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The Study of Developmental Capacity of Vitrified Mouse Blastocysts in Different Straws after Transfer to Mouse Pseudo Pregnant

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Abstract: Vitrification is the commonly used method for long-term storage of pre-implantation mammalian embryos. It is an essential part of assisted reproductive technologies. The re-expansion rate, pregnancy and birth rate of vitrified blastocysts using CPS were compared with OPS and Conventional Straw. Female NMRI mice were injected with Gonadotrophins in order induce them for super ovulation. At that time the mice were sacrified by cervical dislocation and dissected of mouse abdomen. The uterine horns were existed blastocysts were collected in PBS and randomly allocated to four groups: vitrification in CPS, conventional straw, OPS and untreated controls. The vitrification solution was EFS40%. After storage for 1 month in liquid nitrogen, the blastocysts were thawed in 0.5 M sucrose for in vitro culture in M16 medium. After 6 h of culture, the numbers of expanded blastocysts was recorded and ready for transfer to uterus of pseudo pregnant mouse. The re-expansion rate of the CPS group (72.1%) was significantly higher ($p<0.05$) than OPS (52.55) and C.S. (38.6%) groups. The pregnancy (70%) and birth rate (45%) of blastocysts in CPS were similar to those of fresh blastocysts (80% and 45.5%) and the pregnancy (10%) and birth rate (5.1%) in Conventional Straws lower than OPS (20 and 7.5%), but were not significantly different. Mouse blastocysts vitrified using CPS had a better result compared with OPS and Conventional Straw. The value of CPS for vitrification of blastocysts may also merit investigation.

Key words: Vitrification, blastocyst, pulled straw, mouse

INTRODUCTION

The goal of embryo cryopreservation is long term storage and reproducible high survival rates of embryos following warming, leading to the successful establishment of pregnancy and live offspring following embryo transfer (Moore and Bonilla, 2006). Two important parameters determine the success of all cryopreservation protocols: 1) rate of cells regaining equilibrium in response to cooling, 2) speed of freezing (Liebermann *et al*., 2003a). Cryoinjury can occur from formation of intracellular or extracellular ice crystals, chemical toxicity, osmotic injury and/or fracture damage (Kassai *et al*., 2002) and is dependent upon such things as size and shape of cells, membrane permeability, embryo quality and stage, species and embryo origin (*in vivo* derived or in vitro produced) (Vajta and Kuwayama, 2006). There are two main methods for cryopreserving embryos, conventional slow cooling/freezing and Vitrification (Moore and Bonilla, 2006).

Vitrification is a newer method that eliminates both intracellular and extracellular ice formation, producing instead a glass-like state. At sufficiently low temperatures, solutions become very viscous and solidification occurs without ice crystal formation. It does this through dehydration and the extreme elevation in viscosity caused by ultra rapid cooling rates, from 15,000 to 30,000°C min^{-1} (Liebermann *et al*., 2003b).The glass transition state is ~-130°C (Kassai and Mukaida, 2004), but varies depending upon components in the vitrification solution. Vitrification of solution has been known since 1984 (Kauzman, 1984), but was first used for preserving embryos from the mouse in 1985 (Rall and Fahy, 1983). Vitrification, an efficient approach to cryopreservation, has been applied in laboratories to mammalian embryos, the oocytes and embryos of domestic animals and the oocytes and embryos of humans (Yang *et al*., 2006).

A year later the first bovine embryos successfully vitrified were reported (Massip *et al*., 1986). Considerable efforts have been made since the mid 1980's developing simpler protocols and more stable and less toxic solutions for Vitrification (Moore and Bonilla, 2006). Vitrification as an ultrarapid cooling technique is simple, potentially faster and inexpensive (Tucker and Liebermann, 2003;

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Liebermann and Tucker, 2004). Improvements have been made by using toxic and more permeable chemicals, by using a combination of cryoprotectants to reduce toxicity and by using a stepwise approach to equilibration and increasing cooling and warming rates (Vajta and Kuwayama, 2006).

One of the main concerns is related to the toxicity of cryoprotectant that is required for vitrification and can be toxic at very low concentration. Therefore, the aim in any vitrification protocol is to increase the speed of temperature change while keeping the concentration of cryoprotectant as low as possible (Klug *et al*., 2001). Vajta demonstrated that with the Open Pulled Straw (OPS) method the cooling and warming rates can be increased (over 20,000°C min^{-1}) and the toxic and osmotic damage can be decreased (Vajta *et al*., 1998).

Chen *et al.* (2005) attempted to modify the loading of pulled straw into a closed system, called Closed Pulled Straw (CPS). CPS has the characteristics of OPS as a rapid thermal change method and of conventional straws being a non-contact mode.

The greatest advantages of vitrification have been seen in chill-sensitive cells such as blastocysts (Liebermann *et al*., 2003). The main characteristic of the blastocysts is its fluid-filled cavity, the blastocele. It seems that with increasing volume of the blastocelic cavity, the survival rate drops with vitrification. This is thought to be due to insufficient permeation of cryoprotectant into blastocelic cavity, such that residual water may promote ice crystallization during the vitrification process. Several articles report that survival rate in cryopreserved expanded blastocysts could be improved by artificial reduction of the blastocelic cavity (Vanderzwalmen *et al*., 2002, 2003; Son *et al*., 2003; Zech *et al*., 2005; Hiraoka *et al*., 2004). Blastocysts are easier to freeze for two main reasons: the nucleoplasmic ratio is higher and the higher cell number allows embryo recovery even if some cells have been destroyed during the freezing and thawing procedures (Menezo, 2004). Therefore, longer exposure times were favorable for blastocysts which may correspond with the longer amount of water content in the blastocoele cavity. Vitrification requires high levels of cryoprotectant before cryopreservation. A successful vitrification procedure requires optimization of cryoprotectant concentration and exposure time and exposure temperature (Campos-Chillon *et al*., 2006). Blastocysts culture and transfer has been used to increase the implantation rate in IVF treatments. It can reduce the number of embryos transferred and the risk of multiple pregnancies (Gardner *et al*., 1998). The success of frozen embryo transfer requires good synchronization between the age of the embryos and the age of the post-ovulatory uterus. Consequently, cryopreservation of excess blastocysts becomes an important subject. Both the conventional slow-freezing method and vitrification are applied for freezing blastocysts and need more studies to improve their results (Cho *et al*., 2002; Gardner *et al*., 2003; Mukaida *et al*., 2003). Blastocysts have widely been adopted for embryo production cryopreservation and transfer in farm animals, especially in the bovine and ovine. Because blastocysts have passed the critical step of genomic activation and have high developmental capacity (Hiraoka *et al*., 2004). Most effort to improve vitrification focused on media, cryoprotectants, equilibration times and dilution. Less attention has been paid to cooling and warming rates during freezing and thawing (Gayar and Holtz, 2001).

In the present study we decided to study the effects of different containers OPS, CPS and Conventional Straws on re-expansion rates, Pregnancy rates and proportions of live birth of vitrified mouse blastocysts.

MATERIALS AND METHODS

Embryo collection: Female NMRI mice 6-8 weeks of age were induced to superovulate by i.p. injection of 7.5 IU of PMSG followed by 7.5 IU of hCG 48 h later. The following morning (designated day 1 of pregnancy), the females were examined for mating as determined by the presence of vaginal plug. Mated females were euthanized by cervical dislocation at 88 to 90 h after Human chorionic gonadotropin (HCG) injection to collect blastocysts (Chatot *et al*., 1989). The blastocysts were recorded by flushing the excised uteri with PBS medium. The blastocysts were randomly allocated to four groups: vitrification in CPS, Conventional Straws, OPS and untreated controls.

Preparation of solution

Vitrification solution: The vitrification solution used in this study included EFS40% containing 40%(v/v) EG in FS solution, as described by Kassai (Kassai *et al*., 1990). The FS solution consisted of PBI medium containing 0.42 g L^{-1} Ficoll and 243.8 g L^{-1} sucrose.

Diluents: PBI was supplemented with 0.5 M Sucrose to be used as diluents.

Manufacture of the pulled straws: Pulled straws were manufactured as described by Vajta *et al.* (1998a). The plugs of 0.25 mL plastic straws (IMV, L'Aigle, France) were removed 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 inner diameter of the tip was 0.8 mm, with a wall thickness of 0.07 mm. The thin part of each OPS was approximately 2.5 cm long.

Vitrification of blastocysts in OPS: After exposure of blastocysts in EFS40% for 2 min, the embryos were loaded into the narrow end of the pulled straws through the capillary effect by simply touching a micro drop (1-2 μL) of vitrification solution containing blastocysts (Vajta *et al*., 1998a). Then the straw was immediately plunged into liquid nitrogen (LN2) for 1 month. Six embryos were loaded into each OPS.

At warming, the tip of OPS was put into 0.5 mol L^{-1} Sucrose solution. The embryos were expelled from the straws into medium. The recovered embryos were cultured in M16 medium+0.04 g L^{-1} BSA at 37°C in 5% CO_2 in humidified air.

Vitrification of blastocysts in CPS: The blastocysts were loaded in EFS40 for 2 min. Subsequently the tip of the pulled straw was loaded with 2 mm of vitrification medium, 2 mm of air, 2 mm of vitrification medium containing blastocysts, 2 mm of air and 2 mm 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 will not directly contact with liquid nitrogen, which may occur with OPS, the procedures were performed at a room temperature of 22-24°C. The total exposure to EFS (EG 40%) lasted for 2 min. They were then plunged into liquid nitrogen for cooling and storage (Vajta *et al*., 1998).

After storage for 1 month, the CPS was removed from liquid nitrogen for warming. The opposite end of the pulled straw was sealed using the index finger. The content was then expelled into a drop of 0.5 mol L^{-1} sucrose (400 μL) by using increase in air pressure in the tube caused by the thermal change, then incubated in M16 medium+0.04 g L^{-1} BSA at 37°C in 5% CO_2 in humidified air.

Vitrification of blastocysts in conventional straws: The blastocysts were loaded in EFS40 as above using as syringe, the 0.25 mL straw was filled with 1 cm of vitrification medium, 0.5 cm air, 2 cm of vitrification medium containing blastocysts, 0.5 cm of air and 3.5 cm 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 5 sec and then plunged into 37°C water for 10 sec. It was cut with scissors and the contents containing the blastocysts were expelled into 0.5 mol L^{-1} Sucrose (Cates, 1975). Then, it was incubated in M16 medium+0.04 g L^{-1} BSA at 37°C in 5% CO_2 in humidified air. After 6 h in the culture, the numbers of expanded blastocysts were recorded and ready for transfer to uterus of pseudo pregnant mouse.

Definition of morphological survival: Embryos with normal morphology were used for the transfer to pseudo pregnant mouse. The blastocysts were defined as having morphologically survived if the embryos possessed an intact zona pellucida and refraction blastocoels (Yang *et al*., 2006).

Statistical analysis: The re-expansion rates, pregnancy rates and proportions of live young of each group 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 ANOVA was then carried out for comparisons of groups two by two. $p<0.05$ was considered statistically significant.

RESULTS

The re-expansion rates of vitrified mouse blastocysts using CPS, OPS and conventional straws are shown in Table 1. The Re-expansion rate of the CPS group (72.1%) was significantly higher than those of the OPS (52.5%) and CS (38.6%) groups ($p<0.05$). There was no obvious difference between the OPS and CS groups ($p>0.05$). The pregnancy and live birth rate of vitrified blastocysts of three groups and controls after transfer to pseudo pregnant mouse are shown in Table 2. The CPS group had

Table 1: The re-expansion rate of mouse blastocysts after vitrification using CPS, OPS and conventional straws

Method	Vitrified blastocysts	Thawed blastocysts	Re-expansion (n)	(%)
CPS[a]	120	111	80	72.1
OPS[b]	120	101	53	52.5
CS	120	103	39	38.6
Control	-	-	120	100.0

[a]$p<0.05$ compared with blastocysts vitrified in CPS and OPS, [b]$p>0.05$ compared with blastocysts vitrified in OPS and conventional straws

Table 2: The pregnancy and live birth rate of vitrified blastocysts after transfer to pseudo pregnant mouse

Method	Transfer blastocysts (n)	No. mouse	Pregnancy n (%)	Live birth n (%)
CPS	80	10	7/10 (70)	36(45)
OPS	53	10	2/10(20)	4(7.5)
C.S	39	10	1/10(10)	2(5.1)
Control	120	10	8/10(80)	55(45.8)

a higher percentage of pregnancy and live birth similar to the controls and higher than the OPS and Conventional group. In addition, blastocysts vitrified using the convential straw had a significantly smaller percentage of pregnancy and live birth rate than those using CPS and OPS ($p<0.05$). Differences among the OPS and CS were not apparent, but differences between the OPS and CPS were significant ($p>0.05$).

DISCUSSION

Vitrification as an ultra rapid cooling technique is simple, potentially faster and inexpensive, further, it is starting to become clinically established and seems to have the potential to be more reliable and consistent than conventional cryopreservation when carried out properly (Tucker and Liebermann, 2003; Liebermann and Tucker, 2004). Further, the need for controlled rate freezing equipment, which requires routine calibration and maintenance, is eliminated. The cells are placed into the cryoprotectant, then the cells are placed in a very small volume of cryoprotectant on a special carrier and then they are cooled at extreme rates by plunging them directly into LN2. With this method no ice crystals form, avoiding damage to the cells or the tissue (Liebermann and Tucker, 2006).

The volume of vitrification medium and supporting material surrounding the embryos determines thermal change during cooling and warming. Vajta *et al.* (1998) developed OPS to hold bovine oocytes or embryos in a small amount of vitrification solution. Open pulled straws rendered a fast thermal attention that facilitated glass formation and circumvented chilling damage. The straws are then immersed directly into liquid nitrogen allowing direct contact between the two solutions (Vajta *et al.*, 1997). The average cooling rate of the liquid column in the OPS between -25 and -175°C was 22,500°C min^{-1} and between 0 and -195°C was 16,700°C min^{-1} whereas those in conventional straws were 2,550°C min^{-1} (Vajta *et al.*, 1998).

The Re-expansion rate of blastocysts vitrified was 81% by the OPS method. Fracture of the zona pellucida at vitrification is a common phenomenon when embryos are rapidly cooled or warmed in straws (De Paz *et al.*, 1994; Kassai *et al.*, 1996). With the OPS method, despite the increased cooling and warming speeds, fracture of zona is rare as published in Vajta's research work (Vajta *et al.*, 1998).

The disadvantage is the potential hazard of contamination because the embryo holding medium is directly in contact with liquid nitrogen (Tedder *et al.*, 1995). Chen *et al.* (2005) modified the loading of the pulled straws into a close system, called close pulled straws (CPS). Closed Pulled Straws has the characteristics of OPS, as a rapid thermal change method and of conventional straws, as a no contact mode. In the present study we investigated the effects of different containers OPS, CPS and conventional straws on re-expansion rates, pregnancy rates and proportions of live birth of vitrified mouse blastocysts. We found that the mouse blastocysts vitrified using CPS had a higher chance of re-expansion and live birth than those vitrified using OPS or Conventional Straws. The re-expansion rate of the CPS group (72.1%) group was significantly higher ($p<0.05$) than OPS (52.5%) and C.S (38.6%) groups. The pregnancy (70%) and birth rate (45%) of blastocysts in CPS were similar to those of fresh blastocysts (80 and 45.5%) and the pregnancy (10%) and birth rate (5.1%) in Conventional Straws lower than OPS 20 and 7.5%), but the differences were not significant.

Campson-Chillon *et al.* (2006) showed in bovine blastocysts a post thaw Re-expansion rate of 95 with 100% survival after vitrification. In addition, after vitrification of mouse blastocysts using CPS as carrier, pregnancy rate of 28% and live birth rate of 9% have been reported. Re-expansion rates and live birth using OPS as carrier 69.2 and 16.7% were reported respectively (Lazar *et al.*, 2000). Furthermore, after vitrification of mouse blastocysts using OPS as carrier re-expansion rate of 93.5% and Hatching rate of 88.7% have been reported (Vajta *et al.*, 1998). We found that mouse blastocysts vitrified using CPS had a higher chance of re-expansion and live birth than those vitrified using OPS or conventional straws. Blastocysts held in a closed pulled straw for vitrification achieve a faster cooling and warming rate (20,000 and 180,000°C min^{-1}, respectively) than those in Conventional Straws (2,550°C min^{-1}), (Rall and Fahy, 1983; Vajta *et al.*, 1998). Moreover embryos in a small amount of vitrification solution can be directly warmed and immediately thawed into the thaw solution. The property of plastic OPS straws places a lower limit to the diameter of the capillary when the OPS straws are made. When heated, OPS straws are pulled to a diameter under 0.8 mm, the walls of the capillary become fragile and often tear or break near the narrowest point (Kong *et al.*, 2000) that reduce exposure to unsuitable temperatures and concentrated cryoprotectants. In contrast, the conventional straw is warmed in 37°C water and then cut with scissors and then embryos in a larger vitrification volume are expelled into the thaw solution. This allows more time to pass through the inappropriate conditions (Chen *et al.*, 2000b; Hen *et al.*, 2001). It may explain why vitrification of blastocysts using OPS and CPS preserved results better than Conventional

Straws. It is possible that direct contact with liquid nitrogen on a portion of the embryo in OPS may have a negative effect on blastocysts survival (Cates, 1975). In the CPS system, the vitrification medium containing embryos was isolated by two small segments of air and medium. Through this closed loading system of CPS, the embryos will not directly contact with liquid nitrogen, which may occur with OPS. However, the CPS is not actually sealed in both ends of the straw. Therefore, if the CPS system is to be applied to human embryos, the embryos supported by CPS are required to be physically isolated to avoid contamination with pathogens in shared liquid nitrogen storage. This is also essential for the OPS system. With a faster thermal change, CPS reached better results than did conventional straws for blastocysts (Chen *et al*., 2005).

Present results indicate that mouse blastocysts vitrified using CPS had a better result compared with OPS and Conventional Straw. However more research is necessary to confirm the value of CPS for vitrification of human blastocysts.

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REFERENCES

Campos-Chillon, L.F., D.J. Walker, J.F. de la Torre-Sanchez and G.E. Seidel, 2006. *In vitro* assessment of a direct transfer vitrification procedure for bovine embryos. Theriogenology, 65: 1200-1214.

Chatot, C.L., C.A. Ziomek, B.D. Bavister, J.L. Leais and I. Torres, 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. J. Reprod. Fert., 86: 679-688.

Chen, S.U., Y.L. Lien, H.F. Chen, K.H. Chao, H.N. Ho and Y.S. Yang, 2000. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum. Reprod., 15: 2598-2603.

Chen, S.U., T.H. Lee, Y.R. Lien, Y.Y. Tasi, L.J. Chang and Y.S. Yang, 2005. Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocyst, but pregnant with the cytoskeletal stabilizer did not increase blastocyst survival. Fert. Ster., 84: 1156-1162.

Cho, H.J., W.Y. Son, S.H. Yoon, S.W. Lee and H.J. Lim, 2002. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum. Reprod., 17: 2419-2422.

De Paz, P., A.J. Sanchez, J.G. Fernandez, M. Carbajo, J.C. Dominguez, C.A. Chamoro and L. Anel, 1994. Sheep embryo cryopreservation by vitrification and conventional freezing. Theriogenology, 42: 327-338.

Gardner, D.K., P. Vella, M. Lane, L. Wagley, T. Schlenker and W.B. Schoolcraft, 1998. Culture and transfer of human blastocysts increases implantation rates and reduce the need for multiple embryo transfer. Fert. Ster., 69: 84-88.

Gardner, D.K., M. Lane, J. Stevens and W.B. Schoolcraft, 2003. Changing the start temperature and cooling rate in a slow-freezing protocol increases human blastocysts viability. Fert. Ster., 79: 407-410.

Gayar, M.E.L. and W. Holtz, 2001. Technical note: Vitrification of goat embryos by the open pulled-straw method. J. Anim. Sci., 79: 2436-2438.

Hen, S.U., Y.R. Lien, Y.Y. Cheng, H.F. Chen and Y.Sh. Yang, 2001. Vitrification of mouse oocytes Closed Pulled Straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straw, Open Pulled Straw (OPS) and grids. Hum. Reprod., 16: 2350-2356.

Hiraoka, K., M. Kinutani and K. Kinutani, 2004. Case report: successful pregnancy after vitrification of a human blastocyst that had completely escaped from the zona pellucida on day 6. Hum. Reprod., 19: 988-990.

Kassai, M., J.H. Komi, A. Takakamo, H. Tsudera, T. Sakurai and T. Machida, 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution without appreciable loss of viability. J. Reprod. Fert., 89: 91-97.

Kassai, M., S.E. Zhu, P.B. Pedro, K. Nakamura, T. Sakurai and K. Edashige, 1996. Fracture damage of embryos and its prevention during vitrification and warming. Cryobiology, 33: 459-464.

Kassai, M., K. Ito and K. Edashige, 2002. Morphological appearance of the cryopreserved mouse blastocysts as a tool to identify the type of cryoinjury. Hum. Reprod., 17: 1863-1874.

Kassai, M. and T. Mukaida, 2004. Cryopreservation of animal and human embryos by vitrification. Reprod. Biomed. Online, 9: 164-170.

Kauzman, W., 1984. The nature of glassy state and the behavior of liquids at low temperatures. Chem. Rev., 43: 219-256.

Klug, S., H.J. Merker and R. Jackh, 2001. Effects of ethylene glycol and metabolites on *in vitro* development of rat embryos during organogenesis. Toxicol. *In vitro*., 15: 635-642.

Kong, I.K., S.I. Lee, S.G. Cho, S.K. Cho and C.S. Park, 2000. Comparison of Open Pulled Straw (OPS) vs Glass Micropipette (GMP) vitrification in mouse blastocysts. Theriogenology, 53: 1817-1826.

Lazar, L., J. Spak and V. David, 2000. The vitrification of *in vitro* fertilized cow blastocysts by the open pulled straw method. Theriogenology, 54: 571-578.

Liebermann, J., M.J. Tucker and E.S. Sills, 2003a. Cryoloop vitrification in assisted reproduction: Analysis of survival rates in >1000 human oocytes after ultra-rapid cooling with polymer augmented cryoprotectants. Clin. Exp. Obstet. Gynecol., 30: 125-129.

Liebermann, J., J. Dietl, P. Vanderzwalmen and M.J. Tucker, 2003b. Recent developments in human oocyte, embryo and blastocysts vitrification: Where are we now? Reprod. Biomed. Online, 7: 623-633.

Liebermann, J. and M.J. Tucker, 2004. Vitrifying and warming of human oocytes, embryos and blastocysts: Vitrification procedures as an alternative to conventional cryopreservation methods. Methods Mol. Biol., 254: 345-364.

Liebermann, J. and M.J. Tucker, 2006. Comparision of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. Fert. Ster., 86: 20-26.

Massip, A., P. Vanderzwalmen, B. Scheffen and F. Ectors, 1986. Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo-Lett., 7: 270-273.

Menezo, Y.J., 2004. Blastocyst freezing. Eur. J. Obstet. Gynecol. Reprod. Biol., 115: 12-15.

Moore, K. and A.Q. Bonilla, 2006. Cryopreservation of mammalian embryos: The state of art. Biol. Sci., 8: 19-32.

Mukaida, T., S. Nakamura, T. Tomiyama, S. Wada, C. Oka and M. Kassai, 2003. Vitrification of human blastocysts using cryoloops: Clinical outcome of 223 cycles. Hum. Reprod., 18: 384-391.

Rall, W.F. and G.M. Fahy, 1983. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature, 313: 573-575.

Son, W.Y., S.H. Yoon, H.J. Yoon, S.M. Lee and J.H. Lim, 2003. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum. Reprod., 18: 137-139.

Tedder, R.S., M.A. Zuckerman, A.H. Goldstone, A.E. Hawkins, A. Fielding, E.M. Briggs, D. Irwin, S. Blair, A.M. Gorman, K.G. Patterson, D.C. Linch, J. Heptonstall and N.S. Brink, 1995. Hepatitis B transmission from contaminated cryopreservation tank. Lancet, 346: 137-140.

Tucker, M.J. and J. Liebermann, 2003. Oocyte and Embryo Cryopreservation. In: A Color Atlas of Human Assisted Reproduction: Laboratory and Clinical Insight. Patrizio, P., M.J. Tucker and V. Guelman (Eds.). Williams and Wikins, Philadelphia: Lippincott, pp: 137-159.

Vajta, G., P.J. Booth, P. Holm, T. Greve and H. Callesen, 1997. Successful vitrification of early stage bovine *in vitro* produced embryos with the Open Pulled Straw (OPS) method. Cryo Lett., 18: 191-195.

Vajta, G., P. Holm, M. Kuwayama, P.J. Booth, H. Jacobsen, T. Greve and H. Callesen, 1998. Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51: 53-58.

Vajta, G. and M. Kuwayama, 2006. Improving cryopreservation system. Theriogenology, 65: 236-244.

Vanderzwalmen, P., G. Bertin, Ch. Debauche, V. Standaert, E. Van Roosendaal and M. Vandervorst, 2002. Births after vitrification at morula and blastocysts stages: Effect of artificial reduction of the blastocoelic cavity before vitrification. Hum. Reprod., 17: 744-751.

Vanderzwalmen, P., G. Bertin, Ch. Debauche, V. Standaert, N. Bollen and E. Van Roosendaal, 2003. Vitrification of human blastocysts with the hemi straw carrier: Application of assisted hatching after thawing. Hum. Reprod., 18: 1501-1511.

Yang, Q.E., Y.P. Hou, G.B. Zhou, Z.Q. Yang and S.E. Zhu, 2006. Stepwise in-straw dilution and direct transfer using Open Pulled Straw (OPS) in the mouse: A potential model for field manipulation of vitrified embryos. Reprod. Dev., 29: 1-19.

Zech, N.H., B. Lejeune, H. Zech and P. Vanderzwalmen, 2005. Vitrification of hatching and hatched human blastocysts: Effect of an opening in the zona pellucida before vitrification. Reprod. Biomed. Online, 11: 355-361.