ESHRE Campus symposium

Cryobiology & Cryopreservation of Human Gametes & Embryos

Brussels, Belgium

12 and 13 March 2004

CONTENTS

INTRODUCTION

Course description

In ART programs world-wide, there are several topics of concern that impact greatly on patients and are of great concern for clinicians and scientists alike: reducing multiple pregnancies and male and female gamete cryopreservation.

Each topic independently brings its own need for ongoing discussion and research;

(1) the scientific community as well as the public is concerned about high-order multiple births and clearly the only remedy to almost avoid multiple birth is to transfer only one embryo which should result in more embryos being cryopreserved. However, national and international registers indicate that the results obtained with cryopreserved embryos are substantially lower than with fresh ones;

(2) storing the male gamete is currently an accepted clinical procedure. Several questions, however, remain unanswered and deserve further attention such as the male to male variation in sperm freezability;

(3) it is also clear that storing the female gamete can have great impact in reproductive medicine. Despite some successful clinical trials, there are several problems associated with the cryopreservation of the female gamete.

To optimize the cryopreservation of human embryos and gametes clinical embryologists worldwide have empirically modified basic procedures. Often these modifications were introduced without any justification and moreover this has not led to a substantial improvement of the results.

Therefore understanding basic principles of cryobiology and cryopreservation will help scientists and clinicians to optimize protocols for providing multiple options to the patients.

The SIG embryology is offering a two-day course aimed at clinical embryologists, clinical staff and other members with an interest in the fundamental cryobiology and understanding of human embryo and gamete cryopreservation. The course will attempt to describe the scientific properties of cryobiology, how they have been developed and how they are applied. The course will also describe the current status of human embryo and gamete cryopreservation and the factors influencing the cryopreservation outcome.

Learning objectives

At the conclusion of this course, the participant should be able to:

(1) apply the fundamental principles of cryobiology to improve current cryopreservation procedures
(2) to understand the factors that influence the cryopreservation outcome
(3) summarize current cryopreservation practices in ART

COMMITTEES / SPEAKERS

Scientific committee

Prof Dominique Royère (Coordinator of the SIG Embryology)
Dr Geraldine Hartshorne (Deputy member of the SIG Embryology)
Etienne Van den Abbeel (Deputy member of the SIG Embryology)

Speakers

S. Avery (UK)
M. Camus (B)
H. Joris (B)
S. Leibo (USA)
J. Mandelbaum (FR)
D. Roy re (F)
C. Silva (USA)
G. Smith (USA)
J. Van der Elst (B)
P. Van der Zwalmen (B)
G. Verheyen (B)

ESHRE Special Interest Group on Embryology

The Special Interest Group (SIG) Embryology of the European Society for Human Reproduction and Embryology (ESHRE) is aiming to promote interaction between the ESHRE SIG Embryology, clinical embryologists and National Clinical Embryologist Societies. Therefore the board members of the ESHRE SIG Embryology decided to organize on a yearly basis winter symposia for clinical embryologists, clinical staff and all individuals with an interest in some hot topics in embryology.

PROGRAM

Friday, 12 March 2004

8.30 – 9.00: Wellcome and registration

Session I: Fundamental cryobiology
Chair: E Van den Abbeel (B)

09.00 – 10.30: S Leibo (USA) - *Fundamental cryobiology for clinical embryologists (1)*

10.30 – 11.00: coffee break

11.00 – 12.30: S Leibo (USA) - *Fundamental cryobiology for clinical embryologists (2)*

12.30 – 13.30: lunch break

Session II: Human embryo cryopreservation: state of the art
Chair: J Van der Elst (B)

13.30 – 14.00: J Mandelbaum (F) - *Human embryo cryopreservation: past, present and future*

14.00 – 14.30: M Camus (B) - *Human embryo cryopreservation: a review of clinical issues related to the success rate*

14.30 – 15.00: D Royère (F) - *Embryo characteristics and cryopreservation outcome*

15.00 – 15.30: coffee break

Session III: Human embryo cryopreservation: practical aspects
Chair: J Mandelbaum (FR)

15.30 – 16.00: H Joris (B) - *Cryopreservation of biopsied embryos after preimplantation genetic diagnosis or screening*

16.00 – 16.30: P Van der Zwalmen (B) - *Vitrification: an interesting method for the cryopreservation of human embryos?*

16.30 – 17.00: S Avery (UK) - *Safety aspects of the cryopreservation and storage of human embryos*

Session IV: Free communications
Chair: D Royère (FR)

17.00 – 18.00: Four selected free communications
S. Yavin et al - *The effect of chilling on membrane lipid phase transition in human oocytes and zygotes*
V. Frederickx et al - *Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells.*
M. Van den Bergh et al - *First analysis of a cryopreservation programme after the change in the Swiss law, mandating freezing of zygotes only*
N. Naaktgeboren et al - *The impact of cryopreservation and elective single embryo transfer (SET) on the multiple pregnancy and the delivery rate in an IVF/ICSI programme*

Session V: Poster session

18.00 – 20.00: meeting the speakers and poster viewing with wine and cheese

PROGRAM

Saturday, 13 March 2004

Session VI: Cryopreservation of human gametes
Chair: E Van den Abbeel

08.30 - 09.00: J Van der Elst (B) - *Current status of storing the human female gamete*

09.00 - 09.40: G Smith (USA) - *Cryopreservation and compromised Intra-oocyte functions: is vitrification the answer?*

09.40 - 10.00: coffee break

10.00 - 10.30: S Leibo (USA) - *Problems related to the cryopreservation of mammalian sperm cells including the human*

10.30 - 11.00 G Verheyen (B) - *Cryopreservation of testicular sperm*

Session VII:Demonstrations

11.00 - 11.30: G Smith (USA) - *Animated demonstration on a mouse oocyte vitrification protocol*

11.30 - 12.30: *Live demonstrations on the vitrification of mouse oocytes and blastocysts* by C Silva (USA) + lunch

Session VIII: General discussion

12.30 - 14.00: *I. Human embryo cryopreservation today:*

Which procedure?
Which embryos?

II. Is human oocyte cryopreservation a safe procedure?

III. Cryopreservation of the human male gamete today: how?

Panel: S Leibo, J Van der Elst, D Royère, M Camus, J Mandelbaum, G Verheyen
Chair: E. van den Abeel

14.00: Adjourn

Fundamental cryobiology for clinical embryologists

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Introduction

Twenty years ago, the first children derived from the transfer of cryopreserved embryos were born (Zeilmaker et al., 1984). Since then, thousands of other babies have been born as a result of the transfer of frozen embryos. Medical practitioners and societies now consider embryo cryopreservation as an accepted standard of care. The rate of success is sufficiently high so that human embryos are now routinely cryopreserved as an adjunct to standard techniques of assisted reproduction. Numerous reviews of embryo cryopreservation have been published. Among those specifically devoted to human embryos include articles by Bernard and Fuller (1996), Fabbri et al. (2001), Friedler et al. (1988), Ludwig et al. (1999), Paynter et al. (1997), Porcu et al. (2000), and Shaw et al. (2000).
The procedure used to freeze the first human embryos (Trounson and Mohr, 1983; Zeilmaker et al., 1984) was the same as the original one used to cryopreserve cleavage-stage embryos of rodents that resulted in the birth of live young (Whittingham et al., 1972). This procedure utilized dimethyl sulfoxide (DMSO) as a cryoprotective additive (CPA), cooling at a rate of about 0.5ºC/min from –6º to –75ºC, storage of the frozen embryos in liquid nitrogen at –196ºC, and warming at about 10ºC/min until the sample melted. Several years later, it was found that slow cooling could be interrupted at an intermediate temperature of about -35ºC, if the frozen embryos were thawed rather rapidly at about 350ºC/min (Willadsen, 1977). Since then, variations on this approach of Willadsen have been widely used to cryopreserve embryos of 15 or more species (reviewed by Leibo and Songsasen, 2002). An even more innovative approach to embryo cryopreservation was the use of vitrification to cryopreserve embryos under "ice-free" conditions (Rall and Fahy, 1985). This method has now been adapted to cryopreserve a wide variety of reproductive cells, especially oocytes, which are very sensitive to injury caused by cooling them close to 0ºC (reviewed by Vincent and Johnson, 1994; Parks and Ruffing, 1992).
The former methods of cryopreservation by slow cooling are referred to as equilibrium cooling, and the latter rapid methods as non-equilibrium cooling. Although non-equilibrium methods are used with increasing frequency to cryopreserve gametes and embryos, it is instructive to first consider equilibrium methods of cryopreservation, since the same fundamental principles operate to determine survival of embryos cryopreserved by both methods.

When mammalian cells, such as spermatozoa or embryos, are cryopreserved by equilibrium cooling, various factors determine the ultimate survival.

[1] The osmotic response of the cell when suspended in a solution of a permeating CPA.
[2] The concentration of the extracellular solution as the temperature is reduced and as ice forms and grows in the extracellular matrix.
[3] The osmotic loss of water from the cell in response to increasing concentration of the extracellular solution as water is removed in the form of ice.
[4] The rate at which the specimen is cooled to subzero temperatures, primarily from about -5∞ to -35∞ C.
[5] The subzero temperature at which the cell is stored, and its handling during storage.
[6] The rate at which the cryopreserved sample is warmed and thawed.
[7] The osmotic response of the cell when it is removed from the CPA solution.

Osmotic Responses of Cells

With the exception of seeds or spores, all cells (microbes, plants, and animals) remain in osmotic equilibrium with the extracellular milieu. When cells are transferred from an isotonic solution into an anisotonic solution, they respond osmotically either by undergoing dehydration due to water loss or by swelling as water enters the cells. For mammalian cells, an isotonic solution is approximately 300 mOsmoles. When a mammalian cell is transferred into a hypertonic solution (>300 mOsm), it will dehydrate. The rate at which it loses water depends on the cell's hydraulic conductivity (its permeability to water), the temperature coefficient of the permeability, the solution in which the cell is suspended, and the temperature. For a given cell type, e.g. an embryo, different stages of embryonic development have different permeability coefficients. One critical aspect of the cell's response is the question of whether the cell is or is not permeable to the principal solute of which the solution is composed. For example, most mammalian cells are impermeable to electrolytes, such as NaCl or CsCl, and to non-electrolytes, such as glucose or sucrose. However, they are permeable to a variety of other compounds, such as dimethyl sulfoxide or glycerol.

Cryoprotective Additives (CPAs)

Over the course of some fifty years, many compounds have been found empirically to protect mammalian cells against freezing damage. In the case of human embryos, with very few exceptions, most clinics use propylene glycol supplemented with a low concentration of sucrose as the CPA solution for early cleavage-stage embryos and glycerol for blastocyst-stage embryos. Experiments with various animal cells and embryos have demonstrated that effective CPAs include glycerol, ethylene glycol, propylene glycol, and methanol, as well as DMSO. Although the actual mechanisms responsible for this protection have yet to be determined, much is known about the properties of these CPAs. They are all low molecular weight compounds (<150 Daltons), all are completely miscible with water in all proportions

(from 1 to 99%), all reduce the freezing point of aqueous solutions to -50∞ C or lower, all permeate mammalian cells, and all are non-toxic to cells even when present at high concentrations. In this sense, "non-toxic" means that the CPA does not inevitably kill the cell simply as a result of relatively short exposure to the compound. That is not to say that cells may not be damaged or killed when exposed and then diluted out of the CPA. Such damage is the result of osmotic shock. But simple exposure of cells to CPAs does not damage them.

It must be realized, however, that exposure of embryos for >15 minutes to high concentrations of these CPAs at temperatures >20°C means that embryos begin to metabolize in the presence of non-physiological concentrations of these cryoprotectants. This is not evidence of solute toxicity per se, but rather only that embryos may be damaged by developing in the presence of such CPAs. Osmotic shock is the phenomenon exhibited by all mammalian cells in which the intracellular solution has a higher osmotic pressure than the extracellular solution. Water enters the cell more rapidly than an intracellular solute, such as glycerol or propylene glycol, can leave. Depending on the relative permeabilities of the cell to water and to the solute, cell volume may increase to a lytic volume and the cell bursts. Most embryos including human ones are relatively impermeable to glycerol, which renders them especially sensitive to osmotic shock when they are directly diluted out of a glycerol solution into isotonic saline. However, they are more permeable to propylene glycol than to glycerol. That difference in permeability of bovine embryos to glycerol vs. propylene glycol illustrates one important aspect of cryoprotectants. Different CPAs may exert equivalent protection during freezing. But if the embryo is much more permeable to one CPA than to another, then it will be much less sensitive to osmotic shock when diluted out of the former compared to the latter. That is the reason why the use of impermeable solutes such as sucrose or galactose has become an important aspect of the dilution of embryos after cryopreservation. First described for embryos of laboratory species, the use of sucrose during dilution was later introduced for the recovery of bovine embryos. The presence of sucrose in the diluent drastically reduces the susceptibility of the embryo to osmotic shock.

Permeability of Oocytes and Embryos

When oocytes or embryos are exposed to CPAs, they immediately contract osmotically by water loss because of the difference in osmotic pressure between the extracellular and intracellular solutions. But because of the difference in concentration of the CPA between the extracellular and intracellular solutions, the CPA begins to permeate the cell by simple diffusion. Simultaneously, water begins to re-enter the cell to maintain osmotic equilibrium between the extracellular and intracellular solutions. At equilibrium, the cell has the same concentration of CPA as that of the solution in which it is suspended, and the osmotic pressure of the cell cytoplasm is the same as that of the suspending medium.
Several variables determine how quickly this equilibrium is attained. These include the specific solute, e.g. ethylene glycol tends to permeate cells faster than glycerol. Another factor is the concentration of the CPA itself; the

higher the concentration, the faster it will permeate cells. A third variable is the temperature; the higher the temperature, the faster the CPA will permeate the cell. This effect of temperature is profound, and has important consequences. At -5∞ C or below, most mammalian cells and certainly all embryos become virtually impermeable to CPAs. Even methanol, to which embryos are extremely permeable, ceases to permeate embryos at -5∞ C. Other important variables are the biological ones, such as the stage of maturation or of development. The earlier the stage of development, the less permeable are the embryos. For example, morulae are more permeable than 8-cell embryos; these, in turn, are more permeable than 2-cell embryos; and these are more permeable than zygotes.

Almost all human embryos are cryopreserved in a solution of ~1.4 M propylene glycol + ~0.2 M sucrose. Comparisons of the effects on the survival of cryopreserved embryos of adding the CPA in several small steps compared to adding it in one abrupt step have found little or no difference between them. In contrast, when embryos are rapidly diluted directly out of the cryoprotectant after freezing in as single step, they may be injured by osmotic shock. Whether the cells are injured or not depends on the specific CPA, the temperature at which they are diluted, and the rapidity with which they are diluted.

Freezing of Solutions

When aqueous solutions are frozen, water is removed in the form of ice. As a consequence, dissolved solutes become increasingly concentrated as water is sequestered as ice crystals. The amount of ice formed is a function of the temperature, the specific solutes dissolved in the water, and the initial concentration of the solutes. As the temperature is lowered, more and more ice forms, increasing the concentration of the solution of the unfrozen liquid. As the temperature is lowered further, more ice forms and the concentration continues to increase. This process continues until the sample is cooled to the eutectic point of the solution. The eutectic point is the temperature at which the entire system solidifies. For biological solutions such as buffers containing CPAs, there is not a specific eutectic point, but rather a eutectic zone.

Chilling Sensitivity

Another variable that may also affect the survival of cells, especially that of oocytes or spermatozoa, is chilling sensitivity. Although many types of mammalian cells are relatively unaffected by being cooled close to 0∞ C, spermatozoa and embryos are damaged simply by being cooled to that temperature. Although the mechanisms are not completely understood, in the case of oocytes, the meiotic spindle undergoes disassembly as the tubulin dissociates at temperatures near 0∞ C. It is for that reason that cryopreservation of oocytes has been relatively unsuccessful. Although children have been born as a result of fertilization of cryopreserved oocytes, the overall success rate has been less than 5%.

Role of Cooling Rates to Intermediate Subzero Temperatures

The original method to cryopreserve embryos stipulated that they had to be cooled rather slowly at rates <2ºC/minute from about -5º to -75ºC before being plunged into liquid nitrogen (LN2). Then, Willadsen (1977; Willadsen et al., 1978) described a variation on this method in which sheep or bovine embryos were cooled at a rate of 0.3°C/minute but only to -35°C before being placed into LN2. Since the late 1980s, Willadsen's method has become the treatment of choice for most commercial practitioners who freeze embryos of domestic animals. Yet there have been very few studies of the effect of cooling rates on embryo survival. A few years ago, it was shown that cooling bovine embryos from -7º to -35ºC at rates ranging from about 0.2 to 2ºC/minute yielded high functional survival, although there was a significant interaction between the warming rate and the cooling rate that preceded it (Hochi et al., 1996). One important practical consequence of cooling samples from -7° to -35°C at a rate of 2°C/minute vs. 0.3°C/minute is that the latter would require ~90 minutes whereas the former would require <15 minutes. And if the samples were to be cooled from -7º to -75ºC at 0.3ºC/minute, that would require almost 4 hours. Despite that apparent time-saving, very low cooling rates continue to be used to cryopreserve human embryos. This practice seems to be a manifestation of the logic: "Low rates are good; therefore, lower rates must be better." This is not necessarily true.

"Plunge Temperatures"

Considerable attention has been directed to examination of the effects of the temperature at which slowly cooled embryos are plunged into liquid nitrogen. Many experiments have been conducted to compare cooling at very low rates from about -30°C to lower temperatures. In general, the results of these experiments have been inconclusive. This is not surprising in light of the fact that maximum concentration of the extracellular solution, and therefore, maximum osmotic contraction of the embryo will have occurred at much higher subzero temperatures. In short, attention to fundamental principles leads to the conclusion that slow cooling may be terminated over a broad subzero temperature range of about -25° to -40°C with the same overall results. Experimental data have confirmed those predictions. Again, the practical implication is the shortening of the time required for the successful cryopreservation of embryos.

Storage of Cryopreserved Embryos

Although frozen embryos will retain high viability if stored at -80°C for about one day, long-term storage requires that the embryos be stored below about -130°C, the glass transition temperature of water. In practice, the easiest and safest method is to store cryopreserved embryos in liquid nitrogen at -196°C. Mouse embryos stored at that temperature exhibit the same high survival after 24 hours or more than 5 years. In fact, live mice and sheep have been produced from cryopreserved embryos stored for more than fifteen years in liquid nitrogen. In the case of human embryos, children have been born from embryos that were cryopreserved for more than eight

years. It has been formally predicted that cryopreserved embryos stored in liquid nitrogen will remain "alive" for at least 1,000 years.

Warming Rates

Just as there are optimum cooling rates for maximum survival, so are there optimum warming rates. In general, embryos cooled slowly to high subzero temperatures of about -30° to -40°C before being rapidly cooled to -196°C require moderately rapid warming of about 200 to 350°C/minute for maximum survival. Embryos cooled slowly to -60°C or below require rather slow warming of about 25°C/minute or less.

Dilution of Cryoprotectants

The last but often over-looked step of embryo cryopreservation is removal of the CPA from the embryo. Embryos are cryopreserved in CPAs ranging in concentration from 1 to 8 Molar. Virtually all CPAs currently used to cryopreserve the embryos permeate them prior to their being cooled. In general, if CPAs in which embryos have been frozen are rapidly diluted, the embryos will be subjected to osmotic shock, resulting in damage or death. A common practice has been to dilute cryopreserved embryos out of the CPA in a slow step-wise fashion. Although this method works in practice, it is usually rather slow and laborious. A more efficient and shorter method is to use a non-permeating solute such as sucrose as an osmotic buffer to lessen the chance of an osmotic shock. This single-step dilution can be performed within the straw in which the embryo was originally frozen (Renard et al., 1982; Leibo, 1984). A variation on this theme has been to mix sucrose with the CPA to lessen the osmotic shock, and to perform a two-or three-step dilution of the CPA. This method has gained increasing popularity, although there is no unequivocal evidence that it is more efficient that a single-step method using sucrose alone. Undoubtedly, more efficient methods to recover cryopreserved embryos from CPAs will be introduced in the future. In short, application of certain fundamental principles may well improve the efficiency and survival of cryopreserved bovine embryos.

Conclusions

Tens of thousands, if not hundreds of thousands, of children have been born as a result of the transfer of cryopreserved embryos. Embryo cryopreservation is no longer an experimental procedure. Moreover, the techniques used to cryopreserve embryos of humans and animals now rests on a firm mechanistic understanding of the basic principles that lead either to the preservation or to the destruction of the embryos. Application of such understanding and careful attention to details used to cryopreserve embryos can lead to excellent results. The purpose of this lecture is to summarize some of these basic principles.

INVITED CONTRIBUTIONS

Reviews of Embryo Cryopreservation

Bernard A, Fuller BJ. 1996 Cryopreservation of human oocytes: a review of current problems and perspectives. Hum Reprod Update 2:193-207.

Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod. 16:411-416.

Friedler S, Guidice LC, Lamb EJ. 1988 Cryopreservation of embryos and ova. Fertil Steril 49:743-764.

Ludwig M, Al.-Hasani S, Felberbaum R, Diedrich K. 1999 New aspects of cryopreservation of oocytes and embryos in assisted reproduction and future perspectives. Hum Reprod 14 (Suppl 1):162-185.

Paynter S, Cooper A, Thomas N, Fuller B. 1997 Cryopreservation of multicellular embryos and reproductive tissues. In: Karow AM, Critser JK (eds.) Reproductive Tissue Banking. Academic Press, San Diego CA. pp 359-397.

Porcu E, Fabbri R, Damiano G, Giunchi S, Fratto R, Ciotti PM, Venturoli S, Flamigni C. 2000 Clinical experience and applications of oocyte cryopreservation. Mol Cell Endocrinol 169:33-37.

Shaw JM, Oranratnachai A, Trounson AO. 2000 Cryopreservation of oocytes and embryos. In Trounson AO, Gardner DK (eds). Handbook of In Vitro Fertilization, 2nd Ed. CRC Press, Boca Raton FL, pp 373-412.

NOTES

Human embryo cryopreservation: past, present and future

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The use of super ovulation techniques to improve and simplify human in vitro fertilization (IVF) led to the production of large number of oocytes and consequently embryos. In order to avoid the risk of multiple pregnancies without discarding the embryos in excess of the number appropriate for a safe transfer, embryo freezing was developed in humans.

The past: the freezing procedures

Human embryo freezing resulted from the application of animal embryo cryopreservation techniques.

In 1972, Whittingham obtained the first births from transfer of mice morulae, issued from mature oocytes, frozen in **dimethyl sulfoxide (DMSO)** according to a slow freeze-slow thaw procedure and stored at -196°C. The same protocol led to the first reported pregnancy in humans, arising from a frozen 8-cell embryo, owing to Trounson and Mohr in Australia in 1983, followed by the first births of twins in 1984 in the Netherlands (Zeilmaker et al). DMSO is less used now for multicellular embryo freezing. It lengthens the procedure since it involves a slow freeze to - 80°C and either a slow thaw (Trounson and Mohr, 1983) or a rapid one on crushed ice (Van der Elst et al, 1995), without having prove to be any more effective.

In 1984, **propanediol (PROH)** was introduced in embryo freezing in mice and rabbit (Renard et al) and associated with sucrose helping in cell dehydration by its osmotic effect. The protocol using PROH (1.5 mol/l) and sucrose (0.1 mol/l) was reported to be quite successful when embryos were slow cooled to -30°C and thawed rapidly after storage in liquid nitrogen. This protocol, reducing the freeze-thaw procedure to 2.5 hours, was first applied to humans in 1985 (Lassalle et al) and proved to be efficient for 1, 2 or 3 days-old embryos. A widespread application of embryo cryopreservation (CP) was therefore initiated in IVF centres using this procedure.

Cryotechnology was industrially applied to cattle, representing, since 1980, more than 100.000 embryo transfers per year in the world. The blastocyst was the favourite stage and glycerol the cryoprotectant appearing as the most suitable according to the permeability of blastomere membranes at this stage. Human blastocyst freezing was first reported by Cohen et al (1985), using **glycerol** (10%) alone added in a stepwise procedure. Despite only 53% of blastocysts survived, implantation rates per transferred blastocyst

reached 35% on a small series (n=23). Blastocyst CP was nevertheless abandoned for years. Indeed, 25% of zygotes only, were able to reach the blastocyst stage in vitro in usual culture media and results of other groups were far less successful (Hartshorne et al, 1991).

Coculture on feeder cells has allowed obtaining good blastocysts with higher rates of 50-60% (Ménézo et al, 1992). A simplification of the freezing program with exposure to glycerol (9%) in two steps only associated with sucrose (0.2mol/l), allowed to increase the survival rates to 80%. The birth rate per thawed and transferred blastocyst was around 13%. Human blast ocysts are now obtained using sequential media and appear to be half less cryoresistant than the cocultured ones (Mandelbaum and Ménézo, 2001). On the other hands, recent reports claim that human blastocyst is the preferred stage for CP (Anderson et al, 2003; Veeck, 2003). To day, the efficacy of blastocyst culture and CP is still controversial (Kolibianakis, 2002)

"True" **vitrification** procedures allow vitrification solutions to form a solid, non-crystalline phase on cooling and to retain this glass-like state throughout warming. In rapid cooling protocols, crystals can form at any stage during cooling and warming. In the mouse, there has been, over the past ten years, a shift from slow cooling towards vitrification procedures with excellent results (>90% blastocysts; >60% foetuses). Highly efficient protocols have also been developed for other species including rabbits, rats, cattle and sheep and for early cleavage stages as well as blastocysts (Paynter et al, 1997). The success however is dependant upon the type of cryoprotectants used, the equilibration and dilution strategies, the cooling and warming rates. The right protocol for a given embryonic stage and species may considerably vary to be effective. The ultrarapid freezing method developed by Trounson and Sjoblom (1988) allowed embryos to be plunged into liquid nitrogen after a 2 to 3 minute equilibration in media containing DMSO (2-3 mol/l) and sucrose (0.25-0.5 mol/l), with high rates of survival and development in vitro. Pregnancies were reported, using this ultrarapid freezing, but the number of children born has remained small with only 7% deliveries per transfer. Vitrification procedures were applied to multicellular human embryos in exceptional cases, by using ethylene glycol as cryoprotectant. The results were poor. They appear better for morulae and blastocyst and several live births have recently been reported, most involving blastocysts (Vanderzwalmen et al, 2003). As they are inexpensive and simple, rapid procedures will perhaps endly find their place in human embryo freezing.

The present: the efficiency of embryo freezing in humans.

The first level of assessment of the efficiency of a freezing program is **embryo survival.**

Early cleavage stage embryos are considered surviving when they keep at least half of their initial blastomeres intact after thawing (survival index= 50%). The survival rate is the percentage of surviving embryos among all frozen-thawed embryos and represents around 75% of embryos while 50-

60% are totally preserved (100% blastomere survival).
Survival criteria for zygotes usually include their morphological integrity and their ability to further cleave in vitro (80% of thawed zygotes).

Re-expansion of blastocyts in vitro is considered as a good indicator of their post-thaw survival (75% of thawed blastocysts).

Consequently, 20 to 30% of frozen embryos whatever their stage of development at freezing do not survive the cryoprocess. Any improvement in overall survival rates will improve the efficiency of cryopreservation. Moreover, the impact of freezing on the implantation potential of early cleavage stage embryos is clearly related to blastomere loss (BL) in surviving embryos. Intact thawed embryos have the same viability as equivalent fresh embryos. Blastomere loss results in a reduction of 30% in the implantation potential of cryopreserved embryos (Edgar et al, 2000). Does embryo freezing represent an in vitro test revealing the intrinsic developmental potential of embryos, as suggested by El-Toukhy et al (2003) or do we need different strategies of CP in order to avoid blastomere loss? Proposals have been made to diminish the negative impact of BL: laser-assisted removal of necrotic blastomeres from partially damaged thawed embryos (Rienzi et al 2002); in vitro culture to assess the resumption of cleavage after thawing and allow embryos to restore the blastomere assets (Van der Elst et al, 1997); adaptation of the strategy of transfer by increasing the number of transferred thawed embryos in case of BL, with the risk, in transferring more than one embryo to create multiple pregnancies instead of preventing them.

<u>The benefit of embryo CP has also to be evaluated as the ability to enhance the reproductive potential of a single recovery cycle (Jones et al, 1997).</u>

Results expressed as the augmentation of the delivery rate per oocyte harvest vary greatly in the literature: 2% (de Jong et al, 2002), 8-10 % (Mandelbaum et al, 2001; Veeck, 2003), 24% (Titinen et el, 2001), depending on several variables: efficacy of the freezing process, incidence of cycles with cryopreservation in the ART program, criteria for selection of " freezable" embryos, strategy of fresh transfer (SET, DET...). Results can be expressed as pregnancy rates or delivery rates, as implantation rates or birth rates per transferred or thawed embryos. The cryoaugmentation potential may exclude postponed twins (cryoaugmented "first" delivery rate) or include them as welcome (“overall”cryoaugmented delivery rate). The evaluation can be based on "true" results requiring waiting at least several years for their collection or theoretically calculated, which may lead to an overestimation of the cryobenefit.

The evaluation of the impact of embryo CP has to integrate all these variables to optimise cryostrategies. It appears essential to reach a general agreement in the method of evaluation, especially when the single embryo transfer (SET) begins to be proposed as the golden standard for routine IVF/ ICSI procedures (Gerris, 2003).

The future of embryo freezing would greatly benefit from procedures allowing 100 % of frozen embryos to survive and be totally preserved. Frozen embryos could thus reach the same implantation potential as equivalent fresh embryos. Improvements in cryoprocedures and cryoprotective agents have made slow progress since the last 10 years. There is still a large debate on the best stage to freeze. Improvements in cryopreservation however should rather focus on obtaining a good efficacy at any stage and after any ART procedure (ICSI, PGD, embryos issued from in vitro matured oocytes, cryopreserved oocytes...) in order to offer to patients the largest and most flexible range of transfer policies with optimal results including embryo cryopreservation.

Every innovation in CP technology will have to prove its safety. Until now, comparative analyses with pregnancies issued from fresh transfers have not found any differences particularly in respect to foetal development, perinatal risk, obstetrical outcome and congenital anomalies (Wennerholm et al, 1998). Nevertheless, the study by Dulioust et al (1992) in mice, on the long term effects of embryo freezing, with conventional procedures, confirmed that CP did not induce major anomalies but could be responsible for small differences such as particularities in the mandible's morphology, various responses to the behaviour and neurosensorial tests and an 11% increase in the body weight of males at senescence. These features, depending on genotype, sex or age, may result from epigenetic changes induced by embryo freezing. Similarly, CP does affect the normal pattern of gene expression during human preimplantation development (Tachataki et al, 2003). Intact thawed embryos are therefore not equivalent to their non-frozen counterparts. It will be essential, in the future, before introducing any new cryopreservation technology, to rely on extended studies on animal and human embryos, including genetic and epigenetic screening.

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NOTES

Human embryo cryopreservation: a review of clinical issues related to the success rate

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Historically, success rates in ART have intimately been linked to the use of ovarian stimulation protocols, allowing the collection of several oocytes, and subsequently the production of several embryos. This approach allowed a selection of the "best" embryos, based on morphological criteria, to be replaced in the fresh cycle, but also introduced the concept of supernumerary embryos and hence the problematic of their preservation for further use.

The first human pregnancy after embryo cryopreservation was reported in 1983 by Trounson and Mohr, using DMSO as a cryoprotectant on cleaving stage embryos, but turned to be a second trimester miscarriage. The first birth (twins) was subsequently reported by Zeilmaker in 1984, using similar methods.

Later on, pregnancies were reported after freezing of PN stages (1.2 propanediol) or blastocysts (Glycerol).

The ideal strategy in treating patients would be to replace a limited number of embryos in the fresh cycle in order to avoid the occurrence of multiple pregnancies, and to cryopreserve the remaining ones, for further sequential replacements.

Theoretically, the final result would be a reasonable success rate in the fresh cycle, followed by one or several "frozen" transfers, ending in enthusiastic cumulative success rates, together with low multiple birth rates.

Although this initial model certainly appeared attractive, the reality has showed less promising than expected.

Indeed, during the last 20 years, emphasis has always been put on results in the fresh cycles, entailing the production of very high numbers of multiple pregnancies, the major complication of ART technology.

On the other hand (and also explaining the above mentioned problem), success rates of embryo cryopreservation have not improved impressively, whatever the stage of the embryo frozen, the freezing protocol used or the replacement conditions.

As a consequence, the potential of a frozen stored embryo to become a living child lies in the order of 4%, and babies born from cryopreserved embryos do not represent more than 8% of the total of ART babies born in most programs.

Another underestimated problem of human embryo cryopreservation has been the long term management of storage, i.e. the increasing numbers of stored embryos, and a low concern of some patients about their excess embryos, illustrated by the fact that up to 10% of these patients became "untraceable" after 5 years.

Many different parameters potentially influence the efficiency of a cryopreservation program and will be approached during the lecture, attempting at demonstrating the following statements.

1. The implantation potential of human embryos appears to be adversely affected by cryopreservation.

2. The final potential of embryos to survive freezing and thawing procedures is reflected in their ability to implant and to produce an ongoing pregnancy and a living child.

3. Although implantation potential of cryopreserved embryos is lower as compared to fresh ones, transfers of multiple embryos also expose us to the risk of multiple pregnancies.

4. Cryopreservation offers a moderate but significant increase in overall IVF outcome.

5. The stage of development at the time of freezing does not appear to influence significantly the outcome of a FRET cycle.

6. As in "fresh" cycles, advanced maternal age at the time of freezing, negatively influences outcome.

7. There is a correlation between the chance of pregnancy in the fresh cycle and the chance of pregnancy in a subsequent FRET cycle.

8. Mode of preparation of the endometrium or length of cryostorage do not appear to effect the outcome of FRET cycles.

9. Available evidence does not suggest adverse consequences of freezing on health of born babies. Larger follow-up studies are although necessary to reach solid conclusions.

10. The main prognostic factor of human embryo cryopreservation remains the pre-freezing embryo quality.

11. Patients should take responsibility for the fate of their frozen embryos. These embryos should not be frozen for more than 3 to 5 years and patients

should sign contracts in which they decide what should be done with their embryos at the end of the storage period.

NOTES

Embryo characteristics and cryopreservation outcome

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Learning objectives

- to describe the various stages at which embryos may be frozen
- to assess the main embryo characteristics which may modulate embryo freezability
- to stress the value of embryo freezing in embryo transfer strategy

Embryo freezing has been proposed for more than twenty years as a way to increase the chances for couples involved in Assisted Reproductive Technologies to achieve a pregnancy. While the strategy of transfer becomes more and more restrictive in many countries, the income of frozen embryo transfer (FET) needs an even more accurate evaluation. This concerns not only the developmental stage at which embryos may be frozen, but also the methods which might be used for freezing and thawing as well as the way to better evaluate their freezability. Some parameters assessed before the freezing step do influence the embryo survival, while others at the thawing step modulate the chances of implantation of FET. Globally the actual efficacy of embryo cryopreservation will require not only to be evaluated as take home baby rate by thawed embryo but also as additional births to fresh embryo transfers.

Cryopreservation at the pronucleate stage

Cryopreservation at the pronuclear stage was early reported to allow additional pregnancies to be achieved in an ART program (Cohen et al, 1988). While this developmental stage was the sole allowed for cryopreservation in some countries, it was reported to yield the best survival rates and an increased cumulative pregnancy rate (Veeck et al, 1993; Nikolettos & Al-Hasani, 2000; Damario et al, 2000; Senn et al, 2000; Salumets et al, 2003). Among studies comparing the results of different strategies utilizing either zygote or cleavage-stage embryo cryopreservation, some reported a better survival rate for zygotes than for day2 embryos (Senn et al, 2000: 80.4% vs. 71.8%; Salumets et al, 2003: 86.5% vs. 61.7%) while others reported similar results (Horne et al, 1997: 74.4% vs. 77.4%) or lower results (Kattera et al, 1999: 64.4% vs. 73.9%). Reports on pregnancy and implantation rates were also controversial, with some of them arguing for better results after zygote than day2 FET (Senn et al, 2000: 19.5% and 10.5% vs. 10.9% and 5.9%), while others reported on lower results (Kattera et al, 1999: 14.8% vs. 22.8%) and some reported

similar results (Salumets et al, 2003: 20.1% and 14% vs. 21.1% and 14.3% respectively for zygote and day2 FET).

As far as the impact of ICSI fertilization on the post thaw survival and outcome of pronuclear stage embryos is concerned, there are very few reports in the literature with some controversial results. Some reports mentioned no difference between ICSI and IVF in post-thaw survival and pregnancy rate following pronucleate stage FET (Hoover et al, 1997: 93.2% and 14% vs. 94.8% and 17.4% respectively for ICSI vs. IVF; Kowalik et al, 1998; Nikolettos & Al-Hasani, 2000). Conversely lower implantation rates were reported following ICSI derived pronucleate stage embryos after FET compared to IVF (Macas et al, 1998). The faster timing of pronucleate stage following ICSI compared to conventional IVF was proposed to explain this discrepancy, as some ICSI zygotes might have already passed beyond the pronuclear stage which might account for both survival and developmental ability of these embryos.

Contrary to when the supernumerary embryos are frozen at an early cleavage stage, freezing at the pronucleate stage does not allow a selection of the morphologically best-suited embryos for both the transfer and for the cryopreservation. Thus freezing at the pronucleate stage might compromise the fresh embryo replacement cycle. However one comparative study did not mentioned any difference regardless of the time of cryopreservation, whereas higher cumulative pregnancy rates were observed following fresh and frozen embryo transfers for PN stage than for EC stage embryos (Senn et al, 2000: 55.5% vs. 38.6%). However one must keep in mind that the transfer strategy was based on three embryos in this study.

Cryopreservation at the early cleavage stage

Early cleavage stage embryos are most often concerned by cryopreservation and many reports have focussed on the main parameters involved in its efficacy.

Prognostic value of fresh embryo transfer

Fresh embryo transfer outcome was reported to clearly influence the outcome of FET (Lin et al, 1995: birth rate per transfer 17% vs. 5% in pregnant vs. non pregnant patients as fresh ET outcome; Karlström et al, 1997: pregnancy rate per transfer 29% vs. 21%; Wang et al, 2001: pregnancy rate 14.2% vs. 8.6% respectively in pregnant vs. non pregnant patients under 40 years as fresh ET outcome) .

Embryo cleavage stage

The increased pregnancy rate following the transfer of 4-cell embryos after freezing-thawing was clearly stated by many reports (Testart et al, 1986, 1987; Cohen et al, 1988; Hartshorne et al, 1990; Mandelbaum et al, 1998; Edgar et al, 2000). The benefit of 4-cell stage embryo freezing compared to other stages raised the question about growth rate or blastomere number as

the predominant determinant in this issue. The first line of evidence came from the study of Edgar (Edgar et al, 2000) which reported on survival and implantation rates of 4-cell stage embryos depending on the time when they reached the 4-cell stage (from 40 h post-insemination until day 3). The faster this stage was reached the higher the results were (survival rate: 92.5% vs. 72.7%, Fetal heart per embryo transferred: 10.0% vs. 5.5% respectively for fast and slow 4-cell embryos). Such observation argued for the importance of pre-freeze growth rate in embryo freezability. However it does not preclude a possible role for blastomere number in embryo freezability, since higher survival rate as well as overall efficacy of cryopreservation were recently reported for day2 embryos compared to day3 embryos (Salumets et al, 2003, survival rate: 61.7% vs. 43.1% and birth rate per thawed embryo: 7.6% vs. 4.2% respectively), despite similar results in fresh embryo transfer.

Morphological appearance

The prefreeze morphological aspect of the embryos was clearly reported to influence FET outcome for many years. Equality of blastomeres and percentage of cytoplasmic exudates were reported as relevant criteria to predict the embryo survival (Lassalle et al, 1985; Van den Abbeel et al, 1988; Camus et al, 1989).This was later confirmed by other reports (presence of at least one good quality embryo: pregnancy rate 35% vs. 15%, Schalkoff et al, 1993; Mandelbaum et al, 1998)

Embryo staging after thawing

Blastomere survival and ability to further cleave were two post-thawing parameters reported to modulate FET outcome.

Blastomere survival

First reports defined the cut off value of 50% for blastomere survival to allow reasonable chances to get a pregnancy, whereas they did not clearly argue for a detrimental influence of partial survival of blastomeres on pregnancy rates after FET but they were almost all based on low sample size studies (Mohr et al, 1985; Veiga et al, 1987; Hartshorne et al, 1990; Testart et al, 1990). Later reports clearly stated the beneficial effect of full blastomere survival on FET outcome (Van den Abbeel et al, 1997: Birth rate per embryo transferred: 10.4% vs. 2.9% for full vs. partially damaged embryos respectively; Burns et al, 1999: BR/ET 7.5% vs. 2.7%; Edgar et al, 2000: Implantation Rate per Thawed Embryo 11.3% vs. 6.9% ; Guerif et al, 2002: BR/Thawed Embryo 12% vs. 3.2%; El-Thouky et al, 2003: IR per transferred embryo 17.3% vs. 8.1% for fully intact embryos transfers vs. mixed transfers). Additionally both implantation and ongoing pregnancy rate were reported to increase after removal of necrotic blastomeres from partially damaged frozen-thawed embryos before transfer (Rienzi et al, 2002: IR 16.2% vs. 4.3%, Ongoing PR 40% vs. 11.4%). More recently partial survival was reported to be followed by impaired in vitro development to blastocyst stage (Archer et al, 2003).

Resumption of mitosis

While further cleavage was early evaluated to select frozen-thawed zygotes for transfer, few studies have reported on the ability of frozen-thawed early cleavage-stage embryos to resume mitosis. However resumption of mitosis was clearly reported to increase the FET outcome (Van der Elst et al, 1997: IR/transferred embryo 7.7% vs. 2.9% and 1.2% for further cleaved embryos vs. mixed transfers and uncleaved embryos respectively; Ziebe et al, 1998: IR/transferred embryo 10% vs. 4% for further cleave embryos vs. uncleaved embryos; Guerif et al, 2002: BR / thawed embryo 9.5% vs. 6.9% and 1.1% for further cleaved embryos vs. mixed transfers and uncleaved embryos respectively). Moreover both blastomere survival and resumption of mitosis were reported to have additional prognostic values (Guerif et al, 2002: Odds Ratio 3.4 (1.8-6.3), IR / thawed embryo 17.4% vs. 7%, 5.2% and 0% for total blastomere survival with further cleavage vs. total BS without further cleavage, partial BS with further cleavage and partial BS without further cleavage).

Altogether these data support the value of early cleavage stage-embryo cryopreservation to increase the chances for couples to achieve a pregnancy while decreasing the risks of multiple pregnancies. While Single Embryo Transfer (SET) is used with an increasing frequency, the impact of embryo cryopreservation on cumulative pregnancy rate increases as focussed by several reports (Tiitinen et al, 2001; Martikainen et al, 2001).

Cryopreservation at the blastocyst stage

Since the first pregnancy following cryopreserved blastocyst transfer (Cohen et al, 1985) many changes have been done concerning both the way to culture and to cryopreserve blastocysts. The availability of sequential media able to support the growth of healthy embryos has led to an increase in the practice of fresh blastocyst transfers in recent years (Gardner, 1998; Jones et al, 1998). Few studies have reported on the freezing ability of blastocysts which were developed on sequential media. First studies reported lower implantation rate for frozen-thawed blastocysts compared to fresh blastocysts (Hartshorne et al, 1991; Kaufmann et al, 1995).More recent studies have reported better results using frozen blastocysts transfer (Langley et al, 2001; Behr et al, 2002; Gardner et al, 2003; Mukaida et al, 2003; Liebermann et al, 2003).

Slow freezing protocol (Menezo et al 1992, 1997, 1999)

Few data about frozen blastocysts transfer after culture in sequential media are available with clinical relevance. Langley et al reported on comparison of frozen embryo transfer for day 3 (n = 119) versus day 5(n = 72) during a 30 month period. Implantation rate was twice higher after frozen blastocyst transfer (21.9% vs. 10.1%, for day 5 and day 3, respectively). Survival rate was also higher for day 5 (82 %) than for day 3 (68.5%) embryos (Langley et al, 2001). The freezing protocol was classical, except that frozen blastocysts were graded as 3BB or better (Gardner and Schoolcraft, 1999). Although such results look promising, one should notice that the frequency

of embryo freezing is far lower at day 5 as compared to day 3, which may alter the final efficiency of such a procedure. Another report mentioned high implantation rate (16%) after frozen blastocyst transfer in 64 thaw cycles, while survival rate was estimated considering 50% survival of the inner cell mass and trophoblast cells (Behr et al, 2002).

Vitrification procedure (Rall and Fahy, 1985)

Data with clinical relevance remain scarce until now. The study of Choi et al mentioned data about 34 patients, with 37 embryos frozen at the pronucleus or 2-cell stage (8%), whereas 93 blastocysts were frozen (37.5% of those cultured). After thawing, 51.6% blastocysts survived, 38 were transferred, allowing 5 ongoing pregnancies (13.2%). More recently both high survival and pregnancy rates were reported after transfer of vitrified blastocyst using cryoloop technic (Mukaida et al, 2003: SR 79% and PR/Transfer 36%). Thus although simple and cost-less this technique needs more clinical data before it may be recommended for routine use.

Rate of development

The lower implantation rate for day 7 blastocysts after fresh or frozen embryo transfer was already reported for cocultured embryos (Shoukir et al, 1998), despite some recent report on delivery following transfer of blastocyst which were frozen at day 6 and 7 (Sills et al, 2003). Another report mentioned a clear relationship between the cell number at day 3 and the rate of embryo reaching the blastocyst stage at day 5 or 6 (54% for six-cell embryos as compared to 76% for eight-cell embryos), with a clear effect of women age. However results concerning frozen blastocysts transfer did not allow to discriminate between these situations (Langley et al, 2001). It should be of particular interest to verify if recent proposals about graduated embryo scoring for predicting blastocyst formation and implantation, may help to predict the freezability of blastocysts (Fisch et al, 2001; Gardner et al, 2000).

Effect of intracytoplasmic sperm injection

Lower rates for blastocyst formation following ICSI have been reported (recently reviewed by Miller and Smith, 2001). This was followed by poorer quality blastocysts after ICSI as compared to classical IVF, with a relationship with sperm morphology not always confirmed. Beside the possibility of some paternal effect (Janny and Menezo, 1994), one may not exclude the proper effect of ICSI procedure on in vitro embryo development. Recent results on a register data base (Blefco et al, 2001) focussed on lower rates of blastocyst formation (42% vs. 30%) and blastocyst survival after freezing (63% vs. 70%) after ICSI compared to IVF. Thus lower quality and or higher sensitivity of ICSI blastocysts to freezing need to be considered. More recently similar impairment in blastocyst development was reported for fully intact ICSI derived embryos after thawing compared to standard IVF embryos (Archer et al, 2003: 19% vs. 47% respectively).

Effect of incubation time after thawing

One uneasy task is to correctly assess the quality of blastocysts after thawing, since re-expansion of blastocyst may be delayed beyond the usual 3 to 4 hours incubation before transfer was done. If incubation time increases it may be observed a retraction of blastocysts which looked fully re-expanded only few hours after thawing, whereas blastocysts which were initially retracted might expanse later. This may lead to some error in decision making for embryo selection before transfer. We have compared two strategies of transfer, depending on the incubation time before transfer (20 hours = H20 vs. 4 hours = H4), for 80 and 115 thawing cycles respectively. Although the rate of transfer cycles was similar (52% vs. 61%, for H20 and H4 respectively), the percentage of re-expanded blastocysts was far higher in H20 group as compared to H4 group (80 % vs. 36%). Finally the implantation rate per transferred blastocyst was higher in H20 group than in H4 group (23.4% vs. 6.1%). Rather than an indication for better suitability of culture conditions for frozen-thawed blastocysts, these results argue for a better evaluation of blastocyst survival after a longer incubation, which may help to better select the embryos to transfer (Guerif et al, 2003).
If the transfer of one blastocyst may appear as a promising way to reduce twin pregnancy rate, its efficiency will be clearly delineated as far as blastocyst freezing might allow the same chances of getting more pregnancies. Such improvement is related both to the raw number of "supernumerary" blastocysts which are available for freezing and to the efficacy of the freezing procedure.

Conclusion and perspectives

Thus embryo cryopreservation may be considered as a valuable tool to combine a low risk for multiple pregnancies by single embryo transfer with higher cumulative chances to achieve a pregnancy. Whether this strategy will benefit from cryopreservation at zygote, early cleavage-stage or blastocyst-stage needs to be further assessed by studies on cumulative birth rate per oocytes collection.

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NOTES

Cryopreservation of biopsied embryos after preimplantation genetic diagnosis (PGD) or genetic screening (PGD-AS)

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Preimplantation genetic diagnosis was introduced about 15 years ago when evaluation of DNA or chromosomes at the single cell level became clinically applicable. Evaluation of embryos for monogenic diseases, chromosomal aberrations or ploidy status has been performed for an increasing number of cycles over the years (ESHRE PGD consortium,). Although the genetic diagnosis or screening results in fewer embryos available for replacement more genetically normal good-quality embryos than required for replacement in the fresh cycle may be available. Storage of these precious embryos for possible later use seems logic. As compared to storage of embryos obtained after IVF or ICSI, experience in cryopreservation of biopsied embryos is still limited. The aim of this presentation is to summarize experience in cryopreservation of biopsied embryos. The following items will be discussed:

- Cryopreservation of mouse embryos after zona manipulation
- Cryopreservation of biopsied embryos in humans:
    - o Literature
    - o Results of the AZ-VUB
- Conclusions.

Embryo biopsy and cryopreservation in the mouse

In the mouse, several studies looked into the possibility of cryopreserving zona-manipulated embryos. A comparison of the different studies is difficult because of the variety of parameters involved. Some of these differences are: different zona manipulation procedures, different cryopreservation procedures, different definitions of survival and different end-points. Overall, a significant impact of the biopsy procedure on survival rates after freezing and thawing was not found. Nevertheless, when analysing results in detail, some indications for possible problems come forward. In 1991 Depypere et al cryopreserved embryos with 1.5 M PROH and 0.1 M sucrose. They found similar survival rates in control and treatment group. However, when comparing fully intact and partially intact embryos, fewer embryos were fully intact. Krzyminska et al (1991) reported a reduced survival rate of biopsied embryos as compared to intact controls and sham controls. More biopsied

embryos were less than 50 % intact after cryopreservation. Thompson et al (1995) found that blastocyst formation after cryopreservation of biopsied embryos was lower compared to their non-frozen controls. They did not find an influence of the cryoprotectant only. Ludwig et al (1999) concluded that the combination of embryo biopsy and cryopreservation may have an influence on embryo viability.
Others did not observe a negative impact of zona drilling only (Garrisi et al, 1992) zona drilling combined with removal of 1 or more blastomeres on survival, blastocyst formation (Snabes et al, 1993), implantation (Wilton et al, 1989; Liu et al, 1993) or even repeated cryopreservation (Snabes et al, 1993).

Embryo biopsy and cryopreservation in humans

Literature

In humans, after genetic diagnosis or genetic screening, embryos may be considered even more precious as compared to non-biopsied embryos. Cryopreservation of these embryos seems therefore a logical consequence. So far, limited information about results of cryosurvival of human embryos is available. Unfortunately, results available so far do not confirm the results obtained in the mouse. In 1999 Magli et al reported a reduced survival after cryopreservation of aneuploid biopsied embryos as compared to non-biopsied embryos. Cryopreservation was performed using PROH as the cryoprotectant. Fewer embryos were fully intact and less intact blastomeres were observed in partially intact embryos. In our centre, we evaluated the impact of zona drilling with or without blastomere aspiration on day 3 embryos obtained from abnormal fertilization (Joris et al, 1999). Zona drilling only resulted in reduced survival rates. This rate was further influenced by the aspiration of 1 or 2 blastomeres. In this study, DMSO was the cryoprotectant used. Lee and Munné (2000) were the first to report a pregnancy after replacement of frozen-thawed biopsied embryos. Seven euploid day 3 embryos from 1 patient which developed after polar body biopsy were cryopreserved using sucrose only. Two embryos were partially intact after thawing. The replacement of these embryos resulted in a singleton delivery of a healthy girl. Another study evaluated survival rates of embryos obtained after abnormal fertilization using different zona drilling procedures (Ciotti et al, 2000). In this study, PROH was the cryoprotectant used. These authors found a significant lower survival rate of zona-manipulated embryos as compared to their non-biopsied counterpart. Besides this observation, they also found that zona drilling by PZD only as described by cieslak et al (1999), resulted in better survival rates as compared to embryos where this procedure was performed in combination with blastomere aspiration. Wilton et al (2001) reported the birth of a healthy child after replacement of frozen-thawed biopsied embryos. Here cryopreservation of biopsied embryos was required because of the procedure for diagnosis used. Later on, the same group reported improved cryopreservation outcome after modification of their standard procedure (Jericho et al, 2003). After using their standard freezing and thawing procedure using 1.5 M PROH in combination with 0.1 M sucrose, the blastomere survival rate of biopsied frozen-thawed embryos was 46 % as

compared to 76 % for non-biopsied embryos. The modification of their procedure included 1) an increase of the sucrose concentration at the time of freezing, 2) replacement of HSA by a 20 % solution of heat inactivated serum and 3) an increased concentration of sucrose during the thawing procedure. A significantly higher blastomere survival rate was obtained using this modified procedure on a fairly large number of embryos. The replacement of 50 embryos using this modified procedure in 36 embryo transfers resulted in a 12 % implantation rate. According to the information in both papers, the pregnancy reported earlier was obtained using this modified protocol.

Results of freezing and thawing at the AZ-VUB

The first PGD cycle at the AZ-VUB was performed in 1993. In this cycle, more genetically normal embryos than necessary for replacement were available and cryopreserved for later use. The number of cycles with embryo biopsy for PGD or PGD-AS increased over the years. Up until December 2003 this resulted in cryopreservation of 497 biopsied embryos in 191 freezing cycles. Over the years, laboratory conditions changed. Probably, the modification with the largest impact on laboratory procedures was the introduction of sequential media allowing culture embryos up until the blastocyst stage. Over the years, embryos have been cryopreserved at different developmental stages. This required the use of different cryopreservation procedures. Multicellular embryos were cryopreserved using a slow freezing and thawing protocol using DMSO as the cryoprotectant. Compact and blastocyst stage embryos were cryopreserved using the protocol suggested by Ménezo et al using glycerol as the cryoprotectant. In the same period, 147 embryos have been thawed in 38 thawing cycles. Survival rates of embryos in relation to the cryopreservation procedure are summarized in the table.
In our hands, frozen-thawed blastocysts developing after embryo biopsy tend to survive better as compared to multicellular embryos. So far, 1 delivery occurred after replacement of frozen-thawed blastocysts.
An explanation for the different cryosurvival rates may be 1) different cryobiological properties of blastomeres at the blastocyst stage, 2) the different embryo structure with more, tightly packed and smaller cells, 3) an intrinsic better embryo quality of those embryos reaching the blastocyst stage and 4) different properties of the cryoprotectant used in both procedures.

In the past, we observed that blastomere damage in partially intact multicellular embryos occurred more frequently close to the site where the opening was made. We speculated that the opening in the zona results in reduced protection of the blastomeres during freezing and thawing (Joris et al, 1999). Exposing mouse embryos to hyper- and hypo-osmotic solutions and comparing blastocyst development of embryos with or without zona drilling tested this hypothesis. This exposure to solutions of different osmolarities represents to a certain extent the situation of the embryo during freezing and thawing. Exposure to hypo-osmotic solutions represents the situation of the embryo during the thawing procedure. After zona drilling, swelling of blastomeres was not limited to the area under the zona.

Swelling of blastomeres beyond their critical point resulted in lysis. The results from this study confirm previous findings (Mazur and Schneider, 1986; Pedro et al, 1997). However, the impact of a partially intact zona was not yet evaluated.

Conclusions:

To date, most results after cryopreservation of biopsied embryos are disappointing. However, biopsied embryos surviving the freezing and thawing procedure can implant and develop to term. In contradiction to cryopreservation results after ICSI, the creation of a relatively large opening in the ZP of human embryos has a significant impact on cryopreservation outcome. The physical intervention of opening the zona to remove a polar body or 1 or 2 blastomeres has a greater influence than expected from mouse studies. The difference in size between mouse and human embryos probably contributes to this difference. For this specific group of embryos, physical characteristics rather than cryobiological properties of the embryos determine the outcome of freezing and thawing.

In order to improve cryosurvival, further efforts have to be made. Several possibilities may be investigated:

- Evaluation of the possible protective role of macromolecules such as globulins as well as other non-physiological macromolecules (Ficoll, polyvinyl alcohol…)
- Evaluate the use of alternative procedures to close the opening in the zona
- Limit swelling of blastomeres during thawing by modifying the cryoprotectant solution
- Reduce the impact of ice crystal formation by using ultra rapid cryopreservation procedures or vitrification.

Table: Results of thawing in relation to the cryopreservation procedure used

	DMSO	glycerol
Thawed embryos	62	85
Embryos recovered	61	84
Survival		
100 %	8 (13.1)	15 (17.9)
50-99 %	17 (27.9)	31 (36.9)
1-49 %	17 (27.9)	14 (16.7)
0 %	19 (31.1)	24 (28.6)
Percent survival (≥ 50 %)	**41.0**	**54.8**
Embryo replacements	9	17 *
Embryos replaced	18	27

*: One or more frozen-thawed biopsied embryos together with 1 or more fresh biopsied embryos or with frozen-thawed non-biopsied embryos was replaced in 3 cycles.

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NOTES

Vitrification: a promising method for the cryopreservation of human embryos

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1. Vitrification and cryoprotectants

Embryo freezing from the zygote stage till the blastocyst stage is an essential part of any in-vitro fertilisation program. Two different techniques have been developed to cryopreserve embryos: slow freezing (1) and vitrification.(2) During the slow freezing process, the formation of intra-cellular and extra-cellular ice crystals is common and can cause irreversible cell damage. Therefore vitrification could be considered as an alternative technique which avoids the formation of ice crystals in the intra-cellular and extra-cellular space.

Vitrification is the solidification of a solution at low temperature without ice crystal formation, a process achieved by a combination of a high concentration of cryoprotectant (30 – 50% (v/v)) and an extremely high cooling rate. This amorphous, or vitrified, phenomenon can be regarded as an extreme increase of the viscosity. Therefore, during the vitrification process, when the embryos are immersed into liquid nitrogen, the solution solidifies so swiftly that the molecules do not have sufficient time to rearrange themselves into a crystal structure.

The classical methods of slow embryo freezing are based on a progressive dehydration of the embryo during the cooling process. In contrast, for vitrification, the cells are dehydrated mainly just before the cooling process starts, by exposure to the high concentrations of cryoprotectants necessary to obtain a vitrified intra- and extra-cellular state (Fig 1).

Early studies (2., 3, 4) investigated different vitrification protocols in different mammalian species.

After these initial promising papers, numerous other studies followed and concluded that vitrification is the method of choice for freezing embryos of several animal species (5). Recently Shaw and Jones (6) reported that the in-vivo and in-vitro development of many mouse strain embryos cryopreserved by vitrification procedures is statistically the same as control non-frozen and significantly better than for slow cooled embryos.

The necessity to expose embryos to high concentrations of cryoprotectants (toxic injuries) has meant that many ART centres are cautious about using vitrification for clinical application..

However, it is possible to limit cryoprotectant toxicity by using a solution of two different cryoprotectants, thereby decreasing the relative concentration of each. As for the freezing protocols, the cryoprotective media commonly used for vitrification are composed of permeating (ethylene glycol and/or DMSO) and non-permeating (sucrose or trehalose) agents. In some protocols, the vitrification medium is also supplemented with macromolecules such as polyethylene glycol (PEG; MW 8000), ficoll (MW 70,000 or 400,000) or polyvinylpyrrolidone (PVF; MW 360,000). These polymers are generally less toxic and can protect the embryos against cryo-injury, while also increasing the viscosity of the solution. By increasing the viscosity, the macromolecules support vitrification with lowered concentrations of cryoprotectants (7). The viscous matrix in which the embryos are encapsulated also prevents water crystallization during cooling and warming. Kuleshova et al 7 reported increased survival of oocytes after vitrification with macromolecules than with a «classic» vitrification solution containing only permeating cryoprotectants.

It is also possible to reduce the toxicity of the vitrification solution by reducing the length of time the embryos are exposed to it, or by pre-cooling it.

2 Importance of the cooling rate: from the rapid cooling to the ultra-rapid cooling procedures.

2.1. Rapid vitrification procedures

In classical vitrification procedures, 0.25 ml paillettes containing the embryos were immersed in liquid nitrogen, giving cooling rates of up to 2000°C/min (8-9). The cooling is slowed by the thickness of the wall of the straw, by the relatively large volume of liquid contained within the straw and by the boiling of the liquid nitrogen around the outside of the straw. The vapour formed, surrounds the straw and insulates it from the liquid nitrogen. This phenomenon, known as the Leiderfrost effect, can be reduced by using a system (Vitmaster-slush freezing) which decreases the temperature of the liquid nitrogen as low as –210°C, thereby eliminating vapour formation (10).

2.2.Ultra-rapid vitrification

In 1996 Martino et a (11) demonstrated that an accelerated cooling rate improved the cryosurvival rate of bovine oocytes. Ultra-rapid freezing reduces the probability of ice crystal formation during freezing, prevent devitrification during the thawing process and also decreases the problems associated with “chilling” that occur more often with oocytes. This technique can be used with lower concentrations of cryoprotectants.

2.3. Carrier systems for ultra-rapid vitrification procedures

The container used and the total volume of the sample to be frozen determines the size of the contact area with the liquid nitrogen, and thus the cooling rate. In ultra-rapid vitrification techniques the smallest possible

volumes of vitrification solutions are used in specialized carrier or support devices, giving cooling rates approaching 10,000°C/min.to 20,000°C/min.

An early concept of embryo support for ultra-rapid vitrification was to use straws with very thin walls, to decrease the thermal gradient. Vajta et al (12) used the Open Pulled Straw system. Liebermann et al (13) used the Flexipet-denuding pipette to achieve successful vitrification of bovine and murine oocytes.

The second group of carrier systems allowed direct contact between a small volume of vitrification solution and liquid nitrogen

Different carrier systems were developed:

- o electron microscopy copper grids (11,14,15);
- o the "Cryoloop" (16,17,18,19); and
- o the Hemi-Straw (HS) system (20,21)

3. Clinical application of vitrification in ART

Successful pregnancies and deliveries following vitrification of human embryos at different stages of development have been reported.

3.1. Zygotes

Successful vitrification of zygotes, resulting in high survival rates (81-88%), high day 2 cleavages rates (77-85%) and acceptable blastocysts formation (31%), followed by pregnancies, have been reported (13,14,22).

3.2. Cleavage stage embryos

The first birth after vitrification of human 8-cell embryos was reported by Mukaida et al (23), using a mixture of ethylene glycol (40%), Ficoll and sucrose. El-Danasouri and Selman (24) reported also successful pregnancies and deliveries following the vitrification of day 3 embryos with less than 6 cells (79.2% vs 21.1%).

After perfoming 27 vitrification-warming cycles of day 3 human embryos with the hemi-straw system, we observed that although 84% of the embryos appeared totally normal after warming, only 33% reached the morula stage. Seventeen transfers resulted in the establishment of 6 ongoing pregnancies (Vanderzwalmen, unpublished data).

3.3. Blastocysts

Recently, Kuleshova and Lopata (25) reported that vitrification can be more favourable than slow cooling for blastocyst survival, and so must be considered as a viable alternative. Vitrification has been show to gain acceptance for the routine cryopreservation of human blastocysts and several live births have recently been reported (8,15,20,26,27,28) using different carriers (table 1)

3.3.1 Blastocysts vitrification with the hemi-straw carrier.

With the increase of embryo culture to the blastocyst stage in our ART activities, it was necessary to establish a reliable procedure to cryopreserve blastocysts. we established vitrification procedures for blastocysts.

From 1996 to 2000 the blastocysts were vitrified in 0.25 ml “French mini-straw”. In 2000, we designed a system called the Hemi-Straw (Vitro-Plug) that allows a direct contact between the embryo and liquid nitrogen (20)

> According to the level of the blastocoele expansion, blastocysts were first exposed to a solution containing 10 % ethylene glycol (V/V) – 10% DMSO (V/V) for 2 to 4 min. . They were then exposed to the vitrification solution (20 % ethylene glycol (V/V) - 20% DMSO (V/V) 25 µmol/l Ficoll (MW 400,000) and 0.75 M sucrose) for maximum 30 seconds. and deposited on the tip of the Hemi-Straw. The hemi-straw was then instantly plunged into a LN2 and with the aid of forceps inserted in a larger pre-cooled straw. (fig 2)
> For warming, the tip of the straw holding the embryos was immediately immersed into a petri dish containing 3ml of 0.5M sucrose at 37°C. After 3 to 4 minutes, the blastocysts were transferred to 0.25M and 0.125M sucrose at intervals of 2 minutes at 37°C.

At the present time, our results achieved with the Hemi-Straw method demonstrates that human blastocysts can be successfully vitrified. At the present time, out of a total of 167 vitrification-warming cycles, 50 ongoing pregnancies (29)%) after transfer of an average of 2 blastocysts were obtained with an implantaion rate of 19%. (fig 3)

Different parameters, such as the level of expansion of the blastocoele or an hardening of the ZP can affect the results.

Reduction of the blastocoelic cavity: “artificial shrinkage”

Cryopreservation is usually carried out on blastocysts with various degrees of expansion up to a partially or totally hatched state. We and others previously reported that blastocysts with a larger blastocoele are more sensitive to vitrification and that artificially reducing the fluid content within the cavity could improve the efficiency of the vitrification (15,29).

Application of artificial assisted hatching of blastocysts after warming

Cryopreservvation induces hardening of the zona pellucida, which may impair spontaneous hatching after thawing. In our experience, the artificial opening of the Zona pellucida of thawed blastocysts, with partial zona dissection techniques, significantly improved the implantation and pregnancy rates (20) (table 2).

3.3.2.Vitrification with the HS carrier of hatching and hatched blastocysts: an approach to cryopreserve biopsied embryos.(fig 4)

Beside the worldwide increasing use of preimplantation genetic diagnosis attempts and the sensitivity of the biopsied embryos to the cryopreservation

(30,31), there is a need to establish a strategy in order to allow optimal survival after thawing of biopsied embryos. The possibility to vitrify hatching or completely hatched blastocysts may be considered as an approach to cryopreserve embryos especially after PGD.

Therefore we analysed the survival and the further developmental potential of vitrified and thawed blastocysts either in hatching or in completely hatched state. We observed (32) that the presence of a large opening in the ZP of blastocysts has no negative influence on the survival and further development after vitrification using the HS technique (Fig3). One solution could be to cryopreserve PGD embryos after extending the culture of the biopsied embryos to the blastocyst stage before vitrification.

4. Conclusions

Several very significant advances have recently been made in ultra rapid cooling of human embryos, and as a result we may now be at the point where a transition from slow to rapid cooling procedures will start to take place also for the human (6).

This inevitable that both freezing and vitrification methods will improve as a result of directed research, and it is possible that vitrification will prove to be a more favourable technique for the cryopreservation of cold-sensitive oocytes and the more complex cellular system of the blastocyst.

However, an issue which must be addressed immediately in cryobanking is that of bio-containment. The requirement for direct contact between the cryo-medium and liquid nitrogen for successful vitrification introduces a potential difficulty associated with the packaging for storage after vitrification, one which can only be addressed by the development of a technique which ensures the carries are sealed securely into cryocontainers before cryostorage.

Table 1: Vitrification of blastocysts using different embryo supports

Carriers	**Micro-grids** Choi(2000)	**Cryoloop** Mukaida (2003)	**Hemi-Straw** Vanderzwalmen (2003)
Vitrification cycles (n)	34	164	145
Survival (%)	52	87	68 (a)
Transferred blastocysts : average	1.9	2.5	1.9
Ongoing pregnancies / vitrification cycles	5 (25%)	49 (28%)	41 (28%)
Implantation rate (%)	21	20	18

(a) survival after 24 h of culture.

Table 2 : Vitrification-warming of blastocysts using the Hemi-Straw carrier and transfer after 24 h culture: survival, pregnancy and implantation rates in relation to assisted hatching of the ZP

Assissted hatching	**Yes**	**No**
Vitrification cycles (n)	82	64
Survival (%)	73	65
Transferred blastocysts: average	1.9	2.0
Ongoing pregnancies / vitrification cycles	30 (37%) a	14 (22%) a
Implantation rate (%)	20.6	12.6 a

a X^2 $P< 0.05$

Table 3: Results of vitrification and warming cycles after artificial zona opening

Nb. warming cycles	16
Nb of vitrified blastocysts with open ZP	31
Aspect of the blastocysts before vitrification	
Expanded with open ZP	7
Hatching	16
Totally hatched	8
Blastocysts survived after warming	26 (83.9%)
Ongoing pregnancies	7 (43,8%)
Implantation rate	
Per warmed blastocyst	22.6%
Per transferred blastocyst	26.9%

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Fig 1: Step of dehydration in slow and vitrification procedures

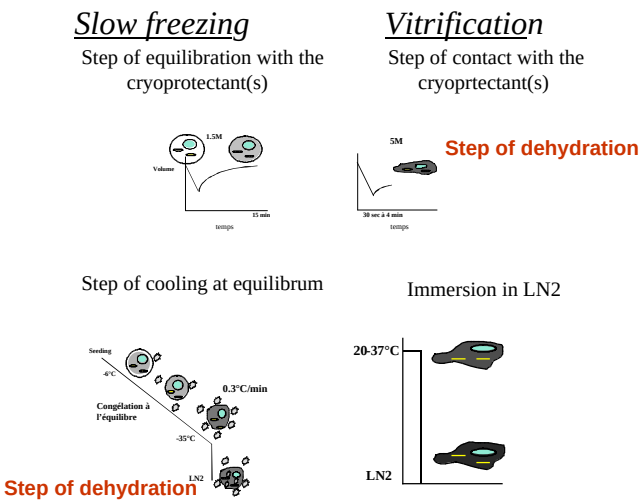
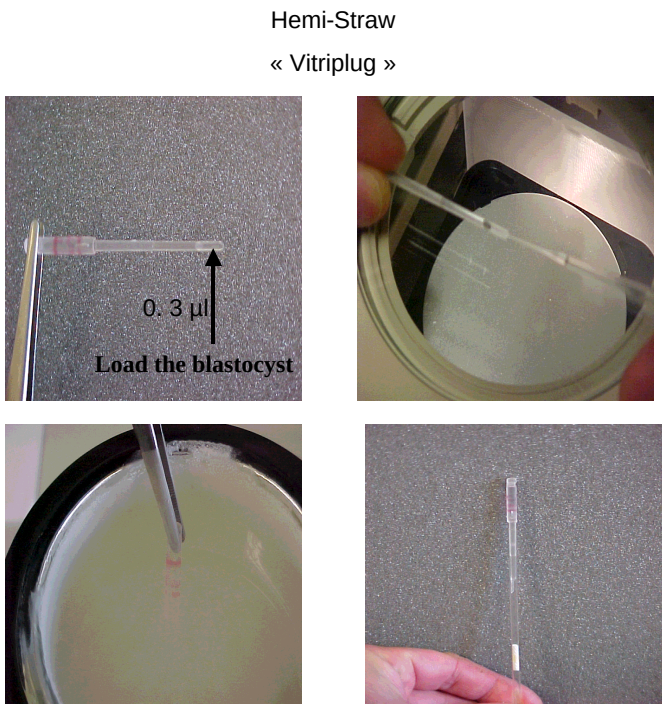
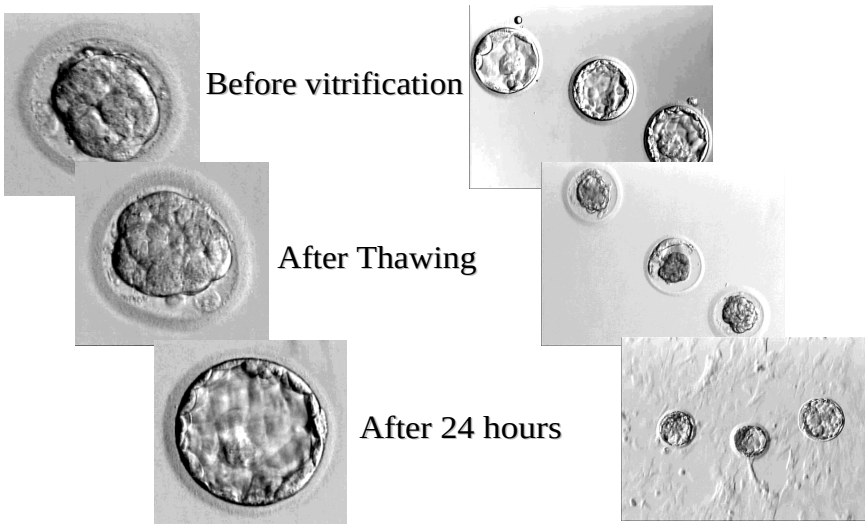


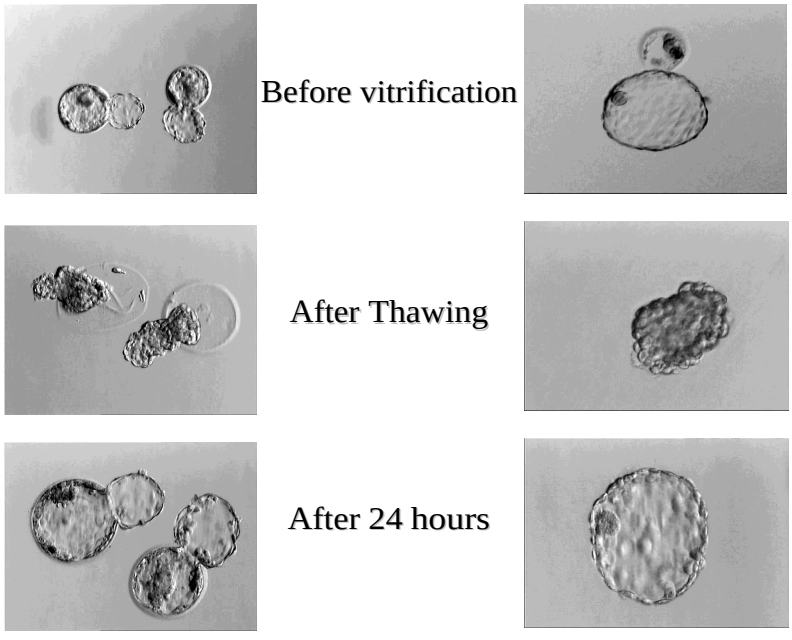
Fig 2: Loading of the embryos on the Hemi-Straw before plunging in LN2



Vitrification of Morulae and blastocysts with the Hemi-Straw (FIG 3)



Vitrification of Hatching and Hatched Blastocyst (FIG 4)



NOTES

Safe cryopreservation

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Introduction

The risk management of storage services for reproductive cells, (prevention of losses, financial or otherwise) is becoming an increasingly sensitive and important issue, particularly with regard to: injury to personnel, sample loss, premature sample thaw and the possibility of transmission of infectious disease between samples (Tedder et al, 1995). Protection of our samples is also required and the following require careful consideration:

I. The relative safety of the containment system (vials or straws)
II. The type of nitrogen storage (liquid v vapour phase)
III. The suitability of equipment to do the job
IV. Witnessing and security of labelling
V. Screening of patients for infectious diseases prior storage
VI. Methods of sample processing used to lessen the risk of transmission
VII. Early warning and monitoring systems e.g. high temperature alarms

Although, this article will discuss a number of these issues, the main focus will be on part VI, ensuring that samples remain frozen and that should any incident occur, appropriate early warning systems are in place to prevent it turning into a catastrophe.

1. Relative safety of the containment system (vials or straws)

In the reproductive field, we are not over-blessed with choice when deciding upon containment for our sperm and embryos. There are basically 3 options, a. PVC straws, b. cryovials or c. Ionomeric resin (CBS) straws. The relative merits of each could be discussed at length and this is not the purpose of this article. What seems reasonably indisputable however is that:

- PVC straws - are fragile after immersion at 'ultra-cold' temperature and have a tendency to break. In addition, as commonly used powder PVA plugs are ineffective, the straws represent an infection risk

- Polypropylene Cryovials - allow ingress of liquid nitrogen due to an ineffective seal. Manufacturers clearly state that they are for use in vapour only, yet centres continue to use them in the liquid phase despite a theoretical infection and explosion risk.

- Ionomeric resin (CBS) straws – Have a more effective sealing method than PVC straws and are less likely to break.

2. Type of nitrogen storage (liquid v vapour phase)

The relative merits of liquid vs vapour storage provide us with an ongoing debate (see Tomlinson and Sakkas, 2000).
As both the national blood service and tissue banking fraternity have moved over to storage in the vapour phase, it is therefore likely that the reproductive field will have to follow suit. This will either require investment in automated (intelligent) freezers or adaptation of existing liquid dewars (see Clarke, 1999). It is important to take into account that the use of vapour phase does not entirely eliminate the risk of transmission of infective organisms (fountain et al, 1997).
However, it is more likely that the argument is largely out of our hands and will most likely be settled by changes in line with the EU directive on the storage of cells and tissues (see http://europa.eu.int/eurlex/en/com/pdf/2002/com2002_0319en01.pdf).

3. The suitability of equipment to do the job

All equipment has a finite lifespan. As nitrogen vessels age their vacuum will slowly diminish until eventually the vessel will hold nitrogen for too short a period for it to be of use. There is usually warning of this (frost on outside of vessel) and plenty of time to provide a replacement. Situations such as this should however be pre-empted by having appropriate capital equipment replacement programs e.g. on a 10-year cycle. Vacuum failure can also be an acute event, for example if welded joint fails, in which case the vessel will lose its nitrogen and begin warming within hours. This is totally unpredictable and is as likely to occur in new vessels as it is in an old one. Clearly in this instance there needs to be in place a further risk strategy to minimise losses. This may be an early warning system as mentioned later in this article, providing spare vessels and/or perhaps spreading the risk by splitting the samples over two (or more) vessels.

4. Witnessing and security of labelling

Centres should ensure that all processes involving transfer of gametes or embryos must be verified e.g. transfer of sperm/cryoprotectant to straws or vials. Labelling of straws or vials must withstand immersion in liquid nitrogen and/or extreme cold. In addition, labelling must be clear and accurate, using appropriate labelling pens and avoiding poor handwriting, which can lead to transcription errors. Automated labellers or barcode generators may certainly reduce operator time and error and help to provide clear and accurate labels.

5. Screening of patients for infectious diseases prior storage

The screening of all patient for HIV and Hepatitis b and c must be implemented by December 2004. This risk reduction strategy has also been implemented by the blood and tissue banking services and will no doubt have

major benefits. However, centres should not become over-reliant on it , particularly as quarantine to cover the 'window of seroconversion' may not be practical and other yet undiscovered pathogens will no doubt cause us a problem in the future. We must therefore reduce our risks further by implementing a combined strategy.

6. Methods of sperm cryopreservation / sample processing to reduce risk

The risks associated with storing sperm and embryos tend to be very different. By the time an embryo is created, a large number washing/processing steps have already taken place, each one reducing potentially contaminating pathogens. On the other hand most sperm storage is undertaken using raw semen without processing of any sort. Potentially this raw semen could harbour a relatively high pathogenic load and as such the risk of transmission within a nitrogen dewar is that much higher. Reductions in seminal viral load to almost undetectable levels have been clearly demonstrated by groups treating HIV discordant couples, simply by sperm washing and density gradient centrifugation to which removes contaminating leucocytes and seminal plasma (Kim et al, 1999). Sperm processing prior to storage therefore represents yet another method of reducing risk.

By complying with the EU directive in 2005, risks associated with processing and storage of gametes and embryos should by reduced as a matter of course. Procurement and validation of materials, equipment and procedures used during storage will all serve to protect the samples and can only be observed as a positive result of the directive.

7. Early warning and monitoring systems for the cryoroom

Well-publicised incidents (both storage and non-storage) in the last 3 years have all done little to increase public confidence in our particular branch of medicine. Indeed the recent storage incident affecting cancer patient samples served as a reminder to us all that the samples we handle are extremely precious and we owe to our service users to do everything possible to keep them in good order. A survey carried out in the UK suggested that only 50% of clinics had alarms of any description on their sperm and embryo freezers. Furthermore, fewer than 25% of centres had alarms linked to external warning systems such as auto-diallers or fire alarm panels to deal with out of hours emergencies.

<u>Alarms</u>

Alarms are generated in liquid vessels only when the temperature is raised above the designated threshold. This does require a cautious approach, as vessels will remain extremely cool with only a few cm of nitrogen in the bottom but will warm rapidly once this has evaporated.

Intelligent freezers generate their own alarm signal in response to a number of conditions, most of which are related to autofilling and liquid nitrogen supply. As they depend highly on autofilling systems there is a tendency to

generate 'non-critical' alarms, quite often because pressure in the nitrogen supply vessel has dropped below the level at which it can deliver nitrogen. Careful cryoroom management and building in appropriate alarm delays should alleviate these problems.

Remote access and system interrogation

Remote access, where it can be developed, has tremendous benefits, especially in the prevention of unnecessary call-out and theoretically, monitoring can take place from almost anywhere providing the software is installed onto a portable PC and an appropriate mobile phone link-up is available.

Out of hours service

Proper management of the system during the week and in normal working hours keeps the need for out of hours call outs to a minimum and out of hours visits to the cryoroom are generally unnecessary. However, it is essential that a system exists to ensure that a member of staff is available to attend in an emergency.

Concluding Remarks

The cryoroom represents an enormous responsibility particularly for scientists involved in reproductive medicine. However, as responsible keepers, we owe it to the users of our service (clinicians and patients) to protect stored sperm, embryos, eggs, tissues etc by whatever means we can. A combination of risk reduction strategies should be implemented and backed up by an early warning system. Any early warning system has to be affordable, manageable, easy to use and implemented alongside other risk reduction strategies..

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NOTES

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Oocyte freezing: here to stay?

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Oocyte freezing is an established technology but, in contrast to embryo freezing, it has very limited application in clinical IVF programmes. Is there a chance that oocyte freezing will become an integrated routine in assisted reproductive technology? The delicate cytological architecture of the oocyte with a cold-sensitive spindle and a hardening zona have made the frozen oocyte 'unwanted' in assisted reproductive technology. Nevertheless, empirical improvements in freezing protocols and the use of ICSI for fertilization have led to an increasing number of live births. This mitigates against a simple ban on oocyte freezing. While efficiency of oocyte freezing can certainly be further improved by basic research, it is clear that there are humanitarian reasons for considering oocyte freezing as a future fully utilized assisted reproductive technology. The storage of the female genome as a particulate entity can provide an alternative in case of moral, ethical, legal or religious concerns about embryo freezing. Oocyte freezing can also offer hope for oocyte donation and preservation of fertility for women facing ovarian loss. The message is one of cautious optimism when looking for a place for oocyte freezing in routine assisted reproductive technology.

Key words: dimethylsulphoxide/ICSI/oocyte banking/oocyte cryopreservation/1,2-propanediol

Introduction

This review will be non-classical in the sense that it will not merely cover literature and provide a list of cryopreservation protocols but will try to answer pending questions on the place of oocyte freezing in assisted reproductive technology. The most intriguing question is whether freezing of unfertilized oocytes such as obtained by oocyte retrieval will ever become a standard IVF routine or remain a local application and, at best, an escape route for suddenly available oocytes? The first success with oocyte freezing was obtained in the mouse in 1977. However, interest was soon lost as the first IVF baby startled the world in 1978 and in 1983 the first human pregnancies and live births from frozen embryos were announced (Trounson and Mohr, 1983; Zeilmaker *et al.*, 1984). Because embryo freezing was not generally morally acceptable, human oocyte freezing was suddenly introduced clinically in 1986 based on the limited experience in mouse oocytes. It soon became clear that human oocyte freezing was compromised by very low efficiency. This drove researchers to investigate the biology of frozen mammalian oocytes. The aim of this review is to take the reader along the pathway of oocyte freezing from the past towards a realistic estimation of the present situation and future prospects.

Are mouse oocytes freezable?

It should be understood from the beginning that oocytes are freezable cells. The question on freezability is rather on the way oocytes can be thawed without damage to the oocyte itself or to the embryo to be.

The first report on successful freezing of a mammalian oocyte was in the mouse (Whittingham, 1977). It was shown that live offspring had been obtained after IVF of mouse oocytes previously frozen with dimethylsulphoxide (DMSO) and stored under liquid nitrogen at –196°C.

Despite this success, several problems with oocyte freezing were discovered. Freeze-thaw induced hardening of the zona pellucida and explained decreased rates of fertilization in frozen–thawed mouse oocytes (Johnson, 1989; Carroll *et al.*, 1990; Vincent *et al.*, 1990; Wood *et al.*, 1992). Inside the oocyte, the temperature-sensitive meiotic spindle seemed to be a target in freezing (Pickering and Johnson, 1987; Van der Elst *et al.*, 1988; Aigner *et al.*, 1992). The phantom of genetic abnormalities completed the picture of failure. Increased polyploidy in first-cleavage mouse embryos from frozen–thawed oocytes fertilized *in vitro* was found to be a major problem (Glenister *et al.*, 1987; Carroll *et al.*, 1989; Bouquet *et al.*, 1992, 1995). For a while, ultrarapid freezing and vitrification were seen as miracle solutions (Surrey and Quinn, 1990) but soon problems of genetic nature or fetal defective development appeared here as well (Van der Elst *et al.*, 1993a; 1998). A single report was published on increased aneuploidy after vitrification of mouse oocytes (Kola *et al.*, 1988). Some reassurance came from publications suggesting that chromosomal abnormalities of maternal origin, such as digynic polyploidy and maternal aneuploidy, were probably procedure-related for cryopreservation protocols with DMSO and thus

avoidable provided that procedures are meticulously respected (Bos-Mikich and Whittingham, 1995; Bos-Mikich *et al*., 1995). Where all of the above had been linked to the use of DMSO or to high concentration cryoprotectant cocktails with DMSO, the danger with the other popular cryoprotectant 1,2-propanediol (PROH) was of a different nature in that it induced parthenogenetic activation in mouse oocytes and thus rendered them non-fertilizable (Shaw and Trounson, 1989; Van der Elst *et al*., 1992b).

Since the major concerns in oocyte freezing such as spindle damage, chromosomal abnormalities and parthenogenesis were all linked to the specific nuclear configuration of mature metaphase II oocytes, hope was put in using immature oocytes in the germinal vesicle stage. Immature oocytes were indeed seen as a clever solution because the chromosome problems were possibly circumvented with the chromosomes protected by the nuclear membrane. But soon it was to be discovered that not the nucleus but the cytoplasm is the problem in immature oocytes (Schroeder *et al*., 1990; Van der Elst *et al*., 1992a; 1993b; Candy *et al*., 1994; Frydman *et al*., 1997; Isachenko and Nayudu, 1999). Nuclear maturation can proceed as a clock-regulated programme and is easy to monitor microscopically by polar body extrusion, but microscopically invisible disturbance of the cytoplasm in the immature oocyte results in defective blastocyst development. For example, it was shown that loss of association between oocyte and cumulus cells was induced by cryopreservation (Cooper *et al*., 1998; Ruppert-Lingham *et al*., 2003). The data indicate that direct physical contact between cumulus cells and the oocyte throughout maturation, as a kind of lifeline, improves subsequent embryo development.

Away from the disappointment with cryopreservation of immature mouse oocytes, scientists returned to exploring preservation of mature oocytes. The mathematical cryobiology of the oocyte as a unique type of cell to freeze was researched in studies trying to optimize mouse oocyte freezing protocols. The temperature dependence of mature mouse oocyte membrane permeabilities in the presence of cryoprotectant was studied (Leibo, 1980; Paynter *et al*., 1997) and theoretically optimized protocols were designed (Karlsson *et al*., 1996). Other researchers varied freezing media by including molecules with cryoprotective value or by substituting for putative nocuous components. The beneficial effect of fetal calf serm on fertilization of frozen–thawed mouse oocytes was demonstrated (Carroll *et al*., 1993). Stachecki and colleagues identified high sodium concentration as possibly detrimental through building up of high solute concentrations and promoted a new freezing medium based on choline chloride and low sodium (Stachecki *et al*., 1998a;b; Stachecki and Willadsen, 2000; Stachecki *et al*., 2002). Increased survival and fertilization rates of cryopreserved mouse oocytes were also attributed to supplementation of freezing media with free radical scavengers (Dinara *et al*., 2001). Cryogenic protection was also related to antifreeze proteins (Rubinsky *et al*., 1992; Arav *et al*., 1993). For ultrarapid freezing and vitrification protocols, several new cryoprotectant cocktails were being mixed (Rayos *et al*., 1994; O'Neil *et al*., 1997; 1998) but outcome remained highly variable. More recently improvement has been announced by new promising technical innovations such as cryoloop vitrification (Lane and Gardner, 2001; Mavrides and Morroll, 2002) and open and pulled straws for vitrification (Chen et al., 2000, 2001), the latter reports being interesting because of high survival rates and good spindle pattern preservation.

From all the above it is clear that the oocyte is a very delicate biological material to freeze. Mouse oocytes can be cryopreserved successfully using slow freezing, ultrarapid freezing or vitrification methods. Success is certainly possible but the outcome is difficult to predict in a reliable way. Much seems ado about protocol variations. The unpredictable outcome of oocyte freezing most certainly prevents a breakthrough and makes it hard to plead for implementation in broad practical application, even in mice. But will these arguments hold against the background of a clinically assisted reproductive technology setting?

Are human oocytes freezable?

The story of human oocyte freezing is similar to that of mouse oocyte freezing. After initial successes, problems emerged with mature oocyte freezing. A solution was sought in immature oocyte freezing which was left in turn to reinvest in workable protocols for mature human oocytes.

The few early successes in human oocyte freezing blinded workers in IVF programmes towards cautionary notes by basic researchers and some clinical IVF teams galloped into human oocyte freezing. Problems linked to mature oocyte freezing had been foreseen by scientists studying electron microscopical morphology as well as fertilizability of frozen human oocytes (Sathananthan *et al*., 1987; 1988). These authors cautioned against integrating oocyte freezing in clinical IVF and advised further assessment of embryos developed from frozen oocytes. Nevertheless, soon after the precocious implementation of oocyte freezing in clinical IVF, the aggravating evidence quickly built up. Low survival rates and polyploidy rates of 20 and 40% were observed using DMSO and PROH as cryoprotectants respectively (Al-Hasani *et al*., 1987; Mandelbaum *et al*., 1988). As in the mouse oocyte, the zona and spindle were cryopreservation targets. Inappropriate exposure to DMSO reduced fertilization rates (Pickering *et al*., 1991). There was devastating irreversible disruption of the meiotic spindle in the human oocyte by transient cooling to room temperature (Pickering *et al*., 1990), and a direct association between temperature-induced spindle damage and chromosomal abnormalities in parthenogenetically activated human oocytes was found (Almeida and Bolton, 1995). Cryopreserved human oocytes, while fertilizable, arrested development during the early cleavage stages and displayed aberrant patterns of cytokinesis (Van Blerkom and Davis, 1994).

Given all the problems encountered with mature human oocytes, it was somehow logical to explore, as had been done for the mouse oocyte, the potential benefits of cryopreservation of the immature oocyte. Several reports (Mandelbaum *et al*., 1988; Toth *et al*., 1994; Son *et al*., 1996; Tucker *et al*., 1998a) showed that immature human oocytes could survive freezing and thawing, mature and be fertilized *in vitro*, but with a very low developmental efficiency. So it seemed that the same problems were encountered in human as in mouse immature oocyte freezing, namely, not the nucleus but the cytoplasm was the problem. Immature human oocyte freezing was quickly abandoned before giving it a chance to turn into a debacle.

As had been the case in mouse oocyte freezing, the setbacks and disappointing results with immature oocytes led a few groups of researchers to re-investigate workable protocols for human mature

oocytes. Some research groups conducted empirical trials in a clinical setting while others put their trust in mathematical protocol design.

In the first successful report of normal development of cryopreserved human oocytes to the hatching blastocyst stage (Gook *et al*., 1995), metaphase II oocytes were cryopreserved using a slow freezing–rapid thawing procedure employing the cryoprotectant PROH. Survival rates were moderate and ICSI was applied to overcome freeze–thaw-induced zona hardening (Gook *et al*., 1995; Kazem *et al*., 1995). Also PROH did not activate the human oocyte as it did with mouse oocytes (Gook *et al*., 1994a) and evidence of lack of chromosomal problems associated with the use of PROH for oocyte freezing was presented (Gook *et al*., 1994b). The first live birth from frozen oocytes was based on Gook's methodology (Porcu *et al*., 1997). Purely cryobiologically, results of optimization studies on exposure of human oocytes to the cryoprotectant PROH showed that commonly used cryopreservation protocols cause potentially damaging cell volume excursions and suggestions for remedy were made (Paynter *et al*., 2001). PROH protocols were further fine-tuned by working on increased sucrose concentrations, on varying exposure time and temperature before freezing and on Na-replacement leading to increased survival (Fabbri *et al*., 1998; 2001; Yang *et al*., 1998; 1999; Chen *et al*., 2002; Quintans *et al*., 2002; Boldt *et al*., 2003). Chromosomal analysis of human embryos obtained from frozen–thawed oocytes for chromosomes 13, 18, 21, X and Y showed no increase in the rate of chromosomal abnormalities, indicating that the technique is safe enough to be further explored and improved (Cobo *et al*., 2001). Nevertheless, publications continue to point to genetic risks of oocyte freezing, even with PROH (Boiso *et al*., 2002) due to a deleterious effect on the organization of the meiotic spindle of cryopreserved human oocytes. Perhaps these difficulties can be circumvented by giving the spindle a chance to restore by adjusting the time between oocyte thawing and ICSI (Eroglu *et al*., 1998; Chen *et al*., 2001; 2002).

Following the success story with PROH, effects of DMSO on human oocytes were further studied but did not make it to the clinic again (Paynter *et al*., 1999). It was found that the permeability parameters of human oocytes for DMSO are higher than those of murine oocytes, suggesting that they require a shorter period of exposure to DMSO with concomitantly reduced toxic effects. Taking into account the volume, hydraulic conductivity and permeability to DMSO of human oocytes, a protocol was designed for addition and removal of DMSO to control the magnitude of volumetric excursions (Newton *et al*., 1999). Moreover, it was found that unfavourable freezing conditions that promote intracellular ice formation can be prevented and survival rates maximized by raising the seeding temperature to –4.5°C, which is above the currently used –7°C (Trad *et al*., 1999). This may be important for protocols with DMSO as well as PROH. In addition to traditional slow freezing, several reports on enhanced survival rates by human oocyte vitrification methods have been published recently (Hong *et al*., 1999; Chung *et al*., 2000).

Several previous reviews and opinions on oocyte freezing have been published (Bernard and Fuller, 1996; Mandelbaum *et al*., 1998; Gook and Edgar, 1999; Ludwig *et al*., 1999; Paynter, 2000; Wininger and Kort, 2002).

Pregnancies from frozen oocytes in clinical assisted reproductive technology programmes

Whatever the caveats were or still are, the reality is that clinical programmes on oocyte freezing are running albeit in a limited number of particular settings in Italy, Germany (embryo freezing prohibition law), USA, South America and Asia. What follows is a chronological listing of human pregnancies from frozen oocytes. Some publications have been mentioned above when discussing freezability of human oocytes, but it seems worthwhile to put together a chronological list of clinical successes. Details on number of oocytes, survival and fertilization rates, embryo cleavage, pregnancies and live births are shown in Table I. This list should give a fair estimate of the number of pregnancies and live births currently (83 pregnancies, 50 deliveries and ongoing pregnancies and 63 children).

In the eighties only a handful of pregnancies from frozen oocytes were reported, all based on DMSO cryopreservation protocols (Chen, 1986; 1988; van Uem *et al*., 1987). Low success rates discouraged the continuation of these clinical programmes.

At the end of the 1990s, oocyte freezing became more common again after the encouraging PROH protocol mastered by Gook and leading to the hatching blastocyst stage from frozen human oocytes (Gook *et al*., 1994a;b; 1995). Establishment of pregnancy was reported 1 year later (Tucker *et al*., 1996) but none of the pregnancies went successfully to term. The first live birth from human oocytes cryopreserved with PROH and fertilized by ICSI was achieved in Italy (Porcu *et al*., 1997). The indications for oocyte freezing were objections against embryo freezing and, most of all, trying to spare oocytes from donor programmes. Some more case reports on oocyte cryopreservation in donor programmes followed (Polak de Fried *et al*., 1998; Young *et al*., 1998) before reports on larger series of successful pregnancies followed (Borini *et al*., 1998; Porcu *et al*., 1998; 1999; 2000; Tucker *et al*., 1998a). In another case report from Germany, unfertilized oocytes were frozen in a case of impressive multifollicular growth, in which the couple did not consent to the freezing of pronuclear stage oocytes for ethical considerations (Wurfel *et al*., 1999). These authors reported a twin pregnancy and parturition of a healthy girl and a healthy boy after ICSI of frozen–thawed oocytes. That a cautious approach remains necessary is shown by a report on a triploid pregnancy after ICSI in frozen oocytes (Chia *et al*., 2000). Induction of polyploidy after oocyte freezing has been described as a consequence of freezing aged oocytes (Gook *et al*., 1994b; 1995). Protocol variations, such as increasing sucrose concentration and varying time and temperature of exposure, led to pregnancies and live births from oocytes so treated (Chen *et al*., 2002; Yang *et al*., 1998; 1999; 2002; Winslow *et al*., 2001). Learning lessons from the Stachecki mouse studies, human pregnancies and live births were obtained using a low sodium choline-based oocyte freezing medium (Quintans *et al*., 2002; Boldt *et al*., 2003). Pregnancies were also obtained from vitrified oocytes (Cha *et al*., 1999; Kuleshova *et al*., 1999; Yoon *et al*., 2000).

For the record, it has to be mentioned that birth after slow-freezing immature oocytes (Tucker *et al*., 1998b) and biochemical pregnancy after vitrification of immature oocytes (Wu *et al*., 2001) were also obtained.

The above list of pregnancies shows that oocyte freezing technology seems to improve day by day (Marina and Marina,

Table I. Clinical results of mature oocyte freezing

Reference	Oocytes thawed	Survived (%)	Fertilized (%)	Cleaved (%)	Pregnancies	Live births
Chen *et al.* (1986; 1988)	50	76	75	60	1 twin 1 singleton	1 twin birth 1 singleton birth
van Uem *et al.* (1997)	28	25	–	–	1 singleton	1 singleton birth
Porcu *et al.* (1997, 1998)	709	56	63	90	9	4 singleton births 1 twin birth
Porcu *et al.* (1999)	1502	54	57	91	16	7 singleton births 2 twin births
Porcu *et al.* (2000)	–	59	64	–	3	1 birth
Borini *et al.* (1998)	129	51	51	94	3	2 births 1 abortion
Tucker *et al.* (1996)	81	25	65	100	3	No term birth
Tucker *et al.* (1998a)	241	31	51	74	5	1 twin birth 1 singleton at 37 weeks
Polak de Fried *et al.* (1998)	10	30	66	100	1 singleton	1 singleton birth
Young *et al.* (1998) Yang *et al.* (1998; 1999)	9	89	100	62	1 triplet	Interrupted
Winslow *et al.* (2001)	324	68	81	95	17	6 singleton births 3 twin births 1 triplet birth
Cha *et al.* (1999)	7	100	100	100	1	1 singleton birth
Kuleshova *et al.* (1999)	17	65	45	60	1	1 singleton birth
Wurfel *et al.* (1999)	4	75	100	100	1 twin	1 twin birth
Yoon *et al.* (2000)	90	63	68	89	3 singleton	2 singleton births 1 ongoing at 25 weeks
Chen *et al.* (2002)	8	100	57	100	1 twin	Ongoing at 9 weeks
Chia *et al.* (2002)	12	83	70	86	1 singleton	Abortion (triploid)
Quintans *et al.* (2002)	109	63	59	100	5 singleton 1 twin	1 singleton birth 1 singleton birth
Marina and Marina (2003)	99	–	–	–	4 pregnancies	3 singleton births 1 twin birth
Boldt *et al.* (2003)	90	74	59	85	3 singleton 1 twin	3 singleton births 1 twin birth

2003). This should give some ground for cautious optimism. The final judgement will be made by following up the outcome of children originating from frozen–thawed oocytes (Wennerholm, 2000).

Is there a future for oocyte freezing in routine clinical assisted reproductive technology?

Oocyte freezing is possible, live children have been born. For workers in the assisted reproductive technology field there is, however, no shadow of a doubt that oocyte freezing is a marginal routine in daily clinical IVF practice.

The current obvious benefit of embryo freezing is very unlikely to be abandoned for the currently still doubtful benefit of oocyte freezing. Moreover it would require a full rethinking and redesign of IVF programmes where patient counselling is still based on both fresh and cumulative pregnancy rates from frozen embryos. Oocyte freezing may look tempting since it would allow fractionated freezing and thawing so that only the required number of embryos are being created. The variable survival rate, necessity of ICSI and limited choice of embryos makes it very unlikely that oocyte freezing will replace embryo freezing. Oocyte freezing does not seem to have a future as first line treatment in assisted reproductive technology.

A possible and most plausible application for oocyte freezing may be found in oocyte donation programmes. Despite the clear treatment advantage (Devroey and Pados, 1998), the mandatory synchronization of donor and receptor cycles is still a burden. Freezing of the oocytes and thawing in relation to the receptor's cycle would be an elegant solution. Supernumerary embryos from these donor cycles would have to go through a second freezing cycle.

The usefulness of oocyte freezing in assisted reproductive technology can also be in saving oocytes at times of unexpected oocyte availability. This can be the case when no sperm or insufficient sperm are available at the time of oocyte retrieval. Unforeseen absence of sperm can be due to a production problem—which should in most cases be discovered during counselling and be remedied by storing semen samples preventively. The most frequent situation will occur in cases of non-obstructive azoospermia where no sperm or insufficient sperm cells can be retrieved and where donor sperm back-up is not an option. It can be argued that in each of these cases freezing of the oocytes might only cause confusion for the couple with respect to

future treatment, but it has to be considered that oocytes can be given up for research—again if counselling on this topic were done before starting the treatment cycle.

Oocyte freezing can certainly offer a solution when there are concerns of moral, ethical, legal or religious nature about embryo freezing. When even pronucleate oocyte freezing is not considered an option, it may be desirable to bank surplus oocytes before IVF to avoid creation of supernumerary embryos. There must, however, be a warning against presenting oocyte freezing as the long-awaited political solution for abolition of embryo freezing.

Oocyte freezing may also offer hope of preservation of fertility for women facing ovarian loss. There are several options of safeguarding fertility such as embryo cryopreservation, oocyte cryopreservation or the currently highly propagated ovarian tissue freezing (Newton, 1998; Gosden, 2000; Oktay *et al.*, 2001). Ovarian tissue freezing does not require ovarian stimulation, but so far there has been no reassurance that fertilizable oocytes leading to term development of a pregnancy can be obtained in this way. For embryo and oocyte freezing, on the contrary, pregnancies are a fact. Both options would require ovarian stimulation, but where embryo cryopreservation requires the presence of a partner or use of donor semen, oocyte freezing can be offered for the single female patient. Since it mostly concerns children or young women facing ovarian loss due to anti-cancer treatment, oocyte freezing could be offered as an alternative when the foreseen treatment allows for ovarian stimulation.

Overall, oocyte cryopreservation is not a valid alternative for embryo cryopreservation at present, but specific areas of application are clearly available such as in oocyte donation programmes, fertility preservation or in case of objection against embryo freezing. These voices will have to be listened to.

Are there spin-offs for oocyte freezing beside classical assisted reproductive technology?

The reader must be aware that most of the conditions set out below are still partly or totally experimental and cannot be researched or implemented in practice without ethical consideration and approval.

Freezing of oocytes in prefertilization genetic screening on oocytes

Preimplantation genetic diagnosis (PGD) for hereditary disorders has gained a place in assisted reproductive technology. The genetic diagnosis is done on cleavage stage embryos and non-affected embryos are replaced or frozen. PGD on the oocyte by polar body biopsy can lead to fertilization only of normal oocytes and avoid the creation of affected embryos (Coutelle *et al.*, 1989; Gitlin *et al.*, 2003). In order to create a limited number of non-affected embryos for replacement, not all oocytes need to be inseminated at the time of retrieval and oocytes could be kept frozen for thawing on call. Another advantage would be that embryo creation and replacement can be done on a time scale independent from the collection cycle. That the survival after oocyte freezing is not optimal can be countered by the fact that cryopreservation of biopsied embryos is also less successful than for standard embryo cryopreservation (Joris *et al.*, 1999).

Oocyte freezing for cytoplast banking in cloning

Nuclear transfer or cloning technology requires a vast source of oocytes. In view of future therapeutic cloning applications, cryopreservation of oocytes can be a way of storing oocytes for later use. Data in the bovine show that frozen oocytes can be used as recipients for nuclear transfer (Kubota *et al.*, 1998; Hochi *et al.*, 2000). The overall objective of the study of different authors was to evaluate the viability as well as the cryodamage to the nucleus versus cytoplasm. Overall, embryo development following IVF or nuclear transfer was poorer when using frozen instead of fresh oocytes. Moreover, the nuclear transfer assay involving enucleation and transfer of a donor nucleus in the empty cytoplast suggests that poorer development of cloned embryos was partly due to cryodamage to the oocyte's cytoplasm.

Banking of activated oocytes (parthenogenote banking)

A totally new approach to cryopreservaton of oocytes is to freeze not the unfertilized oocyte but the parthenogentically activated haploid genome. This approach can combine both the advantage of storing a single genome and protection of chromatin within the shield of a membrane. After thawing, the female pronucleus can be used to build a reconstructed zygote together with a male karyoplast, thus restoring sexual reproduction by hemi-cloning. Evidence from animal studies is given by work with mouse and rabbit parthenogenotes (Levron *et al.*, 1998; Garcia-Ximene and Escriba, 2002).

Translation extract system

In the era of genomics, transcriptomics and proteomics, the oocyte can have a future as an efficient translation system. In animal studies, an equivalent exists in *Xenopus laevis*, in which a highly efficient, cell-free translation/translocation system prepared from *Xenopus* oocytes has been developed (Matthews and Colman, 1991).

Conclusion

Oocyte freezing now has a 25 year history of alternating successes and setbacks. Human oocytes have a delicate architecture but are freezable. In practice, the PROH-based protocol is used as the current standard. Clinical efficiency remains low, but healthy children have been born, indicating that chromosomally normal embryos can originate from frozen oocytes. Freezing protocols are not yet optimal and it is now desirable to combine empirical and theoretical knowledge.

There is clearly no urgent global need for implementation of oocyte freezing in current assisted reproductive technology. There is the powerfully endowed methodology of embryo freezing as a reliable alternative. Perhaps we should not be trying to transform oocyte freezing into a substitute for embryo freezing but rather give it its own clear destiny. There are specific areas where oocyte freezing can be valid, such as in donor oocyte programmes, in the case of threatening ovarian loss or in the case of objection against embryo freezing. In other words, although oocyte freezing cannot currently claim to be a standard assisted reproductive technology

routine, there is certainly a place for oocyte freezing in an individual patient-tailored reproductive medicine.

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NOTES

Cryopreservation and Compromised Intra-Oocyte Functions: Is Vitrification the Answer?

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Introduction

Currently, there are two methods used to cryopreserve mammalian oocytes: "slow-rate" freezing and vitrification (for review see: Bernard Fuller, 1996). Independent of the methodology used for cryopreservation, effects on oocyte functions can compromise abilities to develop normally following the cryopreservation process. These compromised cellular events can be collectively and generally termed oocyte "cryo-damage". Here we will address documented/theoretical specific cellular structures and functions that are/may be compromised by cryopreservation, and subsequent effects on oocyte maturation and subsequent embryonic developmental competence. In this regard we will not attempt to delineate methodology-induced cryo-damage, but instead attempt to view cryo-damage from a cellular structural/functional level, with attention directed toward intracellular organelles and extracellular structures susceptible to cryo-damage.

During cryopreservation, cells are exposed to numerous stresses including mechanical, thermal, and chemical (Mazur et al., 1972; Meryman, 1971), which can lead to compromised cell function and death. In general it has been demonstrated that oocytes are more sensitive to cryo-damage than later embryonic stages (Friedler et al., 1988). "Slow-rate" freezing attempts to control biophysical properties of freezing, like cooling and warming rates, in conjunction with cryoprotectants to minimize adverse cellular events. This method allows cells to be cooled to very low temperatures while minimizing intracellular ice crystal formation, and at the same time attempting to minimize the detrimental influences of increased solute concentrations and osmotic stress (Friedler, et al., 1988). On the other hand, vitrification, a form of rapid cooling, utilizes very high concentrations of cryoprotectant that solidifies without forming ice crystals, a major cause of intracellular cryo-damage. The term vitrification is derived from the Latin word vitreous, which means resembling glass. Vitrification can be considered a non-equilibrium approach to cryopreservation originally developed for mammalian embryos (Rall and Fahy, 1985). The vitrified solids therefore contain the same molecular and ionic distributions as the original liquid state and can be considered an extremely viscous, supercooled liquid (Rall, 1987). In this technique oocytes are dehydrated by

brief exposure to a concentrated solution of cryoprotectant before plunging the samples directly into liquid nitrogen. An excellent review of the history of vitrification and potential advantages is available (Kuleshova et al., 1999).

In consideration of cellular structures and events that can be damaged by oocyte cryopreservation we will consider some specific intracellular organelles within the nucleus and cytoplasm, their normal developmental and homeostatic functions, and the potential consequences of compromising their functions with cryo-damage.

Nucleus

Based on research in the last decade it is inappropriate to consider the nuclear envelope (NE) as just a membrane barrier between the nucleoplasm and the cytoplasm. Indeed the NE, when present, does ensure a temporal and spatial separation of events that take place within the nucleus and cytoplasm. For example, processes such as DNA replication, transcription, RNA processing and ribosomal subunit assembly occur within the nucleus, whereas most protein synthesis takes place in the cytoplasm.

Lamins, which are a major component of the NE, are associated with heterochromatin, at sites of DNA replication, RNA processing, replication proteins, and RNA polymerases (Wilson et al., 2001). This has given rise to the idea that lamins influence gene duplication and expression (review see: Hutchison, 2002). If this hypothesis holds true, one must begin to consider how compromising structural integrity of the NE, and its associated lamins, might influence subsequent DNA replication, transcription, and normal cell function. Because both "slow-rate" and vitrification of oocytes and embryos can disrupt and/or distort the NE the potential exists to interrupt ultrastructural NE protein interactions that may compromise subsequent DNA replication and/or transcription. If such cryo-damage occurs, cells would appear morphologically normal following thawing yet have suboptimal future development.

Oocytes have a profound stage-specific need for protein synthesis, which correlates well with observed times of elevated transcription. This occurs primarily during the oocyte growth phase. At these developmental stages when protein synthesis is high, there must be an adequate cytoplasmic pool of ribosomes. Recall that ribosomal subunits are synthesized from translated rRNA, which is transcribed from rRNA genes by RNA polymerase I. The rRNA genes are present in multiple repeat units called nucleolus organizer regions on the telomeric region of chromosomes. These ribosomal subunits leave the nucleus through pore complexes and associate to form cytoplasmic ribosomes in conjunction with translation of mRNA into protein.

Van Blerkom (Van Blerkom, 1989) demonstrated that while severe nuclear disruptions occurred during dehydration, the majority of nuclear structures returned to a normal state following thawing and rehydration. In most cases this was true for nucleoli. Using time-lapse photography it was observed that the number and nuclear position of nucleoli following thaw were

extremely well preserved. However, fine structural characteristics were noted that distinguished non-cryopreserved from cryopreserved oocytes, specifically nucleolar bodies in the cytoplasm after oocyte thawing and reformation of the NE. Whether this aberrant intracellular nucleoli location influences subsequent development is unknown. While these experiments provide key morphological observations, it is important to remember that issues such as proper enzymatic regulatory protein and fine structure interactions were not assessed. If the entire nucleolar structure can be inappropriately localized within the cell, and are cytoplasmic verses nuclear in location following cryopreservation, what happens to more subtle nucleolar functional/structural interactions like association of RNA polymerase I with the nucleolus? Tethering of enzymes in close association with their substrates (ie. RNA polymerase I and rRNA genes) are important regulatory mechanisms. Whether subsequent function of the nucleolus is influenced by cryopreservation, either slow-rate or vitrification, has not been assessed.

Cytoplasm

The ultrastructural configuration of the cytoplasm is continually changing dependent upon the stage of the meiotic cell cycle. This is especially true in respect to cytoskeletal distribution and/or function. It is important to appreciate that at developmental stages when nuclei exist, cytoskeletal structures are very different compared to the M-phase of meiosis. Thus, potential effects of cryo-damage on cytoplasmic function will inherently be related to stage of the cell cycle.

Proper organization and function of the oocyte cytoskeleton are essential for normal segregation of chromosomes, spindle rotation, cytokinesis, and pronuclei/nuclei formation (Maro et al., 1986; Schatten et al., 1985). One of the predominant components of the cytoskeleton is microtubules. Microtubules consist of polymerized tubulin. In oocytes the major microtubular structure is the spindle, which is responsible for spatial organization and subsequent migration of chromosomes during meiotic divisions. Disruption of the microtubular network within the oocyte can result in scattering and/or displacement of the spindle and thus changes in the chromosomal complement within the cell. This ultimately leads to aneuploidy, which can severely compromise subsequent embryonic or fetal development. Research has demonstrated that exposure of oocytes to cooling (Aman and Parks, 1994; Pickering et al., 1990; Pickering and Johnson, 1987), cryoprotectants (Johnson and Pickering, 1987; Vincent et al., 1989) or the freeze/thaw process (Aigner et al., 1992) can cause depolymerization and disorganization of spindle microtubules resulting in chromosomal scattering and the development of aneuploidy. Recent findings with respect of MII oocyte vitrification and spindle dynamics and chromatin segregation will be discussed.

Zona Pellicida

The zona pellucida surrounds the oocyte and its progeny, the preimplantation embryo. The zona pellucida is known to play a critical role

in the entire fertilization process and in blockage of additional sperm entry (polyspermy) following initial penetration by one spermatozoon. Binding of sperm to the oolemma is believed to trigger the "cortical reaction" which involves exocytosis of cortical granules from the oocyte cortex into the perivitelline space and thus release of cortical granule enzymes. These enzymes result in the block to polyspermy by modifying the zona pellucida (Wolf, 1981). During oocyte "slow-rate" cryopreservation cooling (Johnson, 1989), DMSO (Johnson, 1989; Pickering et al., 1991) and 1,2-propanediol (Schalkoff et al., 1989) exposure have been reported to cause premature cortical granule release and thus compromised sperm penetration and fertilization. Interestingly, very little is known regarding the influence of oocyte vitrification on zona pellucida functional normalcy. Since some vitrification technologies use DMSO, the potential exists to cause premature cortical granule release, reduce the ability of sperm to bind to the zona pellucida and thus interfere with the normal fertilization process. Non-equilibrium approaches of cell freezing, such as vitrification, cause rapid changes in the cell configuration. (Van Blerkom et al., 1994); personal observations). This cell-shape alteration is observed as the cell folding in upon itself and forming a crest moon or concave appearance. "Slow-rate" freezing and thawing conditions such as cooling/warming rates, containers, and cryoprotectants can influence zona pellucida cryo-damage (Van den Abbeel and Van Steirteghem, 2000). We will discuss recent evidence suggesting that vitrification can also compromise zona pellucida function.

Conclusions

The fields of infertility treatment and assisted reproductive technologies are indebted to the numerous cryobiologists, who's basic and translational research has contributed significantly to the current success of oocyte cryopreservation. With this said, there is always room for improvement. Knowledge continues to accumulate regarding intricacies of cell biology, such as the importance of protein structural/functional relationships to normal gene expression, protein translation, intracellular trafficking, epigenetic modifications and cell development. Independent of whether this information is gained through studies on cell lines, somatic cells, gametes, or embryos, ultimately researchers will assess normalcy of these cell functions in relation to cryopreservation. Such an approach will provide data that will lead to optimization of technical procedures and will likely be the avenue by which future oocyte cryopreservation success is improved and refined.

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NOTES

Problems related to the cryopreservation of mammalian sperm cells including the human

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Cryopreseration of testicular tissue: clinical and laboratory aspects

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Since its introduction in 1993 (Schoysman et al., 1993), intracytoplasmic sperm injection (ICSI) with testicular sperm has become a routine procedure for patients with azoospermia, either suffering from obstructive azoospermia with normal spermatogenesis or from non-obstructive azoospermia with testicular failure (NOA). Initially used freshly, attempts have been made to cryopreserve testicular sperm and the first cases of ICSI with frozen-thawed testicular sperm have been reported by Romero et al. (1996). Preclinical data on the use of cryopreserved testicular sperm, however, are very scarce. Verheyen et al. (1997) demonstrated a significant loss of motility and viability of testicular sperm after freezing, thawing and preparation of the thawed suspensions for ICSI. Crabbé et al. (1999) showed that freezing of testicular suspensions with glycerol preserves sperm quality better than freezing of whole biopsies.

Despite the several advantages of cryopreservation of testicular sperm, criteria or limits of testicular sperm freezing are poorly defined. In most cases of obstructive azoospermia (OA) with normal spermatogenesis, testicular sperm can successfully be cryopreserved and used for ICSI (Devroey et al., 1995; Nagy et al., 1995; Friedler et al., 1998; Tournaye et al., 1999; Palermo et al., 1999), and results are almost comparable to those with ejaculated sperm. In patients with non-obstructive azoospermia (NOA), however, sperm retrieval attempts are successful in only ~ 50% of azoospermic patients. In this patient population, it has become clear that the results of ICSI depend on the severity of the testicular failure and the criteria of the IVF centre to select NOA patients for treatment and for freezing. In a large, non-selected patient population in our centre, the results of fertilization and pregnancy rates are significantly lower for NOA than for OA patients, even if expressed per patient with positive sperm retrieval (Vernaeve et al., 2003). Literature data comparing ICSI with fresh and frozen testicular sperm for only NOA patients are very scarce (Oates et al., 1997; Friedler et al., 1997; Ben-Yosef et al., 1999; Kupker et al., 2000; Wood et al., 2002; Sousa et al., 2002). Although freezing of testicular sperm after a diagnostic retrieval in NOA patients has clear advantages, the post-thawing laboratory work at the day of oocyte pick-up may be extremely difficult and time-consuming. The loss of sperm quality after thawing and the risk of not retrieving adequate sperm for injection should be seriously considered. The Centre for Reproductive Medicine in Brussels has

adopted the following attitude towards cryopreservation of testicular sperm and its use for ICSI: (1) Diagnostic testicular biopsies showing at least one motile sperm can be cryopreserved for later use. (2) No preliminary diagnostic thawing of a frozen fraction, as selection criterion for treatment allocation, is carried out. (3) Immotile sperm can exceptionally be injected if no motile sperm are available after extensive search and after digestion of the tissue with collegenase type IV (Crabbé et al., 1997), and if fresh TESE is out of question. (4) A fresh TESE is mostly planned as back-up in case the frozen material is inadequate for use.

While having performed especially 'fresh' ICSI-TESE cycles in NOA patients (n=306) from 1995 on, 97 cycles were scheduled for ICSI with 'frozen' testicular sperm in 69 NOA patients between 1998 and 2002. At histological diagnosis, 34 men showed germ-cell aplasia, 18 maturation arrest and 8 tubular sclerosis or atrophy. Also the nine patients with Klinefelter syndrome showed mainly sclerosis or atrophy at histology. In 20 of the 97 cycles scheduled with frozen sperm, sperm was not found or unusable for ICSI upon thawing. In 77/97 cycles (80%), sperm usable for ICSI was recovered upon thawing.

The results of 77 cycles of NOA patients with frozen-thawed testicular sperm used for ICSI are presented in the following tables, and compared with the results of ICSI with fresh TESE in NOA patients as reported by Vernaeve et al. (2003).

Table I: Number of oocytes and results of fertilization after ICSI with frozen testicular sperm of NOA patients (mean ± SD)

	Frozen TESE - NOA	Fresh TESE – NOA (Vernaeve et al., 2003)
Cycles	77	306
COCa / cycle	9.4 ± 5.0	13.4
Metaphase II / cycle	8.0 ± 4.3	10.5
% 2PN	58.4 ± 26.3	48.5

a Cumulus-oocyte complexes

Table II: Results of pregnancy and implantation rates after ICSI with frozen testicular sperm of NOA patients

	Frozen TESE - NOA	Fresh TESE – NOA Vernaeve et al. (2003)
Pos hCG per cycle (%)	22/77 (28.6)	72/306 (23.5)
per ET (%)	22/64 (34.4)	72/262 (27.5)
Ongoing clinical PR per cycle (%)	16/77 (20.8)	47/306 (15.4)
per ET (%)	16/64 (25.0)	47/262 (17.9)
Implantation (FHB/replaced embryo)	17/150 (11.3)	62/718 (8.6)
Deliveries	14 (13 singletons + 1 twin)	

Thirty-two patients underwent ICSI cycles with fresh (44 cycles) as well as ICSI cycles with frozen testicular sperm (42 cycles). The characteristics of the cycles considering the sperm searching procedure are summarized in table III. The data show that sperm suitable for injection can more rapidly be found in the fresh than in the frozen specimens. The proportions of cycles and the proportion of oocytes injected with motile sperm, however, were comparable. The number of oocytes retrieved and the results of fertilization are presented in table IV. For none of these parameters were differences observed between the cycles performed with fresh and the cycles with frozen testicular sperm for the same patients. Table V shows the results of embryo transfer, pregnancy rates and implantation rates. When fresh testicular sperm was used, significantly more cycles (P=0.028 in the Chi-square test) resulted in embryo transfer than with frozen testicular sperm (93.2% vs 76.2%). The rates of positive hCG and the ongoing clinical pregnancy rates were comparable with the use of fresh and frozen sperm. Similarly, no differences were observed in the implantation rates.

Table III: Comparison of sperm characteristics in the ICSI cycles with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 NOA patients

	Fresh TESE	Frozen TESE	Mann-Whitney
Cycles	44	42	
Search time/cycle (min)	81	110	P = 0.053
Search time/sperm (min)	13	18	P = 0.016
% oocytes injected with motile sperm	82.3	83.7	NS
Cycles injected with only motile sperm (%)	33/44 (75)	31/42 (74)	NSa
Cycles injected with only immotile sperm (%)	3/44 (7)	4/42 (10)	NSa

a Chi-square test

Table IV: Number of oocytes and results of fertilization after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 NOA patients (mean ± SD)

	Fresh TESE	Frozen TESE	Mann-Whitney
Cycles	44	42	
COC / cycle	10.5 ± 6.2	9.3 ± 5.2	NS
Metaphase II / cycle	9.1 ± 5.8	7.6 ± 4.2	NS
% 2PN	58.0 ± 24.2	59.3 ± 25.5	NS
% 1PN	7.0 ± 11.0	7.8 ± 19.2	NS
% ≥ 3PN	3.6 ± 8.3	1.9 ± 4.9	NS

Table V: Results of embryo transfer, pregnancy and implantation rates after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 NOA patients

	Fresh TESE	Frozen TESE	Chi-square
Cycles	44	42	
Transfers (%)	41 (93.2)	32 (76.2)	P = 0.028
Embryos / ET	2.6	2.5	NS
Pos hCG / cycle (%)	9/44 (20.4)	8/42 (19.0)	NS
Pos hCG / ET (%)	9/41 (21.9)	8/32 (25.0)	NS
Ongoing PR / cycle (%)	6/44 (13.6)	6/42 (14.3)	NS
Ongoing PR / ET (%)	6/41 (14.6)	6/32 (18.7)	NS
Implantation rate (%)	8/105 (7.6)	6/81 (7.4)	NS
Deliveries	6 5 singl + 1 twin	5 5 singletons	

Conclusions:

- Testicular sperm can successfully be cryopreserved and used for ICSI in all obstructive and in a subpopulation of non-obstructive patients
- When testicular sperm of NOA patients can be used for ICSI upon thawing, the results in terms of fertilization rate, pregnancy rate, delivery rate and implantation rate are fairly good and comparable to the use of fresh testicular sperm
- Most disappointing in the NOA population are the high rate of failure to find sperm after thawing (21%), and the high rate of cycles without embryo transfer (14%). These findings are, however, related to our attitude to offer each couple the ultimate chance to be treated by ICSI-TESE and to the low restrictive criteria for sperm quality before freezing.
- Diagnostic testicular sperm retrieval followed by cryopreservation can be the first-line procedure. In order to counteract the reasonable risk of not finding sperm or finding only immotile sperm, scheduling a fresh surgery as back-up or counselling the couple for donor sperm as back-up procedure is advocated.

NOTES

The effect of chilling on membrane lipid phase transition in human oocytes and zygotes

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Aims: Biological membrane properties modulate physically and chemically with temperature fluctuations. Decreasing the temperature lowers the thermal energy, which decreases the molecular motion in the membrane's lipid bilayer, resulting in a transition from a "fluid" liquid crystal phase to a "rigid" gel phase. This phase transition may cause direct chilling injury.

Cryopreservation of human oocytes still bears unsatisfactory results, while freezing of 2PN zygotes is routinely and successfully used in IVF programs. Although human oocytes and 2PN zygotes are comparable in size and shape, employing the same freezing protocol for both, yields different results.

The aim of our study was to determine the lipid phase transition temperature of human oocytes and compare it to human one-cell zygotes.

Methods: Oocytes included in this study were donated by patients undergoing IVF-ICSI procedure at the IVF Unit at Sapir Medical Center, Kfar Saba. Sperm injected oocytes that failed to be fertilized and abnormally fertilized oocytes (3PN and 1PN) were included. Each oocyte was compressed between two sapphire windows and placed on the Fourier Transform infra-red (FTIR) microscopy stage. The membrane phase transition was evaluated using the FTIR and Brucker Opus software by measuring changes in the membrane's arrangement whilst gradually cooling the sample from 28ºC to 2ºC at 2ºC intervals. Data was analyzed using the Sigma plot 8 software.

Results: The temperature at which radical changes occur in the physical state of the membrane is called Tm- transition temperature or melting temperature. Lipid phase transition of mature oocytes (n=7) occurred at Tm=18.4±4.5º C, whereas one-cell zygotes (n=7) underwent phase transition at a much lower temperature of Tm=7.7±2.9º C.

Conclusions: Membrane phase transition occurs in human oocytes at higher temperatures than in human zygotes, indicating that oocytes are much more sensitive to a decrease in temperature. This suggests that membrane chilling injury is correlated with relatively poor success rates of oocyte cryopreservation.

Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells.

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Aims: Establishing a successful method for testicular stem cell transplantation of frozen-thawed testicular cells would be of immense benefit to boys with childhood cancer undergoing a sterilising treatment. In this study, we evaluated different cryopreservation protocols in a mouse model by means of testicular germ cell transplantation (TGCT) in order to establish an optimal freezing protocol.

Methods: Prepubertal F1 hybrid males (C57Bl x CbaCA) were sacrificed and their testes were enzymatically digested to obtain a single cell suspension including spermatogenic stem cells. We compared two different cryoprotective agents to evaluate the survival of these cell suspensions and the spermatogonia in specific: dimethylsulfoxide (DMSO) and ethylene glycol (EG). Three independent series of experiments were performed.

In a first series, an uncontrolled protocol versus a controlled long protocol (cooling to –80°C) both with 1.5M DMSO was compared.

In a second series, a shorter protocol (cooling to –40°C) with DMSO as cryoprotectant was applied. Here we compared thawing in a 37°C water bath versus in ice water.
In a last series, the same protocol was evaluated with a different cryoprotectant e.g. EG.
In order to evaluate the functional capacity of the cryopreservad testicular cell suspension, TGCT was performed with fresh and frozen-thawed suspensions. Donor cells of heterozygote transgenic mice which express the E. Colli Lac-Z reporter-gene were injected in the rete testis of busulphan treated recipients. After incubation with X-gal, the donor cells stain blue.

In a second series of transplantation experiments, cryptorchid mice were used in order to obtain a higher proportion of stem cells in the testicular cell suspensions.

Results: The first series of cryopreservation experiments resulted in a better viability with the controlled long protocol (48% versus 36% with the uncontrolled protocol).

We then compared two different thawing methods in either a DMSO based protocol or an EG based protocol and found no differences in viability. In order to evaluate the functional capacity of the cryopreserved testicular suspension, TGCT was performed both with fresh and frozen–thawed suspensions. In 90% of the successfully injected testes spermatogenesis was reinitiated using fresh suspensions. In contrast, this figure was only 12.5% and 22.7% after cryopreservation, for the short controlled EG-protocol and the uncontrolled DMSO-protocol, respectively.

Conclusion: Reinitiation of spermatogenesis is possible after cryopreservation of testicular germ cell suspensions. Although cell survival was acceptable, our results after TGCT show that our protocols need further improvement.

First analysis of a cryopreservation programme after the change in the Swiss law, mandating freezing of zygotes only.

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Introduction: In 2000 a new law regulating assisted reproductive technologies became effective in Switzerland prohibiting culture of more than 3 zygotes and embryo freezing. This created the need for a good cryopreservation programme to minimise a possible reduction of the pregnancy rate caused by the replacement of unselected embryos in the "fresh cycle". A retrospective analysis was made of all zygotes frozen after 01.01.2001 and thawed so far to see if improvement is needed.

Materials and methods: Zygotes were frozen in 0.4 ml of 1.5 M propanediol PBS in Nunc Cryovials, after 30 minutes of equilibration at room temperature. The cooling ramps were - 1 ∞ C/ min. to - 6.5∞ C, followed by 10 minutes of soaking before manual seeding and then further cooling at –0.5 ∞ C/min to -80∞ C. The vials were then removed to liquid nitrogen. Thawing was done by plunging the Cryovials in a 37 ∞ C water bath for about 2 minutes. Propanedial was washed out in 3 consecutive steps (1 M, 0.5 M and 0 M propanediol in PBS) at room temperature. Intact zygotes were cultured for 20 to 24 hours in a glucose free cleavage medium, with glutamine, non essential amino acids and supplemented with Serum Supplement Substitute (Irvine Scientific).

Results: These data correspond to the thawing of 548 zygotes, belonging to 125 different patients. The mean age of the patients at the moment of zygote freezing was 33 ± 3. Only clinical pregnancies with the presence of a embryonic sac are reported. From these 548 zygotes, 6 were not found and 6 were lost due to explosion of the Cryovials. From the remaining 536 zygotes 373 were intact (70%) and 317 out of 373 (85%) cleaved in culture. The percentage of embryos that had undergone the first cleavage 18 to 21 hours after thawing was 37% (117/317). Finally we were able to perform 192 transfers resulting in 43 clinical pregnancies, including 3 twin pregnancies. The clinical pregnancy rate per transfer (CPR/ET) was 22.4 % (43/192) and the implantation rate per thawed-replaced embryo (IR) was 14.5% (46/317). Ten pregnancies ended in an abortion, the final live birth rate per transfer was 17.2 %. There is a 16% risk of no transfer due to zygotes not surviving.

Discussion: The reported pregnancy and implantation rates are above the mean values published by the Swiss National register FIVNAT-CH for the 1977 transfers performed in 2002: CPR/ET = 17.5% and IR = 10.2 %. A critical analysis of our data reveals however that only 55 % (317/548) of the zygotes can be used for treatment and that the implantation rate per thawed zygote does not exceed 10% (46/548). This seems to be the general situation regarding the live birth rates with cryotransfers, reported by other registers DIR, BELRAP and the ESHRE-EIM special interest group, which hardly exceed 20% and seem to have remained unchanged since 1990. Those are sufficient reasons to improve human zygote and embryo cryopreservation especially in countries with restrictive laws and countries which recently adopted selective single embryo transfer.

The impact of cryopreservation and elective single embryo transfer (SET) on the multiple pregnancy and the delivery rate in an IVF/ICSI programme.

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Aim: The delivery rate after SET is lower compared to double embryo transfer. We investigated if the additional value of cryopreservation compensates for this decreased delivery rate in an IVF/ICSI programme.

Methods: In this longitudinal study (1998-2003) the impact of cryopreservation and SET on both the delivery rate per started IVF/ICSI cycle as well as on the multiple pregnancy rate in an IVF/ICSI programme is investigated. Embryo freezing is carried out on day 3 with a final concentration of 1.5M DMSO in Earles medium. CBS straws are used, 75% of the embryos completely survive. In 97% of all thawings transfer is possible.

Results: Since 1998 the average number of embryos per transfer reduced from 2.00 to 1.75 in 2003. The percentage of SET increased from 8% to 29% for all transfers. This is reflected by the decreased pregnancy rate per "fresh" transfer from 38% to 30%. On the other hand the percentage of completed cycles with cryopreservation increased from 22% till 33%. In the SET group the percentage of freezing was 98%. The contribution to the total number of pregnancies by cryopreservation increased from 7% in 1998 to 27% in 2003. This for the most compensated the decreased pregnancy rate due to the application of SET.

The national IVF/ICSI registry of all Dutch centres (www.nvog.nl) show that 6.0% of the ongoing pregnancies from 1998 to 2002 originated from thawing cycles. The results varied for the other centres between 2.0 and 9.0% compared to our results over that period of 10.3%. The average contribution in 2002 was 6.9%. This varied for the other Dutch centres from 0.9-12.8% and was 17.9% in our centre. The total ongoing pregnancy rate per started IVF/ICSI cycle can be calculated from the Dutch registry. There is no central registration of the percentage of multiples after IVF/ICSI in The Netherlands. Thanks to the SET and cryopreservation we were able to reduce in our centre the percentage of multiples from 27,0 % to 9,9 % and at the same time we had the highest overall ongoing pregnancy rate per started cycle in the Netherlands.

Nowadays we reduce the number of embryos per straw; in several cases only 1 embryo is frozen per straw. The pregnancy rate after thawing is about the same as with fresh transfers. The multiple rate after frozen transfers already decreased from 33,3% to 10.4%.

Conclusions: The necessary reduction of the number of transferred embryos to reduce the multiple pregnancy rate leads to a lower pregnancy rate per transfer. With an excellent quality embryo-cryopreservation programme, it is possible to maintain a high delivery rate and at the same time to minimize the number of multiples in a programme with a high percentage of SET.

NOTES

Impact of the introduction of a sequential medium for human IVF embryo culture on the outcome of the embryo-freezing program.

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Aims. The IVF practice in humans produces a large excess of embryos that should be cryopreserved. Many factors can influence the outcome of a cryopreservation program and many efforts were made in our Centre to improve the results of freezing-thawing protocols. Furthermore, a "house made" sequential medium has been introduced, since the year 2000, for day-2 embryo culture in which the glucose is eliminated and non-essential aminoacids are added in day-1 post-fertilisation. We retrospectively analysed the combined effects both of the changes introduced in freezing-thawing protocols and of the culture in the sequential medium on the clinical results of our cryopreservation program.

Material and Methods. Three periods were compared. Period I: embryos cultured up to day 2 in a one step culture medium (modified Earle's Balanced Salt Solution) were frozen-thawed by prolonged exposure to 1,2-propanediol (PROH). Period II: the exposure time to PROH in freezing-thawing procedures was drastically reduced. Period III: the shortened freezing-thawing protocol was combined with embryo culture in the sequential medium.

Results. A total of 541 thawing cycles in which were thawed 1287 embryos, were analysed. The significant increased embryo score observed in Period III (3.64 ± 1.5) in comparison to Period I and II (2.95 ± 1.5, 3.16 ± 1.5) (Mann-Whitney: $P<0.01$), as a consequence of the introduction of the sequential medium, produced a significant increase in the proportion of frozen embryos (30%, 31%, 41% in Periods I, II and III, respectively: c2: $P<0.01$). Furthermore, a significant increase of the embryo survival rate after thawing was observed in Period II (63.9%) and, more markedly, in Period III (80%), in comparison with Period I (47.7%) (c2: $P<0.01$) and of the embryo cleavage rate after thawing (63.1%, 71.1% and 84.6% in Period I, II and III: c2:$P<0.01$). The Implantation Rate per thawing cycle was significantly improved in Period III in comparison to Period I (2.96% vs. 7.20%; c2: $P< 0.01$).

Conclusions. The combined effects on the outcome of our cryopreservation program of minimising the embryo contact with the cryoprotectant and of the changes in composition of the culture medium by day 1 significantly improved the clinical results of our cryopreservation program.

Implantation prognosis and pregnancy outcome of frozen-thawed embryos in relation to ICSI indications

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Aims: Retrospective studies have been published comparing the success rate of embryos obtained either by ICSI or IVF in thawing cycles. In this study, we have analyzed the implantation prognosis in IVF and in differents indications of ICSI such as severe or slight male factor or standard IVF failure.

Methods: Among the 973 attempts with cryopreservation, we have retrospectively analyzed 823 frozen-thawed embryos transfers divided in four groups : group I (ICSI with previous IVF failure, n = 115), group II (ICSI for male factor with sperm count comprised between 1. 106 and 20.106 spermatozoa/ml, n = 241), group III (ICSI with sperm count lesser than 1. 106spermatozoa/ml, n = 103) and group IV (standard IVF, n = 364). For the four groups, the pregnancy (PR) and implantation rates (IR) and the degree of early embryos losses have been evaluated. The results have been analyzed either by X2–test (large series) and Fisher test (< 30) or Student-test (large series) and Wilcoxon test (<30).

Results: As published previously, we have also found a slight diminution of PR when frozen-thawed embryos derived from ICSI procedure (16.0 vs 18.9%). No significant difference has been observed in terms of age (33.2./30.9/31.2/32.4 years) and survival rate (80.7/82.7/86.8/80.4%). The percentage of embryo transfer is very high in the four indications (97.4/97.5/100/98.9%). The number of embryos transferred is more elevated in the group I (2.45 vs 2.23 or 2.33 or 2.32). The PR per transfer is not significantly different (16.9 /15.3/16.5/18.9%). The same observation is noted for the IR (8.7/9.1/8.3/9.7%). But the miscarriage percentage is more increased in the group I (15.8/13.9/0/7.3%, NS). When results are expressed as birth percentages by implanted embryo, (71.4/78.3/100 and 91.4%), significant differences have been described between group I and group III (p = 0.022), group I and group IV (p = 0.017). No significant variation has been noted for the pregnancy duration (39.7/38.7/39.5/38.9 weeks).

We have also analyzed the results during the same period when fresh embryos have been transferred. PR per transfer is significantly reduced compared to embryos derived from ICSI with male factor or from IVF (27.4% vs 33% and 36.5%, p< 0.05). The same observation is noted for the IR (13.29% vs 18% and 19.7%, p= 0.00001). The miscarriage percentage is also increased but not significantly (13.7% vs 9.5% and 12.3%).

Conclusion: The implantation prognosis in thawing cycles seems to be different in relation to ICSI indications. The bad results observed when ICSI has been indicated for IVF failure could suggest the intervention of a female factor in the pregnancy outcome.

The survival rate of large-,medium-and small-sized follicles of mouse vitrified ovaries

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Aims: The survival rate of different size of mice ovarian follicles after vitrification and mechanical isolation was studied to propose the suitable size of the follicles for in vitro maturation.

Methods: The ovaries from adult NMRI mice (4 to 6 week-old) were dissected and transferred to the solution containing 40% (v/v) ethylene glycol, Ficoll70 30% (w/v), 0.5 M sucrose and PBS for 5 min. and then put in liquid nitrogen.

After thawing in room temperature and water (27° C) for 30 sec. , the follicles were isolated by mechanical microdissection using 25G needles.
Follicle diameters (two perpendicular, width and length diameters) were measured at _100 magnification with a precalibrated ocular micrometer under inverted microscope and then the follicles classified to three groups: Large-sized or antral follicles , medium-sized or preantral follicles , small-sized or primordial follicles.

All follicles were stained with 0.4% trypan blue to categorized their viability on the basis of the degree of dye exlusion. Unstained follicles were classified as viable and fully stained follicles as dead. Follicles with medium staining were regarded as damaged.

Results: The percentage of viable, damaged and died follicles were 61.53% , 13.84%, 24.61% for large-sized follicles; 58.90 % , 21.91% , 19.17% for medium-sized follicles ; 60% , 18.57% , 21.40% for small-sized follicles respectively.

The difference between three classes of follicles in respect to viability was not statistically significant.

Conclusion: In respect to our results, we concluded that medium follicles were suitable for In vitro culture.

Success rate of ICSI-thawing cycles with embryos frozen and transferred at different developmental stages

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In recent years efforts to study the factors influencing embryo cryopreservation outcome have intensified. The aim of the investigation was to compare the developmental capacity and implantation rate of embryos frozen and transferred at different developmental stages.

Embryos were frozen on Day 2, 3 and 5 after in vitro fertilization with ICSI. Embryos on Day 2 and 3 were frozen in phosphate buffered solution (PBS) supplemented with 1.5 M 1,2propanadiol (PrOH), 25mg/ml Human Serum Albumin (HAS) and 0.1 M sucrose (S). After seeding at -7oC, the embryos were slowly cooled (0.3oC/min) to -30oC, than they were cooled with higher speed (-10oC/min) to -80oC before plunging into liquid nitrogen (LN2). After thawing, PrOH was removed from the cells in 3 steps with solutions containing PrOH in decreasing concentrations and 0.2 M S.

Blastocysts were frozen on Day 5 in a PBS based solution containing 8% glycerol (G) and 25mg/ml HAS. Glycerol was added to the embryos in 5 steps. After seeding at -7oC, blastocysts were slowly cooled (-0.3oC/min) to -30oC, than they were rapidly frozen (-15oC/min) to -150oC before plunging into LN2. Glycerol was diluted out of the cells in 8 steps in solutions containing the G in decreasing concentrations (8%, 6%, 5%, 4%, 3%, 2% and 1%). Finally, embryos were evaluated and transferred at different developmental stages on Day 2, 3 and 5 into patients (n=53) (2-4 embryos per patient) following a short culture period in incubation medium.

Differences were found in the pregnancy rate (PR) (biochemical /B/ and clinical /C/ pregnancy) between the different groups of patients received frozen embryos on the Day 2, 3 and 5 ($p<0.05$). Blastocysts (Day 5) were transferred into 12 patients and from them 6 become pregnant (6/12; 50%; B=2 and C=4). Out of 30 patients receiving six to eight cell embryos (Day 3) 8 become pregnant (8/30; 27%; B=4 and C=4). Day 2 (two to four cell stage) embryos were transferred into 11 patients out of which 2 become pregnant (2/11;18%; B=0 and C=2). We obtained a total PR (B + C) of 30.2% (16/53;B=6 and C=10) and the “Take-home-baby” rate is 18.86% (10/53).

Our results indicate that the age of the embryos at cryopreservation influence to the success of ICSI-thawing cycles. In our ICSI-thawing cycles the clinical PR of the Day 5 embryos is higher (4/12;33.3%) than that of the Day 3 (4/30;13.33%) and Day 2 (2/11;18%). Our results show that delaying freezing to the blastocyst stage is beneficial and increases the outcome of ICSI-thawing cycles.

First successful pregnancy in Bulgaria following 8-cell embryo vitrification failed to deliver: case report

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Introduction: Because of the progressive speed of developing the innovations in the assisted reproductive technology (ART), freezing of embryos and gametes is not a complicate procedure as several years before. In these days, using vitrification (ultra rapid freezing) technique, it is a question of seconds and minutes to do cryobanking, instead waiting hours with slow cooling protocols. This routine procedure gives a chance to those women, who have spare embryos after their fresh in vitro cycle (IVF), to undergo severalu subsequent embryotranfers in appropriate time without stimulation, stress or expenses.

Material & methods: A 30 years old woman and her husband were referred to our IVF programme in May 2003. The patient was following recovery of 15 mature and 1 immature oocytes after long stimulation protocol with GnRH agonists. Thirteen oocytes were fertilized following conventional IVF. Three fresh embryos were transferred to the uterus, but all failed to result in pregnancy. The ten spare embryos were successfully vitrified on 4-8-cells stage using series of modified cryoprotective solutions [Van der Zwalmen P. et al., 2002], containing PBS–HSA, ethylene glycol, dimethylsulphoxide (DMSO), Ficoll and sucrose. Three months later after thawing all frozen embryos, four viable 8-cells embryos with excellent quality were suitable for transfer in utero. This transfer resulted in one healthy triplet pregnancy. Implantation in this case was 75%. Normal development of the three fetuses was registered. The woman was hospitalized because of the multiple pregnancy, but in the end of the second trimester – 24th week of pregnancy, an obstetric complication (abrubcio placenta) caused sectio parva and fetal death several hours later.

Results: To the best of our knowledge, there have been no reports in Bulgaria of pregnancies following human embryo vitrification. Although this pregnancy did not reach term, three fetuses were completely normal: two boys and one girl. The patient did not accept an embryo reduction, which was necessary in that case.

Conclusion: The embryo transfer of more embryos carries a risk for multiple pregnancy and many obstetric complications. The conclusion for our self is do not exceed the number of the transferred embryos, without matter whether they are fresh or post thawed, the implantation is the same, if the embryos carry potential.

Assessment of epidermal growth factor (EGF) effects on development of vitrified mouse morulae to the blastocyst stage

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Aims: The purpose of this study was to determine if the developmental potential of mouse morulae survived after vitrification could increase using medium containing EGF. Methods and Materials: Mouse morulae were divided into vitrified and non-vitrified groups. Vitrification procedure was carried out using a combination of 40% ethylene glycol, 30% ficoll and 0.5 M sucrose (EFS40) as cryoprotectant and warmed rapidly using 0.5 M sucrose. The survived embryos were cultured either on T6 medium or T6+EGF. Accordingly, the embryos of the non-vitrified group were also cultured. The developmental rates in all groups were daily recorded and compared statistically using Chi-square test.

Results: The results showed that after 4 days of culture, the developmental potential of non-vitrified embryos cultured on T6+EGF was significantly increased. There was no significant difference between vitrified embryos cultured on T6 and T6+EGF medium.

Conclusion: In conclusion, the developmental potential of vitrified-warmed embryos doesn't increase in the medium containing EGF, even though there was significant increased developmental potential of non-vitrified embryos after culture on medium containing EGF. It is needed to do more study about the changes which will probably happen on the embryo EGF receptors following vitrification.

Key Words: Vitrification; Growth factor; Mouse embryo

Intracytoplasmic sperm injection (ICSI) with fresh and frozen-thawed testicular spermatozoa in men with azoospermia.

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Aim: The aim of the study was to compare the fertilization, cleavage and pregnancy outcome after ICSI with fresh and frozen-thawed testicular spermatozoa of infertile couples with the husband suffering from azoospermia.

Methods: One hundred and sixteen (n=116) intracytoplasmic sperm injection cycles performed with testicular extracted spermatozoa were retrospectively analysed. In 93 cycles spermatozoa were fresh and in 23 cycles cryopreserved. The two groups were analysed and compared for fertilization, cleavage, pregnancy and ongoing pregnancy rates. All microinjections were performed with apparently normal spermatozoa - a head with a tail of normal length. At least 2 embryos were available for transfer. Patients in the two groups were comparable with regards to female age and number of embryos transferred.

Results: The overall fertilization, cleavage, clinical pregnancy and ongoing pregnancy rates obtained for the 116 cycles were; 65.0% (585/900), 93.8% (549/585), 30.2% (35/116) and 22.4% (26/116), respectively.

Similar, non significant different outcomes were obtained when cycles using fresh testicular or frozen-thawed testicular spermatozoa were compared. The overall fertilization, cleavage, clinical pregnancy and ongoing pregnancy rates obtained for the 93 fresh testicular spermatozoa cycles were 64.0% (477/745), 98.9% (442/477), 30.1% (28/93) and 20.4% (19/93), respectively and for the 23 frozen-thawed testicular spermatozoa cycles, 69.7% (108/155), 99.1% (107/108), 30.4% (7/23), 30.4% (7/23), respectively.

Conclusion: This study show that the outcome of fresh and frozen-thawed testicular spermatozoa in ICSI is comparable. Spermatozoa form testicular biopsies are therefore performed the day before oocyte retrieval, incubated for 24 hours then used in ICSI and superfluous spermatozoa cryopreserved.

With this regime, most azoospermic patients are treated successfully, irrespective of the use of fresh or frozen-thawed testicular spermatozoa.

Successful Vitrification protocol for cryopreservation of human and mouse embryos

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Aims: Vitrification offers a rapid and cost efficient technique for embryo preservation at different stages of their development. This method is based on high cooling and warming rates that are achieved by using low volume techniques (OPS, Cryoloop, EM grids). However, in these techniques contamination may occur due to a direct contact between the embryos and the Liquid Nitrogen (LN). Our aim was to develop a safe and functional technique in which pulled straws are sealed (SPS), to avoid contact between the embryos and LN, and plunged into LN Slush using the VitMaster apparatus (IMT Ltd, Israel).

Methods: Two pronuclei (2PN) mouse embryos were flushed 12 hours after fertilization and left for further development in culture up to 2-cell, 4-8-cell, and early-blastocysts. For vitrification, embryos were exposed to 10% Vitrification Solution (VS) for 1 minute, transferred into 50%VS and immediately thereafter into a final VS (100%VS containing 38% v/v ethylene glycol (EG), 0.5M Trehalose and 6% BSA in PBS) (table 1). Embryos were then loaded into super open pulled straw (SOPS) that were sealed, and vitrified at a rapid cooling rate (CR) of 15,500∞ C/minute using the VitMaster apparatus. Since blastocysts are most sensitive to decrease in temperature we also tested the effect of slow CR (3,000∞ C/minute) on early blastocysts. Blastocysts were vitrified in 0.25ml sealed straws in LN. Warming of the embryos was performed by plunging the SPS and the 0.25ml straws into the VitMaster warming chamber at 38ºC. Embryos were then immersed in 0.6M Trehalose solution for 4 minutes and transferred though a series of solutions containing decreasing concentrations of trehalose: 0.5M, 0.4M, 0.3M, 0.2M and 0.1M for 2 minutes each. Viability was evaluated by the ability of the embryos to develop into expanded-blastocysts.

In preliminary experiments with human embryos from patients undergoing IVF treatment, thirteen 8-cell embryos and twenty five blastocysts were vitrified. For this purpose, embryos were exposed to VS containing 10% EG, 20% synthetic serum substitute (SSS) and 0.1M Trehalose in PBS, for 2 minutes and then transferred into VS containing 40% EG, 20% SSS and 0.5M Trehalose for 30 seconds. Embryos were loaded into SOPS and plunged into LN-Slush. The day prior to their transfer, embryos were warmed in the VitMaster warming chamber and transferred though a series of solutions containing decreasing concentrations of trehalose, 0.75M, 0.6M, 0.5M, 0.375M, 0.25M and 0.125M for 2 minutes each, washed twice and incubated overnight in culture media. Embryos with the highest morphology rank were selected for transfer.

Results: The results of the mouse embryo experiments are presented in table 1. These results show high survival rates after LN-Slush vitrification of embryos at all stages of their development. Moreover, significantly ($P<0.05$) lower survival rates were observed in blastocysts vitrified by slow CR.
Mouse Embryos

Mouse Embryos

Developmental Stage	N	Cooling Rate [ºC/minute]	Final Vitrification Solution	Blastocyst formation/ Re-Expansion*
Blastocyst	99	3,000	87.5%	24% *
Blastocyst	190	15,000	87.5%	57% *
4-8 Cell	188	15,000	87.5%	65%
2 cell	126	15,000	75%	89%
2PN	168	15,000	87.5%	62%

Table 1 – Mouse blastocyst formation / re-expansion rate (Results were normalized from Control).

Human Embryos
Six of the thirteen 8-cell embryos continued to develop after warming (47%) and were transferred into the uterus of 4 patients, resulting in two clinical pregnancies. One of these pregnancies is still ongoing (12 weeks). Fifteen blastocysts out of twenty five (60%), developed into fully expended blastocysts. Thirteen of them were transferred into the uterus of 9 patients. Unfortunately only two chemical pregnancies were achieved.

Conclusion: These results demonstrate that plunging embryos in SPS into LN-Slush permitted increased cooling rate and successful cryopreservation of mouse embryos at all stages in the presence of low concentrations of vitrification solution. We further demonstrated that this technique can be modified for vitrification of human embryos offering a rapid and simple protocol for embryo cryopreservation in IVF treatments.

Does the type of culture media used effect the results of a cryopreservation program? A preliminary study.

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Introduction: The choice of culture media has a considerable effect on the outcome of human IVF treatments. The aim of this prospective randomized study is to analyse the effect of two different type of culture media on our crypreservation program.

Materials and methods: Records of 428 consecutive fresh IVF cycles and 34 frozen–thawed IVF cycles performed between October 2001 and December 2003 were analysed in this study. Patients were randomized at the time of HCG administration of their first fresh IVF cycle to oocyte and embryo culture in home made Whittingham's T6 or in commercially available Vitrolife IVF medium. If a patient was randomized the same media was used for all further IVF treatment(s). Number of oocytes, fertilized oocytes, transferred and cryopreserved embryos were recorded. Developmental stage and morphological grade of cleaving embryos was assessed before embryotransfer or at cryopreservation and after thawing. Cryopreservation was offered for couples if at least 2 surplus embryos with at least 4 cells and less than 50% fragmentation were available after embryotransfer. Data were analysed with Mann-Whittny U test or _2-test when available. P<0.05 was considered statistically significant.

Results: Records of the 428 fresh IVF cycles are shown in Table I. Significantly lower fertilization rate in T6 media resulted in lower number of surplus embryos appropriate for cryopreservation. The pregnancy rate was higher in T6 group, however, the difference between groups was not significant.

Table I.

	T6	Vitrolife	P-value
No. of cycles	218	210	—
No. of oocytes	1516	1587	—
Fertilization rate %	864/1516 (57.0%)	1035/1587 (65.2%)	<0.0001
No. of embryos transferred	570/864 (66%)	584/1035 (56.4%)	<0.0001
Clinical pregnancy rate (%)	90/218 (41.3%)	72/210 (34.3%)	NS
No of cycles with embryo freezing	34/218 (15.6%)	47/210 (22.4%)	NS
No. of embryos cryopreserved	138/864 (16.0%)	256/1035 (24.7%)	<0.0001
Cell numbera (SD)	6,6 (1,8)	5,8 (1.6)	<0.0001
Fragmentation %a (SD)	18,4 (11.7)	14,9 (10.4)	0.003
No of good quality embryosa (%)	20/138 (14.5%)	51/256 (19.9%)	NS

aCharacteristic of fresh embryos before cryopreservation

Data of the 34 frozen–thawed IVF cycles are shown in table II. Not all of the frozen embryos were thawed and 10 embryos (5.5%) were lost during the thawing procedure.

Table II.

	T6	Vitrolife	P-value
No. of frozen–thawed cycles	9	25	—
No. of frozen embryos	46	136	—
Cell numbera (SD)	6.9 (2.1)	5.9 (1.7)	0.004
Fragmentationa % (SD)	20.4 (12.5)	16.4 (11.8)	0.034
No of good qualitya embryos (%)	5/46 (10.9%)	27/136 (19.9%)	NS
No. of thawed embryos	41	114	
Cell numberb (SD)	3.8 (2.5)	3.9 (2.7)	NS
Fragmentationb % (SD)	37,4 (25.0)	28.9 (21.9)	NS
No of good quality embryosb (%)	4/41 (10.9%)	17/114 (14.9%)	NS
Clinical pregnancy	2/9 (22.2%)	7/25 (28.0%)	NS

aCharacteristic of fresh embryos before cryopreservation
bCharacteristic of fresh embryos after thawing

Conclusion: The higher cell number and more fragmentation before cryopreservation in T6 group indicate a faster development of the embryos in the home made culture media. However, after thawing there was no difference in cell number and embryo quality which refer to a higher frequency of cell injury in T6 culture media. In all, it seems that by using Vitrolife culture media more embryos with less fragmentation will be available for freezing but the real impact of the culture media on the cryopreservation program needs larger studies and further examination.

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NOTES

european society of human reproduction and embryology

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(please read important instructions overleaf)

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❒ psychology and counselling
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❒ safety and quality in ART
❒ stem cells

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