EMBJ.ORG FOR YOUNG DOCTORS

EUROMEDITERRANEAN BIOMEDICAL JOURNAL
2018,13 (25) 109-113
(FORMERLY: CAPSULA EBURNEA)

Original article

POOR-QUALITY FRESH AND FROZEN-THAWED HUMAN CLEAVAGE EMBRYOS MAY HAVE A CLINICAL POTENTIAL AFTER AN EXTENDED CULTURE

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ARTICLE INFO

Article history:
Received 10 September 2018
Revised 27 September 2018
Accepted 12 October 2018

Keywords:
Embryo cryopreservation, Embryo morphology, viability rate, poor quality embryos, ZP birefringence.

ABSTRACT

In many clinics for Assisted Reproductive Technology (ART) cleavage stage embryos are discarded prior to subsequent transfer (ET) and/or cryopreservation for a low morphological score.
The aim of this study was to assess the morphology and viability of fresh and frozen-thawed human cleavage embryos, discarded at the 2nd day of *in vitro* culture and cultured for further 3 days.
Discarded embryos (123) from 57 infertile patients were cultured for 3 days. Their morphology was daily examined. Zona pellucida (ZP) birefringence, assessed by Polscope, and cell viability, by Hoechst/propidium iodide staining, were evaluated at the 5th day.
Top-quality expanded blastocysts successfully developed by poor-quality embryos from both fresh and frozen-thawed embryos. However, a normal ZP birefringence was significantly higher in fresh than in frozen-thawed embryos. Discarded embryos were able to grow up to 5 days, with aviability rate of 60.9%. In addition, the viability rate in fresh embryos was significantly higher than frozen-thawed ones.
In conclusion, poor-quality embryos were capable to develop to the blastocyst stage following an extended culture *in vitro*, thus representing an alternative choice for clinical applications.

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1. Introduction

An accurate selection of human embryos with high implantation potential is essential to improve the success of assisted reproductive technology (ART) programs [1]. In most of ART clinics, the embryo transfer (ET) or cryopreservation is carried out on the second or third day after the oocyte retrieval. The selection of the generated cleavage embryosis mainly based on the application of morphological criteria. The gold standard to evaluate the ultrastructure of oocytes and embryos is the transmission electron microscopy, allowing the analysis of form, distribution and functional status of intracellular elements and cell-to-cell communications [2-7]. However, electron microscopy does not allow to reuse the observed sample and, therefore, is routinely substituted by stereo microscopy, a non invasive, time-saving and cost-effective method [8]. The best quality embryos at the cleavage stage are considered those characterized by a symmetric division, a low percentage of fragmentation (below 10%), even and equal sized blastomeres, a mononuclear status, and a homogeneous and transparent cytoplasm [9].

The ability to store embryos in an out-of-body environment is a matter of great interest for scientists. In fact, embryo cryobiology has greatly progressed over the past two decades, maintaining the reproductive capacity of the infertile couples. Benefits of embryocryopreservation are represented by the possibility to postpone the transfer in the receiving patients when appropriate or to activate the endogenous survival and repair responses [10]. Even if several studies have shown that vitrification technique causes structural and functional changes in the cell organelles, leading to a decrease in the quality of oocytes and development of the embryos [11-14], it was recently demonstrated that poor quality cleavage embryos can be cultured *in vitro* to the blastocyst stage and then transferred after vitrification and warming. These embryos may be capable of implantation and successful delivery of healthy babies [15]. A recent study showed also that low-grade day-3 embryos, after having attained eligibility for fresh transfer or cryopreservation, successfully resulted in viable blastocysts leading to pregnancy [16].
In general, both fresh and frozen-thawed embryos at cleavage stage that do not reach standard inclusive criteria at the morphological assessment

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DOI: 10.3269/1970-5492.2018.13.25
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are discarded due to low survival capability. However, observations by time-lapse imaging of blastocysts with poor conventional morphological score and/or suboptimal morphokinetics, showed that can be successfully transferred and might result in live births[17]. Thus, if low-growth and inappropriate-quality embryos can be improved by *in vitro* culture conditions, the efficiency rate and consequently the chance of pregnancy may increase [18-20]. Therefore, the aim of current study was to examine the morphology, zona pellucida (ZP) birefringence and survival capability of the fresh and frozen-thawed discarded human embryos after an extended culture *in vitro* of three days in appropriate culture condition.

2. Material and methods

Samples

A total of 123 discarded embryos (73 fresh and 50 frozen-thawed) at cleavage stage, considered unsuitable for ET due to low morphological score, were included in this study from January 2018 to July 2018. These embryos were related to 57 patients aged 20 to 40 who were referred for ART cycles. All patients signed the informed consent forms. The work was approved by ethics committee of the Institute for Reproductive Sciences, Yazd, Iran.

Ovarian hyperstimulation protocol

The ovarian hyperstimulation was performed using the GnRH antagonist protocols. Briefly, 150-225 IU/day of FSH (Gonal F, Serono, Geneva, Switzerland) was administered on the second day of the menstrual cycle. When one follicle reached 12mm, GnRH antagonist (Cetrotide, Merck Serono, Darmstadt, Germany) was initiated and continued until reaching 17-18mm. Recombinant hCG (Ovitrelle, Merck Serono, Germany) was, then, administered to trigger final maturation. Approximately 36h later, the oocytes retrieval was performed by transvaginal ultrasonography guide aspiration.

Warming of embryos

Vitrified embryos [1, 12, 14] were thawed and then cultured *in vitro* for three days. To this aim, the Rapid Warm TM Cleave Kit (Vitrolife, Sweden) was used. Shortly, all the solutions were preventively put into incubator to reach 37°C. To do this, the cryotubes were removed from the liquid nitrogen, and vitrified embryos were then placed on the cryotubes and directly transferred to Warm1TM Cleave for 10-30 sec. In the next stage, the embryos were left in Warm 2TMC for 2 min, into Warm 3TMCleave for 2 min and into Warm 4TMCfor 5 min. The embryos were washed several times in the culture medium G-1TMV5 (Vitrolife, Sweden) to be finally incubated in fresh medium at 6% CO_2, 5% O_2 at 37°C.

Assessment and culture of discarded embryos

The morphological score was assessed on the 2nd day according to the degree of fragmentation, number and evenness of the blastomeres. Poor quality fresh and frozen embryos with uneven blastomeres with large cytoplasmic granules and fragmentation higher than 50% (grades C and D)- not suitable for ET - were cultured until day 5. The medium G1TM Ver3 (Vitrolife, Sweden) was used for the development of the embryos up to the eight-cell stage and G2TM Ver3 (Vitrolife, Sweden) for the development from eight-cell to blastocyst stage. During this period, the embryos were observed daily with the Nickon TE 300 Invert microscope to evaluate morphological parameters of embryonic cleavage. The blastocyst grading was classified according to Gardner system. In this system, 6 categories are described for the size of blastocoel expansion, 3 categories for inner cell mass (ICM) and 3 categories for trophectoderm (TE) [21].

ZP birefringence examination

For ZP quality assessment, the embryos were placed individually in an equilibrated 5-mL droplet of buffered medium (GMopsV1; Vitrolife, Sweden) in a glass-bottomed culture dish (WillCo-Dish; Bellco Glass, USA). The embryos were immediately imaged under a TE300 (Nikon, Japan), equipped with an Octax polar AID polarized optical system X400 (Octax, Herbon, Germany), and homogeny of the inner layer of the ZP was done. The embryos were, then, classified as having high (normal) or low (abnormal) ZP birefringence [9].

Assessment of cell viability

After three days of culture, the embryos were examined for degree of degeneration of the blastomeres. Cell survival was determined by using Hoechst 33258 and propidium iodide staining. The embryos were first washed in PBS and then incubated in 20 µl of a freshly prepared and prewarmed staining solution of PI (P4127, Sigma-Aldrich; 300µg/mL) and Hoechst (H 33258, Sigma-Aldrich; 5 µg/mL) in PBS for 30 min. Then, they were washed twice in warm PBS to remove residual dyes. The embryos were then placed in small drops of glycerol. The microscope cover glass was placed on top of the embryos gently. The samples were observed with the BX51 fluorescence microscope (Olympus, Japan). Live cells with intact cell membranes showed a blue stain (Hoechst33258 positive) whilst late apoptotic, necrotic or death cells appeared as red (PI-positive) [22].

Statistical analysis

All data were estimated using Chi-Square, Mann-Whitney U and T-tests in SPSS version 24 software. P value less than 0.05 was considered statistically significant.

3. Results

Evaluation of ZP birefringence

The percentage of high ZP birefringence, evaluated by a uniform and high birefringence to Polscope, was higher in fresh (48%) than in frozen-thawed embryos (24%) ($P<0.05$), showing an irregular and/or low birefringence distribution (Figure 1).

Morphological evaluation

In both groups, after further 3 days of culture from the morphology evaluation, the blastocysts developed up to the expansion stage. There were no statistically differences in the top-quality embryo rates (grades A and B) (38.53% vs. 32%; $P>0.05$) between fresh and frozen-thawed embryos (Figure 2).

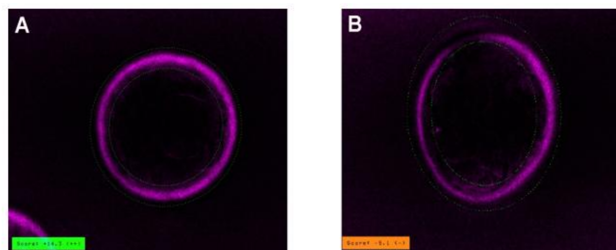

Figure 1 - Evaluation of ZP birefringence of embryo by Polscope. Representative picture of high ZP birefringence in a fresh embryo (positive score) (A) and low ZP birefringence in a frozen-thawed embryo (negative score) (B).

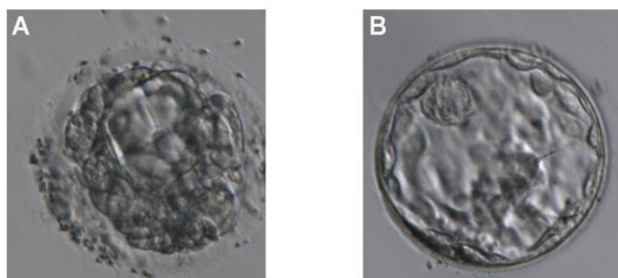

Figure 2 - Evaluation of embryo morphology on day 5. A: Blastocyst Grade C. B: Expanded blastocyst Grade A.

Assessment of cell viability

Overall, Hoechst 33258 and PI staining showed that the discarded embryos were able to grow *in vitro* until the 5th days, with a mean viability rate of 60.9%. However, the viability rate in fresh embryos was significantly higher than in frozen-thawed ones (70.12% vs. 51.81%; $P < 0.05$) (Figure 3).

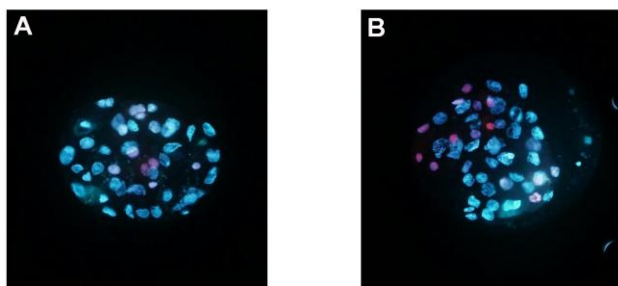

Figure 3 - Embryo viability by Hoechst and PI staining. The living cells are stained in blue and the dead cells in red. A: a representative fresh blastocyst. B: a representative frozen-thawed blastocyst.

4. Discussion

Our results showed that cleavage embryos, discarded according to standard morphological inclusion criteria, were able to develop to the blastocyst stage with a 3-days *in vitro* culture. Discarded embryos can, therefore, be used in cryopreservation program and/or ET. By comparing fresh and frozen-thawed embryos, our data showed that embryo degeneration in the fresh group was lower than in frozen-thawed one, probably due to cryodamages [11-14]. The morphology of embryos in the two groups did not differ significantly, but the mean number of viable cells and the percentage of normal ZP birefringence was higher in fresh than in frozen-thawed discarded embryos.

The most important criterium for the morphological evaluation of human embryos at the cleavage stage includes the number of blastomeres, the degree of fragmentation, the symmetry of the blastomeres, multi-nucleation and compaction [23, 24]. In the present study, there was no significant difference in the embryo morphology between the two analyzed groups, according to the mentioned parameters. This means that, even in presence of morphological alterations normally evaluated as exclusion parameters in ARTs, it is possible to produce viable blastocysts leading to pregnancy [17, 25]. Our data reinforce the concept that embryo morphology cannot be the only predictive value on pregnancy outcomes [26]. Evaluation of other parameters, such as the status of ZP and/or the viability rates, can provides additional useful information, as showed in this study.

Damage to the intracellular organelles and cytoplasm, alterations of intercellular processes, lysis and degradation of oocytes, cumulus cells, granulosa cells and blastomeres were harmful effects of vitrification [2-5, 11-14]. The quality of the embryos before cryopreservation, since connected to the protocols of hyperstimulation, cryopreservation and *in vitro* culture, can influence their morphology and, indeed, pregnancy outcomes [14, 18, 27, 28].

In this study, low quality fresh and frozen-thawed discarded embryos were compared with the aim to increase the efficiency of the embryo selection in ART programs. We did not find any significant differences in the morphology of embryos between the two analyzed groups, therefore embryonic cryopreservation seems to not affect the gross morphology of low-quality embryos. Consequently, frozen-thawed cleavage poor-quality embryos can be used for infertility treatment, if an appropriate *in vitro* culture is applied to monitor their development up to the blastocyst stage.

The survival rate of the blastomeres, mitotic resumption and the total number of blastomeres at the time of ET are the most important parameters for the selection of frozen-thawed embryos [29]. The phenomenon of apoptosis in the early developmental stages of the embryo leads to discarding the damaged cells. Apoptosis is a normal process in the pre-implantation stages, allowing the elimination of hampered cells, but high apoptotic rates can also be attributable to the altered embryo morphology [30]. Embryo freezing can cause cell damage, thus decreasing the survival rate of blastomeres. However, these damages seems not to have a great influence on the embryonic progression up to implantation [30].

5. Conclusion

Extending the culture *in vitro* of embryos up to the blastocyst stage is a feasible strategy in order to optimize the possible application of poor-quality embryos in ART setting, such as the cryopreservation for a subsequent ET usage. Evaluation of the chromosomal status by noninvasive methods on live cells can be done to determine the embryos' health.

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