(53.6%). By culturing the embryos 24 hours further 60.7% of the embryos cleaved after vitrification while 55.6% showed further cleavage with the slow-cooling method. As a result significantly more day 3 embryos were replaced after vitrification (45.6%) than after slow-cooling (30.5%). The pregnancy rate per transfer, although higher after vitrification (22.2%) was not significantly different as compared to that of slow-cooling (13.8%).

We further checked if there was a correlation between the efficiency of vitrification between day 3 and day 5–6 embryos. From a total of 690 embryos vitrified 193 day 3 embryos and 42 day 5–6 embryos were thawed. Of these 74.1% and 76.2% were recovered, respectively. 129 of 143 (90.2%) day 3 embryos survived and this was significantly higher compared to the blastocyst stage (14/32, 43.8%). Consequently, more embryos were available for transfer after day 3 thawing as compared to blastocysts. Pregnancy rate per transfer was higher (22.2%) with day 3 than with blastocysts (14.3%) cryoreplacement, but not statistically significant. Results will be updated in terms of implantation rates.

Conclusion: Vitrification can be a viable alternative to slow-cooling cryopreservation as far as day 3 and day 5–6 embryos are concerned. It is not time consuming and embryos can be stored in a closed system to prevent potential risk of exposure to contaminants in the liquid nitrogen. What further has to be resolved is the loss of embryos due to the method of sealing the cryotips by validation of the sealing procedure.

P-390 Poster Impact of embryo morphology on blastomere lysis and implantation rate of frozen-thawed transferred embryos

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Introduction: We evaluate the impact of embryo morphology on blastomere lysis and implantation rate of frozen-thawed transferred embryos.

Material and methods:

1. Evaluation of blastomere lysis rate of frozen-thawed embryos (n=590) within thawing procedure, according to their morphological aspect defined on the cryopreservation day (48 or 72 hours after fertilization) as follows: Type I: equal sized blastomeres without anucleate fragments (16,6% of freezing embryos), Type II: equal sized blastomeres with 1–20% fragments (54,7%), Type III: unequal sized blastomeres without fragments (16,8%), Type IV: unequal sized blastomeres with 1–20% fragments (11,9%). Frozen embryos had on-time cleavage chronology.
2. Evaluation of implantation rate of transferred frozen-thawed embryos (n=405) according their morphological type and their blastomere lysis rate (0%, 1–25%, 26–50%). Embryos were considered to be suitable for transfer when at least half of the initial number of blastomeres were intact. We could perform 254 thawing-cycles leading to 223 embryo transfers (T): 56 clinical pregnancies (P) were obtained (P/T: 25,1%) with a twin pregancy rate/P of 35,7% (20/56).

Results:

1. Embryo morphology and blastomere lysis within thawing procedure: Embryos showing <50% of blastomere lysis were more frequently observed when no anucleate fragments were present (75% of cases for Types I and III vs 65.1% for Types II and IV, $p<0,02$). Mean blastomere lysis rate was lower for type I et III embryos than for type II and IV (27,9% and 34,8% vs 40,4% et 46,7% respectively, $p<0,05$).
2. Implantation rate (IR) of transferred embryos Among the transferred embryos, 20,6% were type I, 53,4% type II, 17,2% type III and 8,8% type IV.

- When no blastomere lysis occurred, IR was of 19,6%, 22,3%, 25,6% and 10.7% for type I, II, III and IV embryos respectively.
- When blastomere lysis ranged between 1 to 25%, IR was of 21,4%, 21,2%, 23,1% and 0% for type I, II, III and IV embryos respectively.
- When blastomere lysis ranged between 26 to 50%, IR decreased to 3.6%, 14%, 5.5% and 6.6% for type I, II, III and IV embryos respectively.

Conclusion: After freezing-thawing procedure, embryos without anucleate fragments (types I and III) show a lower blastomere lysis rate than embryos with 1–20% fragments. Consequently, these embryos are more often suitable for transfer. Nevertheless, implantation rate is equivalent for transferred type I, II and III embryos. The highest IR is found when these embryos are fully intact or present blastomere lysis ranging from 1 to 25%. IR decreases dramatically when these three type embryos show 26–50% of blastomere lysis. On the other hand, IR of type IV embryos remain low whatever the degree of blastomere lysis. These data might be taken into account when implementing elective frozen-thawed embryo transfer policy.

ART, laboratory: cryopreservation of gametes

P-391 Poster Vitrification of human oocytes: high survival rate and healthy deliveries

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Introduction: Vitrification, an ultra rapid cooling technique, offers a new perspective in the attempts to develop the optimal cryopreservation procedure for the human oocytes and embryos. The method produces a glass-like solidification of cells, completely avoiding intra-cellular ice crystallization during the cooling and warming process. The introduction of such a technique could be performed without the use of expensive equipment and could be completed by one embryologist within a few minutes, providing significant benefits for any busy IVF program. The purpose of the present study was to assess the efficacy of the protocol published by Kuwayama et al. (2005) for human oocytes vitrification by using Cryotops, in the context of further standardization of the method.

Materials and methods: Out of 251 women (fresh Group), seeking pregnancy through IVF, from October 2004 to June 2006, 71 became pregnant after the first attempt; 60 although failed to become pregnant did not come for a second attempt;120 did not become pregnant, came later on for a second chance, to warm their 330 supernumerary vitrified oocytes, (vitrified/warmed Group) and were therefore included in the present study. The denuded Metaphase II oocytes (M II) were incubated in Equilibration Solution (ES) 7.5% Ethylene Glycol (EG) (Sigma-Aldrich, Steinheim, Germany) and 7.5% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) in Ham's F-10 media supplemented with 20% Serum Substitute Supplement (SSS, IrvineScientific, USA) for 5–15 min (according to the time needed for re-expansion of the vitrified material) at room temperature. After an initial shrinkage and recovery they were aspirated and placed into the Vitrification Solution (VS) (15% EG, 15% DMSO and 0.5 M Sucrose) (Merck, Darmstadt, Germany) for less than 60 sec at room temperature. After having observed that cellular shrinkage has been taken place, oocytes were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). No more than two oocytes were placed on each Cryotop. Cooling of the oocytes was done by direct contact with the liquid nitrogen (LN2). Warming of oocytes was performed by placing the Cryotop in Thawing Solution (TS) (1M Sucrose) for less than 60 sec at 37?C and then into Dilution Solution (DS) (0.5 M Sucrose) for three min. followed by other DS of 0.25 M Sucrose for three min. The warmed oocytes were placed 4–5 times into Washing Solution (WS) (Ham's F-10+20% serum) and then placed into the incubator. The ICSI procedure was performed 2 hours later for the survived oocytes. The embryo quality was scored according to Steer et al. (1992). Preparation of the endometrium for the embryo transfer was performed by the administration of the GnRH-A (starting from the 21st day of the previous cycle) and Estradiol Valerate (Schering, Italy) (2 mg three times a day, starting from the 3rd day of the next cycle). An endometrial thickness of 8 mm was considered to be optimal for performing an embryo transfer. Progesterone (Prontogest, AMSA- Italy) administration (50 mg daily) was started 72 hours prior to the embryo transfer.

Results: After the warming procedure 328 out of 330 oocytes survived, giving a survival rate of 99.3%.

The fertilization rate, pregnancy rate and implantation rate per embryo were 92.9%, 32.5% and 13.2%, respectively. Implantation rate per thawed oocyte was 11.8%. Cleavage, pregnancy and abortion rates between the fresh and vitrified/warmed groups were statistically not significant. Until today 12 healthy babies are born.

Conclusions: The results obtained by using this simple procedure has enabled us to achieve a very high rate of oocytes survival,embryo cleavage, pregnancies and healthy deliveries.

P-392 Poster Vitrification, but not slow freezing, maintains the meiotic spindle of sibling human oocytes

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Introduction: The ability to efficiently cryopreserve human oocytes will impact significantly on the practice of IVF. One could even argue that it is an absolute necessity, with governmental legislation in some countries imposing restrictions on the number of oocytes fertilized and embryo cryopreservation prohibited. Recently vitrification has been associated with successful oocyte cryopreservation, but results are still limited. In an attempt to minimize cytotoxicity to the higher concentration of cryoprotectant used in vitrification, most protocols are performed at room temperature. Exposing human oocytes to temperatures below 37°C, however, leads to rapid depolymerization of the microtubules, which compose the meiotic spindle. This may result with incorrect microtubule reformation upon warming, and subsequent mis-segregation of chromosomes. Studies have reported dissolution of the spindle during room temperature vitrification, but it is unclear whether this is due to the temperature at which cryoprotectant exposure and warming are performed or the cooling procedure per se. The objective of this study was therefore to initially assess the meiotic spindle of mouse oocytes, using the new generation Polscope (Oosight^{TM}), following exposure to the vitrification and warming solutions at room temperature (21–22°C) and 37°C. Analysis of the spindle in mouse and fresh human oocytes following the 37°C protocol and subsequent CryoLoop vitrification, was also performed. Finally to directly compare slow freezing and vitrification, sibling human oocytes were cryopreserved using the two techniques and their effect on the meiotic spindle measured immediately following thawing/warming.

Materials and Methods: Spindle images of F1 (C57BL/6×CBA) mouse metaphase II (MII) oocytes were recorded using a Polscope. For the initial set of experiments, images of the spindle were obtained before and after the exposure regimen at room temperature and 37°C. Vitrification of human and mouse MII oocytes was carried out using the CryoLoop. Images of the spindles were taken before vitrification/slow freezing and directly after the final step of the warming/thawing protocol. A retardance value for each spindle image was also recorded.

Results: When compared to before the treatment, mouse oocytes (n=11) exposed to the vitrification and warming solutions at room temperature had a significantly (p=0.0005) reduced spindle retardance (before: 1.87 nm ± 0.19; after: 0.92 nm ± 0.13). This was in stark contrast to oocytes that were treated with the same protocol, but performed at 37°C (n=14). Maintaining the oocytes at physiological temperature during the exposure regimen did not impact statistically (p=0.20) on the retardance of the meiotic spindle (before: 1.71 nm ± 0.17; after: 1.97 nm ± 0.18). Since exposure to the cryoprotectants at 37°C did not significantly alter the spindle, both human and mouse oocytes were subsequently vitrified using the CryoLoop. Following warming, neither human (n=29) or mouse oocytes (n=16) showed statistically different spindle retardance when compared to the value obtained before vitrification. When sibling human oocytes were split between slow freezing (n=7) and vitrification (n=11), the spindle was completely disrupted in all slow frozen oocytes. As before, vitrified oocytes maintained their meiotic spindles (before: 1.55 nm ± 0.09; after: 1.38 nm ± 0.07).

Conclusions: This study on sibling human oocytes reveals that slow freezing disrupts the meiotic spindle, which is in contrast to vitrification. Rather than subjecting the oocytes to prolonged periods at non-physiological temperatures, vitrification can be carried out at 37°C, which does not significantly alter the spindle in either human or mouse MII oocytes. This is of clinical importance since maintaining the meiotic spindle will reduce the chances of chromosomes mis-segregation leading to poly- or aneuploidy. It also means that vitrification permits insemination soon after warming, minimizing oocyte ageing, which is a concern when treating cryopreserved oocytes.

P-393 Poster Oocyte spindle preservation during slow-rate freezing

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Introduction: During oocyte slow freezing, the intracellular ice formation, cryoprotectant toxicity, osmotic stress, non-physiological low temperatures, and pH instability are factors believed to affect the structure of meiotic spindle. Additionally, meiotic spindle is extremely sensitive to the temperature decrease, which has been reported to result in significant spindle disruption. However, the timing and mechanisms of oocyte spindle disruption is still unclear in oocyte freezing and thawing. Therefore, our objective was to investigate the specific changes of oocyte spindle submitted to severe challenges of low temperature with oocyte cryopreservation. To investigate the detail change of meiotic spindle, spindles were examined before, during, and after oocyte cryopreservation in a standardized timely manner. The information herein may provide imperative allusions toward the prevention of spindle disruption on oocyte freezing and thawing.

Material and Methods: All experiments were performed using 8-wk-old BDF1 mice. Superovulation was induced with 7.5 I.U. equine chorionic gonadotrophin (eCG) followed 48 h later with 7.5 I.U. human chorionic gonadotrophin (hCG). After 14 h of hCG, oocytes were freed of cumulus cells by brief exposure to 300 IU/ml of hyaluronidase at 37°C and gentle pipetting. Oocytes were then frozen by slow-rate freeze method with 1.5 M propanediol and 0.3 M sucrose in sodium-depleted solution and thawed by serial dilutions of sucrose (0.5 M, 0.2 M, and 0 M; 10 min for each). To examine the spindle, the oocytes were fixed at 20 min equilibration of freezing solution, 0 min of thawing (thawing frozen oocytes in fixative), and sequential time points during thawing (10 min, 20 min, and 30 min) and after thawing (30 min, 1 h, 2 h, and 4 h), and stained with propidium iodide and FITC-conjugated anti-?-tubulin antibody by confocal microscopy.

Results: A total of 255 oocytes were included in the present study. After 20 min exposure to cryoprotectant in freezing solution, all the examined oocytes were still shown bipolar spindles with metaphase plate alignment (35/35). All oocytes (31/31) sustained a morphologically correct spindle at 0 (zero) min after thaw, in spite that they have been through deep freezing temperature (−196°C). However, the structure of spindle started to diminish during the serial dilution when thawed in 0.5 M (22/22), 0.2 M (19/19), and 0 M (23/23) of sucrose (corresponding to the sequentially elapsing time after thaw). Nine out of 29 oocytes with completely disappeared spindles were found (9/29) when oocytes were incubated in 37°C for 30 min after thawing, and partially disorganized spindles were shown in the rest of them (20/29). The proportion of bipolar spindle organization started to increase gradually when the oocytes recovered in the culture system after thawing 1 h (23/30), 2 h (26/32), and 4 h (34/40).

Conclusions: This study demonstrated that the spindles were well preserved until the thawing process, even though oocytes were exposed to highly subphysiological temperatures for substantial time. This observation indicates that cryoprotectants of freezing solution were able to maintain the spindle structure through the freezing process. The significant alteration of spindle integrity occurred during or after thawing, and it reflects the fact that spindle disorganization is still not avertable in the current setting of cryopreservation. However, our current study may provide clues on how the disruption of oocyte spindle may be prevented by performing modifications on the thaw protocol which can help to improve efficiency of the slow-freezing cryopreservation.

P-394 Poster Artificial microcontainers for cryopreservation of solitary spermatozoa

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Introduction: Some cases of male infertility caused by severe oligozoospermia (cryptozoospermia) or teratozoospermia only leave a few spermatozoa suitable for ICSI fertilization. Isolation of functional gametes from testicular or epididymal aspirates requires considerable workload in azoospermia, while multiple

surgical invasions may entail negative consequences. These problems can be solved by sperm cryopreservation, but there is a high risk of losing unique genetic material in the course of freezing, storing, and thawing. Placing small groups of spermatozoa in microcontainers eliminates these risks and makes conventional cryopreservation protocols applicable. Use of the cryopreservation technique utilizing empty *zonae pellucidae* (ZP's) of human or animal oocytes, which was proposed by J. Cohen in 1997, is limited in common clinical practice due to its potential infection and contamination implications. Furthermore, preparing ZP's requires individual microsurgical evacuation of oocyte cytoplasm; spermatozoa can get stuck on the internal ZP surface; while some substances found in ZP's might induce an acrosomal reaction.

Material and methods: Presented is a technique allowing to obtain an artificial ZP analogue, empty microspheres made of agarose gel and used for cryopreservation of solitary spermatozoa. Such agarose microspheres are inactive and sterile biologically. We used microspheres made of 2% agarose gel and measuring approximately 100 μm in diameter. Motile spermatozoa left after ICSI procedures performed in 18 patients with severe oligoteratozoospermia, were placed in described microspheres (1 to 10 in each), in the same manner as in ICSI and using the same microinstruments, in 10% PVP solution. Microspheres loaded with spermatozoa were then placed in a 1:1 solution, Sperm Preparation Medium (MediCult, 1069/1070) and Sperm Freezing Medium (MediCult, 10670005/10670010), for 5 min. Afterwards, 1 to 5 injected microspheres were put into 250 μL plastic straws, and these straws were frozen in liquid nitrogen evaporation for 10 min and then placed in liquid nitrogen. The straws were thawed at room temperature. Microspheres were washed in five Sperm Preparation Medium drops and incubated at +370C for an hour. Spermatozoon viability was assessed by motility recovery, as well as in an eosin supravital test. There was no medical fertilization of human oocytes in these sperm studies.

Results: In total, 318 motile spermatozoa were frozen in 67 microspheres and 19 straws. Two out of 67 microspheres (3%) containing 7 spermatozoa (2% of the total amount) were lost following thawing. Out of 243 remaining spermatozoa, 311 (78%) recovered motility after incubation. The eosin supravital test demonstrated that 81% of these spermatozoa (251/311) had preserved membrane integrity.

Conclusion: A new technique is presented (The EAPO Pat.No007992. 05.10.2006) allowing to obtain and utilize sterile and biologically safe empty microspheres for efficient cryopreservation of solitary spermatozoa in IVF-ICSI programmes.

P-395 Poster Comparation between human oocyte freezing by vitrification and slow freezing

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Introduction: Cryopreservation of oocytes is highly desirable for severals reasons (preservation of fertility before radio- and chemotherapy, syncronization of donor-recipient, solution of ethical and religious problems of embryo storage). The aim of our study was to compare three different protocols of oocytes freezing.

Materials and Methods: 151 immature oocytes (VG, MI stage) assessed the day of the pick-up without granulosa cells obtained from 68 patients undergoing an ICSI programme were randomly divided for freezing into three groups with different protocols.

For the first and the second group the protocol consisted in a slow freezing method (ice nucleation was induced manually at−7.0°C).

After an equilibration period at room temperature, oocytes in the second solution were loaded in 0.25 ml plastic straws in varius ways (maximum of three oocytes per straw).

In the first group (group A), the oocytes were frozen in a solution of 1.5 mol/l 1,2-Propanediol (PrOH)+0.2 mol/l sucrose; in the second group (group B) the oocytes were frozen in a solution of1.5 mol/l Propanediol (PrOH)+137 mmol/l Choline chloride (ChCl)+0.2 mol/l sucrose.

The third group (group C) had a very short incubation (<30s) in the final vitrification solution prior to being placed in liquid nitrogen without a programmed freezer.

After an equilibration period, oocytes in the second solution were loaded in cryotips and plunged into liquid nitrogen then stored in tanks.

In the third group the oocytes were frozen in a solution of 15% w/v DMSO+15% w/v Ethylene glycol+0.5 mol/l sucrose (vitrification solution) A minimum of 7 days storage elapsed before thawing. The cryoprotectants were removed at room temperature and the oocytes were cultured at 37°C in an atmoshere of 5%CO2. Oocytes were checked for survival 1 h after thawing.

Results: Survival rate for the VG was respectively 80.0% (n=24) for slow freezing-PrOH, 66.6% (n=23) for slow freezing-ChCl and 90.1% (n=38) for Vitrification. Survival rate for the MI was respectively 66.6% (n=23) for slow freezing-PrOH, 40% (n=18) for slow freezing-ChCl and 71.4% (n=28) for Vitrification. VG oocytes were significantly more resistant to the freezing procedure than the MI oocytes regardless of which protocol was used (p=0.01). At each oocyte maturation stage, the vitrification protocol yielded a better survival rate than the slow freezing protocols, although the difference was not significant.

Conclusions: VG oocytes had a better survival rate than the MI oocytes after freezing/thawing. Better results were obtained with vitrification method in our preliminary study. Results will be confirmed in larger groups of samples.

P-396 Poster Solid surface vitrification (SSV) versus slow programmable freezing of human spermatozoa

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Introduction: The method of sperm freezing and thawing, that optimizes motility recovery has not been firmly established. The aim of this study was to compare the efficacy of solid surface vitrification (SSV) and slow programmable freezing of human spermatozoa.

Materials and methods: Semen samples from 30 infertile men with normal sperm count were processed through density gradients. The final pellet was re-suspended and divided into 3 three aliquots: non-frozen control, SSV and slow programmable freezing. In slow programmable freezing, the aliquot was mixed with an equal volume of SpermFreeze (Fertipro, Belgium) at room temperature for 10 minutes. The sperm in cryopreservation medium was loaded into a 0.25-mL straw and put into a programmable freezer. The temperature was decreased at −1C/min from 20C to 5C, hold for 5 minutes, and decreased at −10C/m to −85C. The straw was then taken out of the programmable freezer and dip into a liquid nitrogen tank. In SSV, the aliquot was mixed with an equal volume of cold cryopreservation medium at 4C for 10 minutes. The SSV medium was a modified human sperm preservation medium (HSPM), in which sucrose was replaced by trehalose, and the concentrations of both glycerol and glycine were decreased, together with an increase in the concentration of human serum albumin). The mixture was loaded into a 0.25-mL straw and inserted into a hole in a pre-cooled home-made aluminum block in liquid nitrogen. After 1 week of storage, the samples were thawed in running tap water. Post-thaw samples were assessed for sperm motility and kinematics (Clinical human motility version 12, HTM-IVOS, Hamilton Thorne Biosciences), morphology by strict criteria (Dimensions Morphology, HTM-IVOS), vitality by hypo-osmotic swelling test and supra-vital staining (Eosin Y), and compared with non-frozen control. DNA integrity was assessed by a single cell gel electrophoresis and analyzed by LUCIA Comet Assay program, Laboratory Imaging). Sperm binding was assessed by a hemizona assay.

Results: Post-thawed sperm motility in the SSV group was significantly higher than that in the slow programmable freezing group, but lower than the non-frozen control (50.9%, 31.9% and 72.7%, respectively; p<.001). Sperm vitality was significantly higher in the SSV than the SPF groups, but lower than the control (hypo-osmotic swelling test 60.1%, 44.1% and 77.9%; Eosin Y staining 63.7%, 49.5% and 81.8% for SSV, SPF and control, respectively; p<0.001). There was no significant difference in post-thawed sperm morphology (14.9%, 14.4% and 16%) and sperm head DNA integrity by comet assay (92.7%, 94.3% and 93.1%) in the SPF, SSV and controls. Hemizona assay showed no significant difference in hemizona index (HZI) in the SPF and the SSV groups (p=0.771).

Conclusions: SSV gave superior postthaw sperm motility and cryosurvival compared with conventional SPF. Further study should be done in oligospermic samples.

P-397 Poster Super-rapid cooling using slush nitrogen (SN2) may improve the efficacy of human oocytes after vitrifying/warming

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Introduction: Attempts to develop an human oocyte cryopreservation method have extensively been made after the first success of Chen (1986), who reported on the first live births from frozen human mature oocytes. The clinical application is still limited because of poor viability and quality of oocytes after thawing. The ultrastructure of oocytes is particularly sensitive to the changes of temperature and extracellular osmotic pressure during freezing and thawing, various cellular damage such as cytoskeleton disorganization, chromosome and DNA abnormality, spindle disintegration, premature cortical granule exocytosis related zona pellucida hardening, plasma membrane disintegrity, were also observed. The aim of this study was to improve efficacy of oocyte after vitrifying/warming by applying super-rapid cooling using slush nitrogen (SN2).
Material and Methods: Human fertilization-failed oocytes (FFOs) and immature oocytes were obtained from patients undergoing IVF-ET program under IRB approval. Those oocytes with normal morphology under microscopic observation were chosen for cryopreservation. The oocytes were pre-equilibrated with 1.5M ethylene glycol (EG) for 2.5 min and then equilibrated with 5.5M EG and 1.0M sucrose for 20sec. The oocytes were loaded on to electron microscopic grids and plunged in to SN2 or liquid nitrogen (LN2), and then stored in LN2. Stored oocytes were warmed by a five-step method, and then their survival was observed. Meiotic spindles were observed in these living metaphase II oocytes before vitrification and after warming procedures with the use of a computer-assisted polarization microscopy system (Polscope). Also, apoptosis of cumulus cell (CC) after thawing were examined under microscopic observation and TUNEL assay.
Results: Survival rate after warming of unfertilized ooytes vitrified using SN2 was high compared with those using conventional LN2. And, after 3 hours of warming, the degeneration and apoptosis in CC obtained from immature oocytes in SN2-vitrification was significantly lower than those in CC from in conventional LN2-vitrification. However, there had been no significant difference on the structure of their meiotic spindle between LN2-vitrification and SN2-vitrification groups after warming.
Conclusions: Super-rapid cooling using SN2 may improve the clinical efficiency of human oocyte vitrification and would be a valuable tool for human assisted reproductive technology.

P-398 Poster Factors affecting the outcome of oocyte freezing

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Introduction: The cryopreservation of human oocytes has been proposed as an alternative to embryo freezing. Although the theoretical advantages of this approach are undeniable, the corresponding clinical outcome is still by far inferior to that reported after zygote and embryo thawing. Nonetheless, the interest in oocyte cryopreservation is increasing, especially in those countries where it represents the only option, being zygote and embryo freezing forbidden by local regulations. This promoted the research on factors and strategies that could improve the overall outcome of oocyte freezing. The present study reports on the pregnancy and implantation rate obtained by thawed oocytes in relation to the timing at which the freezing was performed.
Materials and Methods: Between March 2004 and December 2006, 379 thawing cycles were analyzed. Oocytes were cryopreserved using a slow freezing protocol with 1.5M propanediol in 0.3M sucrose. According to the national legislation on IVF, a maximum of three oocytes were inseminated per patients and all the generated embryos were transferred. The results were analyzed retrospectively according 1) to the hours post HCG at which oocyte freezing was performed, and 2) the number of transferred embryos. The thawing cycles were arbitrarily divided in Group 1, 88 cycles, in which the oocytes were frozen less than 40 hours after HCG injection, and Group 2, 291 cycles, in which the oocytes were frozen between 40 and 47 hours post-HCG.
Results: The mean maternal age was similar in the two groups. In the same way, survival, fertilization and cleavage rates showed comparable figures in Group1 (72%, 72% and 89% respectively) and Group 2 (73%, 74% and 88% respectively). The clinical pregnancies were significantly higher in Group 1 (n=22, 29% per transferred cycle, 25% per thawed cycle) than in Group 2 (n=30, 13% per transferred cycle $P < 0.005$, 10% per thawed cycle $P < 0.001$) with an implantation rate of 18.4% and 6.9% respectively ($P < 0.001$). The incidence of spontaneous abortion was 14% in Group 1 and 33.3% in Group 2, leading to an ongoing pregnancy rate of 25% and 9% per transferred cycle respectively, $P < 0.001$. The highest pregnancy rate per transfer was obtained by transferring three embryos both in Group 1 (71% in 17 transferred cycles with an implantation rate of 33.3%) and Group 2 (28% in 50 transferred cycles with an implantation rate of 12%). These differences between the two groups were statistically significant ($P < 0.005$ for both the pregnancy and the implantation rate), while the mean maternal age was comparable. Conversely, when one or two embryos were transferred, the clinical outcome between the two groups did not reach a statistical significance.
Conclusions: According to the present results, the timing of oocyte cryopreservation seems to play a key role in determining the potential development after thawing. The suggested cut-off is between 39 and 40 hours post-HCG, beyond which a significant decrease in embryo implantation is detected. Anticipating the timing of freezing, could predisposes the oocyte to the best conditions for the insemination after thawing. In addition, in consideration of the 3 hours approximately elapsing between thawing and insemination, this strategy could prevent from handling an ageing oocyte.

P-399 Poster Is the pregnancy rate from frozen-thawed oocytes better than from frozen-thawed embryos?

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Introduction: Cryopreservation of oocytes is a technique developed in the early 80's, but only in the late 90's it has been used for many laboratories. The literature presents a great variety of clinical pregnancy rates when frozen-thawed oocytes were transferred. The purpose of this study was to compare the survival, and clinical pregnancy rates of frozen-thawed embryos to frozen-thawed oocytes.
Material and Methods: A total of 52 cycles using frozen-thawed oocytes were compared to 188 cycles using frozen-thawed embryos at Fertilitat–Center for Reproductive Medicine- Brasil during 2004 to 2006. After extensive orientation, the appropriate consent forms were signed. The oocytes not inseminated, were frozen following the Stachecki et al., (Reproductive Biomedicine Online, 11:711–715, 2005) protocol. When the couple decided to thaw their oocytes then the endometrium was prepared using Estradiol Benzoate with an initial dose of 2 mg/day, starting on the 3rd day after bleeding induced by hypophiseal down regulation with nafarelin acetate. The maximum dosage used was 6 mg/day when the endometrium reached a thickness of 8 mm by ultrasound. Three days before the embryo transfer, the patient started using 90 mg progesterone gel daily. Two days before the replacement of the embryos, the oocytes were thawed following again Stachecki's et al., (Reproductive Biomedicine Online, 11:711–715, 2005) protocol. After thawing, the embryos were kept in a 5% CO2 incubator humidified in air, at 37°C during 2 hours before inseminating by ICSI. The cleavage embryos, from 188 cycles, were frozen on the 3rd day after fertilization. The embryos were frozen in 1.5M PrOH and 0.1M of sucrose. When the couple decided to do the FET procedure then, the embryos were thawed in 3 steps, reducing the PrOH and sucrose concentration until their total withdrawn. Following thawing, the embryos were kept in a 5% CO2 incubator humidified in air, at 37°C during 2 hours before transferring. The transfers were performed with Frydman catheter, under ultrasound control. The survival, and clinical pregnancy rates were compared between frozen-thawed embryos and frozen-thawed oocytes. Statistical analysis was performed by chi-square test ($p > 0.01$).
Results: The table shows the results using frozen-thawed embryos and oocytes.

Frozen-thawed	Cycles	Thawed	*Survival (%)	Clinical Pregnancy (%)
Embryos	188	698	534 (77.0)	38 (20.2)
Oocytes	52	486	281 (57.8)	15 (28.8)

*$p < 0.01$

Conclusion: These results showed that although the embryos had a better survival rate, the pregnancy rate was similar for frozen-thawed embryos and oocytes. Demonstrating that the technique for frozen–thawed oocytes is as efficient as the embryo freezing technique. Therefore the patients may choose to freeze oocytes in order to avoid embryo storage keeping similar pregnancy rate.

ART, laboratory: cryopreservation of gonads

P-400 Poster Which tools for evaluating survival and quality of ovarian tissue after cryopreservation?

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Introduction: Cancer treatments, like radiotherapy or chemotherapy can induce premature menopause in women in reproductive age. Ovarian cryopreservation is a way to preserve female gametes and to restore ovarian function and fertility once these patients have been treated. The main problem of cryopreservation is to evaluate the follicles survival and the quality of the ovarian tissue during freezing and thawing protocols. This study investigates the possible predictive value of viability assay, the release of intracellular enzyme lactate dehydrogenase (LDH) as marker of cytotoxicity, the DNA fragmentation and the morphology of follicles of cryopreserved sheep ovarian tissue.
Materials and methods: For assessment of the follicle viability, small follicles (<60 μm) were isolated enzymatically for viability testing. Dead and alive follicles were identified by using trypan blue staining, calcein / ethidium staining; and follicle morphology was examined by histology. For evaluation of the quality of the whole ovarian tissue, the release of LDH was measured; the DNA fragmentation by TUNEL technique in primordial and primary follicles and the morphology of these follicles after freezing and thawing were observed.
Results: No statistically significant difference in follicle viability with trypan blue and calcein / ethidium in fresh and cryopreserved ovarian tissue was observed. There was a significant correlation between the percentage of normal follicles and the rate of viability, either with trypan blue ($R=0.82$, $p<0.05$) or with the calcein /ethidium ($R=0.76$, $p<0.05$) after cryopreservation.

The release of LDH showed an increase in the percentage of cytotoxicity after freezing ($52.2\% \pm 7.7\%$) vs ($21.6\% \pm 1.1\%$) for controls. There was a significant negative correlation between the percentage of follicles with normal morphology and the percentage of cytotoxicity ($p<0.05$). The result of the TUNEL technique showed no significant difference, but the percentage of the TUNEL-positive follicles was slightly higher in the frozen fragments ($38\% \pm 4.5\%$), than in the control group ($26\% \pm 8.2\%$).
Conclusion: For evaluation survival and quality of cryopreserved ovarian tissue, the histological study with viability assay using trypan blue staining must be preferred. LDH release must be done in order to measure cell damage in the whole ovarian tissue and finally DNA fragmentation allows the validation of different freezing/thawing protocols.

P-401 Poster Successful ovarian tissue vitrification in mouse, bovine and human

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Introduction: Recent drastic advances have enabled the cryopreservation of mammalian gametes. Improved "vitrification" methods, have made it possible to preserve pre-fertilized oocytes with little loss of viability. Using a highly efficient vitrification method with ultra rapid cooling (Cryotop), we have established very successful human oocyte banks world wide and have obtained more than 300 babies so far to preserve the fertility of cancer patients after BMT. To preserve fertility in female cancer patients, oocyte vitrification is one solution. However, it does not work for the children of the urgent patients who does not have enough time for one or more IVF cycles. Ovarian tissue cryopreservation has the potential to solve these problems, and to preserve their natural fertility after chemo- and radiotherapy. Similarly to oocyte freezing, ovarian tissue cryopreservation has utilized a conventional slow freezing method, resulting in limited success. However, encouraging good results with ovarian tissue freezing are recently being obtained by using vitrification. Based on the high efficiency vitrification Cryotop method, we have endeavored to develop a practical vitrification method for mouse, cattle and human ovarian tissues.
Material and methods: In mouse, whole, half and 0.2 mm cubes of BDF1 mouse ovary were vitrified according to the Cryotop method (Kuwayama 2005). Ovarian tissues were first equilibrated in m-199 containing 7.5% EG and 7.5% DMSO and vitrified with m-199 containing 15% EG, 15% DMSO and 0.5M sucrose using Cryotop (Kitazato BioPharma, Japan). After thawing samples by plunging in m-199 containing 1M sucrose for 1 min, cryoprotectants were diluted out with 2 steps using m-199 containing 0.5 and 0 M sucrose. Growing oocytes in preantral follicles of vitrified-thawed ovarian tissue were stained by Hoechst and PI to assess their survival. Bovine ovarian tissues were vitrified according to the Cryotop method as an animal model for human clinical use because of the similar size and structure of the ovary. The size of the ovarian tissue was $1\ cm \times 1\ cm$ according to the successful human ovarian tissue transplantation between twins (Silber 2005).
Results: One hundred percent post-thaw survival was obtained for the oocytes of ovarian tissue. Vitrified ovarian tissue was successfully transplanted into the kidney of SCID mouse with normal growth of follicles. Full grown oocytes were obtained from the allotransplant and then matured, fertilized and cultured in-vitro, resulting in normal young after embryo transfer to the recipient. Eighty–eight percent survival was obtained for bovine ovarian tissue after vitrification. In total 8 ovarian tissues were successfully autotransplanted to 4 cattle. Human ovarian tissue from a cancer patient, and from donors for ovarian transplantation (with informed consent) was vitrified according to the cattle method. After thawing, the same high post-thaw survival (89%) was obtained.
Conclusion: These results indicate that the ultra rapid cooling vitrification method in the present study has a potential for clinical use in human ovarian tissue cryopreservation.

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P-402 Poster Perfusion conditions in cryopreservation of complete bovine ovaries

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Introduction: Cryopreservation of an intact ovary (with vascular pedicle) for fertility preservation in cancer patients has the best prognosis for restoring fertility after sterilizing cancer treatment. However, successful freezing and thawing of whole ovaries represents an immense technical challenge. Recently, perfusion and subsequent cryopreservation of human ovaries was described for the first time. Our objective is to optimize the technique to make it an efficacious and safe procedure. We used bovine ovaries to test vessel perfusion, which is crucial for infusing the ovary with a cryoprotectant agent as well as for reperfusion of the ovary after thawing.
Material and methods: Ovaries were obtained from freshly slaughtered cows at a local abattoir. The vascular pedicle was prepared and the A. ovarica cannuled with a venflon, which was anchored with a 2-0 suture. Vessels were flushed with physiological salt with 5000 U/ml heparin and/or with Indian ink with 5000 U/ml heparin for 5 minutes, with a peristaltic pump at 2.5 ml/minute. The ovaries were then fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were cut and stained with haematoxylin and eosin for histological examination. Additional slides were stained with anti-Factor VIII antibody to highlight endothelial cells.