Rienzi et al. [25], in a systematic review of the literature, concluded that their analysis did not entirely support the average opinion about the features of "good" and "bad" quality and respective developmental competence. Nonetheless, Otsuki et al. [26] demonstrated that the presence of SER is associated with lower chances of successful pregnancy and even if normal oocytes from the same

Table 2 Distribution of oocyte morphology and influence of oocyte morphological abnormalities on post-thaw survival rates

Oocyte Morphology	Number of oocytes (%)	RC	R^2 (%)	P value
Normal	122 (29.4)	0.5049	2.2	0.2467
Increased cytoplasmic granularity	96 (23.1)	0.4193	1.6	0.114
Vacuoles in the ooplasm	18 (4.4)	0.5549	2.3	0.276
Aggregates of smooth endoplasmic reticulum	8 (1.9)	−0.089	0.5	0.597
Large perivitelline space size	22 (5.3)	0.0160	0.1	0.919
Perivitelline space granularity	50 (12.0)	0.1526	5.0	0.104
Fragmented first polar body	71 (17.1)	−0.094	1.3	0.403
Zona pellucida abnormalities	12 (2.9)	0.2255	3.5	0.177
Increased cytoplasmic granularity and perivitelline space granularity	16 (3.9)	0.5482	0.9	0.304

RC Regression coefficients

cohort are transferred, pregnancy rates are compromised. Kahraman et al. [27] showed that, despite normal pregnancy rates were obtained using oocytes with centrally located granular cytoplasm, more than 50% of pregnancies resulted in abortions and implantation rate was as low as 5%.

Ebner et al. [28] stated that some major abnormalities of the oocyte are of prognostic value in terms of further development, such as fertilization (meiotic spindle absence and vacuolization), cleavage (meiotic spindle absence, fragmented PB), development to blastocyst stage (fragmented PB, vacuolization and cytoplasmic viscosity) and implantation (SER and ZP defects).

The alpha scientists in reproductive medicine and ESHRE special interest group of embryology concluded that a note of both the perivitelline space and the size of the polar body should only be made if they are exceptionally large. Moreover, the observation of large vacuoles (>14 μm in diameter) should be noted. It was strongly recommended that oocytes presenting SER disks should not be inseminated [29].

Finally, in a recent meta-analysis, Setti et al. [30] demonstrated that the presence of large PB, large PVS, refractile bodies or vacuoles is associated with decreased oocyte fertilisation. Therefore, we hypothesised that morphologically abnormal oocytes could be more affected by the vitrification procedure than normal oocytes. We expected to observe a negative influence of oocyte morphological abnormalities on survival rates post-vitrification and ICSI outcomes. However, our results did not support our hypothesis.

In a similar study, Maldonado et al. [31] analysed the association of dysmorphism with post-thaw survival in 336 oocytes that were vitrified and warmed using the Cryotop method after IVF/ICSI fertilisation failure. Oocytes were divided into six groups based on the following type of dysmorphisms that they contained: vacuolated cytoplasm, smooth endoplasmic reticulum cluster, large perivitelline space, granulated cytoplasm, amorphous cytoplasm and normal morphology. The results demonstrated that the post-thaw survival rate was comparable in all groups. The authors suggested that oocyte morphology is not a limiting factor in predicting the survival following minimal volume vitrification by the Cryotop method.

Oocyte morphology does not predict post-warming survival rates. Moreover, ICSI outcomes such as fertilisa-

Table 3 Influence of oocyte morphology on ICSI outcomes

Oocyte Morphology	Fertilization (RC)	Embryo quality (RC)	Implantation (RC)	Pregnancy (OR – (CI))	Embryo transfer (OR (CI))	P value
Normal	0,6544	0,4511	0,1245	1,02 (0,98–1,11)	1,22 (0,92–1,41)	NS
Increased cytoplasmic granularity	0,0693	0,1787	−0,2752	0,99 (0,96–1,02)	1,01 (0,97–1,06)	NS
Vacuoles in the ooplasm	0,0772	−0,0067	−0,6452	0,95 (0,84–1,07)	1,07 (0,91–1,11)	NS
Aggregates of smooth endoplasmic reticulum	0,0145	−0,2701	0,4288	1,01 (0,98–1,05)	0,98 (0,94–1,02)	NS
Large perivitelline space size	0,2127	−0,0178	−0,0904	0,98 (0,95–1,02)	1,00 (0,94–1,08)	NS
Perivitelline space granularity	−0,0951	−0,1206	−0,0403	0,99 (0,97–1,01)	1,00 (0,96–1,04)	NS
Fragmented first polar body	0,1184	0,0090	−0,0694	1,00 (0,98–1,02)	1,00 (0,96–1,04)	NS
Zona pellucida abnormalities	0,1372	0,2442	0,0247	1,00 (0,97–1,03)	0,98 (0,93–1,04)	NS

Regression coefficients; *OD* Odds ratio; *CI* Confidence intervals; *NS* not significant

tion, embryo quality, implantation and pregnancy were not associated with oocyte morphology. Currently, there is no way to identify characteristics of either oocyte donors or stimulation cycles that predict low oocyte survival after vitrifying and warming. The non-invasive identification of predictive markers for oocyte survival potential remains a difficult task.

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