ORIGINAL ARTICLE

Effect of vitrification on number of inner cell mass in mouse blastocysts in conventional straw, closed pulled straw, open pulled straw and cryoloop carriers

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Abstract

Objective: To compare the effect of using open and closed carriers on count of inner cell mass in vitrified mouse blastocyst after warming.

Methods: The experimental study was conducted at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, from April to September 2010. Forty female NMRI (Naval Medical Research Institute, USA) mice were injected with pregnant mare's serum gonadotropin and human chorionic gonadotropin in order to induce superovulation. Following the latter injection, two or three females were caged with the same-breed male mice. The presence of vaginal plug was examined the following morning. To collect blastocyst embryos, the pregnant females were sacrificed by cervical dislocation at 88-90 hours after the injection and dissected. Blastocysts were collected in phosphate-buffered saline and allocated to four groups: vitrification in conventional straw, closed pulled straw, open pulled straw and cryoloop. The vitrification solution was ethylene glycol, Ficol and sucrose (EFS) 20% and 40%. After storage for 1 month in liquid nitrogen, the blastocysts were thawed in 0.5 M sucrose then cultured in M16 medium. After 6 hours of culture, the number of expanded blastocysts was recorded and stained by double-dye technique. After staining, the number of total cell and inner cell mass was calculated.

Results: The re-expansion rate of blastocysts in the cryoloop group (n=90; 78.26%) was significantly higher ($p<0.05$) than open pulled straw (n=83; 69.16%), closed pulled straw (n=68; 54.83%) and conventional straws (n=63; 51.21%) groups. Significant differences ($p<0.05$) in the number of inner cell mass in blastocysts vitrified in open pulled straws, closed straws and cryoloop with blastocysts cryopreserved in conventional straws.

Conclusion: The re-expansion rate and total cell number of mouse blastocysts vitrified using open system had a better result compared with the closed system. The value of cryoloop and open pulled straws as carriers in vitrification of blastocysts may also merit more investigation.

Keywords: IVF, Cryoloop, Straws, Vitrification, Pregnancy, Blastocyst, Carrier. (JPMA 63: 486; 2013)

Introduction

Vitrification is a promising technology for blastocyst preservation following in vitro fertilisation. Progress in in vitro culture systems for human embryos has enhanced the likelihood of progressively greater blastocyst formation.[1] Embryos transferred at the blastocyst stage further enhance the implantation rate. This permits a reduction in the number of embryos needed for transfer, and tends to minimise the potential risk of multiple gestations.[2,3] During vitrification, the blastocyst is placed in a loading device surrounded by vitrification solution. The device is then placed into liquid nitrogen where it is stored. There are several loading devices available today: closed system like conventional straw and closed pulled straws; open system like open pulled straw and cryoloop. In the open system, the blastocysts come into direct contact with the liquid nitrogen and result in a cooling rate of over 20,000°C/min.[4,5] A study has shown that for an undisturbed embryonic development, a sufficient number of inner cell mass are essential.[6] According to our knowledge, no research has been done on using a variety of carriers on the number of inner cell mass and total cells of vitrified mouse blastocysts after thawing. The objective of the current study was to compare the effect of using different carrier systems on the count of inner cell mass and total cells in vitrified mouse blastocysts after warming.

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Materials and Methods

The study was in accordance with international guidelines and was approved by the Institute Research Ethics and Animal Care and Use Committee of Ahvaz Jundishapur University of Medical Sciences. Every effort was made to minimise the number of animals used and their suffering.

The experimental study was conducted at the Physiology Research Center of Ahvaz Jundishapur University of Medical Sciences (AJUMS) from April to September 2010. A total of 40 NMRI female mice 6 to 8 weeks old were administered intraperitoneal with 5IU pregnant mare serum gonadotropine (PMSG) for superovulation. This was followed 46-48 hours later by the intraperitoneal administration of 5IU

human chorionic gonadotropine (HCG). Following the HCG injection, two to three females were caged with NMRI male mice 10-12 weeks old. The presence of vaginal plug was examined the following morning. To collect blastocyst embryos, the pregnant females were killed by cervical dislocation at 88 and 90 h post-HCG injection. The blastocysts were recorded by flushing the excised uteri with phosphate-buffered saline (PBS) medium and randomly allocated to four groups: vitrification in conventional straws, closed pulled straws (CPS), open pulled straws (OPS) and cryoloop. Before vitrification, all the recovered blastocysts were pre-treated with modified phosphate-buffered saline (PB1) for 5 minutes.

In order to decrease the damage of osmotic and toxic stress, cryoprotectant must be added in a stepwise manner.[7] For this reason the vitrification solution used in this study included ethylene glycol, ficol and sucrose (EFS) 20% and EFS 40% containing 20% and 40% (v/v) EG in FS solution respectively.[8] The FS solution consisted of PB1 medium containing 0.42g.L^{-1} Ficoll and 243.8g. L^{-1} sucrose.

PB1 is a basic isotonic solution that contains phosphate-buffered saline supplemented with 0.33 mM sodium pyruvate, 5.56mM glucose, 3mg/ml bovine serum albumin (BSA), 50µg/ml penicillin and 75µg/ml streptomycin.[7] PB1was supplemented with 0.5M sucrose to be used as diluent.

The blastocysts were loaded in EFS20% for 2 minutes then in EFS40% for 1 min. By using syringe, the 0.25mL straw was filled with 1cm of vitrification medium, 0.5cm air, 2cm of vitrification medium containing blastocysts, 0.5cm of air and 3.5cm of vitrification medium. We sealed both ends of the straw with a plug. It was plunged into liquid nitrogen for cooling and storage for 1 month. For warming, the straw was taken out, held in the air for 30 second and then plunged into 30°C water for 40 seconds.[9] It was cut with scissors and the contents containing the blastocysts were expelled into 0.5 mol L^{-1} sucrose. It was then incubated in M16 medium+0.04 g L^{-1} BSA at 37°C in 5% carbon dioxide (CO_2) in humidified air.

Pulled straws were manufactured as described in literature[10] with some modifications. The plugs of 0.25 mL plastic straws were removed (Figure-1) and the straws were heat-softened at the midpoint over a hot plate and pulled manually. The pulled straws were cut at the tapered end with a razor blade. The thin part of each OPS was approximately 2.5cm long.

The blastocysts were loaded in EFS20% for 2 minutes then in EFS40% for 1 min. Subsequently the tip of the pulled straw was loaded with 2 mm of vitrification medium, 2mm of air, 2mm of vitrification medium containing blastocysts, 2mm of air and 2mm of vitrification medium using a syringe. The vitrification medium containing blastocysts was isolated by two small segments of air and medium. Through this closed loading system of CPS, the blastocysts had no direct contact with liquid nitrogen, which may occur with OPS. The procedures were performed at room temperature of 22-24°C. They were then plunged into liquid nitrogen for cooling and storage.[10] After storage for 1 month, the CPS was removed from liquid nitrogen for warming. The opposite end of the pulled straw was sealed by using the index finger. The content was then expelled into a drop of 0.5 mol L^{-1} sucrose by using the increase in air pressure in the tube caused by the thermal change, then cultured in M16 medium+0.04 g L^{-1} BSA under mineral oil at 37°C in 5% CO_2 in humidified air.

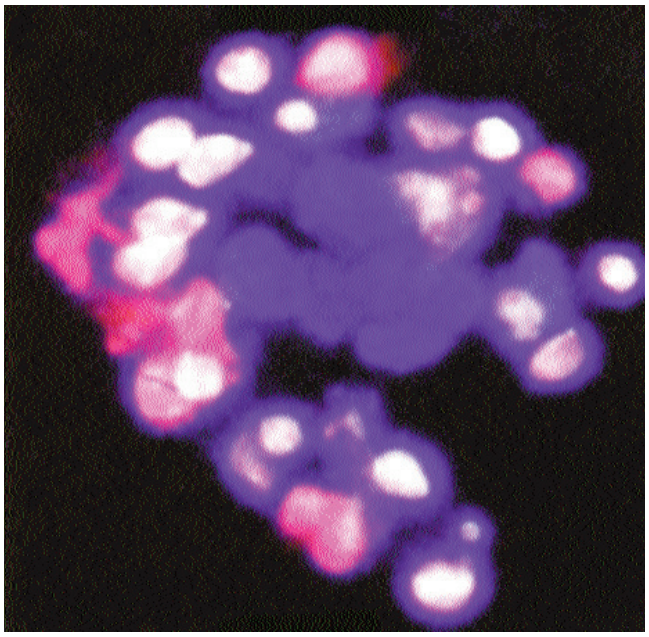

Figure-1: Double stainig of blastocyst (blue, ICM nuclei; pink, trophectoderm nuclei).

At first the blastocysts were exposed to EFS 20% for 2 minutes and to EFS40% for 1 min, then embryos were loaded into the narrow end of the pulled straws by using a syringe. Then the straw was immediately plunged into liquid nitrogen (LN2) for 1 month. At warming, the tip of OPS was put into 0.5 mol L^{-1} sucrose solution. The embryos were expelled from the straws into the medium. The recovered embryos were cultured as already mentioned.

The selected blastocysts for vitrification were exposed to EFS 20% for 2 minutes, and then were transferred to EFS 40% for 1 minute. The cryoloop was placed into EFS 40% in order to make a film. The cryoloop was transferred under stereomicroscope and then 2-3 blastocysts were transferred from EFS 40% to cryoloop by mouth pipette. The loaded cryoloop was plunged into LN2 and screwed on the cryovial

which already was immersed in LN2. After a storage time of 1 month, the cryoloop was removed from the LN2 and placed on top of the dilution medium and as soon as the cryoloop contents were liquefied and the loop was immersed in thawing medium, the recovered blastocysts were transferred to M16 medium+0.04 g L^{-1} BSA under mineral oil at 37°C in 5% CO_2 in humidified air.

After 6 h in the culture, the number of expanded blastocysts (with intact zona pellucida and refraction blastocoele) was recorded and were prepared for staining.

The double dye technique allowed a reliable and obvious distinction between the pink trophoectoderm nuclei and intact inner cell mass in which the nuclei appear blue.

The procedure of Thouas et al[11] was carried out for the differential staining of inner cell mass and trophectoderm cells. Briefly, the blastocysts were treated with a permeabilising solution containing the ionic detergent Triton X-100 and the fluorochrome propidium iodide (cat.NO.P-4170, Sigma). Blastocysts were then incubated in a second solution containing 100% ethanol (for fixation) and the secondary fluorochrome bisbenzimide (cat.NO.B-2261, Sigma). Fixed and stained whole blastocysts were squashed on a slide and observed under a Nikon fluorescence microscope with barrier filter of 410nm and an excitation filter of 365nm. Inner cell mass cells could be recognised by the blue fluorescence of the Hoechst staining, and trophectoderm cells by a red to pink fluorescence due to the accumulated propidium iodide. To enhance the accuracy of counting, all the stained blastocysts were counted by one, and each was repeated twice.

The re-expansion rate, number of inner cell mass, total cell and proportion of inner cell to total cell of blastocysts in the four groups were calculated. A contingency table analysis was performed with several rows and columns for overall difference prior to comparison between individual groups. If it was significant, t-test or one way analysis of variance (ANOVA) was carried out for comparisons. P<0.05 was considered statistically significant.

Results

A total of 497 blastocysts were obtained from 40 adult female mice with an average of 12.4 per mouse. Of the blastocysts, 123,126,128 and 120 were vitrified by using conventional straws, closed, pulled straws and cryoloop respectively (Table-1). The recovery rates of vitrified mouse blastocysts using conventional straws, CPS, OPS and cryoloop was 100%, 98.41%, 93.75% and 95.83% respectively. The re-expansion rate of open system was significantly higher than the closed system (p<0.05) .There was significant difference (p <0.01) between the cryoloop and open groups in term of re-expansion rate.

Table-1: The re-expansion rate of mouse blastocysts after vitrification using different straws.

Type of carrier variable	Vitrified blastocyst (n)	Thawed blastocyst (n)	Re-expansion Number	Re-expansion Percentage
Conventional straws	123	123 (100%)	63:123	51.21
Closed pulled straws	126	124 (98.4%)	68:124	54.83
Open pulled straws	128	120 (93.75%)	83:120	69.16*
Cryoloop	120	115 (95.83%)	90:115	78.26**,†

*P value <0.005. **Value <0.009: There were significant differences with closed system. †Value <0.01: There were significant differences with open pulled straws group.

Table-2: Total cell number and inner cell mass of stained vitrified blastocysts after warming.

Type of carrier variable	Blastocyste stained (n)	Total cells Number (±SD)	Total cells Range	Inner cell mass Number	Inner cell mass Range
Conventional straws	58	82.0±6.3	78-133	15.1±1.2	4-22
Closed pulled straws	60	89.3±5.7	75-112	23.1±1.8°	5-19
Open pulled straws	75	113.5±8.5*	98-145	25.4±2.1°	8-25
Cryoloop	78	123.1±6.4**†	99-149	27.3±2.6‡°	9-27

*P value <0.02. **Value <0.001 There were significant differences with closed system<0.05. ¤P<0.05: There were significant differences with conventional straw. †p=0.04: There were significant differences with open pulled straws group. ‡P=0.001: There were significant differences with closed pulled straws.

The average number of total and inner cell mass in blastocysts vitrified in conventional straws was 82.0±6.3 and 15.1±1.2; in CPS; it was 89.3±5.7 and 23.1±1.8; In OPS, 113.5±8.5 and 25.4±2.1; and in the cryoloop group, it was 123.1±6.4 and 27.3±2.6 (Table-2). The difference between open and closed systems in terms of total cell numbers was significant (p<0.05). Also, the total cell mass in cryoloop group was significantly different compared with the OPS (p=0.04). Significant difference (p<0.05) was also noticed in the average number of inner cell mass among CPS, OPS and cryoloop with the conventional straws group. There was significant difference between the cryoloop and CPS groups (p <0.001) as well.

Discussion

The experiments were carried out in two parts. In the first phase the effect of using open and closed carriers on re-expansion rate of vitrified mouse blastocyst after thawing was examined. It is clear from the results that the re-expansion rate of open systems (cryoloop and OPS) was significantly higher than the closed systems (conventional and CPS). The reduction in re-expansion rate of freeze-thawed blastocyst was probably due to high speed of cooling rate. In open system, two sides of the straw are open and the blastocysts with small amount of vitrification solution come into direct contact with the liquid nitrogen. In agreement with this suggestion, it has been shown that

the average cooling rate of the vitrification solution in the OPS was 16700°C.min^{-1} wherase those in conventional straw was 2550 C.min^{-1}.[10] Fracture of the zona pellucida at vitrification is a common phenomenon when embryos are rapidly cooled or warmed in straws.[8,12]

With the OPS, despite the increased cooling and warming speeds, fracture of zona is rare.[10] We also observed that the re-expansion rate increased in case of using cryoloop. The cryoloop, used as a vessel in vitrification, is a thin nylon loop used to suspend a film of cryoprotectant containing the embryos and directly immerse them in liquid nitrogen. Vitrification of embryos using the cryoloop has advantages over conventional vitrification procedure in that the cryoloop lacking any thermo-insulating layer coupled with the small volume of less than one micro-litre results in both rapid and uniform heat exchange during cooling. The cooling rate with the cryoloop has been reported to be close to 20000°C.min^{-1}.[13-15] High rates of cooling prevent chilling injury to sensitive cells.[16] The rapid cooling rate obtained with the cryoloop substantially reduces the exposure time to the cryoprotectants, thereby reducing their toxicity. The ultimate test for the viability of blastocyste after vitrification is the ability to establish and maintain a pregnancy, but in this study, embryo transfer due to some problems was not performed and, instead, another technique was used to evaluate the embryo development. It was well known that outgrowth of the inner cell mass has been correlated with subsequent foetal development.[16]

In the second phase of the study, the objective was to count inner cell mass and total cell number in embryos vitrified in open and closed carriers. Double-dye technique was used for purpose. This technique was successfully used in mice.[17] In agreement with previous studies, in our investigation the technique proved useful for investigating the presence of inner and outer cell mass of vitrified-thawed mouse blastocyst.

Conclusion

The average number of inner cell mass and proportion of inner cell mass to the total number of cells in blastocysts vitrified in OPS, CPS and cryoloop were significantly higher than in blastocysts cryopreserved in conventional straws. The conventional straw, as such, is not a suitable carrier for cryopreservation of blastocyst.

Acknowledgements

We are grateful to the Research Deputy of AJUMS for financial support, and to Miss Iqbal Zakerhosseini for editorial assistance.

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