Effect of laser-assisted hatching and necrotic blastomere removal on the development of vitrified–warmed four-cell mouse embryos

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Abstract

Purpose To investigate the effect of laser-assisted hatching and necrotic blastomere removal on the development of vitrified–warmed mouse embryos.

Methods The vitrified–warmed four-cell stage mouse embryos were divided into five groups; vitrified intact with no laser-assisted hatching, vitrified intact with laser-assisted hatching, vitrified damaged with neither laser assisted hatching nor necrotic blastomere removal, vitrified damaged with laser-assisted hatching, and vitrified damaged with necrotic blastomere removal. Thereafter blastocyst formation, blastomere and apoptotic cell number within all groups were statistically compared.

Results The rate of blastocyst formation showed a significant improvement in the group vitrified intact with laser-assisted hatching. However, neither laser-assisted hatching nor necrotic blastomere removal can improve a delayed vitrified–warmed damaged embryos in term of blastocyst formation and total cell number. Nevertheless, apoptotic cell number was significantly reduced after application of both techniques.

Conclusions Laser-assisted hatching can improve the development of vitrified–warmed intact four-cell stage mouse embryos, whereas necrotic blastomere removal has no significant effect on the development of vitrified–warmed four-cell stage damaged embryos.

Keywords Laser-assisted hatching ·
Necrotic blastomere removal · Vitrification

Introduction

Cryopreservation of embryos has become increasingly important in assisted reproductive techniques because it offers many potential advantages such as increasing the delivery rate per retrieval cycle, reducing the risk of multiple pregnancies, reducing the verity of ovarian hyperstimulation by delaying embryo transfer, avoiding the destruction of supernumerary embryos, and reducing treatment costs. During the past decade, many studies have been done on different cryoprotectants [1–3] and different cryopreservation techniques [3–5]. However, despite all of these efforts, cryopreservation of embryos still results in the hardening of zona pellucida, which affects the hatching and implantation of blastocyst. Several studies have reported the influence of such zona pellucida damage on in vitro and in vivo development [6–8]. Furthermore, some blastomeres of embryos can also be damaged during the freezing and thawing procedure. The necrotic blastomeres of damaged embryos, because of the release of toxic metabolites, can affect the development and viability of cryopreserved embryos [9–11].

To avoid the adverse effect of necrotic blastomeres, several investigators recommended the techniques of laser-assisted hatching (LAH) and necrotic blastomere removal

Capsule Laser-assisted hatching can improve the development of vitrified–warmed intact mouse embryos. Similar to necrotic blastomere removal, it has no significant effect on the development of vitrified–warmed damaged embryos.

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(NBR) after cryopreservation [12–14]. These investigators mainly focused on slow frozen–thawed embryos and reported better improvement of frozen–thawed embryos.

To our knowledge, there is still no any report on LAH and NBR after vitrification of mammalian embryos. Therefore, this study was set up to evaluate the effect of LAH and NBR on the development of intact and damaged vitrified–warmed four-cell mouse embryos.

Materials and methods

Embryos

Six-to-eight-week-old female Naval Medical Research Institute (NMRI) mice were provided from Razi Institute (Tehran, Iran) and induced to superovulate with an injection of 7.5 IU pregnant mares serum gonadotropin (PMSG; Intervet Inc, Boxmeer, Netherlands) followed by 7.5 IU human chorionic gonadotropin (HCG; Pregnil; Organon, Oss, Netherlands), given 48 h apart. The females were mated with the males from the same strain and inspected for the presence of a vaginal plug the following day. Those with vaginal plugs were considered pregnant and were sacrificed 44–48 h post-HCG by cervical dislocation. Two-cell embryos were flushed from the oviduct with T₆ medium (NaCl: 473, KCl: 11, KH₂PO₄: 50, CaCl₂·2H₂O: 300, MgSO₄·6H₂O: 10, NaHCO₃: 210, Na lactate: 200, Na pyruvate: 3, D-glucose: 100, penicillin G: 6, streptomycin: 5, phenol red: 1 and EDTA: 0.6 mg/100cc deionized water), supplemented with 4 mg/ml bovine serum albumin (BSA: Fraction V, Sigma). Morphologically normal embryos were washed and pooled in fresh G-1 (version 3; vitrolife, Kungsbacka, Sweden) with 10% human serum albumin (HSA, vitrolife, Kungsbacka, Sweden) and developed until the four-cell stage. At this stage of development, the normal embryos were cryopreserved by the vitrification method of closed pulled straws (CPS) as described by Chen et al. [15] with some modification.

Vitrification solutions and procedure

For purposes of vitrification, pretreatment and vitrification solutions were prepared using T₆ medium. The pretreatment solutions contained 1.5 M ethylene glycol (EG, Sigma), whereas the vitrification solution contained 5.5 M EG and 1.0 M sucrose (Sigma). The concentration of salts in the vitrification solution was kept constant during the experiment. The embryos were first pretreated with 1.5 M EG solution for 5 min, and then transferred to the vitrification solution at room temperature for 1.5 min. Then, the dehydrated embryos were loaded within the straw, which was prepared as follows: a 0.25 ml French

straw (IMV, L. Aigel, France) was first heat-softened over a hot plate before being pulled manually until the inner diameter and the wall thickness of the central part were 0.8 and 0.07 mm, respectively. The straw cooled in the air, cut at the narrowest point with a razor blade, and then filled through a tuberculin syringe with 2 mm of vitrification solution, 2 mm of air, 2 mm of vitrification solution containing six embryos, 2 mm of air, and 2 mm of vitrification solution. The loaded straw was immediately plunged into liquid nitrogen.

Warming solutions and procedure

After 1 week, the straws were transferred from liquid nitrogen to room temperature and touched with fingers for 5 s and the contents were transferred into a drop of 0.5 M sucrose in T6 medium supplemented with 20% HSA. The embryos were then diluted in stepwise sucrose solutions (0.5, 0.25, and 0.125 M sucrose; 2.5 min in each solution). After embryo warming, only the surviving embryos (retaining ≥50% of intact blastomeres) were included in this experiment. Fully intact embryos were randomly allocated into laser-assisted hatching group (I-LAH) and nonlaser-assisted hatching group (I-non LAH), whereas damaged embryos (retaining ≤50% of necrotic blastomeres) were randomly divided into three groups. The first and second groups were respectively treated with laser-assisted hatching (D-LAH) and necrotic blastomere removal (D-NBR) technique, and the third group was treated neither with laser nor necrotic blastomere removal (D) techniques.

Laser-assisted hatching procedure

The zona-assisted hatching was performed on a Nikon TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with Hoffman system at ×400 magnification and the Zona Infrared Laser Optical System (Hamilton-Thorne Research, Beverly, MA) utilizing a 1.48 μm infrared diode laser beam. Each embryo was placed on the stage of the microscope at room temperature in HEPES buffered Ham's F₁₀ medium (Gibco, Invitrogen, Life Technologies, Paisley, Scotland) and the laser aligned into the field. The embryo was positioned so that a portion of zona was in the path of the laser beam. The laser beam was then activated and fired several times with a duration of 0.5 ms to open a single hole in the zona (about 12–17 μm).

Necrotic blastomere removal procedure

The necrotic blastomere removal procedure was performed using the Nikon inverted microscope equipped with Hoffman system (at the same magnification as LAH procedure) and Eppendorf hydraulic micromanipulators (Eppendorf, Le Pecq,

Fig. 1 **A** Necrotic blastomere removal procedure. **B** Embryo with necrotic blastomeres extracted

France). Each embryo was first positioned with holding pipette and zona was drilled with laser beam as described above; then, with one blunted pipette about 12–17 μm , necrotic blastomeres were removed. Necrotic blastomere removal was attempted with continued refocusing and rotation of the embryo to avoid damaging the intact blastomeres and to provide access to all necroses (Fig. 1A,B).

Embryo development

After laser assisted hatching and necrotic blastomere removal procedure, all the groups of embryos were washed several times and kept in fresh G_1^{TM} ver3 until the eight-cell stage. Then, the embryos were transferred into G_2^{TM} ver3 droplets for further development. At the end of the experiment (day 5), the number of embryos in expanded blastocyst stages were evaluated in all groups. The quality of the blastocysts was determined using an inverted microscope (Olympus, Tokyo, Japan). Then, the expanded blastocysts were randomly selected for recording the number of blastomeres and incidence of cell death.

Number of blastomeres in blastocysts

The expanded blastocysts were first incubated in 500 μl of solution 1 (BSA-free human tubal fluid (HTF) medium with 1% Triton X-100 (Sigma) and 100 $\mu\text{g/ml}$ propidium iodide (Sigma) for up to 30 s. Blastocysts were then immediately transferred into 500 μl of solution 2 (fixative solution of 100% ethanol with 25 $\mu\text{g/ml}$ Bisbenzamide (Hoechst 33258, Calbiochem, San Diego, USA) and stored at 4°C overnight. Fixed and stained blastocysts were then transferred from solution 2 directly into glycerol (Sigma), taking care to avoid carryover of excessive amounts of solution 2. Blastocysts were then mounted onto a glass microscope slide in a drop of glycerol, gently flattened with a cover slip, and visualized for cell counting. Cell counting was performed on a fluorescence microscope (Olympus BX51) fitted with an ultraviolet lamp and excitation filters (380 and 420 nm for blue and red fluorescence, respectively) [16]. As a result, inner cell mass (ICM) and trophoectoderm (TE) stained blue and red, respectively (Fig. 2A).

Fig. 2 Vitrified-damaged embryos, **A** differential staining, **B** TUNEL staining, Bar 20 μm

Blastocyst TUNEL labeling

Nuclear DNA fragmentation in blastocysts was detected by the TUNEL method using an in situ cell death detection kit (Roche Diagnostics Corporation, Hvidver, Denmark) in the same manner as described previously with some modifications [17]. The blastocysts were removed from the culture medium and washed four times in PBS (pH 7.4) containing 3 mg/ml polyvinylpyrrolidone (PVP; Sigma). The blastocysts were then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature, washed twice in PBS–PVP and permeabilized in 0.1% Triton X-100 for 15 min. Thereafter, the blastocysts were washed in PBS–PVP and incubated with 50 µl of TUNEL reaction mixture (containing fluorescein isothiocyanate conjugated dUTP) and the enzyme terminal deoxynucleotidyl transferase (as prepared by the manufacturer) for 1 h at 37°C in the dark, and mounted in glycerol onto a slide under a cover slip. Finally, the blastocysts were observed under a fluorescent microscope (Nikon, TE 2000) (Fig. 2B), and apoptotic cells were assessed by the observation of a distinct TUNEL reaction of chromatin (bright yellow to green staining). The number of apoptotic cells within each blastocyst was determined.

Statistical analysis

The data from all groups were analyzed with statistical tests. At first, the Kolmogorov Smirnov test was performed for data normalization and then the differences between the percentages of blastocyst development were compared by chi-square analysis. The differences between the total blastomeres, inner cell mass, trophoctoderm, and apoptotic cells were also compared using Tukey HSD test after one-way analysis of variance. A *P* value of <0.05 was considered as significant. All the analyses were carried out using SPSS (Software program, Version 13; SPSS Inc., Chicago, IL, USA).

Results

In this investigation, to obtain enough damaged vitrified embryos, a total number of 2,589 were vitrified, from which 2,431 embryos with ≥50% intact blastomeres were considered as surviving embryos (93.89%); 158 embryos with more than 50% necrotic blastomeres were considered degenerated (6.10%). Of the surviving embryos, 1998 were fully intact (82.18%) and 433 showed ≤50% damaged blastomeres (17.81%). As the statistical analysis showed, out of 1998 intact embryos, only 305 were enough to be included in the experiment and the rest were used for other purposes in the laboratory. They were randomly divided

into I-non LAH (*n*=146) and I-LAH (*n*=159) and compared with all damaged embryos with ≤50% of necrotic blastomeres, which were also randomly divided into D (*n*=151), D-LAH (*n*=134) and D-NBR (*n*=148) groups.

When the intact embryos were drilled by a laser, blastocyst formation rate was significantly higher than those of non-drilled (Table 1). But LAH had no significant effect on blastocyst formation of damaged embryos. Moreover, the effect of NBR on the development of damaged embryos was similar to that of the LAH procedure and, compared with that of the D group, did not show any significant improvement.

LAH had no negative effect on total cell number, ICM, TE, and number of apoptotic cells of intact embryos (Table 2). In vitrified–warmed damaged embryos as compared with intact embryos, total cell number, ICM, and TE were significantly reduced (*P*<0.05), whereas the number of apoptotic cells was significantly increased (*P*<0.05). Neither LAH nor NBR could compensate for the cellularity reduction in damaged ones. But the incidence of cell death significantly decreased after both LAH and NBR as compared with the damaged ones that did not undergo the LAH and NBR procedures (*P*<0.01).

Discussion

Cryopreservation damage of embryos can be induced by several factors, including intracellular ice formation, solution effects, osmotic effects, or physical damage by growing ice crystals [18, 19]. Rall et al. [19] reported that embryos with damaged blastomeres and/or with a cracked zona pellucida were found after thawing. Reports have suggested that partial zona dissection or assisted hatching may be beneficial in improving pregnancy rates following the transfer of frozen–thawed embryos [6, 8], presumably based on the notion that cryopreservation had some effect on the zona pellucida. Tucker et al. [8], Check et al. [6], and

Table 1 Blastocysts derived from intact and damaged vitrified–warmed four-cell mouse embryos

Group	No. of four-cell embryos	Blastocyst formation (%)
I-non LAH	146	114 (78.00) a, d
I-LAH	159	150 (94.33) a, c
D	151	80 (52.98) b
D-LAH	134	81 (60.44) b
D-NBR	148	93 (62.83) b

Values within columns with different letters are significantly different (a and b), (c and d) (*P*<0.05)

I-non LAH Vitrified intact with no laser assisted hatching, *I-LAH* vitrified intact with laser assisted hatching, *D* vitrified damaged with neither laser assisted hatching nor necrotic blastomere removal, *D-LAH* vitrified damaged with laser assisted hatching, *D-NBR* vitrified damaged with necrotic blastomere removal

Table 2 Number of blastomeres and apoptotic cells of blastocysts derived from intact and damaged vitrified–warmed four-cell mouse embryos

Group	Total cells	ICM cells	TE cells	Apoptotic cells
I-non LAH	57.73±5.40a n:65	17.93±2a n:65	39.80±4.30a n:65	0.90±0.30b n: 49
I-LAH	52.27±2a n:80	13.93±0.80b n:80	38.33±1.90a n:80	0.80±0.13b n:70
D	27.70±2.95b n:40	8.77±0.65b n:40	19.15±2.45b n:40	4.40±0.65a n:40
D-LAH	24.42±2.3b n:41	7.49±0.75b n:41	17.88±2b n:41	1.54±0.47b n:40
D-NBR	22.37±2.75b n:50	6.94±1.10b n:50	17.06±2.45b n:50	0.72±0.14b n:43

Data are presented as means±SE. Values within columns with different letters are significantly different ($P<0.05$)

I-non LAH Vitrified intact with no laser assisted hatching, *I-LAH* vitrified intact with laser assisted hatching, *D* vitrified damaged with neither laser assisted hatching nor necrotic blastomere removal, *D-LAH* vitrified damaged with laser assisted hatching, *D-NBR* vitrified damaged with necrotic blastomere removal, *n* number of blastocyst, *ICM* inner cell mass, *TE* trophoectoderm

Tao and Tamis [20], using chemical AH, evaluated the effect of AH after freezing on the pregnancy potential of frozen–thawed embryos. Comparison between AH and non-AH groups revealed a possible positive effect on pregnancy and implantation rates. In a study, Gabrielsen et al. [21] also reported significant results after using LAH in cryopreservation cycles. Balaban et al. [22] showed that the performance of LAH significantly increased implantation (9.9% vs 20.1%) and clinical pregnancy (27.3% vs 40.9%) in embryo cryopreservation cycles. In a more recent study, Valojerdi et al. [23] also showed that performance of LAH significantly increased implantation (4.2% vs 12.8%) and clinical pregnancy (11.1% vs 31.2%) in slow frozen–thawed human embryos. Contrary to all these reports, Sifer et al. [24] indicated that the partial enzymatic digestion of ZP by pronase was not related to any benefit of the frozen–thawed embryo transfer outcome, especially concerning the implantation ability of frozen–thawed embryos (N-AH, 9.6% vs AH, 9.2%) and clinical pregnancy (N-AH, 18.0% vs AH, 17.2%). In a study, Ng et al. [25] found a negative effect of AH on frozen–thawed embryos. Matson et al. [26] also suggested that cryopreservation of embryos is not associated with zona hardening or reducing implantation, making microdissection of the zona in such cases generally unwarranted. However, the results of the present study showed that LAH had a beneficial effect on the blastocyst formation of vitrified–warmed intact embryos without a negative effect on their cellularity and cell death.

On the other hand, some blastomeres of embryos will be damaged in the procedure of embryo freezing and thawing. Several studies have reported that embryos, which have been damaged, are viable, provided at least half of the initial number of blastomeres remain intact [27, 28]. Furthermore, Hartshorne et al. [29] reported that damaged embryos have the same potential for implantation as that of fully intact embryos. However, more evidence of a toxic effect from the damaged blastomeres has been reported. Rulicke and Autenried [9] have reported that the *in vivo* development rate of damaged two-cell mouse cryopreserved embryo group was only 26% compared with 53% in

the intact group. Van den Abbeel et al. [10] reported that the implantation rate of fully intact embryos was significantly higher than those of damaged embryos after cryopreservation (11.4% vs 3.5%, respectively). The further cleavage of cryopreserved embryos was higher when there was 100% blastomere survival as compared with the case when some blastomeres were damaged (79.0% vs 43.7%, $P<0.001$) [11]. The present study also indicates that a damaged blastomere in the vitrified–warmed embryos has a negative influence on further development of the intact ones (D; 52.98 vs I-non LAH; 78.00%). On the basis of these observations and to resolve the deleterious effects of necrotic blastomeres on further development of cryopreserved embryos, microsurgical removal of the degenerating blastomeres was first described in 1993 by Alikani et al. [30]. These investigators and others [14, 30, 31, 32, 33] demonstrated improved viability and increased hatching rate following microsurgical removal of necrotic blastomeres in slow frozen–thawed mouse embryos. This method was also applied in slow frozen–thawed human embryos, and it was reported to be beneficial in terms of pregnancy and implantation rates [14, 30, 31, 32, 33]. In contrast to all of these reports, our finding indicates that blastocyst formation in damaged vitrified–warmed mouse embryos, even after NBR (D-NBR; 62.83% vs D; 52.98%), and a group of damaged embryos with laser hatching (D-LAH; 60.44% vs D; 52.98%) was not noticeably improved. This controversy can be related either to the method of cryopreservation, which was the CPS technique of vitrification in the present study, whereas it was slow freezing for the others. It can also be due to the stage of embryo at the time of cryopreservation, which was at the four-cell stage in the present study, whereas two- and eight-cell stages were used by the other investigators [30, 34], or it can even be due to species differences. However, we should accept that the damage to the embryo because of cryopreservation may also depend on the method of freezing, which is more detrimental for vitrification than slow freezing [35], and cannot even be compensated for by microsurgical removal of degenerating blastomeres or LAH.

Nevertheless, our data revealed that the incidence of cell death decreased after LAH and NBR in a group of damaged vitrified–warmed embryos. This reduction of cell death has a positive effect on embryo development and may explain partly the cause of negligible improvement of our damaged vitrified–warmed embryos in the groups of LAH and NBR, but as blastomere proliferation is another factor for embryo development and did not improve in our vitrified damaged group even after LAH and NBR we could not obtain better result as we expected to be. However, to find a better improvement of embryo development after vitrification, we believed that other techniques such as co-culture system after LAH or NBR may have more beneficial. The reduction of cell death in preimplantation embryos also may have a positive effect on implantation and pregnancy rates as claimed by Freean et al. [27] for human embryos. Thus, it appears that LAH and NBR, because of reduction of apoptotic cell death, could still become an effective strategy to improve implantation rate of vitrified–warmed damaged embryos. However, as NBR is more time consuming, the LAH may be more practicable in the ART lab.

In conclusion, the present investigation indicates that the LAH, without any negative effect on cellularity and cell death, can improve the development of vitrified–warmed intact embryos. In the case of blastomere damage after vitrification, both LAH and NBR techniques can reduce the incidence of cell death but have no significant effect on development and cell number.

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