La crioconservazione


Cryopreservation of human oocytes and embryos is a necessary tool in assisted reproduction treatment that leads to an increased cumulative outcome while decreasing costs. Vitrification is a cryopreservation technique that leads to a glass-like solidification, with rapid cooling of cells or tissues. Nowadays vitrification is claimed to be the future of cryopreservation of human embryos due to improved survival rates and clinical outcomes. This study was conducted at a university clinic to assess the safety and efficiency of vitrification of human zygotes as a routine procedure. A total of 849 pronuclear-stage (PN) zygotes were vitrified between March 2004 and July 2006. During this period, 103 cycles of cryopreserved embryo transfer were completed. In total, 339 PN zygotes were thawed resulting in an 89% survival rate (302 PN zygotes). The mean number of embryos per transfer was 2.2. The pregnancy rate obtained was three times higher (36.9%) than that obtained with the slow-rate freezing method (10.2%) used previously in the same centre. In conclusion, vitrification of human zygotes at the pronuclear stage seems to be a successful and reliable method with favourable outcomes and can be recommended as a routine technique for cryopreservation of human embryos.

M. Antinori, E. Licata, G. Dani, F. Cerusico, C. Versaci, S. Antinori, Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries, in “Reproductive Biomedicine Oline”, 2007, Jan, 14 (1) pp.72-79

Vitrification, an ultra-rapid cooling technique, offers a new perspective in attempts to develop an optimal cryopreservation procedure for human oocytes and embryos. To further evaluate this method for human oocytes, 796 mature oocytes (metaphase II) were collected from 120 volunteers. Since Italian legislation allows the fertilization of a maximum of only three oocytes per woman, there were 463 supernumerary oocytes; instead of being discarded, they were vitrified. When, in subsequent cycles, these oocytes were utilized, 328 out of 330 (99.4%) oocytes survived the warming procedure. The fertilization rate, pregnancy rate and implantation rate per embryo were 92.9, 32.5 and 13.2% respectively. Thus, as already reported in the literature, the vitrification procedure seems to be highly effective, safe (since healthy babies have been born) and easy to apply. In situations where embryo cryopreservation is not permitted (as in Italy), there is now good indication for routine application of the method, once further standardization is achieved.

A. Cobo, M. Kuwayama, S. Pérez, A. Ruiz, A. Pellicer, J. Remohi, Comparison of concomitant outcome achieved served donor oocytes by the Cryotop method, in “Fertility and Sertility”, 2008, Jun; 89 (6), pp.1657-1664

Objective: To evaluate the outcome of oocyte vitrification using the Cryotop method, observed in an egg donation program by simultaneously evaluating embryos derived from vitrified and fresh oocytes coming from the same stimulated cycle. DESIGN: Cohort prospective randomized study. Setting: Instituto Valenciano de Infertilidad (IVI) Valencia,
Spain. **Patient(s):** Thirty oocyte donors and 30 recipients with informed consents. **Intervention(s):** Vitrification by the Cryotop method. Warming 1 hour after vitrification. Microinjection of surviving MII and fresh oocytes, evaluation of fertilization, embryo development, and clinical results. **Main outcome measure(s):** Survival, fertilization, and cleavage rate. Embryo quality, pregnancy rate (PR), and implantation rate. **Result(s):** Survival rate observed was 96.7%. There was no difference in fertilization rates (76.3% and 82.2%), day 2 cleavage (94.2% and 97.8%), day 3 cleavage (80.8% and 80.5%), and blastocyst formation (48.7% and 47.5%) for vitrified and fresh oocytes, respectively. Embryo quality on day 3 and on day 5-6 were similar for vitrification and fresh oocyte group (80.8% vs. 80.5% and 81.1% vs. 70%, respectively). A total of 23 embryo transfers were carried out in the vitrification group. Pregnancy rates, implantation rates, miscarriage rates, and ongoing PR were 65.2%, 40.8%, 20%, and 47.8%, respectively. **Conclusion(s):** The Cryotop method preserves the potential of vitrified oocytes to fertilize and further develop, which is similar, when evaluated simultaneously, to fresh counterparts. Excellent clinical outcome indicates the possible use of this technology for egg donation programs, as well as a high potential for establishing oocyte banking.


Cryopreservation of human gametes and embryos has become an essential part of assisted reproduction. Successful cryopreservation of human blastocysts is increasingly relevant as extended in-vitro culture of human embryos becomes more common, permitting routine use of blastocyst transfer in IVF programmes. This reduces the number of embryos transferred, thereby reducing multiple pregnancies and maximizing cumulative pregnancy rates per oocyte retrieval. The superiority of blastocyst freezing over earlier stage freezing in terms of implantation per thawed embryo transferred improves overall expectations for the cryopreservation programme. Therefore, a reliable procedure for the cryopreservation of blastocysts is needed because, after transfer, only a small number of supernumerary blastocysts are likely to be available for cryopreservation. Since the early 1980s, two common techniques have been used in cryopreservation: the conventional slow cooling method and the more recent rapid procedure known as vitrification. Vitrification has become an attractive alternative to slow freezing, since it appears to result in significantly higher survival and pregnancy rates. The aim of this review is to focus on the cryopreservation of human blastocysts using slow and rapid protocols and to assess the impact of the cryopreservation protocol used on the survival, implantation and pregnancy rates.


**Background:** Recently, interest in oocyte cryopreservation has steadily increased. Newly developed protocols have dramatically improved survival rates, removing perhaps the major hurdle that has prevented this approach from becoming a fully established form of treatment. However, the clinical efficiency of these protocols has not been exhaustively explored and therefore remains controversial. **Methods:** Morphologically normal oocytes
displaying the first polar body were frozen–thawed with a slow cooling protocol that utilized 1.5 mol/l propane-1,2 diol (PrOH) and 0.3 mol/l sucrose. **Results:** A total of 927 oocytes from 146 patients were frozen–thawed, achieving a 74.1% survival rate. Over 76% of microinjected oocytes displayed two pronuclei 16 h post-insemination, while the proportion of embryos at 44–46 h post-insemination was 90.2%. At this time point, the majority (68.3%) of embryos were at the two-cell stage, showing in most cases (78.7%) minimal or moderate fragmentation. Eighteen clinical pregnancies, three of which were twin, were observed, giving rise to rates of 12.3 and 9.7%, calculated per patient and per embryo transfer, respectively. The implantation rate was 5.2%. To date, four children have been born and three pregnancies resulted in spontaneous abortions, while the remaining pregnancies are ongoing. **Conclusions:** Our data indicate that although the combination of slow cooling and high sucrose concentration ensures high rates of oocyte survival, it is not sufficient to guarantee a high standard of clinical efficiency.

G. Coticchio, L. De Santis, G. Rossi, A. Borini, D. Albertini, G. Scaravelli, C. Alecci, V. Bianchi, S. Nottola, S. Cecconi, Sucrose concentration influences the rate of human oocytes with normal spindle concentration and chromosome configurations after slow-cooling cryopreservation, in “Human Reproduction”, 2006, num. 21, pp. 1771-1776

**Background:** Recently described slow-cooling cryopreservation protocols involving elevated sucrose concentration have improved survival frequencies of human oocytes, potentially overcoming a major hurdle that has limited the adoption of oocyte storage. Because implantation rates of embryos from frozen oocytes remain generally low, it is still debated whether, irrespective of survival rates, this form of cryopreservation leads inevitably to the disruption or complete loss of the metaphase II (MII) spindle. **Methods:** Human oocytes with an extruded polar body I (PBI) were cryopreserved using a slow-cooling method including 1.5 mol/l propane-1,2-diol (PrOH) and alternative sucrose concentrations (either 0.1 or 0.3 mol/l) in the freezing solution. Fresh control and frozen-thawed survived oocytes were analysed by confocal microscopy to evaluate MII spindle and chromosome organizations. **Results:** Of the 104 oocytes included in the unfrozen group, 76 (73.1%) displayed normal bipolar spindles with equatorially aligned chromosomes. Spindle and chromatin organizations were significantly affected (50.8%) after cryopreservation involving lower sucrose concentration (61 oocytes), whereas these parameters were unchanged (69.7%) using the 0.3 mol/l sucrose protocol (152 oocytes). **Conclusions:** Partial disruption of the MII spindle and associated chromosomes accompanies inadequate cryopreservation during slow cooling. However, protocols adopting higher sucrose concentration in the freezing solution promote the retention of an intact chromosome segregation apparatus comparable in incidence to freshly collected oocytes.

M. Kuwayama, Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method, in “Theriogenology”, 2007, num 67, pp.73-80

Vitrification is frequently referred to as a novel technology of cryopreservation in embryology, although some young embryologists were born after its first successful application. Unfortunately, in spite of the accumulated evidence regarding its enormous potential value, most domestic animal and human laboratories use exclusively the
traditional slow-rate freezing with its compromised efficiency and inconsistency. The purpose of this paper is to clarify terms and conditions, to summarize arguments supporting or disapproving the use of vitrification, and to outline its role among assisted reproductive technologies. To provide evidence for the potential significance of vitrification, achievements with the Cryotop technology, an advanced version of the “minimal volume approaches” is analyzed. This technology alone has resulted in more healthy babies after cryopreservation of blastocysts than any other vitrification technique, and more successful human oocyte vitrification resulting in normal births than any other cryopreservation method. The value of this method is also demonstrated by achievements in the field of domestic animal embryology. A modification of the technique using a hermetically sealed container for storage may help to eliminate potential dangers of disease transmission and open the way for widespread application for cryopreservation at all phases of oocyte and preimplantation embryo development in mammals.


**Background:** The aim of the present study is to investigate cryopreservation of oocytes in patients refusing embryo cryopreservation for ethical reasons, patients from whom no sperm could be retrieved and patients with enough oocytes to yield a number of fresh and cryopreserved embryos to transfer. **Methods:** A total of 2900 oocytes out of 6216 retrieved were cryopreserved in 286 patients undergoing 303 cycles. The reasons for cryopreservation were because no sperm was found in 16 cycles, for ethical or personal reasons in 80, and in 207 only supernumerary oocytes were frozen. In 159 cycles, the oocytes were thawed and the surviving metaphase II oocytes microinjected. **Results:** A total of 1087 oocytes were thawed, 760 (69.9%) survived and 687 were microinjected. We obtained 368 (53.5%) normally cleaved embryos, 331 were transferred and 37 were cryopreserved. One hundred and forty-five transfers (range 1–3 embryos/patient) were performed and 18 (12.4%) pregnancies were obtained. Twelve patients delivered 13 healthy children, and six first trimester abortions were observed (33.3%). **Conclusion:** Although a low implantation rate was observed and a higher abortion rate than in fresh cycles, our results show that in sibling oocytes, the process of cryopreservation apparently does not affect the fertilization and cleavage rate. In this group of patients, producing a large number of mature gametes, oocyte cryopreservation gives the couple extra chances to achieve a pregnancy within a single retrieval and is a good effort towards reducing the number of embryos cryopreserved and enhancing our experience in this new technology.


**Background:** We studied the ultrastructural characteristics of human mature oocytes frozen/thawed (F/T) using different concentrations of sucrose. Fresh human mature oocytes were used as controls. **Methods:** The oocytes (n = 48) were fixed in 1.5% glutaraldehyde at sampling (n = 16) or after freeze/thawing performed using a slow cooling
method with propane-1,2-diol 1.5 mol/l and sucrose at either 0.1 mol/l (n = 16) or 0.3 mol/l (n = 16) in the freezing solution. The oocytes were then processed for electron microscopy observations. **Results:** Fresh and F/T oocytes belonging to both study groups were regularly rounded in sections, with a homogeneous cytoplasm and an intact zona pellucida (ZP). Organelles (mainly mitochondria—smooth endoplasmic reticulum aggregates and mitochondria—vesicle complexes) were abundant and uniformly dispersed in the ooplasm. The amount and density of cortical granules appeared to be abnormally reduced in some F/T samples, independently of the sucrose concentration in the freezing solution: this feature was frequently associated with an increased density of the inner ZP, possibly related to the occurrence of zona ‘hardening’. Furthermore, slight to moderate microvacuolization was revealed in the ooplasm of some F/T oocytes, particularly in those treated with sucrose 0.3 mol/l. **Conclusions:** Freeze/thawing procedures are associated with ultrastructural alterations in specific oocyte microdomains, presumably linked to the reduced developmental potential of mature cryopreserved oocytes. Further work is needed to determine whether or not a high concentration of sucrose plays a role, at least in part, in producing the above alterations.


**Objective:** To describe the first birth achieved after intracytoplasmic sperm injection (ICSI) of cryopreserved human oocytes. **Design:** Case report. **Setting:** University of Bologna Hospital, Department of Obstetrics and Gynecology, Reproductive Endocrinology Unit, IVF and Infertility Center. **Patient(s):** One patient undergoing IVF. **Intervention(s):** Transvaginal ultrasound-guided oocyte retrieval followed by oocyte freezing. Artificial preparation of the endometrium with E2 and P, oocyte thawing, and ICSI. **Result(s):** Four of 12 cryopreserved oocytes survived; using ICSI, 2 underwent normal fertilization but only 1 cleaved. One good-quality 4-cell embryo was transferred. A single gestation was confirmed by ultrasound at the 7th week. Amniocentesis was performed at the 16th week and demonstrated a normal female karyotype of 46,XX. After a normal pregnancy, a healthy female infant was born at the 38th week of gestation. **Conclusion(s):** The combination of ICSI and oocyte cryopreservation is a new tool in assisted reproductive technology.


The success of human oocyte cryopreservation depends on morphological and biophysical factors that could influence oocyte survival after thawing. Various attempts to cryopreserve human oocytes have been performed with contrasting results. Therefore the effect of some factors, such as the presence or absence of the cumulus oophorus, the sucrose concentration in the freezing solution and the exposure time to cryoprotectants, on human oocyte survival after thawing were investigated. The oocytes were cryopreserved in 1,2-propanediol added with sucrose, using a slow-freezing-rapid-thawing programme. After thawing, the oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and the
outcomes of insemination and subsequent embryo development were also recorded. The post-thaw cryosurvival rate was not different for the oocytes cryopreserved with their cumuli partially removed mechanically (56%) when compared with those cryopreserved with their cumuli totally removed enzymatically (53%). On the contrary, a significantly higher survival rate was obtained when the oocytes were cryopreserved in the presence of a doubled sucrose concentration (0.2 mol/l) in the freezing solution and the survival rate was even higher when the sucrose concentration was tripled (0.3 mol/l) (60 versus 82% \( P < 0.001 \)). Furthermore, a longer exposure time (from 10.5 to 15 min) to cryoprotectants, before lowering the temperature, significantly increased the oocyte survival rate (\( P < 0.005 \)). Intracytoplasmic sperm injection produced a good fertilization rate (57%) of thawed oocytes and a high embryo cleavage rate (91%) and a satisfactory embryo morphology was observed (14 and 34% for grade I and grade II embryos respectively).


Le tecnologie di crioconservazione riproduttiva rappresentano importanti strategie per la conversazione della fertilità. Infatti, malattie, cicli di chemio e radioterapia e la predisposizione genetica possono ridurre il potenziale riproduttivo degli individui. La crioconservazione degli gameti necessita di protocolli che prevedono l’uso di crioprotettori e di particolari procedure di congelamento e scongelamento. La crioconservazione degli ovociti umani permette di superare problemi etici, morali e legali legati alla crioconservazione degli embrioni e di evitare i problemi associati alla sindrome da iperstimolazione ovarica. Il protocollo di crioconservazione prevede l’esposizione degli ovociti ad un crioprotettore permeante (1,2 propandiolo) e ad uno non permeante (saccarosio) la membrana cellulare ed un programma di congelamento lento/scongelamento rapido utilizzando un congellatore programmabile. La crioconservazione degli spermatozoi è stata la prima tecnologia utilizzata per la conservazione della fertilità maschile. I protocolli di crioconservazione prevedono un congelamento rapido in vapori di azoto oppure un congelamento lento, utilizzando o meno congellatori programmabili, dopo l’esposizione ad un crioprotettore. Nei pazienti in età prepubere, per i quali la crioconservazione degli spermatozoi non è possibile, l’alternativa è quella di crioconservare le biopsie testicolari da cui si prelevano spermatozoi immaturi che verranno successivamente maturati in vitro. Un’altra ipotetica alternativa è il reimpianto del tessuto testicolare per ripristinare la spermatogenesi in vivo.


In March 2004, a new law was introduced in Italy to regulate assisted reproduction; at present it is impossible to use more than a maximum of three oocytes per IVF cycle, nor can embryos or prezygotes (2PN cells) be selected or cryopreserved. The prohibitions introduced by the new law have, on the one hand, reduced the expectations of success of current techniques and, on the other hand, stimulated clinicians and embryologists to work
on new therapeutic strategies so as to offer the highest chances of success with the lowest risks. In-vitro maturation (IVM) of oocytes fits very well with these new requirements: ovarian stimulation is avoided and the handling of spare oocytes is facilitated. The IVM protocol is an intriguing alternative to conventional IVF techniques, since it removes the side-effects of drug stimulation, especially ovarian hyperstimulation syndrome, and it also reduces the costs of the entire procedure, both in terms of 'time consumption' and 'patient/society costs for drugs'. In the authors' IVF centre the IVM technique has been used for more than a year, with significant success in terms of maturation and fertilization rates, percentage of embryo transfers, number of pregnancies and, finally, healthy babies born.

F. Gandolfi, A. Paffoni, E. Papasso Brambilla, S. Bonetti, T.A. Brevini, G. Ragni, Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models, in “Fertility and Sterility”, 2006, April, 85, Suppl. 1, pp.1150-1156

Objective: To compare the efficiency of equilibrium cooling and vitrification for cryopreservation of human ovarian tissue and to determine the best experimental model for developing new protocols. Design: Experimental prospective study. Setting: An academic research environment. Patient(S) and Animal(S): Human ovarian biopsy specimens were obtained from three women undergoing operative laparoscopy for ovarian cyst enucleation. Adult cow and pig ovaries, collected at the abattoir. Intervention(S): Ovarian tissue fragments of three individuals for each species were cryopreserved by using two protocols, either for equilibrium cooling or vitrification. Main outcome Measure(S): Comparison between fresh and cryopreserved tissue of primordial, primary, and secondary follicle morphology, graded in three classes. Result(S): Human and bovine follicles responded in the same way to the two equilibrium cooling protocols, whereas pig tissue was more cryoresistant. Both vitrification protocols caused extensive damage to the tissue of all species. Human tissue showed a response to vitrification that was different from that of both animal species. Conclusion(S): Bovine is a good animal model for the development of human ovarian tissue cryopreservation protocols by equilibrium cooling procedures. Vitrification is less efficient than equilibrium cooling, and at present, neither bovine nor pig can be considered relevant animal models for human tissue.


Background: The aim of the present study was to verify the feasibility of cryopreserving testicular tissue during the first diagnostic biopsy and then using thawed sperm to inseminate the partner's oocytes. The expected advantages are: (i) minimal risk of not having spermatozoa available at the time of intracytoplasmic sperm injection; (ii) no repeated surgical interventions, and (iii) programming the treatment cycle at the couple's convenience. Materials and Methods: Between May 1996 and May 1998, 64 azoospermic patients underwent investigative testicular biopsy combined with cryopreservation of spermatozoa which were retrieved in a simultaneously examined fresh
sample. Testicular tissue cryopreservation was carried out in 43 cases (67%) for later intracytoplasmic sperm injection attempts. **Results:** In all, 23 couples underwent 26 assisted conception cycles; the fertilization rate was 64% with spermatozoa (139/218, 24 cycles), 40% with round spermatids (2/5, 1 cycle), and 69% with elongated spermatids (9/13, 1 cycle). The embryo cleavage rate was 84%. The mean number of embryos replaced in 24 patients was 2.7 +/- 0.7. In 2 cases, embryo quality was very poor, and they were not transferred to the patients. Eight clinical pregnancies resulted (35%/patient and 33%/transferred cycle) with an implantation rate of 14.1%; 2 patients have already delivered and 6 pregnancies are ongoing normally. **Conclusions:** Testicular tissue cryopreservation during the first diagnostic biopsy is an alternative to repeated surgical interventions. Patients can initiate an ovarian stimulation cycle, confident of having spermatozoa available. Moreover, since only one straw is routinely used for each intracytoplasmic sperm injection cycle, the frozen tissue remains as a sperm source for multiple attempts. Copyright (C) 2000 S. Karger AG, Basel.