

POSTER SESSION

ART, laboratory: cryopreservation of embryos

P-386 IVF children after vitrification

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Introduction: Vitrification of human embryos is an efficient and simple method of cryopreservation but it requires increased concentrations of permeable cryoprotectants (40% v/v). However, none of the studies analyze the course of pregnancies and the health of the infants born after vitrification. The MA-MA clinic practices vitrification of human embryos since 2001. According to the 2002–2005 statistics, the total number of births is 34. This study undertakes to summarize the available data on pregnancies and labor after vitrification.

Materials and methods: We use the vitrification protocol conventionally to store supernumerary embryos obtained via IVF. Only promising embryos on day 2 or 3 with normal or subnormal morphology are selected for cryopreservation. The embryos are sequentially exposed to vitrification solutions, containing ethylene glycol, trehalose, ficoll, hyaluronan and HSA. Total exposure time is 5 min. Conventional freezing straws (0.25 ml) are filled with a 0.05 ml droplet of vitrification solution with embryos, plugged immediately and plunged directly into liquid nitrogen. The storage is effected in liquid nitrogen, 1–2 embryos per straw. Thawing and transfer of the embryos is planned in cycles favorable to patients. Vitrified embryos are thawed quickly by placing straws in a water bath at 37°C. Embryos were transferred in a few hours or the next day after flushing them. To evaluate pregnancy, labor and the perinatal period, the following criteria were used: spontaneous miscarriage rate; ectopic pregnancy rate; premature delivery rate; singleton or twin; male/female ratio; delivery method (spontaneous delivery or cesarean section); pregnancy term at delivery; newborn weight and length; Apgar score rating; major and minor birth defects; perinatal mortality; whether or not babytherm care and intensive care are necessary within 24 h on delivery.

Results: In 2001–2005, we thawed 465 embryos, stored for subsequent transfer. Four hundred and thirteen (88%) of thawed embryos maintained normal morphology of all blastomeres and showed no signs of post thawing injury. Three hundred and seventy-six embryos were selected for transfer to 204 patients, which resulted in 63 (31%) clinical pregnancies, of which 13 (21%) ended in miscarriage before 12 weeks term and 2 (3%) were ectopic. Nineteen women continue pregnancy, 29 have concluded pregnancy. The total of singleton births is 18. Three pregnancies resulted in twins. There are no detailed records on eight childbirths that took place in 2004 (6 singletons and 2 twins). As a result, we have reliable data on 18 singleton pregnancies and 3 twins. Overall, there are 14 boys and 10 girls. Nine (43%) women delivered via cesarean section owing to aggregate indirect indications, the rest (12) went through spontaneous delivery (57%). Preterm delivery rate is 14% (3 cases). Average term at time of delivery is 38 weeks. The newborns weighed in the range from 2850 to 4200 g with length in the range from 47 to 53 cm. Average Apgar score rating is 8.2 grade. In eight cases (33%) the newborns required intensive care owing to respiratory compromise of various origins. No perinatal mortality cases were registered. In one case, a newborn appeared to have single kidney. An additional examination of the baby's father was conducted to reveal the same kind of urinary tract pathology.

Conclusions: The average characteristics of pregnancies and labor as well as perinatal health exhibited by infants born as a result of embryo transfer after vitrification are no different from the overall population average.

P-387 The effect of vitrification on the physiological characteristics of human embryos

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Introduction: This study was performed to investigate physical and physiological alterations and the embryonic survival of human blastocysts cryopreserved in LN2.

Materials and methods: All the blastocysts which were derived from 1PN- or 3PN-zygotes for this study (January 1, 2003 to October 31, 2004) were allowed by Institutional Review Board in Maria Infertility Hospital. The expanded blastocysts produced on day 5 or day 6 were vitrified using EM-grid following artificial collapse of blastocoel. The total cell number was labeled with Hoeschst 33258 and the presence of viable mitochondria was identified by Rhodamine 123. The relative amount of H₂O₂ per embryo was quantified by the fluorescence intensity of 2',7'-dichlorofluorescein (DCF). Finally, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) was employed to quantify the DNA fragmentation.

Results: The survival and hatching rates of the vitrified-recovered blastocysts were significantly higher (94%: 183/194 and 77.6%: 142/183, respectively) than those of the frozen-thawed blastocysts (57.6%: 102/177 and 57.8%: 59/102, respectively) (p<0.05). Also, the total cell number of the vitrified-recovered blastocysts (n=25) was 78.0±12.9, which was significantly higher than that (44.0±13.5) of the frozen-thawed blastocysts (n=19) (p<0.05). In addition, mitochondria quantity showed to be 49.0±6.2 in the vitrified-recovered blastocysts (n=20), which was higher than that (31.0±5.8) in the frozen-thawed blastocysts (n=17) (p<0.05). However, the concentration of H₂O₂ per vitrified-recovered blastocyst showed to be significantly lower (p<0.05) than that per frozen-thawed blastocyst (38.0±6.2 vs. 62.0±6.4). Finally, the proportion of DNA fragmentation was significantly lower (p<0.05) in the vitrified-recovered blastocysts (n=15) than that in the frozen-thawed blastocysts (n=13) (14.2%±3.5 vs. 22.9%±3.8).

Conclusion: These results suggest that vitrification is superior to general freezing in cryopreservation of human blastocysts. The embryonic freezing must be more subjected to physical and physiological alterations, such as cell death, destruction of mitochondria and increase of reactive oxygen species (ROS) compared to vitrification.

P-388 The removal of ZP from blastocysts before vitrification increased their survival and also their implantation rates

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Introduction: The first pregnancy after vitrification of human blastocyst was reported in 2000 by Yokota. Since then several techniques using different cryoprotectants and various embryo carriers were developed. However, the surviving and pregnancy rates after human blastocyst vitrification are still non-consistent. Some of the factors affecting the success of the vitrification are the rate of blastocyst expansion and the volume of the blastocoel. Several studies showed a better surviving of vitrified blastocysts after artificial collapse of the blastocoelic cavity or after microsuction of the blastocoelic fluid. Moreover, a better surviving rate was observed after vitrification of hatched blastocysts comparing to non-hatched, expanded day 5 blastocysts. This phenomenon could be explained by a higher degree of the blastocoelic collapse during vitrification and a quicker removal of the highly viscous and toxic vitrification solutions during warming in case of zona pellucida (ZP)-free blastocysts. The purpose of our study was to examine the effect of an artificial ZP removal on the survival rate of vitrified human blastocysts and on the pregnancy rate of zona-free vitrified blastocysts after embryo transfer.

Materials and methods: We have compared the surviving and implantation rate of ZP-intact and artificially denuded blastocysts of day 5 culture. One hundred and fifty-eight expanded blastocysts were included into the study. Control group consisted of 89 ZP-intact blastocysts. Naturally hatched or partly hatched blastocysts were excluded from the study. Study group consisted of 63 artificially denuded blastocysts. For denudation, blastocysts were put in acidic Tyrode's solution (Sigma, Steinheim, Germany) until ZP was dissolved (2–5 s)

and afterwards they were washed in five drops of culture medium (Blastocyst protein Plus, Sage, Trumbull, CT, USA) under mineral oil (Sage, Trumbull, CT, USA). For vitrification, the VitriFreeze Medium and VitriThaw Medium (FertiPro, N.V., Beernem, Belgium) were used in combination with Vitri-plug carrier for embryos (Astro Med Tec, Salzburg, Austria), according to the manual. Briefly, blastocysts were placed in preincubation medium for 2 min and afterwards transferred to VitriFreeze 1 medium for 2 min. The procedure was performed at room temperature. Finally, blastocysts were transferred into VitriFreeze 2 medium and within 30 s were placed to the tip of the through of the VitriPlug and immersed into liquid nitrogen. Warming procedure was as follows: VitriPlug was transferred directly into pre-warmed Thawing Medium 1, blastocysts were washed out and left in medium for 3 min. Subsequently, blastocysts were transferred into Thawing medium 2, 3 and 4, each time for 2 min at 37°C. Finally, blastocysts were placed into culture medium. Surviving of embryos was controlled after 3 h and re-expansion after 24 h after warming. Embryo transfers were performed 24 h after warming.

Results: We have observed quicker and higher degree of blastocoele collapse in VitriFreeze Medium 1 in case of zona-free blastocysts comparing to zona intact blastocysts. The surviving rates were 73% (65/89) vs. 85.7% (54/63) and re-expansion after 3 h was 90.8% (59/65) vs. 100% (54/54) for ZP-intact and ZP-denuded blastocysts, respectively. Pregnancy rates were 27.8% (10/36) vs. 44.8% (13/29) and implantation rates were 21.5% (14/65) vs. 35.2% (19/54) for ZP-intact and ZP-denuded blastocysts, respectively.

Conclusions: We have observed higher surviving rates and significantly higher pregnancy rates after artificial removal of ZP before vitrification of expanded human blastocysts.

P-389 The effect of carrier systems on blastocyst survival and developmental potential after vitrification

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Introduction: Recent advance in *in vitro* technology have made it possible to produce blastocyst efficiently. Therefore, the cryopreservation of blastocysts will surely play an important role in assisted reproductive technology (ART). Cryopreservation by vitrification was described as a simple method of directly submerging embryos into liquid nitrogen after brief exposure to a high concentration of cryoprotectant. There are several reports of successful pregnancies following human blastocyst stage embryos vitrified with Cryoloops. The disadvantage of vitrification by using Cryoloops is that embryos with the vitrification solution directly contact with the liquid nitrogen during cooling or storage, which makes the possibility of contamination on embryos. The purposes of this study were to test the effectiveness of Cryoloops or Closed Pulled Straw (CPS) when the mouse blastocysts were cryopreserved by vitrification and to find a vitrification system that prevents directly exposure of blastocysts to liquid nitrogen and still provides acceptable post thaw outcomes. **Materials and methods:** Day 3.5 blastocysts were collected from ICR mice and divided into three groups as follows: Group 1: no treatment (control); Group 2: vitrified blastocysts with Cryoloops; Group 3: vitrified blastocysts with CPS. The mouse blastocysts were vitrified by a two-steps protocol at room temperature; embryos were initially place in HEPES containing 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO) for 3 min. Embryos were then transferred to HEPES containing 15% EG, 15% DMSO and 0.65M sucrose for 30s before placed on the Cryoloops or loaded into CPS and submerged in the liquid nitrogen for storage. After thawing, embryos were expelled from the Cryoloop or CPS and moved through dilutions of sucrose solution. Subsequent embryo survival through re-expansion and viability through embryo transfer to surrogate were used to determine the developmental potential of post thaw embryos.

Results: The post thawing re-expansion rates and hatching rates of vitrification using CPS [55.6% (60/108), 76.9% (83/108)] were similar to that of vitrification using Cryoloops [43.9% (47/107), 69.2% (74/107)], but in comparison with that of the control, CPS and Cryoloops showed significant reduction. The proportion of live pups from vitrified blastocysts using CPS was higher than that of vitrified blastocysts using Cryoloops [53.2% (59/111) vs. 38.7% (43/111), $p < 0.05$], but no difference from that of non-vitrified controls [53.2% (59/111) vs. 50% (19/38), $p > 0.05$].

Conclusions: The CPS and Cryoloops method of vitrification are both successful, easy to perform and demonstrate that both carriers are useful to vitrified blastocysts. However, the CPS method provided not only high post thaw pregnancy rates but also an elimination of liquid nitrogen contamination during cryopreservation.

POSTER SESSION

ART, laboratory: cryopreservation of gametes

P-390 Dose-dependent effect of cryoprotective agents on post thaw human sperm motility, morphology, lipid peroxidation and DNA integrity

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Introduction: Human sperm freeze-thaw process induces lethal and sublethal damage that leads to loss of viability, acrosome degradation, DNA fragmentation and poor fertilization. Reactive oxygen species, lipid peroxidation (LPO) and loss of antioxidant activity are considered as a mode of sublethal cryo-damage to human sperm during cryopreservation. The aim of this present study was to assess the effect of addition of GSH, acetyl-L-carnitine and ascorbic acid individually in freezing media at different concentrations on motility, DNA integrity, morphology and LPO status of cryopreserved human spermatozoa.

Materials and methods: Prospective study of semen samples of men attending the Andrology Laboratory. Each semen sample after liquefaction was divided into two equal parts (control and test). Neat semen samples were frozen in aliquots with cryoprotective agent (CPA, semen:CPA at 1:1 dilution) containing additives like acetyl-L-carnitine (final concentrations, 175 and 87.5 μ M, nos 23 and 20, respectively), reduced glutathione (final concentrations, 5 and 2.5 mM, nos 18 and 16, respectively), ascorbic acid (final concentrations, 150 and 75 μ M, nos 24 and 21, respectively) along with controls. DNA integrity was assessed both before and after cryopreservation using acridine orange fluorescence. After thawing, sperm motility and LPO were assessed in both controls and tests using Makler counting chamber and malonaldehyde assay (MDA), respectively.

Results: Cryoprotectant supplemented with acetyl-L-carnitine at a final concentration of 175 μ M was found to improve post thaw progressive motility from 16.4 \pm 11.9 (control) to 25.4 \pm 17.8% ($p < 0.05$) and normal DNA integrity from 59.3 \pm 20.5 (control) to 72.8 \pm 15.2% ($p = 0.007$). However, ALC did protect only DNA integrity when used at a final concentration of 87.5 μ M ($p < 0.05$). No improvement was noted in morphology or LPO values at both the concentrations. No difference of % motility, progressive motility, LPO status was observed between control and tests frozen with or without GSH at 5 and 2.5 mM concentrations. However, 5 mM GSH group showed 69.2 \pm 19.5% normal DNA integrity ($p = 0.030$) and 53.0 \pm 7.2% normal morphology ($p = 0.040$) compared to the control. There was no statistically significant improvement in post thaw sperm characteristics studied when ascorbic acid was included in the CPA at final concentrations of 150 and 75 μ M, respectively.

Conclusion: Since human sperm cryopreservation process reduces the innate antioxidant capacity of human semen and spermatozoa and thereby augmenting LPO and DNA damage, inclusion of antioxidants like GSH or ALC in freezing media may prove beneficial. Pre-freeze washing and direct insemination without processing the freeze-thawed samples have become common nowadays which may lead to fertilization by spermatozoa with fragmented DNA and thereby miscarriages. Samples frozen as neat semen with protective environment of seminal plasma along with supplemented antioxidants can withstand cryo injuries, especially during post thaw processing involving dilution and centrifugation steps.