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## ARTICLE

# The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting

## Alpha Scientists in Reproductive Medicine <sup>1,\*</sup>

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Alpha Scientists in reproductive medicine is a non-profit organization that provides an international forum for scientists in reproductive medicine. Alpha's objectives are to advance the art and science of clinical embryology for the benefit of the public worldwide, through international promotion of education, communication and collaboration.

**Abstract** This proceedings report presents the outcomes from an international workshop designed to establish consensus on: definitions for key performance indicators (KPIs) for oocyte and embryo cryopreservation, using either slow freezing or vitrification; minimum performance level values for each KPI, representing basic competency; and aspirational benchmark values for each KPI, representing best practice goals. This report includes general presentations about current practice and factors for consideration in the development of KPIs. A total of 14 KPIs were recommended and benchmarks for each are presented. No recommendations were made regarding specific cryopreservation techniques or devices, or whether vitrification is 'better' than slow freezing, or vice versa, for any particular stage or application, as this was considered to be outside the scope of this workshop. 

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**KEYWORDS:** benchmarking, consensus meeting, cryopreservation, cryosurvival, key performance indicators, quality improvement

## Introduction

Key performance indicators (KPIs) are essential for evaluating the introduction of a technique or process, as minimum standards for proficiency, for monitoring ongoing performance as part of a quality management system, both internal quality control or external quality assurance, and for benchmarking and quality improvement. Almost all KPIs commonly used in gamete and embryo cryopreservation, whether by slow freezing or vitrification, relate to cryosurvival. They are typically assessed immediately post thawing/warming, being evaluated through the return of apparently normal function or morphological development. For comparability between laboratories, KPIs require precise definitions and objective, standardized methods for their determination.

Since all assisted reproduction laboratories have perceptions of how well gametes or embryos should survive cryopreservation, it was anticipated that there would be moderate levels of general agreement among practitioners and laboratories and that these might allow the establishment of specific standards for application across all laboratories. Consequently, at the Alpha Biennial Meeting held in Budapest in May 2010, consensus values for the most common indicators of cryosurvival for oocytes, zygotes, cleavage-stage embryos and blastocysts, cryopreserved using either slow freezing or vitrification were sought, via administration of an interactive questionnaire to the delegates.

The questionnaire included 16 suggested KPIs, with space for delegates to respond separately for slow-freezing and vitrification methodologies. These KPIs were as follows.

### Oocytes

- (1) Proportion of oocytes that should be intact post cryopreservation.
- (2) Fertilization rate of cryopreserved oocytes by intracytoplasmic sperm injection (ICSI).
- (3) Cleavage rate for ICSI-fertilized cryopreserved oocytes.

### Zygotes

- (1) Proportion of oocyte retrieval cycles that have zygotes for freezing.
- (2) Proportion of zygotes that appear intact post cryopreservation.
- (3) Proportion of post-cryopreservation zygotes that cleave during overnight culture.

### Cleavage-stage embryos

- (1) Proportion of oocyte retrieval cycles that have embryos suitable for freezing.
- (2) Proportion of embryos with  $\geq 50\%$  blastomeres intact post cryopreservation.
- (3) Proportion of embryos with all blastomeres intact post cryopreservation.

- (4) Proportion of post-cryopreservation embryos that cleave during overnight culture.
- (5) Implantation rate for post-cryopreservation embryos (women <38 years).

### Blastocysts

- (1) Proportion of oocyte retrieval cycles that have blastocysts suitable for freezing.
- (2) Proportion of blastocysts that are more-or-less intact post cryopreservation.
- (3) Proportion of blastocysts that re-expand within 3 h post cryopreservation.
- (4) Proportion of early blastocysts post cryopreservation that expand during overnight culture prior to embryo transfer.
- (5) Implantation rate for cryopreserved blastocysts (women <38 years).

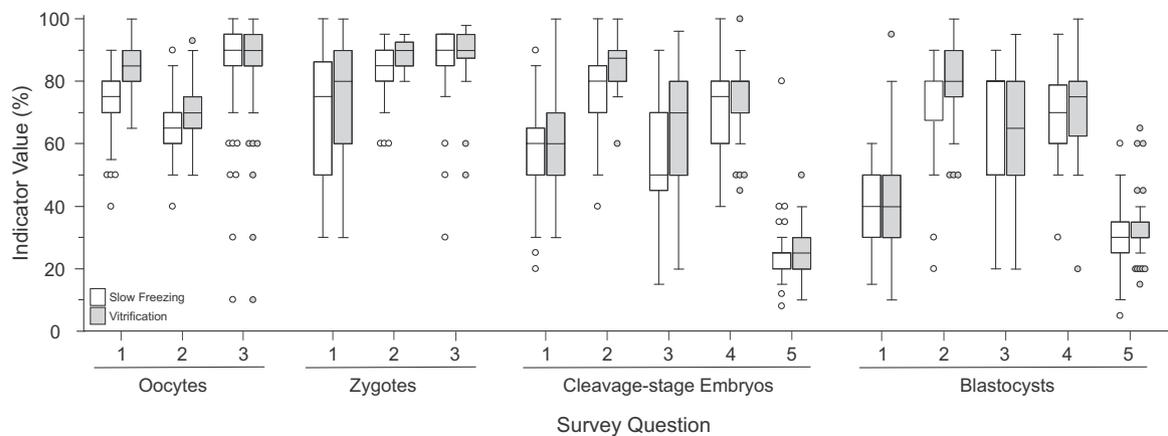
Completed questionnaires were collected at the end of the session, with 82 audience members providing responses to at least some of the questions for slow freezing and/or vitrification. In addition, respondents provided comments or opinions in the following general areas:

- (i) Impact of female age;
- (ii) Impact of stimulation regimen, including poor responders, cases with unusually high numbers of oocytes retrieved, and cases of incipient or manifested ovarian hyperstimulation syndrome;
- (iii) Careful definition of endpoints, e.g. implantation rate based on fetal sac c.f. fetal heart;
- (iv) Meaningfulness of some KPIs, owing to the biological differences between patients, c.f. an industrial process;
- (v) Selection criteria for cryopreservation suitability, including a need for standardized grading of embryos to select those suitable for cryopreservation;

Data were entered into an Excel spreadsheet and analysed (**Figure 1**) using MedCalc version 11.1 (MedCalc Software, Mariakerke, Belgium). Only two specific conclusions could be derived from the delegates' responses: (i) the ranges of values perceived to be appropriate for each suggested KPI were generally very wide, particularly for expected post-cryopreservation cleavage rates for oocytes and zygotes; and (ii) there were generally similar expectations for cryopreservation outcomes using either slow freezing or vitrification.

Overall, the widely variable expectations for these KPIs precluded the derivation of consensus values, due to several possible reasons: (i) genuine differences between the respondents in their perceptions of adequate cryosurvival and post-cryopreservation functional ability of oocytes, zygotes, cleavage-stage embryos and blastocysts; (ii) differences in efficacy between the various slow-freezing and vitrification methodologies; and (iii) inadequate specificity in the definitions of the proposed KPIs.

From these results, it was clear that a more carefully constructed exercise to define KPIs for oocyte, zygote and embryo cryopreservation was required. It was agreed that



**Figure 1** Cryopreservation key performance indicators (KPIs): range of respondents' estimates for each question. Box-and-whiskers plots of the respondents' estimates for each question of the KPI survey (see text for the specific questions asked regarding each developmental stage). The box represents the values from the lower to upper quartile, with the middle line representing the median. The limits of the bars show the minimum and maximum values excluding outlying values (smaller than the lower quartile minus  $1.5 \times \text{IQR}$  and larger than the upper quartile plus  $1.5 \times \text{IQR}$ ). Outlying values are presented individually.

for optimum efficiency and practicality, such an exercise should be undertaken within the format of a consensus workshop by an expert panel, such as was done for the consensus workshop on embryo morphology. This report presents the results of this Expert Panel consensus meeting, held in Istanbul 11–12 November 2011.

## Workshop presentations

### Comparison of cryopreservation by slow freezing or by vitrification (Stanley Leibo)

Since 1972, oocytes and embryos of more than 30 mammalian species have been successfully cryopreserved, resulting in the birth of millions of normal offspring. Moreover, hundreds of thousands of normal human babies have been born from cryopreserved oocytes and embryos. Oocytes and embryos of all of these species have been cryopreserved by methods often referred to either as slow freezing or as vitrification. Vitrification is the reversible transition of a liquid into an amorphous non-crystalline glass. The method of slow freezing uses relatively low concentrations of  $\sim 10\%$  cryoprotective additives (CPAs), cooling rates of  $\leq 1^\circ\text{C}/\text{min}$ , and warming rates of  $\sim 250^\circ\text{C}/\text{min}$ . In contrast, the various methods of vitrification specify the use of high CPA concentrations of 30–40%, use of saccharides as supplements, cooling rates much higher than  $1000^\circ\text{C}/\text{min}$  and very rapid warming rates. Often, extremely high cooling rates of  $> 10,000^\circ\text{C}/\text{min}$  are used. Recent results achieved with vitrification, especially the cryopreservation of human oocytes, have been very successful, with functional survival of cryopreserved oocytes, i.e. the ability to undergo fertilization and embryo development, approaching 100%. One limitation of many vitrification methods is that human specimens, to be cooled at high rates, are immersed directly into liquid nitrogen. This may expose them to potential contamination by viruses or microbes. Nevertheless, comparisons of results attained by vitrification with results from

standard equilibrium cooling methods have almost always demonstrated that vitrification is as good as, and usually better than, standard slow freezing.

Vitrification presumably requires high concentrations of CPAs and very high cooling rates to produce the glassy state. One criterion considered to be essential if the best results are to be achieved is that the extracellular solution itself must form a glass when cooled at high rates. However, recent results and re-examination of older results reveal that this may not be so. The key to survival is whether the intracellular contents vitrify. Ultimate survival of cryopreserved cells and tissues may be more dependent on the rate at which the specimen is warmed, rather than the rate at which it is cooled (Leibo and Pool, 2011). Furthermore, the fundamental principles of cryobiology suggest that the mechanisms leading to high survival of vitrified cells are similar, if not identical to the mechanisms resulting in survival of slow-frozen cells.

### Media and device: comparing apples with apples (Pierre Vanderzwalmen)

Vitrification is a cryopreservation procedure in which the biological material and the surrounding liquid solution are converted into a glass-like amorphous solid, free of any crystalline structures when plunged in liquid nitrogen (LN; Rall and Fahy, 1985). With the introduction of several commercial vitrification kits, there is increasing interest in vitrification of oocytes and embryos at different stages of development.

Since the development of the first vitrification solution, which contained dimethylsulphoxide (DMSO), 1,2-propanediol (PrOH) and acetamide (Rall and Fahy, 1985), numerous CPAs have been found to be effective. At present, the most widely used CPAs are composed of agents that penetrate the cell (such as ethylene glycol (EG), DMSO, PrOH or glycerol) as well as low-molecular-weight non-penetrating CPAs

(such as sucrose or trehalose). Some CPA solutions include high-molecular-weight polymers such as Ficoll (PM 70 or 400).

Many 'open' and 'closed' carrier devices are currently utilized for loading the biological material before storage in LN, and their design can indirectly affect outcomes by causing alterations in the cooling and warming rates, so laboratories have developed their own protocols (AbdelHafez et al., 2011; Valbuena et al., in press). However, the existence of numerous vitrification methods using different carrier devices and hypertonic CPAs may be the source of problems in the future. These problems will be serious when oocytes or embryos at different stages of development are transferred to other assisted reproduction centres that routinely use different techniques to perform vitrification. Ideally, then, a universal cryopreservation protocol for use with oocytes, zygotes, cleavage-stage embryos and blastocysts that successfully accommodates different CPAs and carrier devices needs to be developed.

The success of a vitrification protocol depends on: (i) an optimal process of dehydration when cells are exposed to hypertonic conditions; and (ii) an ideal penetration rate of a non-toxic CPA, sufficient to generate an intracellular environment that will vitrify and remain vitrified for a defined cooling–warming rate. Addition as well as removal of CPA causes successive phases of shrinkage and re-expansion due to movement of water and CPA across the cell membranes that are linked with several biophysical parameters.

### Intrinsic and extrinsic factors governing cryopreservation protocols

Both intrinsic and extrinsic factors influence the outcome of oocyte and embryo cryopreservation and both types of factors must be considered when deriving cryopreservation protocols.

Intrinsic factors are the inherent cryobiological properties of the cell membrane at different stages of development, such as its ability to cope with osmotic stress during vitrification/warming and its water and CPA permeability.

Extrinsic factors are related to technical practice such as the type of carrier device or adaptation of the protocol to the stage of embryo development (Vanderzwalmen et al., 2002).

### Permeability and pathway of water and CPAs through the cell membrane

#### *Exposure to CPA solutions: an essential step before cooling.*

Before being plunged into LN, oocytes or embryos are exposed to a non-vitrifying CPA solution, containing only permeable CPAs, then to a vitrifying CPA solution, containing both permeable and non-permeable CPAs. The CPA composition and concentration differ between commercial kits.

Upon exposure to the non-vitrifying CPAs, the cell immediately adjusts its osmolarity by losing water, and shrinks. The CPAs then enter the cell more slowly, due to their low membrane permeability (Schneider and Mazur, 1984). The time of exposure to the non-vitrifying solutions at a defined temperature ( $T^\circ$ ) is of critical importance, as it

determines the intracellular concentration of CPA. Exposure time may range between 3–15 min, depending on the CPA used and the type and developmental stage of the biological material.

The biological material is then exposed to the vitrifying CPAs for 30–90 s. This solution causes further cellular dehydration, which concentrates the intracellular CPA.

The overall objective of this procedure is to create an intracellular environment that will remain vitrified for a defined cooling–warming rate.

#### *Intrinsic factors that influence the process of osmotic response. Measurement of the permeability of the cell membrane to water and CPAs.*

The movement of water and CPAs across cell membranes is crucial for cell survival during cryopreservation, and the degree of permeability depends upon the stage of embryo development. These events can be optimized when the following biophysical factors are known:

- (i) Ratio of membrane surface area ( $A$ ) to cell water volume ( $V_w$ );
- (ii) The stage-specific membrane permeability for water (hydraulic conductivity,  $L_p$ );
- (iii) The stage-specific membrane permeability for CPAs ( $P_s$ ) in relation to their respective activation energies ( $E_a$ ) for  $L_p$  and  $P_s$ .

While it is possible to visualize the shrinkage and swelling of cells under the stereomicroscope, it is not possible to quantify the proportion of CPA that enters the cells. Only full expansion of oocytes or zygotes representing full equilibration with CPA may be assessed.

#### *Pathways for the movements of water and CPAs across the cell membrane.*

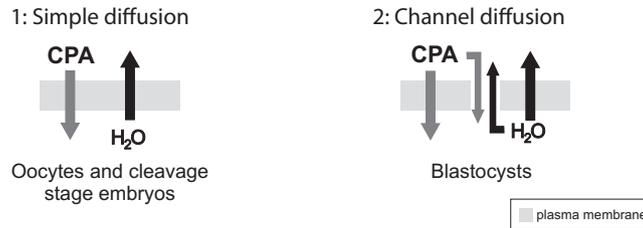
As shown in **Figure 2**, there are two pathways for the movement of water and CPAs across the plasma membrane of oocytes and embryos. One is simple diffusion through the lipid bilayer, in which case permeability is low and  $T^\circ$  dependency ( $E_a$ ) is high. The other pathway that plays a role in CPA transport lies in the aquaporins, a variety of water channels expressed by mammalian oocytes and embryos (Edashige et al., 2006; Verkman et al., 1996). The permeability via channels is high and  $T^\circ$  dependency ( $E_a$ ) is low (Kasai and Edashige, 2010).

##### (a) Surface/volume ratio

The flow of water across each unit of cell surface as a function of time,  $L_p$ , is related to the cell volume (Agca et al., 1998). Oocytes and zygotes have the lowest surface/volume ratio, so they are less efficient than cleavage-stage embryos at losing water and taking up CPAs and thus are more susceptible to intracellular ice formation if the exposure time to CPAs is not well adapted (Maneiro et al., 1991). As a consequence, to create an adequate environment that will vitrify during the cooling step, the incubation time, during which water is removed from the intracellular compartments and 'loading' with CPAs takes place, should be longer than for smaller cells.

(b) Permeability of cell membrane to water and CPAs: lessons from the rodent model

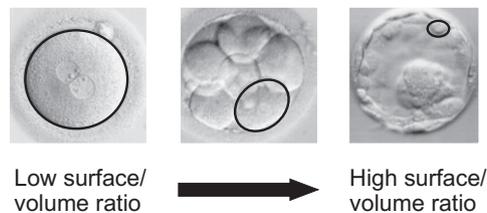
## A: Membrane permeability and dehydration relative to developmental stage



## B: Membrane permeability and dehydration relative to CPA type



## C: Change in cell size and surface/volume ratio relative to developmental stage



**Figure 2** Pathways for the movement of water and cryoprotective additives (CPAs) across the plasma membrane. The movement of water becomes more efficient with embryo development to the blastocyst stage, as the Arrhenius activation energy declines. (A) Movement of CPAs and water through the plasma membrane is via simple diffusion in the early developmental stages (A1) and via channel diffusion (aquaporins) by the blastocyst stage (A2). (B) Movement of water and CPAs by simple diffusion or via channels also depends on the type of CPA. (C) The efficiency of dehydration and CPA uptake is also influenced by cell size, increasing with increasing surface/volume ratio (cell size highlighted).

To design suitable cryopreservation protocols, it is necessary to consider the pathway for the movement of water and CPA. This pathway differs according to maturational/developmental stage, resulting in varying permeability (Ford et al., 2000).

## (i) Permeability according to the stage of development

Permeability can be classified according to the  $P_s$  coefficient. Low permeability to CPA is typical for metaphase-II (MII) oocytes and early stages of development whereas higher permeability is representative for morulae and blastocysts (Pedro et al., 2005).

In MII oocytes and cleavage-stage embryos, water and CPAs move slowly through the lipid bilayer by simple diffusion. Longer exposure to CPAs is necessary in these stages to allow adequate permeation into the biological material (Hunter et al., 1992; Leibo, 1980). Although there is no comparative study showing the superiority of one method to the other, the general principle is to add the CPA solution gradually, especially for MII oocytes (Quinn, 2010).

Stepwise addition of CPAs during the exposure step is now a standard procedure in vitrification (Vajta and Nagy, 2006). Practically, gradual addition of CPA is the preferred method before plunging MII oocytes in LN. One method consists of placing oocytes into smaller volume drops and then

merging the drops with increasing concentrations of CPA before cooling in LN (Kuwayama, 2007).

The duration of exposure to the CPA solutions may differ between the stages of development. For example if ultra-rapid vitrification (cooling rate  $>20,000^\circ\text{C}/\text{min}$ ) using open carrier systems is applied, an average time of 5–15 min is necessary to protect the oocytes from cryoinjury compared with maximal 5 min for blastocysts (Vanderzwalmen et al., 2007).

## (ii) Permeability according to the type of CPA

Today, the most commonly used CPAs are DMSO, EG, PrOH and glycerol. Each CPA has its own cell penetration characteristics, and so for slow freezing each developmental stage has its own CPA mix: PrOH for zygote and cleavage-stage embryos, DMSO for cleavage-stage embryos, and glycerol for day-5 embryos.

For vitrification, mixtures of DMSO/EG or PrOH/EG are used for oocytes as well for early stages of development and blastocysts. Glycerol permeates mouse and bovine zygotes at low rates but the permeability increases as embryos progress to the morula stage. It is possible that expression of specific aquaporins in embryos increases with the progression of embryo development. At the blastocyst stage, a change in the type of aquaporins expressed in embryos was observed; aquaporins 8 and 9, which are more

specific for glycerol, were additionally expressed (Cobo et al., 2011).

Another example for different behaviour of CPA in the various stages of development is that, in human morulae and blastocysts, glycerol, EG and DMSO move rapidly through the cell membrane by facilitated diffusion through channels whereas PrOH enters only slowly by simple diffusion (Kasai and Edashige, 2010).

#### Extrinsic factors: impact of closed aseptic carrier devices in vitrification

The conversion of a solution into a glassy state is simply obtained when the temperature is very rapidly lowered beneath the glass transition temperature. To achieve ultra-rapid cooling rates of more than 20,000°C/min, open embryo carriers (e.g. electron microscopy grid, open pulled straw, Cryoloop, McGill Cryoleaf, Hemi-Straw, Cryotop, Cryolock) were designed to allow direct contact of the biological material with LN (Quinn, 2010; Vajta and Nagy, 2006). The benefit of ultra-rapid cooling is that although the cells are exposed to increasing CPA concentrations for a short period of time to permit the extraction of the intracellular water (e.g. 3 min for blastocysts), only a limited amount of CPA permeates into the cells.

#### Crucial considerations when reducing the cooling rate.

The use of the open carriers involves direct exposure of oocytes and embryos to LN. The possible risks of (cross) contamination of the biological sample (Bielanski, 2012; Morris, 2005) or presence of toxic compounds during cooling as well as during long-term storage (Yan et al., 2011) remain contentious. Nevertheless, the European Union directive dictates that cooling and storage of embryos must be performed in a way that minimizes the risk of contamination.

As a consequence, the use of sterilized LN (Cobo et al., 2011; Parmegiani et al., 2010) and the storage of the straws in vapour (Cobo et al., 2010a) are seen as options if open carrier devices are used. Another alternative is the development of a vitrification protocol for hermetically closed carrier devices (Vanderzwalmen et al., 2009) allowing a total isolation and protection of the sample from direct contact with LN during the cooling procedure and subsequent long-term storage, while preserving acceptable rates of survival.

In the establishment of a vitrification protocol for an aseptic carrier the critical point is the reduction of the cooling rate. The sample is separated from the LN by an outer straw which inevitably leads to a reduction in the cooling rate from about 20,000°C/min to <1500°C/min. During cooling, the sample has to cross a temperature zone of high risk for ice crystal formation before reaching the glass transition temperature. The quicker this temperature range is crossed, the lower the probability for ice crystal formation. Therefore, due to the reduced cooling rate, the possibility of ice crystal formation occurring in aseptic carriers can only be overcome by increasing the intracellular concentration of CPA and viscosity. Consequently, the step of exposure of the embryo to the CPA before cooling is a crucial one and has to be modified.

#### Modification of the vitrification protocol for aseptic carriers.

The key to successful vitrification in aseptic closed devices is to find the optimal balance between cooling and warming rates and the quantity of CPA needed to achieve vitrification without cytotoxicity. A microcinematographic evaluation observed the shrinking–swelling response of embryos to increasing concentrations of CPA and identified those that allowed the embryos to remain within the volume variation limits ( $\leq 30\%$  of the initial volume) and were compatible with good survival rates (Vanderzwalmen et al., 2009). When longer exposure times to the CPA solutions is required, a gradual exposure in at least three steps induces less shrinkage-swelling stress to the cell than does a two-step addition.

In summary, to date, there is no agreement on a universal vitrification procedure and progress towards such a procedure is not yet realistic, based on the range of different cryobiological and technical factors involved in the process.

#### Clinical outcomes related to oocyte morphology before freezing and after thawing (Veronica Bianchi)

##### Introduction

The slow-freezing technique was the first procedure applied in IVF laboratories to cryopreserve gametes and embryos. It is based on slow dehydration of the cell(s), carried out in a controlled machine that automatically decreases the temperature. Oocytes are equilibrated in a mixture of CPAs that penetrate the membrane and permeate the cytoplasm, which also protects its ultrastructure.

##### Morphological selection

Pre-freeze morphological selection is important since oocytes must not only survive the thawing process, but also maintain their competence to be fertilized and yield viable embryos. Strict pre-freeze morphological selection may lead to better post-thaw recovery, but what exactly is a morphological selection, and is it really needed?

Although information about possible damage to subcellular structures caused by exposure to low temperatures is limited, it has been addressed in some studies (Bianchi et al., 2005; Bromfield et al., 2009; Coticchio et al., 2006). Furthermore, not all protocols and techniques have been tested, so it is not yet possible to draw an evidence-based conclusion.

The effect of oocyte dysmorphism on IVF outcome remains contentious, but it is generally accepted that oocyte abnormalities may negatively influence fertilization and embryo development. However, since abnormal oocytes are usually discarded, little is known about their potential for fertilization and development.

Currently, oocyte selection is based mainly on morphological characteristics (including shape of the oocyte and polar body, cytoplasm colour and vacuolization, presence of perivitelline debris; Ebner et al., 2003). Nevertheless, there are structures in the eggs that can be damaged by the freezing process but cannot be investigated without losing oocyte viability. For example, Nottola et al. (2008) analysed the effect of freezing and thawing on mitochondria

associated with the smooth endoplasmic reticulum. After thawing, the mitochondria appeared smaller, underdeveloped and disorganized, with a shortening in the microvilli length. The degree of oocyte vacuolization after thawing also seemed to be increased, with the larger vacuoles localized in the inner ooplasm, while the smallest were in the cortical area. However, the report of a term singleton pregnancy following the transfer of three poor-quality vacuolated embryos suggests that the presence of a large vacuole may not necessarily compromise the outcome (Fancovits et al., 2011).

The meiotic spindle of the MII oocyte can be visualized without losing oocyte viability thanks to the Polscope. This optical microscopy system allows the observation of highly ordered subcellular structures such as the spindle and the inner layer of the zona pellucida. By measuring the retardance, it can give a rough evaluation of the number of microtubules present in the oocyte. The Polscope can be used before freezing, to exclude oocytes that do not display a signal, or after thawing, to evaluate spindle recovery following return to physiological conditions. Nevertheless the presence or absence of the spindle is not a guarantee of successful outcome, and it should be noted that the spindle undergoes a transient disappearance during freezing and reappears after thawing (Bianchi et al., 2005). The absence of the spindle after thawing, though, does not necessarily compromise fertilization or subsequent embryo development (Sereni et al., 2009).

Recently, attention has focused on aspects of cryosurvival not directly related to oocyte appearance, such as the timing of insemination after thawing. Normally, oocytes are cultured for several hours prior to insemination, as oocyte nuclear and cytoplasmic maturation is reached by about 40 h after human chorionic gonadotrophin (HCG) administration. One of the concerns related to cryopreservation is the possibility of generating discontinuity in the oocyte's development during its most critical period, i.e. between recovery and fertilization. However, there is only a small amount of data on the timing of cryopreservation in the literature, based on retrospective studies. Parmegiani et al. (2008) reported significantly improved embryo quality and clinical outcome when oocyte cryopreservation was performed within 2 h of retrieval, and Lappi et al. (2009) found that oocytes frozen within 40 h of HCG injection resulted in significantly higher pregnancy and implantation rates compared with those cryopreserved more than 40 h after HCG administration. However, this difference was not observed when a lower sucrose concentration was used in the freezing medium. In a cohort of 325 patients, under 35 years of age, and 375 thawing cycles, the final outcome was not compromised by the length of time the oocytes were in culture before freezing, with the length of post-thaw culture found to be more important (Bianchi et al., 2011). This result is most likely associated with the protective effect that cumulus cells exert on the oocyte limiting its ageing; thus, denuded oocytes might be more sensitive to damage.

In conclusion, although morphological selection of oocytes may play a role in raising the recovery rate, little is still known about which features are critical. Moreover in certain groups of patients, i.e. poor responders or

patients with low quality oocytes, oocyte selection is not an option.

### Clinical outcomes with slow freezing

The first protocol used to slow freeze human oocytes was exactly the same as that utilized to cryopreserve embryos (Lassalle et al., 1985). Since then, the major change applied to the whole slow-freezing process has been related to the amount of sucrose in the freezing medium. It has been clear since the beginning that the presence of sucrose during freezing led to a higher survival rate after thawing. Unfortunately, few studies have addressed the ultrastructural effects caused by the extent of dehydration related to the high amount of sucrose used. It is likely that increased water loss before cooling, in response to increased sucrose concentration, accounts for the improved rates of oocyte survival but excessive cell shrinkage may result in loss of viability. This hypothesis was confirmed when good biological data (Fabbri et al., 2001) did not correspond to satisfactory clinical outcomes (Borini et al., 2006), highlighting the need to balance the rate of post-thaw recovery with the take-home baby rate.

Following studies on cell permeability (Paynter et al., 2005), the sucrose concentration was lowered in the freezing solutions but maintained in the thawing solutions, leading to increased survival rates and a pregnancy rate comparable to that from fresh cycles. A retrospective study of the differences between the two freezing protocols, using 0.1 M and 0.3 M sucrose, highlighted that while the survival and fertilization rates were significantly improved using the higher sucrose concentration, pregnancy and implantation rates were higher when the lower sucrose concentration was used (De Santis et al., 2007).

When the cryopreservation protocol was modified so that the freezing solution contained 1.5 M PrOH and 0.2 M sucrose to reduce the impact of shrinkage during cooling, while the thawing solutions contained 0.3 M sucrose, the survival rate was 76.0%, with a fertilization rate of 76.2% and an embryo cleavage rate of 93.7% (Bianchi et al., 2007). The pregnancy rates were 21.2%, 18.9% and 21.8% per embryo transfer, thaw cycle and patient, respectively, and the implantation rate was 13.4%. Pregnancy and implantation rates per thawed oocyte were 4.9% and 6.9%, respectively.

These data have now been confirmed in a higher number of cycles (Bianchi et al., in press). In a series of 443 thawing cycles involving 2458 MII oocytes, the survival, fertilization and cleavage rates were 71.8%, 77.9% and 94.5%, respectively. A total of 907 embryos were replaced in 394 transfers, resulting in 90 pregnancies. The pregnancy rate was 22.8% per transfer and 26.3% per patient. Ultrasound assessment confirmed the presence of 122 gestational sacs, 93 of which had fetal heart activity, giving an implantation rate of 13.5% per embryo transferred and 9.4% per oocyte injected. To date, 77 babies have been born: 35 males and 42 females.

An age-dependent analysis showed no differences for biological parameters such as survival, fertilization and cleavage rates, but better outcomes in younger patients, as expected.

This slow-cooling protocol maintains the survival, fertilization, cleavage rates and embryo quality reported for previous protocols (Fabbri et al., 2001; Gook and Edgar, 2011) while pregnancy and implantation rate are significantly improved. Oocyte slow freezing performed with a modified protocol can give good and consistent outcomes.

### Clinical outcomes related to oocyte morphology before vitrification and after warming (Lodovico Parmegiani)

During assisted reproduction treatment, the administration of exogenous hormones results in the recruitment of oocytes of different maturation and quality. With mature (MII) oocytes, the detection of specific cytoplasmic dysmorphisms associated with reduced developmental competence is currently used to select the 'ideal' oocyte for ICSI. For the same reason, a strict oocyte selection based on morphological features (zona pellucida thickness, size of perivitelline space, oocyte shape, cytoplasm colour and granularity, presence of vacuoles and first polar body morphology) before cryopreservation is also helpful to optimize the clinical outcome of oocyte thawing/warming cycles. High-quality MII oocytes suitable for cryopreservation are colourless and of regular shape, with regular zona pellucida and small perivitelline space without debris, homogeneous cytoplasm and no vacuoles or granulations (De Sutter et al., 1996; Xia, 1997; Ebner et al., 2003; Parmegiani et al., 2008, 2011; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b). Amongst high-quality oocytes, the presence of an intact, round or ovoid polar body with smooth surface should be considered as a selection criterion (Ebner et al., 2000). Specifically, polar body quality and perivitelline space size seem to be related to oocyte ageing which may be an intrinsic feature of the collected MII oocyte or may be caused by a prolonged culture before cryopreservation. With fresh oocytes, the timing of ICSI is a critical factor in determining embryo viability and implantation, as the developmental capacity of the oocyte declines 41 h after HCG administration (Dozortsev et al., 2004; Yanagida et al., 1998). Similarly, with cryopreserved oocytes, metabolic ageing at ICSI depends not only on the time of retrieval after HCG administration and on the incubation time before cryopreservation, but also on the post-thawing culture before insemination. Furthermore, excessive extension of the culture period before freezing could affect oocyte competence after cryopreservation, since the oolemma permeability characteristics change over time (Hunter et al., 1992). For these reasons, oocyte cryopreservation should be carried out as soon as possible after retrieval to avoid injecting 'aged' oocytes after thawing and this seems to be extremely critical especially in the case of slow freezing (Parmegiani et al., 2008).

Some oocyte features change during the cryopreservation procedure, such as dehydration–rehydration or shrinkage–recovery, and these represent important indicators of the process. In particular, during human oocyte vitrification there are some specific oocyte behaviours that may predict the outcome of the future warming procedure (Kuwayama et al., 2005). For example, during exposure to equilibration solution, the oocytes should be completely shrunken after

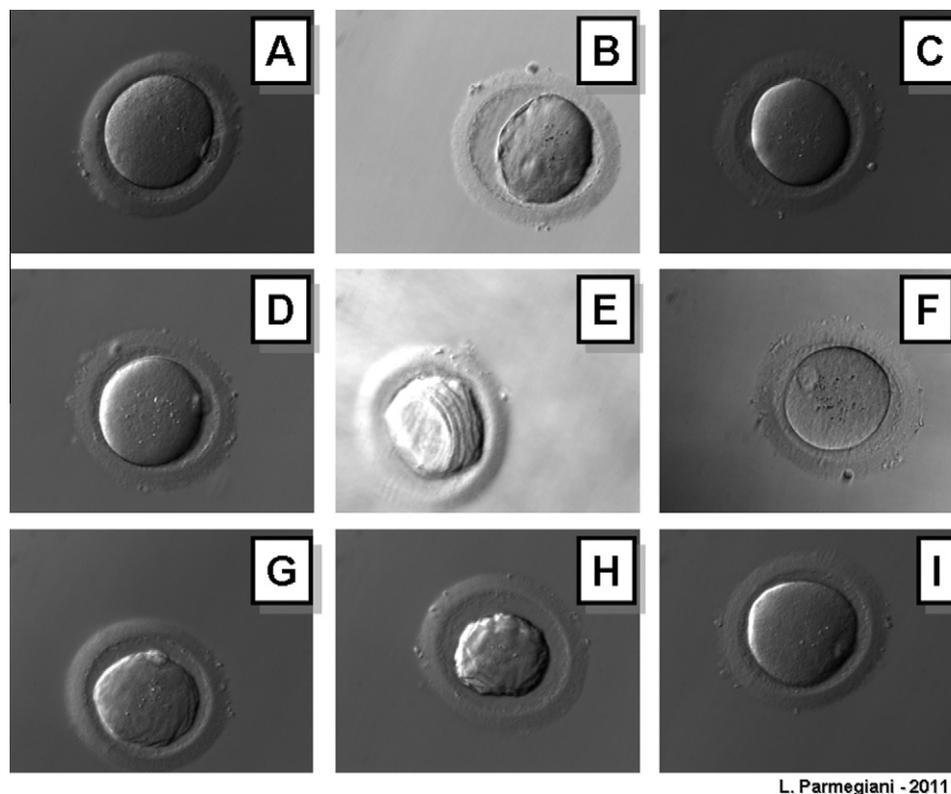
3–6 min, depending on the dilution of the equilibration solution. After this, oocyte shape starts to recover, with full recovery expected within 9–15 min. For human oocytes, full-size recovery seems to be extremely important for the success of the warming procedure: if they are still smaller than before the process, an additional equilibration time is strongly recommended. During exposure to the vitrification solution, the oocytes first shrink very quickly and float on the surface. They then partially recover but are not able to completely reshape before being loaded onto the carrier and vitrified.

During both vitrification and cryostorage, particular attention should be paid to biosafety and protecting the vitrified oocytes against temperature shocks to avoid contamination and damage (McDonald et al., 2011; Parmegiani and Rienzi, 2011; Parmegiani et al., 2010, 2011).

The first warming step is generally performed at 37°C in a 1 M sucrose solution; as soon as the oocytes are released from the carrier, they tend to float and they appear 'vitreous'. In the following warming solution, generally 0.5 M sucrose, the oocytes shrink and appear clear and very bright. In the subsequent warming steps, 0.25 M sucrose and/or subsequent washings, the oocytes slowly recover their original shape. If during warming the oocytes appear dark, flat or they quickly recover their shape during the first stages of warming, they are probably damaged and will degenerate. After post-warming culture, oocytes are considered to have survived in the absence of negative characteristics such as dark or contracted or extremely expanded ooplasm, massive vacuolization, cytoplasmic leakage, abnormal perivitelline space or cracked zona pellucida. Any discrepancies from the typical oocyte features (Figure 3) during vitrification–warming should be checked and recorded in order to have as much information as possible to select the 'ideal' oocyte and to optimize the warming cycle outcome.

### Clinical outcomes following oocyte vitrification and warming (Ana Cobo)

Oocyte cryopreservation has been one of the most fascinating challenges of assisted reproduction treatment and its incorporation into clinical practice has been a goal for many years. Despite the early disappointing results, recent advances in cryobiology have significantly improved the clinical efficiency of cryopreserved oocytes, including the birth of healthy children, especially when employing different vitrification approaches. There are several indications for oocyte cryostorage that include both subfertile and fertile patients. For example, women at risk of losing their reproductive function due to oncological treatment or premature ovarian failure, and fertile women electing to delay childbearing, could benefit greatly from this practice. In addition, oocyte cryopreservation may increase the flexibility of the options offered by current assisted reproduction strategies, especially in cases where embryo transfer must be delayed due to the risk of ovarian hyperstimulation syndrome, low response to gonadotrophins or failure to obtain a sperm sample on the day of oocyte retrieval. One of the most interesting advantages of oocyte cryostorage is the greater flexibility granted to oocyte donation pro-



L. Parmegiani - 2011

**Figure 3** Metaphase-II human oocyte features during vitrification/warming. (A–E) Vitrification: (A) MII oocyte before equilibration; (B) oocyte shrinking at equilibration; (C) oocyte recovery at equilibration; (D) full-size recovery at equilibration; (E) shrunken oocyte in vitrification solution before loading on the carrier device. (F–I) Warming: (F) oocyte at first step of warming (1 M sucrose solution) on release from the carrier device; (G–H) oocyte shrinking during the second step of warming (0.5 M sucrose solution); (I) oocyte recovery during final washing.

grammes, removing the need for synchronization between donor and recipient and allowing a quarantine period before the utilization of the oocytes. The aim of this presentation is to depict the experience of this study group in the routine clinical application of oocyte vitrification for over 4 years.

An efficient oocyte cryopreservation method is mandatory for the establishment of a successful egg-banking programme, and a pilot study demonstrated that oocyte vitrification did not impair subsequent embryo development (Cobo et al., 2008). Although there have been a number of reports showing good clinical outcomes following oocyte cryopreservation (Kuwayama, 2007; Nagy et al., 2008; Noyes et al., 2010; Paffoni et al., 2011), there is still a need for large controlled studies evaluating the effectiveness of egg cryo-banking. In a prospective, randomized, triple-blind, single-centre, parallel-group controlled, clinical trial comparing the outcome of vitrified-banked oocytes to the gold standard of fresh oocytes, with 300 randomized patients per arm, the ongoing pregnancy rate (OPR) per intention to treat population was 43.7% and 41.7% in the vitrification and fresh groups respectively (Cobo et al., 2010b). Although the odds ratio of OPR was 0.921 in favour of the vitrification group, the 95% CI was 0.667–1.274, so there was no significant difference ( $P = 0.744$ ). The proportion of top-quality embryos was also similar between groups, whether per inseminated oocyte (30.8% versus 30.8% for day-2 embryos; and 36.1% versus 37.7% for day-3 embryos,

respectively) or per cleaved embryo (43.6% versus 43.8% for day-2 embryos, and 58.4% versus 60.7% for day-3 embryos, respectively). This trial confirmed the effectiveness of oocyte cryostorage by demonstrating no difference in the OPR between fresh or vitrified oocytes. These findings may open a new range of possibilities in assisted reproduction treatment.

The study centre has a well-established oocyte donation programme supported by its oocyte bank (Cobo Cabal et al., 2011). Over 4 years, a total of 1856 ovum donation cycles following oocyte cryobanking have been performed, with oocytes from 1187 donors aged  $27.1 \pm 3.4$  years, with body mass index  $22.5 \pm 3.1$  kg/m<sup>2</sup>. A total of 22,741 MII oocytes ( $12.2 \pm 3.9$  per donation cycle) were obtained and donated to 1602 recipients (aged  $41.1 \pm 4.4$  years, body mass index  $23.7 \pm 4.3$  kg/m<sup>2</sup>). The overall cryosurvival rate was 90.1%, with 20,699 oocytes ( $11.1 \pm 3.9$  per donation cycle) then inseminated by ICSI. Fertilization, day-3 cleavage, blastocyst and implantation rates were 73.4%, 83.8%, 58.9% and 41.9% respectively, with 52.6% of day-3 embryos and 36.5% of blastocysts classified as top-quality embryos. To date, 1607 embryo transfers (86.6%) have been performed; 47% with day-3 embryos and 53% with blastocysts. The ongoing pregnancy rate was 41.3% per donation cycle. There were supernumerary embryos in 1181 (63.6%) cycles, with a mean number of  $1.9 \pm 2.0$  embryos revitrified. A total of 423 cryo-transfers of revitrified embryos have been carried out, with

a mean of  $2.2 \pm 0.7$  embryos replaced and the birth of a total of 520 babies from 395 deliveries. Other oocyte donation programmes have reported similar outcomes following oocyte vitrification (Nagy et al., 2009).

Oocyte vitrification has also been applied successfully in infertile patients, a very different population than healthy young donors. In this study centre, there are several clinical indications for oocyte vitrification, including impaired endometrial thickness or presence of polyps, in addition to those patients at risk of ovarian hyperstimulation syndrome and with failure of semen collection on the day of oocyte retrieval, as well as for fertility preservation. To date, 308 patients (mean age  $35.1 \pm 4.3$ ) have undergone IVF cycles with vitrified oocytes at this centre. A total of 3104 (mean  $10.7 \pm 5.0$ ) oocytes have been warmed, with a survival rate of 85.8%. The clinical pregnancy rate was 51.2% and 42.6%, with an ongoing pregnancy rate of 44% and 36.6%, per embryo transfer and per cycle, respectively. Similarly, Rienzi et al. (2010) conducted a prospective randomized sibling-oocyte study that included 120 fresh and 124 vitrified sibling oocytes from 40 infertile patients (mean age  $35.5 \pm 4.8$  years). The cryosurvival rate was 97%, with a fertilization rate after ICSI of 77% (95/124) per warmed oocyte and 79% (95/120) per warmed/inseminated oocyte. There was no difference in fertilization rates between the fresh and vitrified oocytes, and the proportions of excellent-quality embryos were the same in each group: 52% (52/100) from fresh oocytes and 52% (49/95) from vitrified oocytes. The clinical pregnancy rates were 38% per cycle and 39% per embryo transfer, with ongoing pregnancy rates of 30% per cycle and 31% per embryo transfer. In another study, the cumulative outcome after the transfer of embryos derived from fresh and vitrified oocytes from a single ovarian stimulation cycle was calculated (Ubaldi et al., 2010). The study included 182 ICSI cycles which oocyte vitrification was also performed. The cumulative CPR was calculated when after failing a fresh embryo transfer, the patient underwent a second embryo transfer using embryos derived from the vitrified oocytes. The cumulative ongoing clinical pregnancy rate per stimulation cycle was 53%. In this study, maternal age (>40 years old) was the only characteristic found to influence the reproductive outcome (Ubaldi et al., 2010).

A longitudinal cohort multicentre study to assess the efficacy and reproducibility of oocyte cryopreservation, as well as the effect of age and cycle parameters on the delivery rate has been conducted very recently (Rienzi et al., in press). The study included 486 cycles performed in 450 couples, in which 2721 oocytes were warmed and 2304 survived (84.7%). Of the 2182 oocytes inseminated by ICSI, the fertilization and developmental rates to top-quality embryos were 75.2 and 48.1%, respectively. There was a total of 128 deliveries (26.3% per cycle and 29.4% per transfer) in 450 patients (28.4%), with 147 liveborn babies from 929 embryos transferred (15.8%). The number of oocytes vitrified was correlated with outcome, with a higher delivery when >8 oocytes were vitrified (22.6 versus 46.4%). Furthermore, when fewer oocytes were available in women aged >38 years, the delivery rate was dramatically reduced (12.6 versus 27.5%). Conversely, when >8 oocytes are available, blastocyst culture represents the most efficient policy

(62.1% delivery rate; data from one centre only). These results demonstrate the reproducibility of oocyte vitrification and should be of value when counselling patients for fertility preservation.

All of this evidence suggests that oocyte vitrification may be a useful strategy for fertility preservation in oncology patients, and to date in this study centre, 223 women have undergone a stimulated cycle for oocyte vitrification before cancer treatment (Domingo et al., 2009, 2012). Non-cancer patients could also benefit from oocyte vitrification when they wish to delay motherhood for 'social reasons', including career, absence of partner or adverse social and economic conditions (Domingo et al., 2011).

### Clinical outcomes related to zygote morphology before and after cryopreservation with slow freezing and vitrification (Thomas Ebner)

Several reports have suggested that slow freezing at the zygote stage gives improved results as compared with cleavage stage (Fugger et al., 1988; Horne et al., 1997; Senn et al., 2000). This is probably due to the fact that a zygote is a single cell, like the oocyte, but without the problems arising from the sensitive meiotic spindle. One feature of zygotes that make KPIs feasible is that at the zygote stage there is no partial survival: the cell is either completely damaged or it survives, as demonstrated by the first mitotic division. Since prognostic factors in terms of blastocyst formation are rare at the zygote stage, slow freezing of blastocysts rather than zygotes may result in higher rates of implantation and pregnancy, although the survival rate might be lower.

Microscopic examination of zygotes after slow freezing and thawing showed that certain parameters used for scoring were affected by freezing, resulting in lower cumulative pronuclei scores (Senn et al., 2006). This might be due to a negative effect of cooling on microtubule organization, e.g. depolymerization (Mandelbaum et al., 2004). In particular, pronuclei proximity and orientation and presence of a cytoplasmic halo, as well as number and polarization of nucleolar precursor bodies, were affected. Since embryos derived from frozen-thawed zygotes showed acceptable rates of implantation and clinical pregnancy (Senn et al., 2006) it seems that a proportion of zygotes actually do recover from slow freezing-induced damage.

Vitrification at the zygote stage is somewhat different. Unique cryopreservation techniques have been established by altering the concentration of CPA, cooling rate, and/or carrier devices (Kumasako et al., 2005; Park et al., 2000). Zygote vitrification is no longer experimental (Jelinkova et al., 2002) and has become a routine method in assisted reproduction treatment (Al-Hasani et al., 2007), with survival rates at least as high as with slow freezing. However, vitrification and warming can have a significant impact on zygote morphology; around 15% of zygotes do not show pronuclear integrity after warming, which has been associated with poorer outcome (Isachenko et al., 2008). This type of morphological change is different from those seen after slow freezing. In conclusion, it would appear that, for zygotes, slow freezing is equivalent to vitrification.

### Clinical outcomes related to cleavage-stage embryo morphology before freezing and after thawing (Lisbet Van Landuyt)

Before discussing the selection criteria that influence the outcome of embryo slow freezing, the importance of uniform reporting (Jones et al., 1995) must be emphasized. A true assessment of a cryopreservation programme cannot be performed without information on the cycle cryopreservation, fresh embryo transfer and embryo cryopreservation rates.

The following aspects of embryo morphology before freezing were demonstrated to influence the post-thaw outcome in terms of survival and/or implantation ability: cell stage before freezing, cell symmetry, cleavage pattern (synchronous versus asynchronous cleavage) and percentage of anucleate fragments (Camus et al., 1989; Edgar et al., 2007; Hartshorne et al., 1990; Lassalle et al., 1985; Salumets et al., 2006; Solé et al., 2011).

Embryo survival rate is the primary parameter used to assess the efficiency of a freezing programme. The survival rate is expressed as a function of the number of embryos thawed, as assessed by morphological evaluation immediately after thawing. When embryos are subsequently cultured overnight to assess further cleavage, survival can also be defined by the transfer rate, expressed as the number of embryos transferred per thawed embryo. The final selection for transferring a thawed embryo can be based on a range of factors, including the number of blastomeres intact after thawing. Early publications on day-2 embryo freezing showed that it was possible to obtain pregnancies with damaged embryos and used a threshold of 50% blastomere survival (Hartshorne et al., 1990; Testart et al., 1988; Veiga et al., 1987). Later reports demonstrated the importance of obtaining fully intact embryos, as implantation rates were significantly higher than for embryos with cell loss (Burns et al., 1999; Edgar et al., 2000; Guerif et al., 2002; van den Abbeel et al., 1997). However, for day-2 embryos, other studies reported no impact on the implantation rate when the embryos were >50% intact (Edgar et al., 2007; Gabrielsen et al., 2006). For day-3 embryos, several reports concluded that implantation rates similar to those for fully intact embryos could be obtained for embryos in which up to 2 cells had been lost (Pal et al., 2004; Solé et al., 2011; Tang et al., 2006; Zheng et al., 2008).

Resumption of mitosis is another indicator for the outcome after frozen transfer. Several reports demonstrated increased implantation rates for day-2 embryos in which at least 1 cell cleaved post thaw (Guérif et al., 2002; Salumets et al., 2006; Van der Elst et al., 1997; Ziebe et al., 1998). Others found that implantation rates were higher when at least 2 cells cleaved post thaw for both day-2 (Edgar et al., 2007; Gabrielsen et al., 2006) and day-3 embryos (Solé et al., 2011).

The total number of blastomeres in the embryo at the time of transfer, resulting from either cryosurvival or resumption of mitosis, has also been correlated with outcome. Guérif et al. (2002) reported higher implantation rates for thawed day-2 embryos with at least 6 cells at transfer, while Zhang et al. (2009) found a positive correlation between the number of blastomeres at the time of

transfer in thawed day-3 embryos and the clinical pregnancy rate, regardless of the initial number of intact cells.

Finally, based on data from the Centre for Reproductive Medicine, UZBrussels, from 7664 frozen–thawed day-3 embryos, the proportion of blastomere survival was similar for the different cell stages before freezing (6–8 and >8 cells). The ability of the 4935 surviving embryos to resume mitosis was analysed according to cell stage before freezing and cell loss. It was concluded that an intact embryo, regardless of the cell stage, had a chance of at least 85% to cleave further. Partially damaged embryos had a lower cleavage rate, but the impact of 1 or 2 cells damaged was different for embryos with <8 cells than for embryos with at least 8 cells. Using single-embryo transfer, when an embryo with further cleavage was transferred, there was no negative impact of damage of 1 or 2 cells, for all cell stages, on the implantation and live birth rates. It was concluded that the number of cells intact post thaw was the determining factor for further cleavage and that the primary aim of a cryopreservation programme should be to obtain fully intact embryos.

### Clinical outcomes related to cleavage-stage embryo morphology before vitrification and after warming (Ana Cobo)

The first report documenting a successful pregnancy resulting from the transfer of cryopreserved human embryos was published almost 30 years ago (Trounson and Mohr, 1983). Since this landmark publication, cryopreservation of human embryos has played an important role in assisted reproduction treatment. For several years, reduction in the number of transferred embryos has been suggested as a way to reduce the incidence of multifetal pregnancy. Moreover, elective single-embryo transfer, which was first reported in 1999, is frequently used to prevent twin pregnancy. As the number of redundant embryos has increased, so has the demand for cryopreservation, and a simple and convenient method is needed. Worldwide, slow freezing is still the most common method used for cryopreservation, but vitrification is becoming increasingly common. The aim of the current presentation is to illustrate the experience of this study group with the application of vitrification to its embryo cryopreservation programme.

Based on 3150 warming cycles (6019 embryos), the overall survival rate was 95%, with 93% (95% CI 90.1–95.3) of day-2 and 95% (95% CI 94.3–95.7) of day-3 embryos fully intact upon warming. The delivery rate/warming cycle was 32.5% (95% CI 30.9–34.2). There were slight differences in the survival rate relative to developmental stage: 94.9% (95% CI 93.0–96.8) for day-2 embryos; 94.2% (95% CI 93.4–94.9) for day-3 embryos; 95.7% (95% CI 94.5–96.9) for day-5 embryos; and 97.6% (95% CI 96.9–98.6) for day-6 embryos ( $P < 0.05$ ). There was no significant difference between number of cells, mean fragmentation, mean symmetry index, multinucleation or mean compaction index before vitrification and after warming. Similarly, no differences in blastocyst morphology were observed after re-expansion post warming for day-5 or day-6 embryos. Overall implantation, clinical pregnancy, ongoing pregnancy and live birth

rate per warming cycle were 35.5% (95% CI 33.5–38.5), 41.7% (95% CI 39.9–43.4), 32.6% (95% CI 31.0–34.2) and 38.1% (95% CI 36.4–39.8), respectively. A linear regression model considering embryo developmental stage, oocyte source (donor or patient's), and method for endometrial preparation (hormone replacement therapy or natural cycle), showed no impact on the delivery rate ( $P < 0.05$ ; OR 1.179; 95% CI 0.912–1.277). These data confirm that an efficient embryo cryopreservation programme can be carried out with the help of the vitrification technique.

### Clinical outcomes related to blastocyst morphology before freezing and after thawing (Matthew 'Tex' VerMilyea)

With the ever-increasing production of mid- to fully expanded and hatched blastocysts on the 5th, 6th and 7th day of embryo culture, it is imperative that blastocysts are of good quality before undergoing cryopreservation (Sunkara et al., 2010). Several factors are known to be involved in the damage of cells during freezing and thawing. In slow controlled-rate freezing—rapid thawing processes, intracellular ice formation and osmotic stresses are key causes of blastomere damage. Although varying protocols have been developed in an attempt to curb such injury, the extent of damage can vary for the various stages of blastocyst development.

It is commonly accepted that blastocysts which exhibit a clear, well-defined inner cell mass (ICM) and an adequate total cell count are of better quality and deemed more viable by morphological assessment. In addition, many studies have shown in human assisted reproduction, the time an embryo takes to achieve the blastocyst stage is a positive indicator of embryo viability. Arguably, others consider that embryo viability is determined more by the stage of blastocyst development and is not dependent upon the time spent in culture at which the embryo reaches a certain morphological stage (Liebermann and Tucker, 2006). Both theories are applied when selecting blastocysts for cryopreservation. For example, embryos frozen at the expanded blastocyst stage had the same viability, implantation potential and pregnancy outcome, whether they were frozen on day 5 or day 6 (Richter et al., 2006). However, the implantation and pregnancy rates for expanded day-7 blastocysts were lower than those for day-6 blastocysts, even though they were much higher than previously reported for day-7 fresh embryo transfers (McVeary et al., 2004). These data reinforce the importance of freezing developmentally competent expanded blastocysts to ensure viability potential post thaw.

Frozen—thawed blastocysts undergo multiple morphological changes that include the collapse of the blastocoele cavity along with cellular lysis and degeneration. Morphological assessments of blastocysts post thaw are also necessary to estimate the survival of individual cells and the embryo in its entirety. By visual examination of the extent and locale of cellular degeneration, a skilled embryologist can estimate the proportion of viable cells. This estimate of total embryo survival is correlated with the implantation potential of the given embryo. The probability of implantation is relatively high when the majority of, if not all, cells survive, but begins to drop as cell survival declines below

95%. When fewer than 80% of cells survive, implantation potential appears minimal (Shipley et al., 2006).

Whatever the approach to cryopreservation, the objective of a successful blastocyst cryostorage programme should be to maximize the viability of the embryos transferred whilst reducing the number of embryos thawed in order to produce a child. By selecting embryos which fulfil morphological criteria that are not dependent upon their day of development, along with accurate viability estimations of cryodamage, one can greatly improve the outcomes of a cryopreservation programme. As technology continues to develop in the field of cryobiology, its purpose in assisted reproduction treatment becomes more pertinent. With the advent and application of modern techniques such as vitrification, perhaps the cryosurvival rates of blastocysts could be doubled. It is therefore critical to continue to investigate and agree upon a consensus that will enable near-perfect survival outcomes to be achieved in the near future.

### A continued role for slow freezing of blastocysts (Virginia Bolton)

Blastocyst culture was introduced at Guy's Assisted Conception Unit, London, in 2006, as part of a strategy to implement a selective single-embryo transfer policy to minimize multiple births. Before the introduction of blastocyst culture, all cryopreservation was carried during cleavage stages, almost exclusively on day 3 of development. On introduction of blastocyst culture, the freezing strategy was changed immediately, so that freezing takes place exclusively on days 5 and 6. Even when patients have embryo transfer on day 2 or 3 (i.e. those patients with fewer than two—four 7- or 8-cell-stage embryos with good morphology available on day 3, depending on their age), spare embryos are cultured to day 5 or 6 and assessed for suitability for freezing. Blastocysts are frozen provided they develop at least to grade 3 expansion, with ICM and trophoctoderm (TE) both at least grade C, and with no signs of degeneration, according to Gardner's grading system (Gardner and Schoolcraft, 1999).

With the adoption of this strategy, 33% of cycles result in freezing, including both those where embryo transfer is carried out on day 2 or 3 and those where it is carried out on day 5, with an average of 4.5 blastocysts frozen (range 1–13; mode 2).

Blastocysts are frozen individually in straws, using glycerol as the CPA and Quinn's Advantage reagents (SAGE; Cooper Surgical). A Cryobath controlled-rate freezer commences cooling at 19°C, then cools at a rate of  $-2^{\circ}\text{C}/\text{min}$  to  $-7^{\circ}\text{C}$  when seeding is carried out. After holding for 5 min, cooling resumes at a rate of  $-0.3^{\circ}\text{C}/\text{min}$  to  $-30^{\circ}\text{C}$ , when straws are plunged into LN. A single freezing procedure is carried out simultaneously for all embryos frozen each day. It takes approximately 2 h to complete and with, electronic witnessing, can be carried out by a single practitioner, with a second witness present as embryos are loaded into the straws.

This slow-freezing protocol, with the defined selection criteria for blastocysts considered suitable to freeze, achieved a clinical pregnancy rate of 37% per cycle, 38% per frozen embryo transfer and an implantation rate of

32% per thawed embryo transferred following 288 thaw cycles of 547 blastocysts in 2010. This compares with a current implantation rate of fresh blastocysts of 35–40%, depending on factors such as patient age.

In light of these results, this study centre has been reluctant to change from slow freezing to vitrification. Although progressively more reports from other centres suggest that vitrification is the better approach for cryopreservation, data has still to be seen to convince the centre that a switch in strategy is indicated.

### Clinical outcomes related to blastocyst morphology before vitrification and after thawing (Tammie Roy)

With the increasing prevalence of culturing human embryos to the blastocyst stage, in part due to improvements in sequential culture media, there has been a corresponding increase in the application of vitrification technologies for embryo cryopreservation (Youssry et al., 2008). Gardner et al. (2000) reported that blastocyst culture combined with comprehensive embryo assessment yielded high pregnancy rates. Similarly, a comprehensive embryo assessment before vitrification and post warming will yield high pregnancy rates in cryopreserved embryo transfer cycles.

Globally, clinics are developing models based on blastocyst culture and single-embryo transfer to minimize risks associated with multiple pregnancy, and consequently there is a requirement for laboratory methods and protocols to yield high pregnancy rates for thawed/warmed embryos. As the human embryo develops from the morula stage to the blastocyst there are complex structural changes. The blastocoel cavity forms, continuing to expand as the embryo moves through the expanded blastocyst stage and eventually emerges from the zona as a hatching blastocyst. The presence of the ICM and TE cells combined with a relatively high water content requires optimized concentrations of CPA and minimal final volumes of vitrification solutions (Morimoto, 2011).

Blastocyst vitrification was initially introduced at Genea's Sydney clinic in 2006, using the Cryologic hook protocol and device. In 2010 the vitrification protocol was modified to incorporate the use of the Cryotop device to further minimize the volumes for vitrification. At Genea the majority of patients have an embryo transfer on day 5. All supernumerary blastocysts that have developed to at least grade 2 in expansion, with ICM and TE both at least grade B, and with no signs of degeneration, according to Gardner's grading system (Gardner and Schoolcraft, 1999) are vitrified on day 5. Any remaining embryos are assessed on day 6 and vitrified if they have reached at least grade 4 in expansion, with ICM and TE both at least grade B, and with no signs of degeneration. These assessment criteria result in 36% of cycles having at least 1 blastocyst cryopreserved.

Blastocysts are vitrified individually, using equilibration and vitrification solutions containing DMSO, EG and trehalose at room temperature. Blastocysts are initially exposed to a 8% DMSO/EG solution for up to 15 min for equilibration followed by a 60–90 s exposure to a 16% DMSO/EG with 0.68 M trehalose vitrification solution before being vitrified in minimal volume.

This vitrification protocol yielded a clinical pregnancy rate (defined as the presence of fetal heart) of 41% for single blastocyst transfers following 679 thaw cycles of 709 blastocysts (blastocyst survival rate of 96%) between March 2010 and November 2011. These results are the same as for fresh transfers of single blastocysts of similar quality and development. Furthermore, there was no significant difference in viability, implantation potential or pregnancy outcome between blastocysts, of similar quality and development, vitrified on day 5 or 6.

Preimplantation genetic diagnosis (PGD) is utilized in many assisted reproduction clinics for the analysis of single-gene disorders through to molecular karyotyping of embryos via comparative genome hybridization microarray techniques. To facilitate the application of microarray analysis of embryos, laboratories are employing blastocyst biopsy followed by vitrification, prior to analysis and transfer of embryos in a later cycle (Wells et al., 2008). Optimization of vitrification techniques combined with rigorous assessment of embryo morphology has allowed clinics to implement a blastocyst-based single-embryo transfer model whilst maximizing cumulative pregnancy rates in combination with advanced PGD technologies (Traversa et al., 2011).

### Basic principles of defining and using benchmarks (David Mortimer)

The steps in the process of defining a KPI for use with benchmarking (Mortimer and Mortimer, 2005) are:

- (i) Define the biological or technical process to be monitored;
- (ii) Identify the endpoint of interest;
- (iii) Identify relevant qualifiers, e.g. female age;
- (iv) Identify confounders, i.e. factors extrinsic to the process of interest, e.g. clinical practices;
- (v) Define the KPI fully, based on all of the above;
- (vi) Establish the appropriate periodicity for calculation/ updating;
- (vii) Specify the data to be collected, and how they will be used to derive the KPI (calculation formula).

A well-defined KPI should be amenable to routine application in any IVF lab performing the procedure in question, regardless of geographical location.

Because benchmarks are derived from population-based data in order to remove the effect of interindividual variability in the KPI being measured, they are intended for use with similarly derived data, e.g. monthly statistics. Benchmarks should not therefore be applied to individual patients. For example, if a benchmark is 75% then this does not mean that a particular case with a result of <75% is 'abnormal' or 'a failure'. However, the proportion of cases with results  $\geq 75\%$  (or <75%) could be taken as a further KPI.

Based on the experience gained from the Budapest 2010 exercise and subsequent web-based questionnaire (see Introduction), the primary goals of the present Expert Group consensus were 2-fold. Firstly, to establish definitions that could be applied objectively for KPIs related to oocyte and embryo cryopreservation by either slow-freezing or vitrification techniques. Secondly, to provide both minimum

performance level and aspirational benchmark values for each KPI, allowing the setting of competency and best practice goals respectively.

No recommendations of specific cryopreservation techniques or devices, or whether vitrification is 'better' than slow freezing, or vice versa, for any particular stage or application were considered. It was felt that premature attempts to do so would detract from the workshop's real purpose of establishing KPIs and benchmarks.

Achieving consensus is a collegial activity undertaken by a group of peers who are willing to listen, think, discuss and make some compromises so that the majority of participants are comfortable with each consensus point. Reaching unanimous agreement on everything is either necessary nor realistic – if there was a 'right answer' then a consensus would not be needed.

For each defined cryopreservation KPI, the group was tasked with reaching a consensus on:

- (i) Minimum performance (basic competency) values for labs using slow freezing;
- (ii) Minimum performance (basic competency) values for labs using vitrification;
- (iii) Aspirational benchmark values (best practice goals) for labs using slow freezing;
- (iv) Aspirational benchmark values (best practice goals) for labs using vitrification.

### Defining KPIs for cryopreservation of oocytes and embryos in clinical assisted reproductive technology (David Edgar)

Before attempting to reach consensus on KPIs/benchmarks for cryopreservation within an assisted reproductive technology laboratory, a number of key considerations should be emphasized. Most importantly, any KPI should be based on an endpoint which is specific to cryopreservation and is controlled for other variables, many of which will differ between laboratories.

The suggestion that the proportion of oocyte collection cycles that incorporate cryopreservation and/or the proportion of all oocytes/embryos that are cryopreserved should be included as a KPI demands close scrutiny (Edgar and Gook, 2007; Gook and Edgar, 2009). These endpoints are influenced by a large number of variables which are not related to cryopreservation *per se* and may only be useful as an in-house benchmark.

Assessment of post-thaw survival of oocytes and embryos at the structural level should be relatively objective but KPI/benchmarks should reflect clinically relevant endpoints e.g. the relevance of thresholds such as 50% blastomere survival should be subject to evaluation based on clinical evidence. The establishment of interlaboratory benchmarks for survival at the structural level should, however, be possible but may need to be specific for the individual method used.

Assessment of survival at the functional level, i.e. post-thaw retention of developmental potential, is dependent on a number of factors related to variations in practice between laboratories, particularly selection criteria prior to

freezing (Edgar et al., 2005). These factors, and the relevant controls required to allow for them, make the establishment of inter-laboratory benchmarks more challenging. KPIs for post-thaw development should include reference to the relevant controls.

KPIs/benchmarks for clinical outcomes following cryopreservation, i.e. pregnancy, implantation, miscarriage and birth rates, are subject to variables associated with clinical practice. The age of the woman at the time of cryopreservation is critical in defining KPIs but pre-freeze and post-thaw selection/utilization of oocytes and embryos also vary widely between clinics. Such differences may, potentially, be partly overcome by assessing implantation and/or birth on a 'per thawed oocyte/embryo' basis.

In all cases involving post-thaw development and implantation, the outcomes from equivalent fresh oocytes/embryos within the individual clinic are a crucial reference point. For example, if clinic A and clinic B have blastocyst development rates per fertilized fresh oocyte of 30% and 60% and fresh implantation rates of 25% and 40% respectively, it would not be valid to apply the same KPIs for development and implantation of thawed oocytes to both clinics.

In summary, when defining a KPI for cryopreservation in assisted reproductive technology, it is also essential to define the biological material which is being cryopreserved and the way in which it is being applied clinically (see Edgar and Gook, *in press*).

## Consensus points

### General comments

When a centre is defining any KPI, it is the scientific or laboratory director who should decide if, and what, age definition or qualifier is necessary. Similarly, for the calculation of implantation rate KPIs, the scientific or laboratory director should document variations in embryo transfer techniques used, as well as operator variability, to verify how much of the centre's data can be incorporated into the calculation.

All KPIs are 'general' – in other words, they do not take into account possible local regulatory requirements that might influence practice. Hence, the existence of any such issues must be specified, to allow for comparability. It is recognized that no international KPIs exist for fresh IVF/ICSI, and hence many of the cryopreservation KPIs will need to be established with reference to the local (centre-specific) fresh KPIs. Furthermore, it was acknowledged that no KPIs can be enforced, since KPIs will be influenced by the patient population, medical practice, legislation, etc. For example, while the European Union's Tissues and Cells Directive has a requirement to minimize the risk of cross-contamination (although there is no evidence base for this risk), there also needs to be regard for the efficacy of the procedure. Thus KPIs must always be considered within each centre's own quality management system.

For vitrification, and possibly slow freezing, operator confidence/competence must be comparable; otherwise, the quality manager must be aware that inter-operator variation is possible and can affect overall performance, and

hence the KPI values. Standardization of protocol details and training are essential, e.g. preventing LN vapour from adjacent container(s) from cooling dishes; defining, and recording, local room temperature; and using an adequate volume of warming solution to maintain warming rate. Other technical issues include the following:

- (i) All cryopreservation base media should be as physiological, and as close to the corresponding embryo culture medium, as possible to minimize deleterious effects upon oocytes/embryos;
- (ii) While DMSO is considered safe to use in vitrification solutions, the stability/shelf life of vitrification solutions should be defined;
- (iii) During warming, it is important to get from  $-196^{\circ}\text{C}$  to the solution's melting point as quickly as possible to avoid crystallization damage; the higher the cooling rate used, the higher the warming rate needed;
- (iv) There is a risk of crystallization damage during handling, including during audits, especially for vitrified specimens, and even more so for minimum volume vitrification. It was agreed that closed systems, which have an insulating layer of air, afford a measure of protection;
- (v) During transportation of cryopreserved specimens between clinics/laboratories, the temperature in the shipping device must remain below  $-132^{\circ}\text{C}$  (ideally below  $-150^{\circ}\text{C}$ ), and this should be verified by a logging device.

In all publications and technical reports, it is critical to specify the full and exact cryopreservation protocol used, including, for oocytes, denudation timing and method

With regard to the recommended KPIs, it was recognized that some centres cryopreserve embryos on day 4. However, it was felt that the general experience with morula cryopreservation is restricted to too few centres to support the derivation of any generalizable KPIs. In addition, endometrial preparation issues were felt to be beyond the scope of this discussion, and so have not been addressed. It should be noted that all of the recommended KPIs refer to cases without any manipulation for PGD.

In the KPI recommendations below, 'Equivalent to comparable fresh x' is intended to refer to the equivalent to all measured parameters of x quality in the centre for women of comparable age at the time of cryopreservation. When the recommended KPIs refer to 'absolute' percentage values, e.g. '10% (absolute)', this refers to percentage points. In other words, no more than 10% (absolute) lower than 65% would be a value that is 55% or higher. When the recommended KPIs refer to 'relative' percentage values, e.g. 10% (relative), this is a proportional difference – so a 10% (relative) decrease from 70% would be 63%.

## Oocyte KPIs

These KPIs assume that the oocytes to be cryopreserved are morphologically normal MII oocytes, and that abnormal oocytes, such as those with smooth endoplasmic reticulum disks, or giant oocytes, will be discarded ([Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group](#)

[Embryology, 2011a,b](#)). At present, there are no validated methods for the cryopreservation of germinal-vesicle or metaphase-I oocytes, and so they should not be included in the KPI calculation.

It was the consensus that the current best practice is to denude oocytes immediately before cryopreservation. For slow freezing, this should be completed by 38 h post HCG, with ICSI performed 2–3 h post thaw to allow the spindle to reform. For vitrification, oocytes should be vitrified 38–40 h post HCG, with ICSI performed at the equivalent of 40–41 h post HCG (i.e. between 1 and 3 h post warming).

It should be noted that the recommended KPI values for oocyte cryopreservation are applicable in the absence of any oocyte selection, such as that required by legislation.

### O1: Morphological survival

This KPI was defined as the proportion of morphologically intact oocytes, based on intention to inject, at the time of ICSI ([Table 1](#)). That is, if the oocyte is judged to be suitable for injection, then it is considered to be morphologically normal for the purposes of this KPI. The consensus was that oocytes with an abnormal oolemma or ooplasm at the time of ICSI should not be excluded.

Because this KPI could be affected by the caseload and/or by practitioner experience, different values were assigned for the acquisition of competency and for benchmarks for both slow freezing and vitrification. The competency values are those that should be achieved by any practitioner judged competent to perform cryopreservation, while the benchmarks are aspirational targets.

### O2: Fertilization rate

Fertilization rate ([Table 1](#)) was defined as the proportion of oocytes with 2 pronuclei at the time of fertilization check ( $17 \pm 1$  h post insemination; [Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b](#)).

### O3: Embryo development

Embryo development ([Table 1](#)) was defined as the proportion of embryos at the expected developmental stage and grade for the time of observation (i.e. 2-cell stage at  $26 \pm 1$  h post ICSI, 4-cell stage at  $44 \pm 1$  h post insemination, 8-cell stage at  $68 \pm 1$  h post insemination, morula stage at  $92 \pm 2$  h post insemination and blastocyst stage at  $116 \pm 2$  h post insemination; [Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b](#)).

It was the consensus that the rate of embryo development after oocyte vitrification should be the same as for the comparable population of fresh embryos at the centre. For oocytes cryopreserved by slow freezing, it was the consensus that the embryos may exhibit some delay in the rate of development, and so different values were assigned for the acquisition of competency and for benchmarking.

### O4: Implantation rate

Implantation rate was defined as the proportion of fetal hearts relative to the number of embryos transferred ([Table 1](#)). It was the consensus that the implantation rate

**Table 1** Oocyte key performance indicator values.

KPI		Competence	Benchmark
O1	Morphological survival	Freezing $\geq 50\%$ Vitrification 70%	75% 85% (95% for donors <30 years)
O2	Fertilization rate	No more than 10% (absolute; i.e. 10 percentage points) lower than that for the comparable population of fresh oocytes at the centre	
O3	Embryo development rate	Freezing Vitrification	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre The same as for the comparable population of fresh embryos at the centre
O4	Implantation rate	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre	

should be no more than 10–30% (relative) lower than the comparable population of fresh embryos at the centre.

### Zygote KPIs

For inclusion in the KPI calculation, the zygotes selected for cryopreservation should meet the consensus criteria for an optimal fertilized oocyte, i.e. spherical, with two polar bodies and two centrally located, juxtaposed pronuclei that are even-sized, with distinct membranes. In addition, the pronuclear score should be 'symmetrical', which is equivalent to Z-scores Z1 and Z2 (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b).

The KPI values assume that there was no other pre-selection of zygotes, or enforced cryopreservation of abnormal zygotes, and that there are no requirements for the subsequent transfer of any abnormal embryos post thaw/warming.

It was the consensus that for zygotes derived from ICSI, observations made during the ICSI procedure regarding oocyte quality should always be recorded. This would allow possible later analysis of the prevalence of oolemma/ooplasm abnormalities that might have been caused by the cryopreservation procedure.

#### Z1: Morphological survival

This KPI was defined as the proportion of morphologically intact zygotes immediately upon thawing/warming relative to the number of morphologically normal zygotes cryopreserved prior to nuclear envelope breakdown (Table 2). A morphologically intact zygote is one that is similar in appearance to a fresh zygote. It was the consensus that the same survival rate should be achieved by slow freezing as by vitrification.

Different values were assigned for the acquisition of competency and for benchmarking. The competency values are those that should be achieved by any practitioner judged competent to perform cryopreservation, while the benchmark is an aspirational target.

#### Z2: Cleavage rate

This KPI was defined as the proportion of thawed zygotes that divide to form a cleavage-stage embryo (Table 2). It

was the consensus that the cleavage rate should be the same as for the comparable population of fresh embryos at the centre.

#### Z3: Embryo development

Embryo development (Table 2) was defined as the proportion of embryos at the expected developmental stage and grade for the time of observation (i.e. 2-cell stage at  $26 \pm 1$  h post ICSI, 4-cell stage at  $44 \pm 1$  h post insemination, 8-cell stage at  $68 \pm 1$  h post insemination, morula stage at  $92 \pm 2$  h post insemination and blastocyst stage at  $116 \pm 2$  h post insemination; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b).

It was the consensus that the rate of embryo development should be the same as for the comparable population of fresh embryos at the centre.

#### Z4: Implantation rate

Implantation rate was defined as the proportion of fetal hearts relative to the number of embryos transferred (Table 2). It was the consensus that the implantation rate should be no more than 10–30% (relative) lower than the comparable population of fresh embryos at the centre.

### Embryo KPIs

For inclusion in the KPI calculation, it was the consensus that the embryos selected for cryopreservation should meet the consensus criteria for an optimal cleavage-stage embryo (i.e. 4-cell stage at  $44 \pm 1$  h post insemination, 8-cell stage at  $68 \pm 1$  h post insemination with <10% fragmentation, stage-specific cell size and no multinucleation; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011a,b). The question of whether specific KPIs for day-2 and day-3 embryos should be developed was discussed, but it was agreed that the same KPI values were valid for both day-2 and day-3 embryos (Table 3).

It was noted that the recommended KPI values are based on the current standard methodology for embryo freezing. However, higher cryosurvival rates have been reported using a revised method (Edgar et al., 2009), and if this method is used, then alternative KPI values should be developed. Sim-

**Table 2** Zygote key performance indicator values.

KPI	Competence	Benchmark
Z1 Morphological survival	70% <sup>a</sup>	85% <sup>a</sup>
Z2 Cleavage rate	The same as for the comparable population of fresh embryos at the centre	
Z3 Embryo development rate	The same as for the comparable population of fresh embryos at the centre	
Z4 Implantation rate	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre	

<sup>a</sup>The KPI value is calculated as the proportion of morphologically normal zygotes cryopreserved.

**Table 3** Embryo key performance indicator values.

KPI	Competence	Benchmark
E1 Morphological survival: fully intact	Freezing 40% Vitrification 70%	55% 85%
E2 Morphological survival: $\geq 50\%$ intact	Freezing 60% Vitrification 85%	85% 95%
E3 Post-thaw development (including implantation rate) for fully intact embryos	$\leq 10\%$ (relative) lower than that for the comparable population of fresh embryos at the centre	The same as for the comparable population of fresh embryos at the centre

The KPI values are calculated as the proportion of all thawed/warmed embryos.

**Table 4** Blastocyst key performance indicator values.

KPI	Competence	Benchmark
B1 Survival rate	Freezing 70% Vitrification 80%	85% 95%
B2 Transfer rate	Freezing 70% Vitrification 80%	85% 95%
B3 Implantation rate	$\leq 10\%$ (relative) lower than that for the comparable population of fresh embryos at the centre	The same as for the comparable population of fresh embryos at the centre

The KPI values are calculated as the proportion of all thawed/warmed blastocysts.

ilarly, it was acknowledged that because the vitrification KPI values are based on more recent and relatively limited experience, they may need to be revised in the future.

### E1 and E2: Morphological cryosurvival

Traditionally, KPIs dealing with embryo cryosurvival are based on the proportion of thawed embryos with (i) 100% and (ii)  $\geq 50\%$  of the blastomeres intact. It was suggested that based on published evidence (e.g. Edgar et al., 2007), the lower rate of cryosurvival could be  $\geq 67\%$  or  $\geq 75\%$ , rather than  $\geq 50\%$ , but it was the consensus to retain the traditional definitions for the development of the current KPIs, with a future KPIs to be the proportion of intact blastomeres across all embryos thawed. Accordingly, the competence and benchmark KPI values for morphological cryosurvival were based on the proportion of frozen and vitrified cleavage-stage embryos in which all of the blastomeres were intact (E1, Table 3) and for embryos with at least 50% intact blastomeres (E2, Table 3).

It should be emphasized that these KPI definitions do not preclude the transfer of embryos with a blastomere survival rate of  $< 50\%$ , as this may be necessary for clinical expediency.

### E3: Post-thaw development

It was the consensus that only those embryos with 100% morphological cryosurvival should be included in the calculation of post-thaw development KPIs. Post-thaw development includes further cleavage and development to the blastocyst stage, as well as the implantation rate, defined as the proportion of fetal hearts relative to the number of embryos transferred.

It was the consensus that post-thaw/warming development of embryos should be no more than 10% (relative) lower than that of the comparable population of fresh embryos at the centre, with a benchmark of equivalent rates of development to that of fresh embryos (Table 3).

## Blastocyst KPIs

Because the rate of development *in vitro* is substantially affected by stimulation and culture system parameters, it was the consensus that there would be no differentiation between blastocyst stage (early, blastocyst, expanded, hatching) or day post insemination within the blastocyst KPIs. Similarly, there was no recommendation regarding blastocoel collapsing, as this is protocol dependent and would be accounted for within each centre through reference to the results with equivalent fresh blastocysts.

It was the consensus that reported outcomes for biopsied embryos cryopreserved at the blastocyst stage are substantially better following vitrification than slow freezing, using the current protocols.

### B1: Survival rate

It was the consensus that blastocyst cryosurvival be defined as at least 75% of cells perceived to be intact after thawing/warming, with the KPI calculated as the proportion of surviving blastocysts as a function of the total number of blastocysts thawed (Table 4).

The proportion of thawed/warmed blastocysts that re-expand within 2 hours was recommended as an extra KPI for survival, but it was noted that there is not always time to conduct this assessment.

### B2: Transfer rate

This KPI was defined as the proportion of thawed/warmed blastocysts that are of sufficient quality to transfer (Table 4). This parameter assumes that the decision to transfer is not subject to legislative oversight.

### B3: Implantation rate

Implantation rate was defined as the proportion of fetal hearts relative to the number of blastocysts transferred (Table 4). It was the consensus that for thawed/warmed blastocysts, the implantation rate should be  $\leq 10\%$  (relative) lower than that for the comparable population of fresh blastocysts at the centre and that the benchmark implantation rate should be equivalent to that for the comparable population of fresh blastocysts at the centre.

## Further considerations

The workshop participants recognized the existence of possible device- or operator experience-dependent issues resulting in the failure to recover oocytes or embryos after thawing/warming. It was the consensus that since such failures should be a rare event, non-recovery rates should be stated in any reports of studies or programme analyses and in any device issue reported to the appropriate regulator.

The group also recognized the absence over the past two decades of significant research and development towards the optimization of slow-freezing methodology.

Finally, it was noted that although serial cryopreservation, such as cryopreservation of embryos from thawed/warmed oocytes, is being performed, there is insufficient evidence available to ascertain any deleterious

effect. It was the recommendation that these events be recorded so that the practice can be monitored.

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