

# Vitrification of human early cavitating and deflated expanded blastocysts: clinical outcome of 474 cycles

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## Abstract

**Purpose** The present study was undertaken to evaluate and compare the post thaw survival, implantation and pregnancy rates of vitrified human early cavitating blastocysts with deflated expanded blastocysts.

**Material and methods** Supernumerary blastocysts were vitrified in 30% ethylene glycol-dimethyl sulphoxide based solution using cryoloop. Fully expanded blastocysts were deflated by gentle aspiration of the blastocoelic fluid using a micromanipulator until the cavity collapses prior to vitrification.

**Results** Of the 576 vitrified blastocysts, 545 (94.61%) survived thawing in the early cavitating blastocyst group which was significantly higher than deflated expanded blastocyst group, in which only 370 survived thawing out of 459 (80.62%). However, no significant difference was observed in implantation and pregnancy rates between early cavitating and deflated expanded blastocyst groups.

**Conclusions** Early cavitating blastocyst would be the ideal stage for cryopreservation of human blastocysts as it has higher survival rate and avoids additional invasive procedures like deflation of the blastocoele.

**Keywords** Embryo stage · Early cavitating blastocyst · Vitrification · Cryoloop · Survival rate

## Introduction

Research during the last decade in the area of assisted reproduction has resulted in significant improvements in stimulation protocols and culture conditions resulting in better quality and number of blastocysts available for embryo transfer. Blastocysts are generally considered to be preimplantation embryos which have successfully passed the genomic activation and have better developmental potential [1]. In addition, physiologically the uterine environment on day 5 post oocyte retrieval is ideally suited for embryonic implantation, hence blastocyst transfer has become a better option to enhance the overall pregnancy rates [2]. Further, transferring the embryos at the blastocyst stage makes it feasible to reduce the number of embryos transferred per cycle while still attaining an acceptable pregnancy rates with low multiple pregnancies [2, 3]. This shift in policy by most of the IVF clinics worldwide to transfer fewer embryos to decrease multiple gestations resulted in plenty of untransferred embryos for cryopreservation. Hence the necessity of a robust cryopreservation method for freezing these surplus blastocysts has become a priority.

Cryopreservation of human blastocysts using the traditional slow freezing method was first reported in 1985 by Cohen et al. [4]. Later modifications by Menezo et al. resulted in a better clinical pregnancy rates using supernumerary co-cultured blastocysts [5]. Subsequent to these initial break throughs, the survival and pregnancy rates achieved using this protocol remained static and less than the rates achieved with fresh embryo transfers. In 1996, Martino et al. used electron microscope grids to vitrify bovine oocytes, which enabled very rapid cooling and warming rates [6]. Later in 1999, Vanderzwalmen et al. reported the first successful pregnancy using vitrified human blastocysts. However, these initial attempts of

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**Capsule** Survival during freeze thaw is stage specific and early cavitating blastocyst is the ideal stage for vitrification.

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blastocyst vitrification using cryostraws have not improved the existing pregnancy rates [7, 8]. Later modifications by various workers using different cryoprotectants and better carriers like cryoloop [9], electron microscope grids [10], cryotop [11] enabling faster heat transfer has improved the pregnancy rates. This ultrarapid approach significantly improved the survival and pregnancy rates of human blastocysts.

Vitrification is a relatively new procedure introduced for cryopreservation of human embryos. Extensive work has been done in the past few years to optimize the protocol for the best possible results. Several studies were done with modifications in cooling rates, concentrations and combination of cryoprotectants, and carriers used for vitrifying human embryos to achieve better results. However, very limited studies were undertaken to evaluate the ideal stage for vitrifying the embryos which could play a major role in both increasing survival and pregnancy rates. Few studies have stated that vitrification of blastocysts yield better results compared to earlier stage embryos [12, 13]. Considering these previous observations the present study was conducted on different stages of blastocysts to evaluate (i) the survival rates of vitrified early cavitating blastocysts (ii) compare the survival and pregnancy rates of the same with deflated expanded blastocysts.

## Material and methods

### Patients

Krishna IVF clinic is a private outpatient facility for infertility management with an ongoing IVF/ICSI programme. All the surplus embryos after transfer of fresh embryos are vitrified. In the present study the patients whose embryos were cryopreserved were randomized into two groups. Group I comprising of patients whose embryos were frozen at the early cavitating blastocysts stage and Group II whose embryos are frozen at the fully expanded blastocysts after deflating the cavity. The study was initiated in May 2007 and lasted up to March 2009. The vitrified embryos after thawing were transferred to patients who failed to conceive after the fresh embryo transfers during this period. A total of 474 patients underwent vitrified blastocysts transfer during this period. Informed consent was taken from all the patients and were explained about vitrification and assisted hatching (AH) procedures. The study was approved by Institutional Review Board (IRB), Krishna IVF Clinic.

### Stimulation protocol, embryo culture and grading

Down regulation was initiated using intramuscular injection of Decapeptide 3.75 mg (Ferring Pharmaceutical GmbH, Kiel, Germany) on day 21 of the cycle. Adequacy of down regulation was confirmed by measuring E2 (<50 pg/ml) and

LH levels (<1 ng/ml). Controlled ovarian stimulation was achieved using recombinant FSH (Recagon; Organon, Hyderabad, India) and the dose was adjusted on the basis of individual response by monitoring the follicular size and estradiol levels. Human chorionic gonadotropin at a dose of 10,000 IU was administered after two follicles of 18 mm diameter or more were visualized in the ultrasound scan. Oocyte retrieval was scheduled 36 h later by transvaginal ultrasonography (TVS)-guided aspiration. Oocytes were fertilized using either conventional IVF or ICSI and incubated in fertilization media (Sage Biopharma, USA). After 16–18 h oocytes were examined for fertilization. Oocytes with two pronuclei (2PN) and having a second polar body were considered as normally fertilized. The fertilized oocytes were washed twice and cultured in cleavage media (Sage Biopharma) for 48 h. On day 3, the morphology of the embryos was assessed and transferred to blastocyst media (Sage Biopharma) for further culture to the morula (day 4). The embryos were routinely transferred on the day 4 post oocyte retrieval. The surplus embryos on day 4 and day 5 were graded based on the morphological features outlined by Fiel et al. [14] and Gardner and Schoolcraft [15] respectively. The embryos were vitrified at the specific stage as per the groups assigned.

### Vitrification of early and deflated expanded blastocysts

The protocol for the vitrification used was a modified version of the cryoloop vitrification protocol reported by Mukida et al. [16]. Briefly, the cryoprotectant solutions are made with a base media consisting of MOPS and bicarbonate buffered media with human serum albumin (G-Gamete, Vitrolife, Sweden). The cryoprotectant solution A consists of 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in base media and cryoprotectant solution B consists of 15% EG, 15% DMSO, 1 mol/L sucrose, 10 mg/ml ficoll. Cryoprotectant solutions and media were warmed briefly in an incubator at 37°C. Both vitrification and warming procedures were performed on a heating stage maintained at 37°C. After rinsing in base media, the blastocysts were placed in solution A and incubated for 2 min. Embryos were then transferred to solution B for 30 s. Cryoloop was dipped into the same media to create a thin film of vitrification solution (3–5 µL) on the nylon loop. Two blastocysts (Fig. 1) were then immediately loaded onto the nylon loop and directly plunged into a cryovial containing liquid nitrogen. These cryovials were closed tightly and stored in liquid nitrogen tanks.

### Assisted hatching for expanded blastocysts

The expanded blastocysts in Group II patients were deflated prior to vitrification [15]. The blastocysts were placed in a



**Fig. 1** Early cavitating blastocysts before vitrification



**Fig. 2** Immediate post thaw survival of early cavitating blastocysts

petri dish containing 50  $\mu$ l of pre-equilibrated blastocyst media overlaid with mineral oil. Using a micromanipulator the blastocyst is held with the help of a holding pipette such that inner cell mass (ICM) is at 12 or 6 o'clock position, an injection needle was then pushed into the blastocoele cavity from 3 o'clock position and gentle aspiration was applied until the cavity collapsed. Immediately the blastocysts were vitrified as per the procedure described above.

#### Warming of embryos and assessment of survival

The blastocysts were warmed by passing them through sucrose solutions of decreasing osmolality to remove the cryoprotectant and rehydrate the embryos. Warming solutions and media were equilibrated in an incubator at 37°C. With the cryovial submerged in liquid nitrogen, the vial was opened and the loop containing the embryos was removed from the liquid nitrogen and plunged directly into 1.0 M sucrose solution for 2.5 min at 37°C. The embryos were then transferred through different concentrations of sucrose solution (i.e., 0.5 M, 0.25 M, and 0.125 M) for 2.5 min at each step at 37°C. After warming, the embryos are rinsed in blastocyst media and transferred into fresh blastocyst media for further culturing till transfer.

The blastocysts were examined under inverted microscope immediately and 3 h after warming. Survival was assessed based on re-expansion of blastocoele, integrity of inner cell mass and trophoctoderm cells (Figs. 2 and 3). Degeneration or arrest development was characterized by no signs of re-expansion and darkening of cytoplasm 3 h post thaw. The survived blastocysts were graded as per the criteria described by Gardner and Schoolcraft [15].

#### Blastocyst transfer and pregnancy confirmation

For vitrified-warmed blastocysts transfers, patients were subjected to a controlled cycle using GnRH analogue (Lupride; Sun Pharmaceuticals India Ltd., Vadodara, India) and estrogen replacement (Progynova; Cadila Healthcare Ltd., Ahmedabad, India) for the preparation of the endometrium. On an average, two blastocysts were transferred to the patient. Luteal support was given by progesterone vaginal suppositories at a dose of 600 mg in three divided doses per day for 2 weeks (Uterogestan; Laboratories Besins International, Paris, France). Serum hCG was measured after 2 weeks of blastocyst transfer for confirmation of pregnancy and a diagnosis of clinical pregnancy was made after visualization of fetal heart pulsation 4 weeks later by trans vaginal sonography (TVS).



**Fig. 3** Reexpansion of early cavitating blastocysts 3 h post thaw

## Statistical analysis

Retrospective data for the present study were obtained from the patient data base system (File maker pro; FileMaker Inc, Santa Clara, CA) from May 2007 to March 2009. The data were examined for any differences by student's *t*-test and  $\chi^2$  (Chi square) analysis. A *P* value of <0.05 is considered as statistically significant.

## Results

There was no significant difference in the mean maternal age, duration of infertility, number of failed transferred cycles, number of IVF and ICSI cycles between the two groups (Table 1). There was also no significant difference in mean number of oocytes retrieved, number of embryos achieved, mean number of embryos transferred and number of blastocysts frozen per cycle in either group (Table 2).

The survival, implantation and pregnancy rates of early and deflated expanded blastocysts vitrified by cryoloop are summarized in Tables 2 and 3. A total of 611 patients underwent vitrification of surplus embryos out of which embryos of 474 patients were thawed. Among the 474 thawed cycles, 281 cycles contained early cavitating blastocysts and 193 cycles contained deflated expanded blastocysts respectively. The survival rate in early and deflated expanded blastocyst groups after warming was 94.61% (545/576) and 80.62% (370/459) respectively. The mean number of embryos transferred per cycle was 1.93 in early blastocyst group and 1.91 in deflated expanded blastocyst group. Of the 545 transferred blastocysts, 23.11% (126/545) implanted 4 weeks after transfer in the early blastocyst group and 21.89% (81/370) in deflated

expanded blastocyst group. Of the 281 embryo transfers, 107 resulted in clinical pregnancy, the pregnancy rate per cycle being 38.07% in the early blastocyst group and 62 resulted in clinical pregnancy with a pregnancy rate of 32.12% in deflated expanded blastocyst group respectively.

From the pregnancies achieved the total number of deliveries, ongoing pregnancies and miscarriage rate in early blastocyst group was 68(24.19%), 14(4.98%) and 23 (8.18%), where as in the deflated expanded blastocyst group it was 40(20.72%), 05(2.59%) and 16(8.29%) respectively. Two patients were lost for follow up in early cavitating blastocyst group and one patient in deflated expanded blastocyst group. Statistically significant difference was observed in survival and degeneration rate between early and deflated expanded blastocyst groups (*P*>0.05). There was no significant difference observed in terms of implantation, pregnancy and miscarriage rates between early and deflated expanded blastocyst groups.

## Discussion

Traditionally most of the IVF laboratories transfer the embryos on day 3. But with a shift in policy of some IVF clinics to transfer lesser embryos and improvement of the embryo quality due to use of sequential media, more number of good quality blastocysts are available for cryopreservation. Hence the need for developing the right protocol for cryopreservation of these blastocysts and the appropriate stage to do so has become a prime concern for these clinics.

Several animal experimental studies were conducted to evaluate the effect of the stage of development on embryonic survival during cryopreservation. Studies on

**Table 1** Mean age, duration of infertility, number of failed transferred cycles, number of IVF and ICSI cycles and cause of infertility between the two groups

Variables	Early cavitating blastocyst group (n=364)	Deflated expanded blastocyst group (n=247)	<i>P</i> -Value
Age	32.14±4.72	31.67±4.65	NS
Duration of infertility	3.2±0.3	3.0±0.2	NS
Mean no of failed transferred cycles	1.5±0.4	1.4±0.5	NS
No of IVF	151 (42%)	89 (36%)	NS
No of ICSI	213 (58%)	158 (64%)	NS
<b>Cause of infertility</b>			
PCOD	68 (18.68%)	38 (15.38%)	NS
Endometriosis	23 (6.31%)	17 (6.89%)	NS
Tubal Factor	42 (11.50%)	27 (10.93%)	NS
Uterine Factor	15 (4.12%)	12 (4.86%)	NS
Male Factor	171(46.9%)	120 (48.5%)	NS
Combined male & female factor	45 (12.36%)	33 (13.36%)	NS

IVF In vitro fertilization, ICSI Intra cytoplasmic sperm injection, NS Not significant Values are expressed as mean ± SD

**Table 2** Embryo data of early cavitating and deflated expanded blastocyst groups

Parameters	Early cavitating blastocyst group	Deflated expanded blastocyst group
No. of oocytes retrieved per cycle	10.26±2.94	10.02±2.45
No. of embryos achieved per cycle	8.34±1.76	8.52±1.88
Mean no of embryos transferred	1.92±0.6	1.94±0.5
Total no of blastocysts frozen	1385	890
No. of total frozen cycles	364	247
No. of blastocyst frozen per cycle	3.80±0.7	3.62±0.8
No. of frozen cycles transferred	281	193
No. of blastocysts thawed	576	459
No. of blastocysts survived	545 (94.61%)	370 (80.61%)*
Mean no of frozen blastocysts transferred	1.93±0.3	1.91±0.5
No. of blastocysts degenerated	31 (5.38%)	89 (19.38%)*

Values are expressed as mean ± SD

\*  $P < 0.05$

cryopreservation of mouse embryos at different developmental stages by Massip et al. suggested that the post thaw survival rate was higher with early blastocysts among all the developmental stages and also concluded early blastocysts withstand osmotic changes better with improved survival during freeze thaw procedure [17]. This is further supported by experiments conducted on different developmental stages of equine blastocysts by Slade et al. with higher post thaw survival and pregnancy rates in early blastocysts compared to expanded blastocysts [18]. Later studies by Hochi et al. confirmed the influence of relative embryonic volumes on the survival of frozen thawed equine blastocysts. Blastocysts of different sizes <200 μm (early blastocyst), 200–300 μm (partially expanded blastocyst) and >300 μm (hatched blastocyst) were cryopreserved in straws by slow freezing method with an increase in overall viability and developmental ability after post thaw with early blastocyst compared to expanded and hatched blastocyst stages. It was also observed embryonic coats were less damaged after post thaw in early blastocysts than hatched blastocysts [19]. Further experiments conducted by Hochi et al. using equine blastocyst of different developmental stages by vitrification procedure also confirmed expanded blastocysts are more sensitive to vitrification than early blastocysts [20]. A recent

study by Zhou et al. on vitrification of mouse embryos of different developmental stages by open pulled straw suggested that early blastocyst stage is the most feasible stage for embryo cryopreservation [21]. All the above animal experimental studies revealed that survival during freeze thaw is stage specific and the early blastocyst stage yielded the best results with both slow freezing and vitrification procedures.

For any successful cryopreservation protocol of embryos, efficient intracellular permeation of cryoprotectant is a prerequisite to avoid the formation of ice crystals, which is detrimental to the embryo. Investigations on mammalian embryos revealed that every development stage has its own mechanism with respect to the permeation of cryoprotectants and the extent of dehydration when exposed to cryopreservation solutions [22–24]. The rate of permeation of cryoprotectant is more stage specific and increases as the development stage progresses [25]. In the cleavage stage embryos the movement of water through plasma membrane is by simple diffusion and requires more time for dehydration and equilibration with cryoprotectant. In the later stage embryos like morula and blastocysts the water/cryoprotectant permeability is by facilitated diffusion via channel process. This process enables rapid permeation resulting in less time

**Table 3** Clinical parameters of early cavitating and deflated expanded blastocysts in vitrified thaw cycles

Parameters	Early cavitating blastocyst group	Deflated expanded blastocyst group
Implantation rate of frozen blastocysts (%)	126 (23.11)	81 (21.89)
No. of clinical pregnancies (% per embryo transfer)	107 (38.07)	62 (32.12)
No. of deliveries (% per embryo transfer)	68 (24.19)	40 (20.72)
No. of miscarriages (% per embryo transfer)	23 (8.18)	16 (8.29)
No. of ongoing pregnancies (% per embryo transfer)	14 (4.98)	05 (2.59)
No. lost follow up (% per embryo transfer)	2	1

for equilibration with the cryoprotectants [26]. As exposure time to the high concentration of cryoprotectant solution is a crucial factor for the success of a vitrification protocol any process which decreases the equilibration time results in better survival of embryos. Hence, the ideal stage for embryo vitrification would be that stage which is more developmentally competent and which allows rapid equilibration of water/cryoprotectant system. The early blastocyst stage which has numerous small volume blastomeres with high nucleocytoplasmic ratio is an ideal stage for vitrification because of the shorter equilibration time needed. Further even loss of a few blastomeres during freezing has less impact on subsequent progression and implantation [27, 28]. However, with the cleavage stage embryos it is more difficult to assess their developmental competence, takes longer time to equilibrate and loss of even a few blastomeres after thawing has significant impact on subsequent implantation potential [29, 30].

The different maturation stages of blastocyst also have an impact on the success of vitrification. Early blastocyst with a small blastocoele cavity and no expansion in overall size structurally differs from the expanded blastocyst. The small volume of blastomeres forming the blastocysts in which water is intracellular allows rapid permeation of cryoprotectant and equilibration before freezing. In addition, small blastomeres withstand osmotic stress better during thawing procedure [27]. Whereas an expanded blastocyst is significantly larger than the early blastocyst and has an enlarged fluid filled cavity i.e. the blastocoele. Due to the presence of this large proportion of intra-blastocoele water which is extracellular equilibration of cryoprotectant takes longer time resulting in longer exposure to toxic cryoprotectants and higher chance of ice crystal formation. This may damage the blastomeres with decreased embryo viability. So an additional procedure of artificial reduction of blastocoele cavity using either needle or laser is advocated before vitrification to avoid this problem and enhance the post thaw survival rate which already reported in both animal and human studies [16, 31, 32]. However, to enable the procedure, instruments like a micromanipulator and technical expertise are required, which is not possible in some of the ART clinics where only IVF procedures are conducted.

Studies on different developmental stages of human embryos by Vanderzwalmen et al. suggested that the efficiency of vitrification was dependent on the stage of embryo development and post thaw survival was related to volume of the blastocoele [31]. Similar studies conducted by Mukida et al. on early blastocysts stage embryos in comparison with expanded blastocysts without deflation of blastocoele cavity concluded that early blastocysts would be the ideal stage for cryopreservation [16]. In the present study too, the post thaw survival rate of early blastocyst (94.61%) is higher than the deflated expanded

blastocyst (80.62%) which was significant. These results show a higher survival rate of early cavitating blastocysts compared to those of Vanderzwalmen et al, where a survival rate of 71.8% was achieved. This may be attributed to the changes in protocols with reference to changes in concentration and duration of exposure of embryos to the various cryoprotectants [31]. However, no significant difference was observed in implantation and pregnancy rates of survived early and expanded blastocysts after transfer to the recipients. This is an agreement with previous study by Mukida et al. who reported significant difference in survival rate between early and expanded blastocysts which are not deflated but no difference in implantation and pregnancy rates [16]. Based on the above factors, it is apparent that once the embryos survive the vitrification procedure either early or expanded blastocysts have similar implantation and developmental potential.

In conclusion, the present study is one of the few studies involving a large cohort of embryos confirming that early cavitating blastocysts would be the preferable stage for selection and cryopreservation of human embryos compared with the deflated expanded blastocysts. Vitrification of early cavitating blastocysts has a higher survival rates and is a simple procedure, avoiding additional invasive procedures like deflation of the blastocoele cavity needed for expanded blastocysts to achieve acceptable results.

## References

1. Menezo Y. Blastocyst freezing. *Eur J Obstet Gynecol Reprod Biol.* 2004;1:12–5.
2. Gardner DK, Vella P, Lane M, Wagley L, Schlenkar T, Schoolcraft WB. Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil Steril.* 1998;69:84–8.
3. Yoon HG, Yoon SH, Son WY, Kim JG, Im KS, Lim JH. Alternative embryo transfer on day 3 or day 5 for reducing the risk of multiple gestations. *J Assist Reprod Genet.* 2001;18(5):262–7.
4. Cohen J, Simons RF, Edwards RG, Fehilly CB, Fishel SB. Pregnancies following the frozen storage of expanding human blastocysts. *J In Vitro Fert Embryo Transf.* 1985;2(2):59–64.
5. Ménéz Y, Nicollet B, Herbaut N, André D. Freezing co cultured human blastocysts. *Fertil Steril.* 1992;58(5):977–80.
6. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod.* 1996;54(5):1059–69.
7. Vanderzwalmen P, Zech H, Prapas Y, Nijs M, Vandamme B, Segal-Bertin G, et al. Pregnancy and implantation rates after transfers of fresh and vitrified embryos on day 4 or 5. *J Assist Reprod Genet.* 1999;16:147.
8. Yokota Y, Yokota H, Yokota M, Sato S, Araki Y. Birth of a healthy baby following vitrification of human blastocysts. *Fertil Steril.* 2001;75:1027–9.
9. Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil Steril.* 2001;76(3):618–20.

10. Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. Successful birth after transfer of blastocysts derived from oocytes of unstimulated woman with regular menstrual cycle after IVM approach. *J Assist Reprod Genet.* 2002;19(11):541–3.
11. Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online.* 2005;11(5):608–14.
12. Veeck LL. Does the developmental stage at freezing impact on clinical results post thaw. *Reprod Biomed Online.* 2003;6(3):367–74.
13. Anderson AR, Weikert LM, Crain JL. Determining the most optimal stage for embryo cryopreservation. *Reprod Biomed Online.* 2003;8(2):207–11.
14. Feil D, Henshaw RC, Lane M. Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers. *Hum Reprod.* 2008;23(7):1505–10.
15. Gardner DK, Schoolcraft WB. In vitro culture of human blastocyst. In: Jansen R, Mortimer D, editors. *Towards reproductive certainty: Infertility and genetics beyond pp.* Camforth: Parthenon; 1988. p. 378–88.
16. Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, et al. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. *Hum Reprod.* 2003;18(2):384–91.
17. Massip A, Vanderzwalmen P, Leory F. Effect of stage of development on survival of mouse embryos frozen thawed rapidly. *Cryobiology.* 1984;21:574–7.
18. Slade NP, Takeda T, Squires EL, Elsdon RP, Seidel GE. A new procedure for the cryopreservation of equine embryos. *Theriogenology.* 1985;24(1):45–58.
19. Hochi S, Ogasawara M, Braun J, Oguri N. Influence of relative embryonic volumes during glycerol equilibration on the survival of frozen-thawed equine blastocysts. *J Reprod Dev.* 1994a;40:243–9.
20. Hochi S, Ogasawara M, Braun J, Oguri N. Pregnancies following transfer of equine embryos cryopreserved by vitrification. *Theriogenology.* 1994b;42:483–8.
21. Zhou G-B, Zhu S-E, Hou Y-P, Jin F, Yang Q-E, Quan G-B, et al. Vitrification of mouse embryos at various stages by open-pulled straw (OPS) method. *Anim Biotechnol.* 2005;16:153–63.
22. Vanderzwalmen P, Gaurois B, Ectors FJ, Massip A, Ectors F. Some factors affecting successful vitrification of mouse blastocysts. *Theriogenology.* 1988;30:1177–83.
23. Valdez C, Albas-Mazni O, Takahashi Y, Hishinuma M, Kanagawa H. Effects of equilibration time, precooling, and developmental stage on the survival of mouse embryos cryopreserved by vitrification. *Theriogenology.* 1990;33:627–36.
24. Zhu S, Kasai H, Ootoge T, Sakurai T, Machida T. Cryopreservation of expanded mouse blastocysts by vitrification in ethylene glycol based solution. *J Reprod Fertil.* 1993;98:139–45.
25. Mazur P, Rigopoulos N, Jakowski SC, Leibio SP. Preliminary estimates of the permeability of mouse ova and embryos to glycerol. *Biophysical Journal.* 1976;16:232a.
26. Verkman AS, van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, et al. Water transport across mammalian cell membranes. *Am J Physiol.* 1996;270:C12–30.
27. Tachikawa S, Otoi T, Kondo S, Machida T, Kasai M. Successful vitrification of bovine blastocysts, derived by in vitro maturation and fertilization. *Mol Reprod Dev.* 1993;34:266–71.
28. Menezo Y. Cryopreservation of IVF embryos: which stage? *Eur J of Obs & Gyn.* 2004;113S:S28–32.
29. Van Den Abbeel E, Van Steirteghem A. Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and in-vitro viability. *Hum Reprod.* 2000;15(2):373–8.
30. Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod.* 2000;15(1):175–9.
31. Vanderzwalmen P, Bertin G, Debauche Ch, Standaert V, van Rosendaal E, Vandervorst M, et al. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod.* 2002;17(3):744–51.
32. Chen SU, Lee TH, Lien YR, Tsai YY, Chang LJ, Yang YS. Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocysts, but pretreatment with cytoskeletal stabilizer did not increase blastocyst survival. *Fertil Steril.* 2005;84:1156–62.