Journal of Animal and Veterinary Advances 11 (2): 200-206, 2012
ISSN: 1680-5593
© Medwell Journals, 2012

Comparison Between Direct Plunging and Vitrification Techniques on Development of Mouse Embryos at Various Preimplantation Stages

A.B. Ainul Bahiyah, W.E. Wan Khadijah and R.B. Abdullah
Institute of Biological Sciences, Faculty of Science, University of Malaya,
50603 Kuala Lumpur, Malaysia

Abstract: This study evaluated the efficiency of direct plunging and vitrification techniques in preserving mouse embryos at various preimplantation stages. Embryos were from superovulated ICR female mice. In direct plunging, embryos were equilibrated in EG (4.0 M), sucrose (0.25 M), BSA (4%), loaded into straw and plunged into LN_2. The straw was thawed in water bath (37°C) and embryos were equilibrated in sucrose (0.5 M), BSA (4%). In vitrification, embryos were equilibrated with EG (7.5%), DMSO (7.5%) with EG (15%), DMSO (15%) and loaded onto cryoleaf. The cryoleaf was plunged into LN_2. Embryos were warmed in sucrose (1.0 M), sucrose (0.5 M), washing solution and in modified WM medium. Frozen-thawed/vitrified-warmed embryos were cultured in modified WM medium in 5% CO_2 incubator (37°C). Some embryos were transferred into pseudopregnant CBA/ca females for *in vivo* viability assessment. Vitrification gave higher survival rates than direct plunging technique at all developmental stages studied. No significant differences in the percentage of live-birth from direct plunging (22.40±4.40%), vitrification (29.80±9.00%) and control embryos (28.83±3.25%).

Key words: Cryopreservation, direct plunging, embryo, mouse, vitrification, Malaysia

INTRODUCTION

Cryopreservation of embryos is a common practice now-a-days within the livestock industry and human *in vitro* fertilization programs as the embryos could be stored without losing their viability until further needed. Experience gained over the last three decades has produced numerous modifications in the cryopreservation process. Most efforts have been directed toward improving the survival rate and simplifying the procedures. Various methods for embryo cryopreservation have been developed. Among the methods, direct plunging and vitrification offer simple, rapid, practical and economical advantages when compared to the conventional slow freezing.

Cryopreservation by direct plunging technique was first reported by Takeda where straw containing embryos was directly plunged into LN_2 after equilibration in the Cryoprotectant (CPA) without seeding or equilibration in LN_2 vapor phase. The rate of cooling >3000°C min^{-1} which resulted in the formation of very small intracellular ice crystals which are harmless to the cells (Niemann, 1990). Vitrification of mouse embryos was first described by Rall and Fahy (1985). When embryos are cryopreserved by vitrification, ice crystal formation is prevented by the use of high concentration of CPA and high cooling and warming rates. It is generally known that the formation of ice crystals during the cryopreservation process reduces embryo survival. This important characteristic has made vitrification a widely used technique and now regarded as a good alternative to slow freezing. Traditionally, embryos were vitrified via closed system where there was no direct contact with LN_2 (sealed 0.25 mL insemination straw). However, an open system (which required direct contact with LN_2) was favored nowadays as it increased the speed of cooling and warming (electron microscope grid, cryotip, cryotop and cryoleaf). Although, many researchers reported a high survival rate of these two techniques (Nowshari and Brem, 1998; Cseh *et al*., 1999), the overall outcome is still inconsistent and warrant further investigation especially for cryopreservation of earlier preimplantation stages. The objective of this study was to evaluate the efficiency *in vitro* and *in vivo* of two ultra-rapid cryopreservation techniques, namely direct plunging and vitrification in preserving various preimplantation stages of mouse embryos.

MATERIALS AND METHODS

Experimental animals and chemicals: Mice of ICR strain were used for collection of embryos while CBA/ca strain mice were used for embryo transfer. All mice were bred in the animal house, Institute of Biological Sciences, University of Malaya. They were kept in cages that were covered with wire lids. Bedding consisting of ground wood shavings was changed once a week while

Corresponding Author: W.E. Wan Khadijah, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

commercial pellet feed for mice and clean drinking water were made available to the animals *ad libitum*. Mice were bred at room temperature (27°C) with natural light; Dark cycle (12L:12D). All animals were used for experiments according to the guidelines of Institute of Biological Sciences. All chemicals were purchased from Sigma-aldrich chemical (St. Louis, MO, USA) unless otherwise stated.

Superovulation and embryo collection: Female ICR mice (8-12 weeks) were superovulated via intraperitoneal injection with 5 IU of Pregnant Mare's Serum Gonadotrophin (PMSG; Intervet) followed 48 h later by 5 IU of human Chorionic Gonadotrophin (hCG; Intervet) and were mated to male of the similar strain. Successful mating was indicated by the presence of a vaginal plug the following morning. Embryos at the 2 cell stage were flushed from the oviducts at 36-39 h post-hCG. For cryopreservation of 2 cell stage, the embryos were washed in three changes of modified WM medium and were used immediately after collection. For cryopreservation of 4 and 8 cell and compact morula stages, the embryos were cultured in modified WM medium in 5% CO_2 incubator at 37°C up to the developmental stages required. Non-cryopreserved embryos were used as control in both cryopreservation techniques employed (direct plunging and vitrification).

Cryopreservation procedures: In direct plunging technique, freezing medium used consists of PBS, EG (4.0 M), sucrose (0.25 M) and BSA (4%) (w/v). Embryos were equilibrated in 3 changes of freezing medium (100 μL each) at room temperature (27°C) for a total time of 5 min. The embryos were quickly loaded into the middle column of the insemination straw (0.25 mL) (IMV, L'Aigle, France) containing freezing medium (20 μL) using a hand-controlled micropipette toward the end of the equilibration time. The straw was heat sealed and then directly plunged vertically into LN_2 in a styrofoam container at the end of the equilibration time. The straw was kept in LN_2 for at least 30 min. The straw was thawed in air (10 sec) followed by water bath (37°C, 15 sec) and wiped dry. The embryos were expelled from the straw and were equilibrated in thawing medium consisting of PBS, sucrose (0.5 M) and BSA (4%) (w/v) for 5 min for dilution of intracellular CPA and were rinsed in WM medium prior to *in vitro* culture in modified WM medium in 5% CO_2 incubator at 37°C. In vitrification technique, the general vitrification procedure was a modification from Vajta *et al.* (1998). Embryos were equilibrated in Equilibration Solution (ES) consisting of EG (7.5%) (v/v) and DMSO (7.5%) (v/v) for 5 min followed by Vitrification Solution (VS) consisting of EG (15%) (v/v) and DMSO (15%) (v/v) for 1 min and were loaded onto cryoleaf with minimal volume (0.1 μL) of vitrification solution. The cryoleaf was plunged into LN_2 in a styrofoam container and kept in LN_2 for at least 30 min. Embryos were warmed at room temperature (27°C) in Thawing Solution (TS) consisting of sucrose (1.0 M) for 1 min followed by Diluent Solution (DS) consisting of sucrose (0.5 M) for 3 min and Washing Solution (WS) consisting of HWM medium and BSA (20%) (w/v) for 5 min. Finally, the embryos were rinsed in modified WM medium prior to *in vitro* culture in modified WM medium in 5% CO_2 incubator at 37°C.

Assessment of embryo survival and development *in vitro*: Survival was based on the re-expansion and intactness of the blastomeres and zona pellucida after thawing or warming procedures. The *in vitro* cultured embryos were observed daily under the inverted microscope (40× magnification) and development up to the blastocyst stage was recorded.

Embryo transfer: Embryo transfer procedure was performed based on Hogan. Only morphologically normal embryos at the morula stage developed from frozen-thawed, vitrified-warmed or control embryos at 4 or 8 cell stage were transferred into the uterus of day 3 (2.5 dpc) pseudopregnant CBA/ca females between 0800-1100 h. The recipients were mated earlier to vasectomized ICR males. The presence of a vaginal plug at 13-16 h post-hCG was designated as day 1 of pseudopregnancy. Only females with vaginal plug present were chosen for the embryo transfer experiment. Live offspring after embryo transfer were delivered naturally.

Statistical analysis: The data obtained in the present study were presented as mean and Standard Error of the Mean (mean±SEM). All data were analyzed using one-way Analysis of Variance (ANOVA). Differences in the blastocyst formation rate and live offspring obtained between stages of development as well as the cryopreservation techniques were further analyzed using the Duncan Multiple Range Test (DMRT). Significant differences among factors were determined where the p<5% ($p<0.05$). The analysis was carried out with SPSS (Statistical Packages for Social Sciences) for windows, Version 11.0, SPSS Inc. in 2002, USA.

RESULTS AND DISCUSSION

Effect of cryopreservation technique on survival and cleavage rates of cryopreserved mouse embryos: The percentages of embryos that survived were significantly higher in the vitrification technique at all stages of cryopreservation than that of direct plunging. The percentages of embryos that survived when cryopreserved at 2, 4 and 8 cell and compact

morula stages in direct plunging and vitrification were 76.77±4.80, 68.45±2.89, 62.72±4.77 and 53.68±3.40% vs. 94.67±2.59, 81.33±6.01, 87.83±3.63 and 87.86±3.36%, respectively. Except at the 2 cell stage, the survival of vitrified-warmed embryos at 4 and 8 cell and compact morula stages were significantly lower when compared with those of control.

The survival rates of embryos cryopreserved using direct plunging technique were significantly lower than the control embryos at all stages of cryopreservation. Figure 1 shows the graphical presentation of survival rates of embryos cryopreserved by direct plunging and vitrification.

Development into blastocysts also displayed a similar trend where higher percentages of blastocysts were obtained in vitrification compared with direct plunging at all stages of cryopreservation while significant differences were detected when embryos were cryopreserved at 8 cell and compact morula stages by vitrification technique (57.10±4.64 and 77.61±7.02%, respectively) compared to direct plunging (33.18±4.66 and 27.90±3.57%, respectively).

The *in vitro* developments of embryos to blastocysts after cryopreservation by direct plunging and vitrification are shown in Table 1.

Morphological changes of embryos when subjected to the vitrification process are shown in Fig. 2 while the *in vitro* development of frozen-thawed or vitrified-warmed 4 cell embryos is shown in Fig. 3.

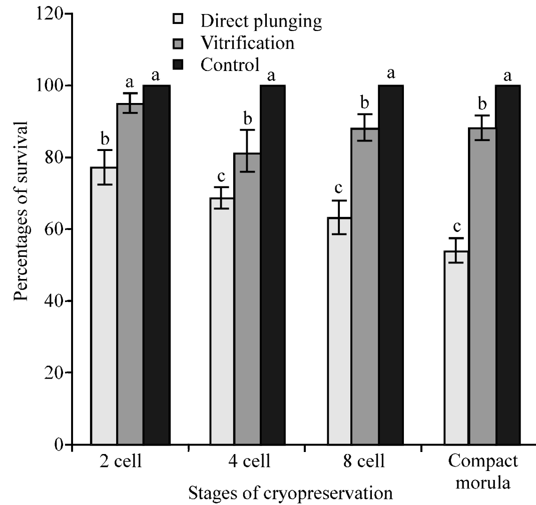

Fig. 1: Percentages of survival of embryos cryopreserved at different developmental stages by direct plunging or vitrification technique compared with control (non-exposed to CPA, non-cryopreserved)

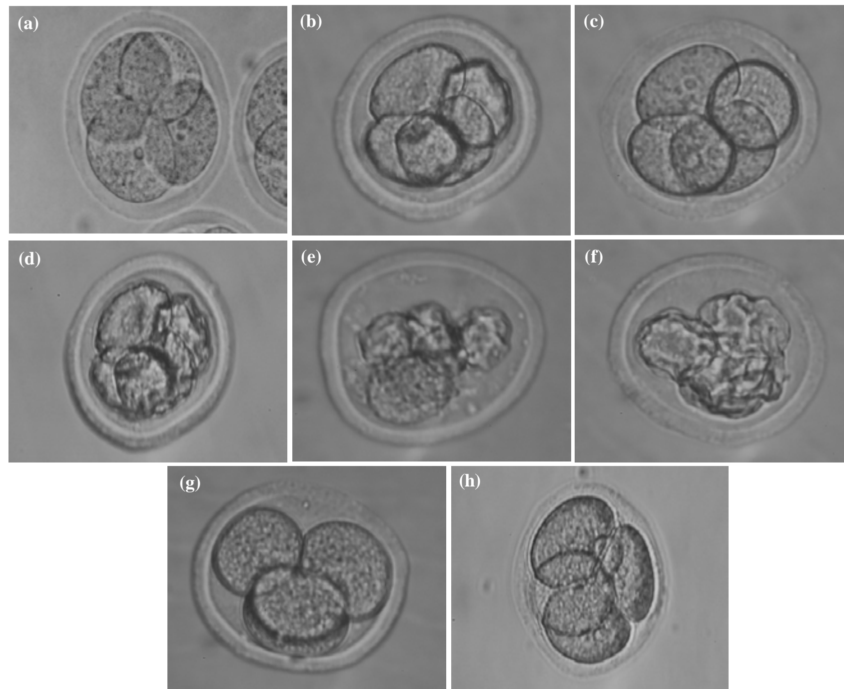

Fig. 2: Morphological changes of embryos during exposure to vitrification and warming solutions; a) normal 4 cell embryo prior to vitrification procedure; b) embryo shrank after equilibration in ES for 1 min; c) embryo swelled back to its original shape after equilibration in ES for 5 min; d) embryo shrank after exposure to VS for 1 min; e) embryo in TS; f) embryo in DS; g) embryo regained its original shape in WS and h) embryo after 1 h culture in IVC medium

Table 1: *In vitro* development to blastocysts (%; mean±SEM) of embryos cryopreserved at different stages of development using direct plunging or vitrification technique

	Stages of embryos used (cell)			
Treatments	2	4	8	Compact morula
Direct plunging	40.92±5.99$^{a, x}$ (n = 83)	30.91±6.68$^{a, y}$ (n = 81)	33.18±4.66$^{a, y}$ (n = 109)	27.90±3.57$^{a, y}$ (n = 72)
Vitrification	48.57±8.65$^{b, x}$ (n = 74)	43.44±6.19$^{b, xy}$ (n = 56)	57.10±4.64$^{b, x}$ (n = 68)	77.61±7.02$^{a, x}$ (n = 112)
Control	52.66±7.10$^{b, x}$ (n = 21)	56.72±8.44$^{b, x}$ (n = 30)	72.17±7.56$^{ab, x}$ (n = 35)	86.67±7.87$^{a, x}$ (n = 46)

Control embryos were non-exposed to CPA and non-cryopreserved; n: No. of embryos at each stages of development; ^{ab}In a row: Means with different superscripts were significantly different (p<0.05); ^{xy}In a column: Means with different superscripts were significantly different (p<0.05)

Table 2: Percentages of offspring (mean±SEM) obtained after transferring morula stage embryos cryopreserved by direct plunging or vitrification technique

Treatments	No. of recipients	No. of pregnant recipients	No. of embryos transferred*	Percentage of offspring
Direct plunging	9	5	100.00±0.00^{a} (n = 77)	22.40±4.48$^{b, x}$ (n = 17)
Vitrification	9	5	100.00±0.00^{a} (n = 93)	29.80±9.00$^{b, x}$ (n = 29)
Control	10	6	100.00±0.00^{a} (n = 114)	28.83±3.25$^{b, x}$ (n = 33)

*No. of embryos transferred into female recipients which subsequently became pregnant; n: No. of embryos or offspring for each group; ^{ab}In a row: Means with different superscripts were significantly different (p<0.05); ^{x}In a column: Means with similar superscript was not significantly different (p>0.05)

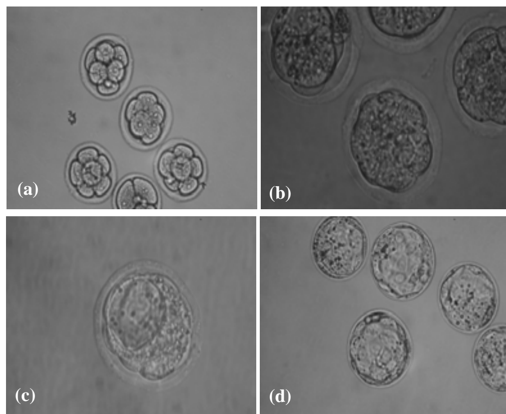

Fig. 3: *In vitro* development of frozen-thawed or vitrified-warmed 4 cell embryos; a) 8 cell; b) compact morula; c) expanded blastocyst and d) fully expanded blastocyst

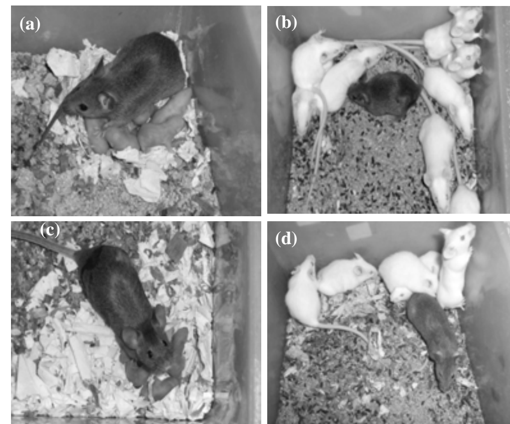

Fig. 4: Healthy offspring born from vitrified embryos; a) 1 day old; b) 12 weeks old offspring with their foster mother. Healthy offspring born from frozen embryos; c) 1 day old; d) 12 weeks old offspring with their foster mother

Production of offspring after transfer of frozen-thawed or vitrified-warmed embryos: There were no significant differences in the percentages of live-birth obtained from direct plunging, vitrification and control embryos which were 22.40±4.40, 29.80±9.00 and 28.83±3.25%, respectively (Table 2). For vitrification, 4 offspring out of 29 died at 2 weeks old. The healthy offspring produced from transferring cryopreserved embryos are shown in Fig. 4. Results obtained from this study demonstrated that mouse embryos at 2, 4 and 8 cell and compact morula were able to survive and develop *in vitro* when subjected to cryopreservation by the procedures of direct plunging and vitrification techniques. However, the percentages of embryo survival and blastocyst rate were significantly higher (p<0.05) in the vitrification compared to direct plunging at all initial stages of cryopreservation. The present results are in agreement with Men *et al.* (1997) where vitrification of oocytes from Kunming mouse were demonstrated to yield higher rates of survival and development into 2 cell stage (after IVF) than the oocytes frozen by direct plunging technique.

The differences of the results produced by vitrification and direct plunging techniques in the present study could be explained by the different principles on how these two techniques functioned in achieving cryopreservation. It was well known that two of the major limiting factors for achieving optimal cryopreservation were chilling injury (Vajta and Kuwayama, 2006) and ice formation (Mazur, 1984; Jin *et al.*, 2008). Chilling injury mainly affects cytoskeleton (Pickering *et al.*, 1990) and cell membranes (Ghetler *et al.*, 2005). Vitrification technique minimizes these two major factors by the use of very high cooling rates (Liebermann *et al.*, 2003).

Cooling speed depended upon the vitrification solution volume such that the smaller the sample volume, the higher the cooling rate. In addition, direct contact of the vitrification solution containing samples with LN_2 also increases the cooling rate (Vajta *et al.*, 1998; Morato *et al.*, 2008). The present study employed commercially supplied

cryoleaf as a carrier device. The device allows embryos to be loaded in a very small volume of vitrification solution (e.g., 0.1 μL) onto a fine thin strip of polypropylene film followed by direct immersion into LN_2. Small volume of vitrification solution coupled with direct contact with LN_2 permit an extremely fast cooling rate (23,000°C min^{-1}) to be achieved, thus avoiding chilling injury (Kuwayama *et al*., 2005a, b; Morato *et al*., 2008). Furthermore, the extremely small volume also helps achieve a faster warming rate (42,000°C min^{-1}) thereby avoiding ice crystal formation during warming (Morato *et al*., 2008).

In contrast, direct plunging technique employed conventional insemination straw (0.25 mL) as a carrier device. Embryos were loaded into the middle of the straw containing freezing medium (20 μL). Since, larger volume of freezing medium was used without direct contact of freezing medium with LN_2, lower cooling rate was achieved (2500-3000°C min^{-1}) (Niemann, 1990; Palasz and Mapletoft, 1996) compared to vitrification technique. The lower cooling rate resulted in the formation of very small intracellular ice crystals (sizes ranging from 0.1-1.0 μm in diameter) which were proposed to be harmless (Niemann, 1990; Valdez *et al*., 1990). However, based on the lower results of direct plunging compared to vitrification in the present study, the occurrence of these ice crystals could indeed have detrimental effects on the embryos as it has been suggested its absence would certainly be beneficial and might increase further the embryo survival rates (Niemann, 1990; Valdez *et al*., 1990). The lower percentages of survival in the direct plunging compared with vitrification could be attributed to the insufficient permeation of CPA into the cells during the fixed equilibration time of 5 min as employed in this study. Sufficient permeation of CPA was very important as it protects the cells during freezing.

Rayos *et al*. (1992) reported that a shorter equilibration period (5 min) in EG (3.0 M) yielded a significantly lower survival rate of 1 cell embryos compared to longer equilibration period (10 min) and suggested that the lower survival rates were due to insufficient intracellular concentration of EG prior to freezing. This was in agreement with Szell and Shelton (1987) where they suggested that the survival of rapidly frozen embryos depends upon the period of equilibration and the effect of the equilibration period is due to cryoprotectant permeation into cells which was necessary for protection before freezing. However, prolonged equilibration could have resulted in the permeation of a very high concentration of CPA into the cells, making the embryos susceptible to osmotic shock caused by the rapid influx of water when the cryoprotectant is diluted out after thawing (Leibo, 1989; Mazur, 1990). To maintain viability of cryopreserved embryos after recovery, several obstacles must be circumvented such as the toxic effect of CPA, physical and chemical injuries caused by extracellular ice, the formation and growth of intracellular ice and fracture damage. However, even if the embryos could avoid these obstacles, they are still at risk of injury after warming by osmotic swelling when diluted from the CPA solution and returned to a physiological solution (Pedro *et al*., 1997). In this study, the incidence of the osmotic swelling was frequently observed in direct plunging rather than vitrification. The higher frequency of swelling in direct plunging embryos could be due to rapid influx of water into the cells during thawing process as single-step thawing procedure were carried out in direct plunging as opposed to two-steps in vitrification. The CPA that permeated into the blastomeres probably could not diffuse out rapid enough to prevent sudden influx of water into the cells (Leibo, 1989), thus causing excessive swelling of the blastomeres which sometimes lead to disintegration during the dilution process.

Earlier stages of embryo development were shown to be more sensitive than the later stages. This finding has been confirmed by the previous study (Rall and Fahy, 1985). The lower survival and blastocysts rates in the direct plunging may be due to the insufficient permeation of CPA into the cells. In vitrification, the high *in vitro* development of morula when compared to other developmental stages in this study is in agreement with the results of De Mello *et al.* (2001). Although, the overall results for vitrification are better than direct plunging, the values are relatively low when compared to other report (Kasai *et al*., 1992). Therefore, study on the optimal procedure for a specific embryo stages were in need.

The *in vivo* survival rates (live-born offspring) between the cryopreservation groups and control group were not significantly different ($p>0.05$). However, the rates are relatively low when compared to other reports (Nowshari and Brem, 1998; Kuleshova *et al*., 2001) but comparable to the results obtained by De Mello *et al.* (2001). The low *in vivo* survival rates obtained was probably due to the different protocols employed, strain of mice used, researcher's skill and the quality of the recipients.

Although, mouse embryos at all preimplantation stages had been successfully cryopreserved by slow cooling (Whittingham *et al*., 1972), the present results

indicated that a variant sensitivity of embryos at various stages to freezing is observed when ultra-rapid cooling techniques (direct plunging and vitrification) were used. Since, the optimum conditions required to freeze mammalian embryos at various stages were different (Massip *et al*., 1984; Miyamoto and Ishibashi, 1986), cooling and thawing or warming conditions should be adapted to the embryonic stage to obtain high survival. These observations indicated that different embryonic stages were differently affected by the cryopreservation procedures. Optimal results for each embryonic stage could be achieved by manipulating factors such as the concentrations of CPAs, temperature of exposure and equilibration time to increase the penetration of permeable CPA into cells thus protecting the embryos during cryopreservation process.

Further studies were needed to better understand the causes for different survival rates of embryos cryopreserved at different developmental stages using the ultra-rapid procedures such as direct plunging and vitrification.

CONCLUSION

In this study both protocols are potentially suitable for cryopreservation of mouse embryos whereby vitrification gives higher developmental competence than the direct plunging technique, especially for the later stages of embryonic development.

ACKNOWLEDGEMENTS

This research was funded PJP VOT F, University of Malaya. The researchers wish to thank all the ABEL members for their contributions in the research process.

REFERENCES

Cseh, S., W. Horlacher, G. Brem, J. Corselli, J. Seregi, L. Solti and L. Bailey, 1999. Vitrification of mouse embryos in two cryoprotectant solutions. Theriogenology, 52: 103-113.

De Mello, M.R.B., V.S. Queiroz, A.S. de Lima, L.M.T. Tavares, M.E.O. D'Avila Asumpcao, M.B. Wheeler and J.A. Visintin, 2001. Cryopreservation of mouse morulae through different methods: Slow freezing, vitrification and quick-freezing. Braz. J. Vet. Res. Anim. Sci., 38: 160-164.

Ghetler, Y., S. Yavin, R. Shalgi and A. Arav, 2005. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum. Reprod., 20: 3385-3389.

Jin, B., K. Kusanagi, M. Ueda, S. Seki, D.M. Jr. Valdez, K. Edashige and M. Kasai, 2008. Formation of extracellular and intracellular ice during warming of vitrified mouse morulae and its effect on embryo survival. Cryobiology, 56: 233-240.

Kasai, M., M. Nishimori, S.E. Zhu, T. Sakurai and T. Machida, 1992. Survival of mouse morulae vitrified in an ethylene glycol-based solution after exposure to the solution at various temperatures. Biol. Reprod., 47: 1134-1139.

Kuleshova, L.L., J.M. Shaw and A.O. Trounson, 2001. Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. Cryobiology, 43: 21-31.

Kuwayama, M., G. Vajta, O. Kato and S.P. Leibo, 2005a. Highly efficient vitrification method for cryoprervation of human oocytes. Reprod. Biomed. Online, 11: 300-308.

Kuwayama, M., G. Vajta, S. Ieda and O. Kato, 2005b. Vitrification of human embryos using the CryoTip™ method. Reprod. Biomed. Online, 11: 608-614.

Leibo, S.P., 1989. Equilibration and non-equilibration cryopreservation of embryos. Theriogenology, 31: 85-93.

Liebermann, J., J. Dietl, P. Vanderzwalmen and M.J. Tucker, 2003. Recent development in human oocyte, embryo and blastocyst vitrification: where are we now? eprod. Biomed. Online, 7: 623-633.

Massip, A., P. Van der Zwalmen and F. Leroy, 1984. Effect of stage of development on survival of mouse embryos frozen-thawed rapidly. Cryobiology, 21: 574-577.

Mazur, P., 1984. Freezing of living cells: Mechanisms and implications. Am. J. Physiol. Cell Physiol., 247: C125-C142.

Mazur, P., 1990. Equilibrium, quasi equilibrium and nonequilibrium freezing of mammalian embryos. Cell Biophys., 17: 53-92.

Men, H.S., J.C. Chen, W.Z. Ji, E.Y. Shang, S.C. Yang and R.J. Zou, 1997. Cryopreservation of Kunming mouse oocytes using slow cooling, ultrarapid cooling and vitrification protocols. Theriogenology, 47: 1423-1431.

Miyamoto, H. and T. Ishibashi, 1986. Liquid nitrogen vapour freezing of mouse embryos. J. Reprod. Fertil., 78: 471-478.

Morato, R., D. Izquierdo, M.T. Paramio and T. Mogas, 2008. Cryotops versus open-pulled straw (OPS) as carriers for the cryopreservation of bovine oocytes: Effects of spindle and chromosome configuration and embryo development. Cryobiology, 57: 137-141.

Niemann, H., 1990. Cryopreservation of bovine embryos in the field. Embryo Trans. Newslett., 8: 5-7.

Nowshari, M.A. and G. Brem, 1998. Effects of cryoprotectants and their concentration on post-thaw survival and development of expanded mouse blastocyst frozen by a simple rapid-freezing procedure. Theriogenology, 50: 1001-1013.

Palasz, A.T. and R.J. Mapletoft, 1996. Cryopreservation of mammalian embryos and oocytes: Recent advances. Biotechnol. Adv., 14: 127-149.

Pedro, P.B., S.E. Zhu, N. Makino, T. Sakurai, K. Edashige and M. Kasai, 1997. Effects of hypotonic stress on the survival of mouse oocytes and embryos at various stages. Cryobiology, 35: 150-158.

Pickering, S.J., P.R. Braude, M.H. Johnson, A. Cant and J. Currie, 1990. Transient cooling to room temperature can cause irreversible disruption to the meiotic spindle in the human oocytes. Fertil. Steril., 54: 102-108.

Rall, W.F. and G.M. Fahy, 1985. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature, 313: 573-575.

Rayos, A.A., Y. Takahashi, M. Hishinuma and H. Kanagawa, 1992. Quick freezing of one-cell mouse embryos using ethylene glycol with sucrose. Theriogenology, 37: 595-603.

Szell, A. and J.N. Shelton, 1987. Osmotic and cryoprotective effects of glycerol-sucrose solution on day-3 mouse embryos. J. Reprod. Fertil., 80: 309-316.

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

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.

Valdez, C.A., O.A. Mazni, H. Kanagawa and S. Fujikawa, 1990. Confirmation by cryoelectron microscopy of the absence of crystallization using a vitrification solution. Cryo-lett., 11: 351-358.

Whittingham, D.G., S.P. Leibo and P. Mazur, 1972. Survival of mouse embryos frozen to -196°C and -269°C. Science, 178: 411-414.