Review

Are programmable freezers still needed in the embryo laboratory? Review on vitrification

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Abstract

The predictable answer to the provocative question of whether programmable freezers are still needed in the embryo laboratory is an even more provocative 'no'. However, such a radical statement needs strong support. Based on the extensive literature of the past 5 years, the authors collected arguments either supporting or contradicting their opinion. After an overview of the causes of cryoinjuries and strategies to eliminate them, the evolution of vitrification methods is discussed. Special attention is paid to the biosafety issues. The authors did not find any circumstance in oocyte or embryo cryopreservation where slow freezing offers considerable advantages compared with vitrification. In contrast, the overwhelming majority of published data prove that the latest vitrification methods are more efficient and reliable than any version of slow freezing. Application of the proper vitrification methods increases the efficiency of long-term storage of stem cells and opens new perspectives in cryopreservation of oocytes, both for IVF and somatic cell nuclear transfer. However, lack of support from regulatory authorities, and conservative approachs regarding novel techniques can slow down the implementation of vitrification. The opinion of the authors is that vitrification is the future of cryopreservation. The public have the final say in whether they want and allow this future to arrive.

Keywords: *cryopreservation*, *embryo*, *oocyte*, *vitrification*

Introduction

Widespread practical application of reproductive technologies in any species is facilitated by and sometimes depends on the success rate of cryopreservation of both gametes and embryos. Over the past 60 years, evidence supports this statement, including the cryopreservation of bovine sperm for artificial insemination and bovine embryos for transfer. In contrast, in pigs the lack of a standardized and internationally accepted method of embryo cryopreservation with high success rates restricts the applications of embryo transfer. Additionally, differences in the ability to store male and female gametes limit the possibilities in animal breeding and preservation. In the human, this creates a distinct difference in reproductive choice between men and women, providing extreme flexibility for males and severe, in some situations tragic, restrictions for females.

To resolve these problems, considerable efforts have been expended in oocyte and embryo cryopreservation, both in the animal and human field. Initial diverse approaches and inconsistent results have eventually converged and led to an alternative and very promising strategy. Unfortunately, these achievements have remained mostly neglected in everyday practice. Meanwhile, slow freezing, in spite of its well-known limitations, has become highly standardized with a considerable industrial and commercial background. Exhibition stands of international conferences offer a huge variety of sophisticated, attractive and expensive instruments. Programmable freezers are offered with precisely determined parameters and ready made media for freezing and thawing. User friendly techniques have led to cryopreservation becoming a routine standard step that can be performed after a short training period and without any knowledge of basic

physical and biological events (Vajta and Kuwayama, 2006). Moreover, pressing commercial considerations in animal breeding as well as scrupulous legal restrictions in human assisted reproduction provide very little space for innovation. Those who pioneer new methods have to manoeuvre carefully through bureaucratic barriers as well as the ambivalent support from the business side.

Recently, however, signs of a long-awaited change can be detected. This article attempts to summarize the main features of this progress and to outline the future perspectives. In contrast to the usual 'politically correct' atmosphere of many cryopreservation reviews, where a careful balance between the traditional and new approaches is maintained, this article is strongly biased in favour of the latter ones. According to the authors' own experiences and the overwhelming evidence provided by the scientific literature (listed or summarized below), it appears that slow freezing has very little if any future in the field of embryology, and the rate of advancement in oocyte and embryo cryopreservation will depend on the rate by which embryologists and decision-makers adopt the new approaches. It is understood that this sharp statement may not be agreeable to everybody, but an open dispute based on scientifically sound arguments from both parties may be more helpful to find the right solutions than a polite and respectful silence.

Regarding the definitions of terms, we refer almost entirely to the excellent paper of Shaw and Jones (2003). The fundamentals of cryobiology in reproductive medicine were thoroughly reviewed by Fuller and Paynter (2004) and others (Kasai and Mukaida, 2004; Smith and Silva, 2004; Stachecki and Cohen, 2004). This review therefore deals only with the differences in techniques.

Damage, prevention and reparation during cryopreservation

With the rare exception of certain species, the body temperature of mammals is strictly regulated. Cooling of oocytes and embryos of domestic animals and humans to subzero temperatures is a situation they never meet under physiological conditions. Consequently, the defence mechanism to survive such conditions is inappropriate and requires external support.

Injuries may occur at all phases of the cryopreservation procedure. Understanding the causes and mechanisms of damage may help the development of cryopreservation methods to avoid lethal or irreversible injuries.

During cooling, three types of damage may be distinguished according to the different temperature ranges the cells pass through. At relatively high temperatures between +15 and –5ºC, the chilling injury is the major factor, damaging predominantly the cytoplasmic lipid droplets and microtubules including the meiotic spindle (Aman and Parks, 1994; Leibo *et al*., 1996; Martino *et al*., 1996a; Zenzes *et al*., 2001). While the latter damage may be reversible, the former is always irreversible and contributes to much of the death of cryopreserved lipid-rich oocytes and embryos of some species. Between –5 and –80ºC, extracellular or, predominantly, intracellular ice crystal formation is the main source of injury, while between –50 and –150ºC fracture damage to the zona pellucida or the cytoplasm (Rall and Meyer, 1989) are postulated to occur (although the mechanism and the actual temperature of occurrence is not entirely defined). However, it is unlikely that zona fracture could occur as a simple consequence of osmotic stress, as suggested by Smith and Silva (2004).

Storage below –150ºC (typically in liquid nitrogen, at –196ºC) is probably the least dangerous phase of the cryopreservation procedure. Importantly, accidental warming is probably the most frequent form of injury. The effect of background irradiation seems to be less harmful than supposed, and is not a significant source for DNA injury in a realistic time interval, i.e. years, decades or even centuries (Rall, 2001). However, there is increasing concern regarding possible disease transmission between the stored samples mediated by the liquid nitrogen. At warming, the same types of injuries may occur as at cooling, obviously in inverse order.

Apart from these processes, there are some partially understood injuries including damage of intracellular organelles, cytoskeleton and cell-to-cell contacts (Vincent and Johnson, 1992; Massip *et al*., 1995; Dobrinsky, 1996).

All oocytes and embryos suffer considerable damage during cooling and warming. Fortunately, they also have a remarkable, sometimes surprising ability to repair fully or partially this damage, and in the best case to continue normal development. The purpose of the cryopreservation procedures is similar to that of medicine: to minimize the damage and to help to regenerate.

Almost all cryopreservation strategies are based on two factors: cryoprotectants and cooling–warming rates. The common feature of cryoprotectants is the ability to decrease cryoinjuries. A wide range of materials fit this definition, including simple, defined, low molecular weight solvents such as ethanol; or complex, partially undefined biological compounds like sera or egg yolk. They may either enter the cell (permeable cryoprotectants) or remain outside (non-permeable cryoprotectants). The main supposed effect of permeable cryoprotectants is to minimize ice formation. A similar mechanism, osmotic dehydration, may play a considerable role in the protective effect of non-permeable cryoprotectants, although there are very few examples in which non-permeable cryoprotectants actually protect cells against freezing damage. However, both permeable and non-permeable cryoprotectants may also have additional protective mechanisms, for example to stabilize intracellular structures and the cell membrane. Unfortunately, most cryoprotectants have some negative effects, including toxicity and osmotic injuries. Toxicity is usually proportional to the concentration of the substance and to the time of exposure (at physiological temperatures).

Cooling rates may vary according to the applied method, from moderate or stepwise cooling between the physiological temperature to –4ºC, highly controlled cooling rates to –40 or –80ºC, followed by plunging to liquid nitrogen, to either rapid (around 200ºC/min) or ultrarapid (up to 20,000–100,000ºC/min) rates throughout the whole temperature range (Rall, 2001; Kasai and Mukaida, 2004). Warming

may be also performed stepwise, with highly controlled or just slightly delayed increase of the temperature, or (more typically) rapidly, including the commonly achievable highest rates of temperature change described above. Cryoprotectant composition, addition, concentration and removal, as well as warming rates, are more or less determined by the selected cooling rates.

The mechanism and reasons for damage during cryopreservation as well as the precise protective mechanisms of cryoprotectants are poorly understood because of the inappropriate investigation methods. Morphological observations of the intracellular structures during the actual phase of cooling (especially at subzero temperatures) are difficult, functional analysis of specific processes at a given moment is almost impossible. Apart from some sophisticated and very expensive equipment (which in fact add relatively little to our understanding), the most frequently applied approaches are to investigate the effect of cryoprotectants without cooling and warming, or make retrospective conclusions based on the damage that can be observed after warming. However, the effects of a given cryoprotectant may substantially differ at physiological and at low temperatures; thus, the retrospective analysis of damage may result in faulty conclusions. Consequently, the interpretation of cryoinjuries (including the significance of specific mechanisms) and cryoprotective effects is highly variable between investigators.

Considering these uncertainties, it is not surprising that vast majority of existing cryopreservation techniques were established empirically, based on rough morphological changes observed under a stereomicroscope, and have been justified by the outcome, i.e. in-vitro and in-vivo survival. The underlying complex mechanisms were explained only later by hypotheses based on analogue models and calculations. Paradoxically, in some cases, even these hypotheses fail to reveal a logical explanation, and there are still situations where oocytes and embryos survive cryopreservation and develop afterwards, although the damage induced by the applied technique should have killed them, for example in the case of ultrarapid freezing (see later).

Main cryopreservation strategies

The first successes of mammalian embryo cryopreservation occurred in the 1970s (Whittingham *et al*., 1972; Wilmut, 1972; Wilmut and Rowson, 1973; Bank and Maurer, 1974; Willadsen, 1976) and first human pregnancies were achieved relatively soon after (Trounson and Mohr, 1983; Zeilmaker *et al*. 1984) becoming just the ninth mammalian species with normal offspring following the transfer of cryopreserved embryos to foster mothers (Rall, 2001). During the past decades, two major groups of methods can be separated: slow freezing and vitrification. Storage, warming and rehydration, i.e. removal of cryoprotectants differ only slightly between the two procedures (with some exceptions), the main difference exists in the addition of cryoprotectants and cooling.

Slow freezing can be interpreted as an attempt to create a delicate balance between various damaging factors including ice crystal formation, fracture, toxic and osmotic damage. Embryos and oocytes are typically equilibrated in 1–2 mol/l solutions of permeable and non-permeable cryoprotectants, loaded into 0.25 ml straws, sealed and cooled relatively rapidly to –6ºC by placing the straws into the controlled-rate freezer. At –6ºC, seeding, i.e. ice crystal formation is induced in the solution preferably far from the embryo or oocyte. The subsequent steps are entirely or almost entirely performed by the machine. There are slight variations in the subsequent cooling rates, but values are between 0.3 and 1ºC/ min. The controlled rate cooling then continues to around –30ºC. At these temperatures, straws are immersed to liquid nitrogen for final cooling and storage.

In slow freezing, the toxic and osmotic damage caused by the relatively low concentration of cryoprotectant solutions may not be too serious. However, this concentration is insufficient to avoid ice crystal formation; therefore an additional manipulation is required to minimize the damage. It is the slow cooling and seeding that result in controlled growth of ice in the extracellular solution; consequently, a considerable increase of the concentration of ions, macromolecules and other components, including cryoprotectants, occurs in the remaining fluid. The slow rate of the procedure allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing; Mazur, 1990). Although the concentration of these components, especially of cryoprotectants, seems to be dangerously high at the final phases, it happens at low temperatures, where the real toxic effect is minimal. On the other hand, this elevated concentration minimizes ice crystal formation, allowing solidification of intracellular water in a glass-like form.

This phenomenon fulfils the physical criteria of vitrification. However, in cryobiology this definition is mainly reserved for the other major group of cryopreservation methods, where the whole sample including both the extracellular and intracellular solutions vitrify. The name refers to the macroscopic appearance, where at vitrification the solution should remain entirely clear and transparent.

Vitrification may be regarded as a radical approach, as one of the main sources of injuries, ice crystal formation, is entirely eliminated. However, a negative consequence of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation. To achieve vitrification of solutions, a radical increase of both the cooling rates and the concentration of cryoprotectants is required. The higher the cooling rate, the lower the required cryoprotectant concentration is, and *vice versa*. With the extreme increase of cooling rate (to approximately 10^7°C/s), vitrification can also be achieved in pure water, but the usual limits in embryology are far below this rate (Rall, 1987). The balance required in vitrification is between (i) establishment of a safe system for maximal and reliable cooling (and warming) rates while avoiding consequent damage including fracture of the zona pellucida or the cells, and (ii) elimination or minimization of the toxic and osmotic effects of high cryoprotectant concentrations needed to obtain and maintain the glass-like solidification. Cell shrinkage caused by non-permeable cryoprotectants and the incomplete penetration of permeable components may cause a relative increase of intracellular

concentration of macromolecules that is enough to hamper intracellular ice formation. Accordingly, vitrification belongs to the group of non-equilibrium cryopreservation methods.

Vitrification was long restricted to experimental laboratories for various reasons. Although vitrification was first applied to embryo cryopreservation by Rall and Fahy in 1985, 13 years after the reported successes of slow freezing, most practitioners disregarded its potential advantages and regarded it as an experimental procedure; others may remain insufficiently informed or sceptical of its benefits. At times, those working with vitrification established their own unique procedures, and attempted to prove its superiority. Additionally, commercial interest in marketing slow freezing (equipment and material) was not motivated to propagate a method that can be performed in a simple foam box and with some primitive handmade tools. Last, but not least, vitrification has suffered from two drawbacks: the lack of possibility for direct transfer (i.e. warming and transferring the embryos without the need of microscopic control, preferably by in-straw dilution that may be important at on-field situations in domestic animal embryology) and the concerns regarding possible disease transmission mediated by liquid nitrogen. These points provided arguments against the method and have hampered its application.

Eventually, practical solutions were found for both problems, attracting several commercial producers. Meanwhile, as the result of a kind of natural selection, some vitrification methods started to dominate the publications while others remained restricted to the laboratory that introduced them, or have become forgotten entirely. A selected few have really proved their superiority compared with the other vitrification methods, and, more importantly, to the slow freezing, in certain applications.

It should also be mentioned that there is a small, rather controversial group of cryopreservation techniques called ultrarapid freezing where ultrarapid cooling is applied with cryoprotectant concentrations insufficient to establish vitrification (Wilmut, 1972; Leibo *et al*., 1978; Kasai *et al*., 1980; Wood and Farrant, 1980). This approach has been established entirely empirically and does not meet the supposed requirements of cryopreservation in embryology. In spite of the definite signs of ice formation in the solution, under certain circumstances embryos and oocytes may survive and develop (Trounson *et al*., 1987, 1988; Shaw *et al*., 1988). The general explanation for this phenomenon is that extracellular ice formation does not necessarily result in intracellular freezing, and that the former is less harmful for the cells; and even a moderate ice formation inside the embryos or oocytes may be tolerated and may allow normal development following regeneration. Probably as the consequence of the uncontrollable processes, the results of ultrarapid freezing techniques are generally less predictable than those of slow freezing or vitrification, and due to this inconsistency, application is rather restricted. On the other hand, according to Kasai and Mukaida (2004), some of the early experiments described as ultrarapid freezing were in fact vitrifications, including those published by Barg *et al*. (1990) and Feichtinger *et al*. (1991) for human embryos.

Vitrification approaches

Cryoprotectants

In general, vitrification is mostly performed with the use of cryoprotectants that have previously been applied in slow freezing methods. However, selection of the appropriate composition is different. As a consequence of the high concentrations required, research in the first decade was focused on decreasing the toxic and osmotic damage. Various approaches were used, first of all to find less toxic and more permeable cryoprotectants. As a result, ethylene glycol has become an almost standard part of all present vitrification protocols. Another successful approach was to use a combination of two, or more frequently three cryoprotectants, to decrease the individual specific toxicity. At least one of these cryoprotectants had to be permeable (for that, ethylene glycol was the obvious choice), and one or two impermeable. Additional permeable components including propylene glycol, acetamide, glycerol, raffinose and dimethylsulphoxide (DMSO) were tested in various combinations (dela Pena *et al*., 2001; Kasai and Mukaida, 2004), eventually the mixture of ethylene glycol and DMSO seems to be the most acknowledged choice (Ishimori *et al*., 1992a,b, 1993). According to some investigations, the permeability of the mixture is higher than that of the individual components (Vicente and Garcia-Ximenez, 1994).

Regarding the non-permeable cryoprotectants, mono- and disaccharides including sucrose, trehalose, glucose and galactose are the primary candidates (Ali and Shelton, 1993; Kasai, 1997; Wright *et al*., 2004). Recently, sucrose has become almost as standard a component of vitrification mixtures as ethylene glycol, although it was for a long time challenged by trehalose. Interestingly, nearly all comparative investigations proved the superiority of trehalose; nevertheless, it seems to be less popular in recent publications. Sucrose as well as other sugars may not have any toxic effects at low temperatures, but may compromise embryo survival when applied extensively to counterbalance embryo swelling after warming (Kasai, 1986; Kasai *et al*., 1992; Vajta *et al*., 1997a), although this effect was not always realized (Kuleshova *et al*., 1999b). A recent approach, injection of trehalose into the cytoplasm of oocytes, has resulted in improved survival after cryopreservation (Eroglu *et al*., 2002). The trehalose is rapidly eliminated from the cytoplasm of the developing embryo and does not seem to impair further developmental competence (Eroglu *et al*., 2005)

Although different polymers were also suggested for the purpose, including polyvinyl pyrrolidine, polyethylene glycol, Ficoll, dextran and polyvinyl alcohol (Leibo and Oda, 1992; Naitana *et al*., 1997; Ohboshi *et al*., 1997; Shaw *et al*., 1997; Kuleshova *et al*., 2001; Asada *et al*., 2002), so far the only widely used compound is Ficoll, predominantly in combination with ethylene glycol and sucrose (Kasai *et al*., 1990). Unfortunately, the fascinating theory of replacement of penetrating cryoprotectants by polymers (Kuleshova *et al*., 2001) resulted in only a partial success: non-permeable cryoprotectants either applied alone or in combination could diminish only moderately the required concentration of permeable compounds. Various forms of

protein supplementation have also been used including egg yolk, but its optically dense appearance made the microscopic manipulation rather difficult. High concentrations of sera of different origin as well as serum albumin preparations (Rall, 1987) are common additives. In the bovine model, recombinant albumin and hyaluronan were also effective (Lane *et al*., 2003).

During the past 15 years, many reports were published on the positive effects of antifreeze proteins isolated from arctic animals or analogue synthetic compounds having a specific potential to decrease ice crystal formation (Rubinsky *et al*., 1992; Eto and Rubinsky, 1993; Wowk *et al*., 2000). These effects are not disproven; however, the application of these proteins has not become routine.

Other strategies to minimize toxic effects are the stepwise addition of cryoprotectants, and the use of lower temperatures at about 4°C at high levels of cryoprotectant (Rall, 1987; Vanderzwalmen *et al*. 1989; Saha *et al*., 1994; Széll and Shelton, 1994). While the former strategy has become an almost constant element of the present vitrification methods, the latter has abandoned in newer technologies, partly because of the slight technical problems in practice, and also the potential (but never proven) risk of increase of chilling injury under these circumstances. Although some authors suggested multistep exposure to increasingly concentrated cryoprotectants, generally a two-step strategy was used, where the first solution contained 20–50% of the final cryoprotectant concentration. In the past few years, however, a new tendency in incubation times has emerged. While earlier methods predominantly focused on the shortest possible exposure restricting the first incubation to 1–3 min, and the subsequent final one to seconds, although recently a long (5–15 min) pre-incubation with a considerably diluted first cryoprotectant solution followed with an approximately 1-min incubation in the final solution (Papis *et al*., 1999, 2000; Kuwayama *et al*., 2005a,b). This approach may increase slightly the toxic effect, but provides a much better protection for the whole cell, and may be especially beneficial in the case of large objects with a low surface/volume ratio including oocytes or early stage embryos. However, even in the case of blastocysts, not always the shortest incubation is the best. For example a 25 s incubation with the final cryoprotectant solutions has resulted in excellent survival rates after open pulled straw (OPS) vitrification of porcine blastocysts (Vajta *et al*., 1997b; Holm *et al*., 1999), but pregnancies were obtained only when another team, finding the short incubation technically difficult to perform, extended it to 60 s (Berthelot *et al*., 2000; F Berthelot, UMR, 6073 INRA-CNRS F Rabelais University, France: personal communication).

Traditional tools

Early vitrification experiments have been performed in the traditional vessels of cryopreservation, i.e. 0.25-ml plastic insemination straws or cryovials. These tools were not designed for the special purpose, had a thick wall and required a relatively large amount of solution for safe loading. Accordingly, the theoretically achievable cooling and warming rates were quite limited (approximately 2500°C/min for straws; Palasz and Mapletoft, 1996; and even less for cryovials). This relatively low rate was still hazardous to perform, as direct immersion into liquid nitrogen at cooling, and transfer to a water bath at warming induced extreme pressure changes in the closed system and frequently led to the collapse or explosion of the straws and loss of the sample. Even if the container remained intact, there was a high probability of fracture damage that could be explained by the scissor-like effect of the partially solidified solution fragments shearing under the extreme pressure changes. Most protocols therefore included a transitional step, typically cooling in liquid nitrogen vapour (on a Styrofoam boat floating on the surface, or by a slow vertical immersion of the sample into the liquid nitrogen), and warming first in air for 10 s or more, before the immersion into the 37–39°C water bath (Rall and Meyer, 1989; Kasai *et al*., 1996; Kasai, 1997). These manipulations resulted not only in decreased but also inconsistent rates: the temperature of the vapour of liquid nitrogen is variable, depending on many factors, and the definition of 'room temperature' laboratory air may mean 5–7°C differences, even at the same place on the same day. Consequently, a minimum 5–7 mol/l cryoprotectant concentration was required, and chilling injury could not be lowered to the level occurring at slow freezing.

Small volume–direct contact approach

Curiously, during the first decade of vitrification in embryology, almost all experts respected the limits offered by these traditional tools, although the potential benefits of a higher rate were widely acknowledged and demonstrated on sophisticated and expensive cryomicroscopes, not appropriate for the routine work. The most logical way to increase the cooling rate is to use the smallest possible volume of cryoprotectant medium surrounding the embryo and to establish a direct contact (without any thermoinsulating layer) between the solution and the liquid nitrogen. The small volume may also offer a special advantage: it prevents heterogeneous ice formation (Rall, 1987)

Table 1 summarizes the most important approaches to break the traditional frontiers of vitrification in mammalian embryology. The earliest documented attempt used the simplest way, and dropped the sample without any container directly into the liquid nitrogen (Landa and Tepla, 1990; Riha *et al*., 1994; Yang and Leibo, 1999; Papis *et al*., 1999, 2000). However, it was soon revealed that this simple solution is probably not the optimal one. To form a drop requires a relatively large amount of solution (approximately 5 µl). Moreover, once the drop reaches the liquid nitrogen, it will not sink immediately but remains floating and starts bizarre cruises on the surface for several seconds. The explanation of this phenomenon is quite simple: the liquid nitrogen in the containers is usually just at its boiling point, at –196°C. Anything that is warmer and contacts it induces an extensive boiling and induces strong evaporation at its surface. The vapour functions as an insulating layer decreasing the cooling of the sample. Additionally, this vapour coat did not allow the sample to sink, decreasing the cooling rate even further.

The first method that has fully utilized the enormous potential of the small sample-direct contact approach was the application of copper electron microscopic grids as a carrier

Table 1. Innovative vitrification techniques in mammalian embryology. Only first descriptions were included. For further references, see text.

System	*Reference*
Direct dropping into liquid nitrogen	Landa and Tepla, 1990
Electron microscopic grids	Martino *et al*., 1996b
Open-pulled straw (OPS)	Vajta *et al*., 1998b
Glass micropipettes (GMP)	Kong *et al*., 2000
Super-finely pulled OPS (SOPS)	Isachenko V *et al*., 2000
Gel-loading tips	Tominaga and Hanada, 2001
Sterile stripper tip	Kuleshova and Lopata, 2002
Flexipet denuding pipette (FDP)	Liebermann *et al*., 2002a
Fine diameter plastic micropipette	Cremades *et al*., 2004
100-µl pipetting tip	Hredzak *et al*., 2005
Closed-pulled straw (CPS)	Chen *et al*., 2001
Sealed open-pulled straws	Lopez-Bejar and Lopez-Gatius, 2002
Cryotip	Kuwayama *et al*., 2005b
Cryoloop	Lane *et al*., 1999a
Nylon mesh	Matsumoto *et al*., 2001
Minimum drop size (MDS)	Arav, 1992
Minimum volume cooling (MVC)	Hamawaki *et al*., 1999
Hemi-straw system (HSS)	Vanderzwalmen *et al*., 2000),
Cryotop	Kuwayama *et al*., 2005a
Vitmaster	Arav *et al*., 2000
Solid surface vitrification (SSV)	Dinnyes *et al*., 2000

for the sample (Steponkus *et al*., 1990; Martino *et al*., 1996b; Choi *et al*., 2000; Cho *et al*., 2002). In this ingenious approach, the size of the drop surrounding the sample was extremely small, as after loading, most of it was removed by placing the grid on a filter membrane. The thermoconductive metal grid also increased the cooling and warming rates. Surprisingly, the solidified cryoprotectant solution fixed the sample safely to the grid during cooling and storage, and released it easily after warming. Storage can be performed in cryovials filled with liquid nitrogen (Son *et al*., 2005).

However, the straw as an idea was still much more acceptable for those working in this field. That is probably why the OPS technology has achieved widespread attention and (together with its analogues) is probably the most widely used approach for ultrarapid vitrification (Vajta *et al*., 1997b, 1998a,b; Arav *et al*., 2000, 2002; Chen *et al*., 2000; El-Danasouri and Selman, 2001; Selman and El-Danasouri, 2002; Isachenko V *et al*. 2003a).

The OPS is based on a very simple idea, and a very simple technique. Its unexpected additional benefits making the method safe and easy to use have only been discovered subsequently. The idea was to minimize the required volume of the sample by minimizing the diameter of the common insemination straw. Similar to glass capillary production, straws were warmed and pulled by hand, then cut at the thinnest point with a razor blade. As the result, the diameter and the wall thickness of the straw decreased to approximately half of the original (**Figure 1**), while the required amount of solution to form a safe column decreased from 5 µl to less than 1 µl, leading eventually to a 10-fold increase in the achievable cooling rate, thus allowing a 30% decrease in cryoprotectant concentration required for safe vitrification. An additional benefit was related to the open system: no explosion of the straws occurred and the fracture damage (with some precautions) could be entirely eliminated.

Further unexpected benefits included the simple loading with the capillary effect (**Figure 2**), simple dilution connected directly to warming, and simple expelling of the sample from the straw using the dilatation of the warming gas in the empty part of the straw. Storage can be based on tools used for storage of other straws. The only problem, the floating of straws, can be easily resolved by inserting a standard plastic plug into the wide end. Moreover, the transparent walls allow microscopic follow-up during loading and expelling, and the glass-like solidification can be detected by naked eye. In animal embryology, the possibility of semidirect transfer after in-straw dilution (described in detail later) means a considerable benefit.

The principles of the OPS were used in many versions (see **Table 1**) including handmade or prefabricated tools, and also the specially prepared sophisticated Cryotip system. In some versions, no direct contact between the cryoprotectant and the liquid nitrogen occurs. So far, no comparative study has proven the unquestionable superiority of any one of these subclone techniques regarding oocyte or embryo survival and development. The commercially available sterile pulled straws have eliminated the only major disadvantage of using the original OPS method, the technical problems in tool production.

The Cryoloop is the third approach using the small volume–

direct contact principle. A small nylon loop attached to a holder and equipped with a container. It has been used for cryopreservation in crystallography and is now used widely for oocyte and embryo cryopreservation (Lane *et al*., 1999a,b; Mukaida *et al*., 2001, 2003). The solution film bridging the hole of the loop is strong enough to hold the oocyte or the embryo, and with this minimal solution volume, the achievable cooling rate may be extremely high, up to an estimated 700,000°C/min (Isachenko E *et al*. 2003). Using this tool, safe cryopreservation can be achieved even in the vapour of liquid nitrogen (Larman *et al*., 2006).

For mass vitrification of oocytes in domestic animals, a nylon mesh technique (a hybrid of the Cryoloop and the electron microscopic grid method) was successfully applied (Matsumoto *et al*., 2001).

Another successful strategy was the minimum drop size (MDS) method of Arav (1992) consisting of a small droplet of vitrification solution containing the oocyte or embryo placed on a solid surface that is immersed into liquid nitrogen. The approach was used later with some modifications called the Minimum Volume Cooling (MVC; Hamawaki *et al*., 1999) or in the hemi-straw system, (HSS, Vanderzwalmen *et al*. 2000), where the carrier tool was a cut-open straw.

The latest version of these minimum volume procedures is the Cryotop, where the carrier tool, a thin film attached to a plastic holder is equipped with a protective plastic tube (Kuwayama and Kato, 2000; Kuwayama *et al*., 2005a,b) (**Figure 3**). Oocytes or embryos are loaded on the film, the solution is almost entirely removed by aspiration (**Figure 4**), and the sample is immersed into liquid nitrogen. Subsequently, for safe storage, the plastic tube is pulled over the film. The method is easy to learn and perform, the cooling and warming rates are higher than those achievable with OPS, and the simple manipulation decreases the risk of inconsistency.

Elimination of vapour

Other high-rate vitrification techniques focus on decrease or total elimination of the vapour coat that arises around the sample in the liquid nitrogen. One possibility is to use liquid nitrogen slush instead of liquid nitrogen for cooling. At 10ºC below its boiling point, liquid nitrogen behaves differently: the nitrogen escapes from the fragile boiling temperature zone, and the sample immersed into the semi-solid slush creates just a minimal evaporation, consequently the cooling rate gets considerably higher. (A simple subjective but very convincing proof is to immerse the sample into either liquid nitrogen or nitrogen slush and listen to the volume and length of the 'sssssh' noise created by the evaporation.) Nitrogen slush can be produced from liquid nitrogen by using the basic principles of physics: under vacuum, part of the liquid evaporates and the rest of it cools down. With the introduction of the VitMaster (IMT, Israel), this technique is now available commercially (Arav *et al*., 2000, 2002; Huang *et al*., 2004).

The other possibility of eliminating the vapour is the use of pre-cooled metal surfaces instead of liquid nitrogen for cooling. Originally, a metal block immersed into liquid nitrogen was used (Dinnyes *et al*., 2000), but eventually a commercially available form has also been produced (CMV; Cryologic, Australia). The few comparative data do not provide entirely convincing evidence regarding the superiority of these vapour-minimizing or vapour-free approaches compared with the other vitrification procedures.

Disease transmission problems

Probably the strongest argument against the use of vitrification in domestic animal and especially human embryology is the potential risk of liquid nitrogen mediated disease transmission. To achieve a healthy balance between the real dangers and the potential benefits, we try to summarize the facts and outline the available ways to minimize or fully eliminate the risks.

(i) Semen and embryo collection, processing and cryopreservation protocols are not sterile procedures (Bielanski *et al*., 2003), consequently, the contents of virtually all stored straws and cryovials may be a source of infection.

(ii) In domestic animal and also human embryology, liquid nitrogen may also be contaminated by the surface of straws, cryovials, racks and other tools that are usually not handled fully aseptically. Accordingly, the presence of infective agents is not strictly related to leaky or open containers. In the majority of laboratories, there is no systematic and regular cleaning of containers and samples. It is technically very difficult, and in most situations impossible to perform, but in most laboratories it is not considered to be important, either.

(iii) Seemingly sterile containers may not be as safe as supposed. Infection may occur in common straws in slow freezing (through the holes of incomplete sealing, or pores of the plastic walls), and most cryovials do not have secure caps. A possible source of infection may also be the inappropriate decontamination of the outer walls of straws before loading and expelling.

(iv) Consequently, liquid nitrogen in storage tanks likely contains a number of commensal and potentially pathogenic environmental micro-organisms (Bielanski *et al*., 2003).

(v) There were documented cases of liquid nitrogen mediated disease transmission (Tedder *et al*., 1995; Fountain *et al*., 1997; Berry *et al*. 1998).

(vi) According to the experiments of Bielanski *et al*. (2000), cross-contamination may also occur during storage between OPS straws if one of them is artificially infected. This is, without doubt, applicable to any open systems.

In conclusion, the danger of liquid nitrogen mediated disease transfer exists and traditional tools and methods of cryopreservation are vulnerable, as well.

On the other hand, no documented case of liquid nitrogen mediated disease transmission related to embryo transfer activities has been reported yet. The few published disease transmissions happened between blood specimens and carcasses, between volumes approximately 10^3–10^4 larger than samples in embryology. These samples had incomplete wrapping with large leaks or no wrapping at all. According to our knowledge, there may be very few if any confirmed disease transmissions related to embryo transfers (either by using frozen

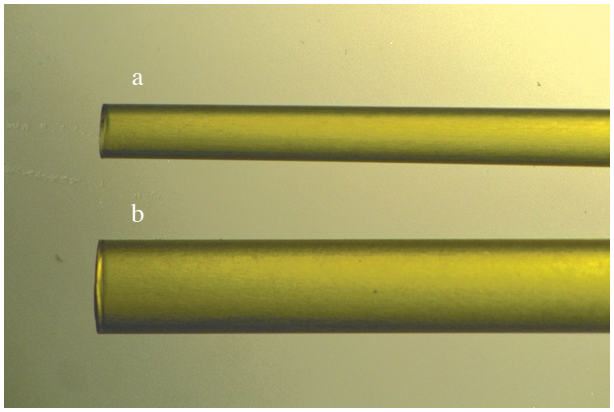

Figure 1. An open pulled straw (OPS) (**a**) with approximately 50% diameter of a standard 0.25-ml insemination straw (**b**).

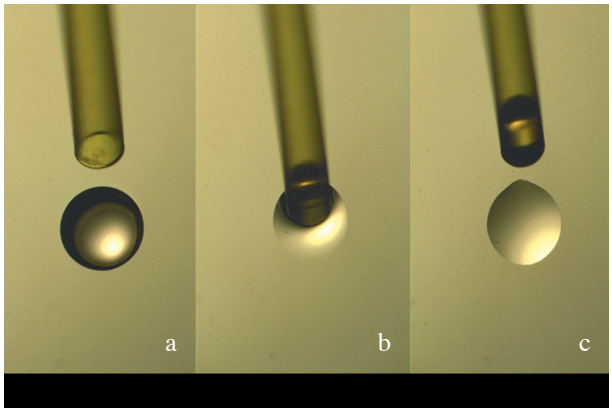

Figure 2. Loading of the OPS straw. (**a**) Oocytes or embryos are placed into an approx. 1 µl droplet of cryoprotectant medium; (**b**) the drop is touched with the OPS straw; (**c**) as the result of the capillary effect, the medium with the oocytes or embryos enters the straw.

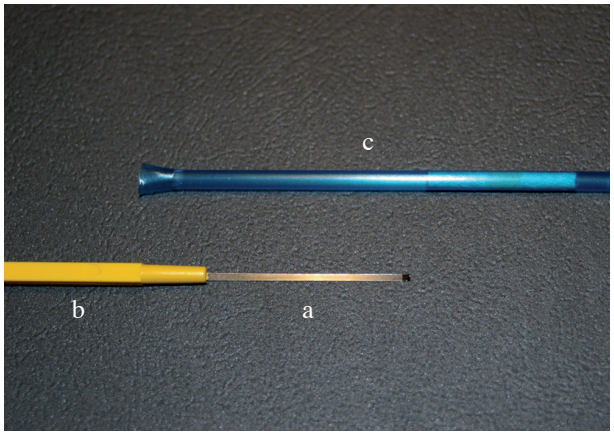

Figure 3. The Cryotop vitrification tool. A narrow filmstrip (**a**) is attached to a handle (**b**). After vitrification, a plastic tube (**c**) is used to protect the filmstrip from mechanical damage during storage.

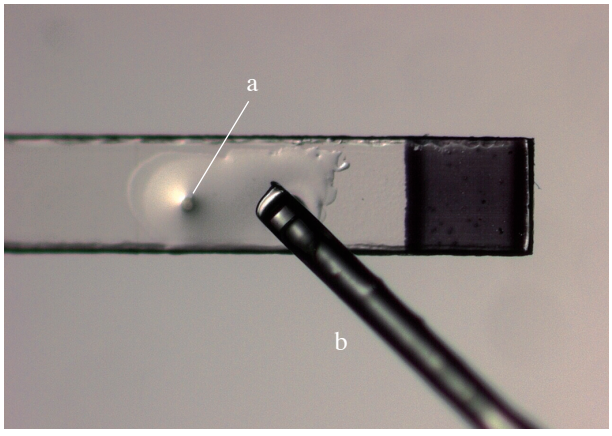

Figure 4. Loading of the Cryotop. The oocyte or embryo (**a**) is loaded in a small drop onto the filmstrip, then the excess solution is removed with a capillary (**b**), leaving only a thin layer of cryoprotectant to cover the sample.

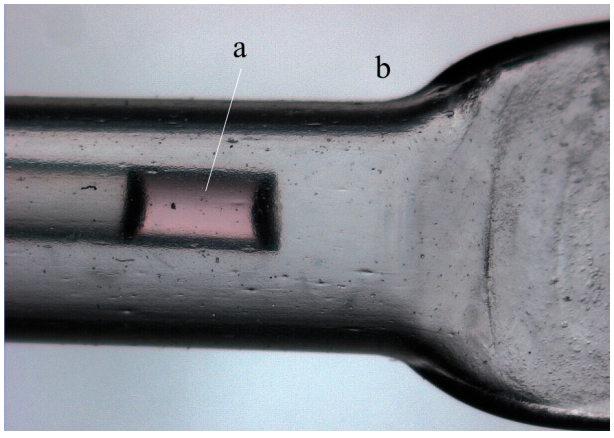

Figure 5. Sterile application of the OPS vitrification: after cooling, the OPS (**a**) is sealed into a 0.5-ml straw (**b**).

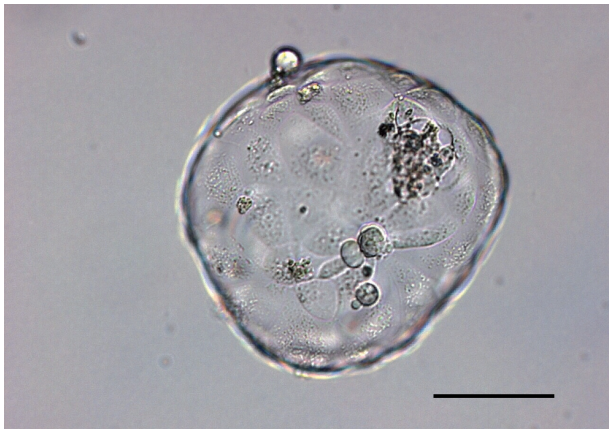

Figure 6. Porcine blastocyst produced by parthenogenetic activation and in-vitro culture of oocytes after delipation, by centrifugation, removal of the zona pellucida by pronase digestion and Cryotop vitrification. Bar represents 100 µm. (Dr Yutao Du, unpublished).

or fresh embryos). It may mean that the oviduct and uterus has an appropriate defence system to eliminate infectious agents in the quantity they may be transferred during common embryo transfer.

Of course, systems that are resistant even to the theoretically existing hazards should be used. From this point of view it is important to emphasize that most recent vitrification methods offer at least as high or higher level of aseptic handling of samples than common slow freezing procedures.

A common feature of the different approaches to achieve aseptic vitrification is the separation of the two phases of work, cooling and storage. The cooling must be performed in factory-derived (if possible aseptically produced, but it is not always an option), aseptically stored and handled liquid nitrogen. UV illumination and filtration may also be applied. The probability of infection from this treated nitrogen is equal to or lower than that originating from a medium prepared and filtered in the laboratory.

Some of the recent techniques do not even need the direct contact with the liquid nitrogen. The Cryoloop method can successfully be performed by cooling in the vapour of liquid nitrogen (Larman *et al*., 2005), the 'sealed open pulled straw', the CPS and the Cryotip techniques are basically closed systems, and the methods using metal surfaces for cooling as CMV do not expose samples to liquid nitrogen.

For aseptic storage of OPS and related techniques, a detailed protocol was published in 1998 by Vajta *et al*., using pre-cooled 0.5 ml leak-proof Cryo-Bio (IMV, L'Aigle, France) straws to wrap the pulled straws in after cooling (**Figure 5**). The special sealer provided with the straws may also mean an increased level of security. The large straw provides appropriate surface for well visible pre-labelling, as well. Tools and containers are now commercially available for easy application (VitSet; Minitüb, Germany; Video 1). Subsequently, similar approaches were also applied for the SOPS, HSS and FDP methods, as well (Jelinkova *et al*., 2002; Liebermann *et al*., 2002a; Vanderzwalmen *et al*., 2003).

Regarding the other option, i.e. storing the 'naked' vitrified samples in liquid nitrogen, there may be serious concerns about its safety. Even the vapour phase may contain infective agents (Fountain *et al*., 1997). Although the chances of cross contamination may be negligible (Bielanski, 2005), there is a definite danger of accidental warming. Liquid nitrogen vapour is not a completely homogenous system, and many factors may influence its temperature (Stachecki and Cohen, 2004). Samples in the ultrarapid vitrification systems are extremely small. While a normal straw can be held for seconds in room temperature air without the danger of irreversible warming, the vitrified samples in the new tools may not tolerate more than a fraction of second. The hermetically sealed container may function as a buffer providing stability similar to that of the normal straws, and permitting the safe transfer of the sample from one container to the other. On the other hand, with this protective container, samples may be stored either submerged or in the vapour of liquid nitrogen (Bielanski *et al*., 2003). Although most cryovial producers strongly discourage submerging their product into liquid nitrogen (the cap may not be completely airtight, and the vial may explode upon warming) storage in the vapour may mean an increased risk for accidental warming over the safe temperature zone (Fuller and Painter, 2004).

It should be noted that recently OPS straws were used by Kuleshova and Shaw (2000) and later by Isachenko V *et al*. (2005a,b) for a strange hybrid of previous cryopreservation procedures. As mentioned earlier, in the past several ultrarapid freezing techniques were applied where the cryoprotectant concentrations were not appropriate for achieving vitrification. Here, the contrary happens: cryoprotectant concentrations as well as the applied tool (OPS straw) are appropriate for safe vitrification. However, the rate of cooling is diminished to 400 or even 200°C/min (100 times less than that achievable with the original OPS method, and more than 10 times less than achievable in sealed 0.25 ml common insemination straws) by wrapping the OPS straws into the 0.5 ml protective straws before cooling. Moreover, in this situation the cooling rate between +30 and −5°C (that is critical from the point of chilling injury) may be even lower (A Arav, Institute of Animal Science, The Agricultural Research Organization, Bet Dagan, Israel: personal communication). Consequently two major achievements of the recent vitrification methods, the elimination of chilling injury and the low cryoprotectant concentration (i.e. less toxic and osmotic injury) are sacrificed for biosafety. It should be mentioned that there is a considerable difference between the observations of the two groups: while Isachenko still emphasizes the importance of rapid warming, Kuleshova and Lopata (2002) states that straws can be kept '... inside a second protective container throughout vitrification, storage and warming...', consequently authors do not regard it as important to maintain the high rate of temperature changes at warming, either. One may doubt if this method is really the optimal compromise between the various requirements in vitrification. Concerns may also be raised regarding the applicability in case of chilling sensitive objects including cattle oocytes and early stage embryos, porcine blastocysts, or human oocytes (Ghetler *et al*., 2005), regarding not just in-vitro survival rates, but in-vitro development, pregnancies and birth of healthy offspring.

Warming

Warming after vitrification is performed almost the same way as after most slow freezing procedures, i.e. by direct immersion into a solution at the core temperature of the given species, although this is not always true since Rall (1987) demonstrated that, depending on the cryoprotectant, high survival of embryos can be achieved even with rather slow warming. Generally, it seems to be advisable to keep the samples for 1–3 s in air to avoid fracture damage caused by gas bubbles occurring in the too-rapidly immersed samples. Closed systems are usually immersed into water baths, while open systems can be directly submerged into the medium, this way the warming and the first dilution is performed in a single step. Although a slight devitrification (occurrence of ice crystals) may occur, especially when the cryoprotectant level is kept at the minimum level, this transitional change is usually restricted to a part of the embryo-containing medium and most probably does not involve intracellular crystal formation, and consequently does not cause significant harm in the embryos or oocytes (Shaw *et al*., 1991). In routine warming protocols of vitrified oocytes and embryos, the

dilution is a multistep procedure with decreasing concentration of osmotic buffers (usually sucrose) to counterbalance the swelling caused by the permeable cryoprotectant that leaves the cells relatively slowly.

Direct transfer, i.e. one-step dilution of the cryoprotectant at warming without the need of a microscope and immediate transfer of the embryo to the foster mother, may not be critical in human reproduction but is very important in domestic animal embryo transfer practice. Theoretically, the osmotic shock and the inappropriate dilution of the toxic cryoprotectants in the small volume provided by the embryo transfer tools might hamper this application. Surprisingly, the practice contradicted concerns. One-step dilution without significant decrease of in-vitro survival was reported in cattle (Kuwayama, 1994; Saha, 1994; Vajta *et al*., 1997a, 1999) and pig (Cuello *et al*., 2004). Direct transfer or analogue methods after ultrarapid vitrification of embryos resulted in offspring after transfer in cattle (Tecirlioglu *et al*. 2004) and sheep (Isachenko V *et al*., 2003b).

Factors influencing outcome

Species, genotype

There are well known, but only partially understood, differences in sensitivity to cryoinjuries between different species in mammals. An approximate estimation of sensitivity can be established according to the microscopic view. Transparent oocytes and embryos are usually more resistant, dense dark ones are more fragile, due to the increased lipid content. Accordingly, cryopreservation of light mouse embryos is a relatively easy task, darker bovine embryos require more attention, and the cryopreservation of dense pig embryos is one of the most demanding challenges in cryobiology. As lipid content increases chilling sensitivity, it is not surprising that vitrification may mean the only solution for pigs. However, the problem should not be oversimplified, as extremely dense feline embryos can also be cryopreserved by slow freezing (Pope, 2000).

The role of the accumulated lipid droplets is not fully understood. Some authors suppose an important role in the accelerated metabolism of the developing embryo (Leibo *et al*., 1996; Zenzes *et al*., 2001). However, this theory does not really explain the minimum 100-fold differences between species: are porcine embryos 100 times more active than murine ones? Moreover, delipidation of porcine oocytes and embryos does not impair their further development (Esaki *et al*., 2004). The delipidation may happen with centrifugation-micromanipulation, or in a non-invasive way, without or with trypsin zona digestion and centrifugation followed by Cryotop vitrification. In parallel with the lighter appearance of the cytoplasm, considerably increased survival rates were detected after both slow freezing (Nagashima *et al*., 1994) and vitrification (Dobrinsky *et al*., 1999; Nagashima *et al*., 1999; Beebe *et al*., 2002; Esaki *et al*., 2004, Du *et al*., 2006). This approach also improves in-vitro survival of vitrified porcine blastocysts produced by somatic cell nuclear transfer (Du *et al*., 2006; Li *et al*., 2006). Moreover, up to 30% blastocyst rates can be achieved from delipated, zona digested and vitrified oocytes after parthenogenetic activation (Y Du, Department of Genetics and Biotechnology, Danish Institute of Agricultural Sciences, Tjele, Denmark: personal communication; **Figure 6**).

The fact that porcine in-vivo-derived blastocysts become more resistant to cryoinjuries after exposure to cytoskeleton stabilizers including cytochalasin B and taxol (Dobrinsky *et al*., 2000; Fujihira *et al*., 2004, 2005) shows that apart from the lipid content, there are other possible explanations for the increased sensitivity to cryopreservation of porcine embryos.

It should be also noted that apart from the differences between species, in mouse, differences between genotypes in the ability to develop after vitrification were also observed (Dinnyes *et al*., 1995a,b).

Developmental stage

During the approximately 6–8-day period of development from the unfertilized oocyte to the hatched blastocysts stage, the change in the size and shape of the cells is unprecedented in mammalian development. A relatively simple spherical shape protected by an acellular outer layer develops to a complicated multicellular structure without external protection. Predictably, the extreme differences in morphology also result in considerable differences in sensitivity to cryoinjuries.

Generally, the earlier the development stage (starting from the germinal vesicle stage), the more sensitive oocytes and embryos are. In the case of the oocytes, the low surface/volume ratio may negatively influence the rate of penetration of the cryoprotectant, although the permeability characteristics of a cell are primarily due to properties of the cell membrane. Accordingly, the outer layers may suffer toxic injury while the core areas are still unprotected from ice crystal formation. However, although there is only a minimal difference between the size and shape, the immature oocytes are usually more sensitive to cryopreservation than mature (MII phase) oocytes (Leibo *et al*., 1996; Men *et al*., 2002; Ghetler *et al*., 2005). The contrary might be supposed, based on the known sensitivity of the meiotic spindle to chilling. Additionally, a very remarkable difference exists between the chilling sensitivity of unfertilized and fertilized human oocytes. A possible explanation for this phenomenon is the increased chilling sensitivity of membranes: the lipid phase transition at room temperature storage in human germinal vesicle and MII stage oocytes is 10 times higher than that of human pronuclear embryos (Ghetler *et al*., 2005).

In cattle and in pigs, the higher the cell number of the developing zona intact embryo is, the higher the survival rates, as expected (Vajta *et al*., 1997b; Berthelot *et al*., 2001). After hatching, there may be a slight decrease in cryotolerance; however, present in-vitro culture systems are suboptimal for these stages, and so compromised development might not reflect solely the damage during the cryopreservation. In human, the survival rates after slow freezing are not significantly different between zygotes, cleavage stage embryos and blastocysts (between 75 and 80% for each; Veeck, 2003; Pool and Leibo, 2004).

The complex structure of blastocysts may give rise to additional problems. In humans, mechanical reduction of the blastocoele by puncturing or repeated pipetting improved survival and pregnancy rates (Vanderzwalmen *et al*., 2002; Son *et al*., 2003; Hiraoka *et al*., 2004). The usual explanation is that the large blastocoel may not be protected appropriately from ice crystal formation (Vanderzwalmen *et al*., 2002). However, other feasible mechanisms may include the inappropriate dilution

of the accumulated cryoprotectants after warming (Vajta, unpublished) or increased protection against cryoinjuries due to stress-induced biochemical changes (Pribenszky *et al*., 2005).

In-vitro versus in-vivo produced

The origin of the embryos may also be an important factor. In domestic animals, in-vivo-produced embryos are generally more resistant to injuries, including cryoinjuries than in-vitro fertilized or somatic cell cloned ones (Massip *et al*., 1995). There might be some correlation between the increased lipid content of embryos produced in some in-vitro systems. In general, the less morphological difference from the in-vivo counterpart is detectable in the in-vivo produced embryos, the small the expected difference in survival after cryopreservation. Although total elimination of these differences is still impossible, according to the joint conclusion of many publications, vitrification seems to be especially appropriate to counterbalance this handicap (**Figures 7** and **8**).

Evidence supporting the use of vitrification

Domestic, experimental and wild animals

There is an extensive literature of comparative experiments between slow freezing versus vitrification (some examples may include Mahmoudzadeh *et al*., 1994; Wurth *et al*., 1994; Dinnyes *et al*., 1995; Reinders *et al*., 1995; Agca *et al*., 1998; Lane *et al*., 1999a,b). The overwhelming majority of these papers prove the superiority of vitrification for the given purpose. Probably less than 10% did not find significant differences, and, according to our knowledge, no publication stated that results achieved by vitrification were significantly worse than those obtained by slow freezing. Moreover, there are situations where vitrification is uniquely or predominantly suitable to achieve the goal: most of these areas are summarized in **Table 2**.

Humans

Oocytes

Probably more reviews and definitely more scientific papers have been published about human oocyte cryopreservation than babies born worldwide. This fact can be regarded as evidence of the importance of the problem. The application fields are diverse, most of them extremely important (Kuwayama *et al*., 2005a; Paynter, 2005), and all of them are crucial to eliminate the severe difference in the storage possibilities between female and male gametes. However, in spite of the fact that the first baby born after oocyte cryopreservation was reported 20 years ago (Chen, 1986), until recently the efficiency of the procedure has remained low. Apart from some isolated experiments with low number, only 0.6–6% of cryopreserved oocytes developed to term (Porcu *et al*., 2000; Stachecki *et al*., 2004; Wright *et al*. 2004). Apart from oocyte size, which has been discussed in the previous section, the following mechanisms may play a role in these compromised results: oolemma alterations, including low permeability to water, sensitive structural components including the zona pellucida, and spindle as well as mitochondrial damage (Jones *et al*., 2004; Paynter, 2005), absence or presence some structural/functional elements such as cortical granules. On the other hand, the osmotic effect may not be as serious as supposed according to the level of deformation and births have resulted from cryopreserved oocytes. Additionally, no chromosomal abnormalities to date have been detected (Stachecki and Cohen, 2004), even though depolymerization of spindle resulting in cytogenetic alterations was an earlier concern (Stachecki *et al*., 2004). Spindle re-organization after oocyte freezing–thawing (slow method) can now be better investigated using a non-invasive technique called PolScope (Rienzi *et al*., 2004). Although more recent studies do not confirm that spindle damage is the only (or main) drawback of slow freezing, one may postulate that vitrification (which can circumvent this obstacle due to elimination of egg exposure to non-physiological temperature) would be advantageous. The

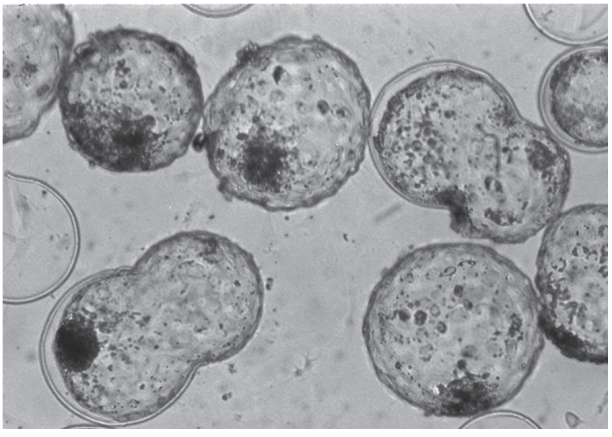

Figure 7. Bovine in-vitro-produced blastocysts 8 days after fertilization and 1 day after OPS vitrification and warming. Bar represents 100 μm. (G Vajta, unpublished).

Figure 8. Sheep produced by somatic cell nuclear transfer and born after OPS vitrification of the blastocyst stage. (Dr Teija Peura, Sydney IVF, Sydney, NSW, Australia: unpublished).

Table 2. Examples in mammalian embryology where first success in cryopreservation was achieved by vitrification. Embryos and oocytes were not treated mechanically or chemically to prepare them for the vitrification. Full-term developments were reported except where otherwise indicated.

Species, stage, system	*Reference*
Bovine immature oocytes for IVF	Vieria *et al*., 2002
Bovine in-vitro-matured oocytes for IVF	Martino *et al*., 1996b; Vajta *et al*., 1998a
Bovine in-vitro-matured oocytes for somatic cell nuclear transfer	Hou *et al*., 2005
Bovine cytoplasts for embryonic cell nuclear transfer	Booth *et al*., 1999
Bovine early stage IVF embryos	Vajta *et al*., 1998a; in-vitro study
Bovine zona-included blastocysts generated by somatic cell nuclear transfer	French *et al*., 2002
Bovine zona-free blastocysts generated by somatic cell nuclear transfer	Tecirlioglu *et al*., 2004
Bovine transgenic blastocysts generated by somatic cell nuclear transfer	French *et al*., 2003
Ovine zona included embryos generated by nuclear transfer	Peura *et al*., 2003
Porcine immature oocytes for ICSI	Fujihira *et al*., 2004; in-vitro study
Porcine in-vitro-matured oocytes for ICSI	Fujihira *et al*., 2005; in-vitro study
Porcine in-vivo-derived blastocysts	Kobayashi *et al*., 1998
Porcine in-vivo-derived morulae	Berthelot *et al*., 2001
Porcine in-vitro-produced blastocysts	Men *et al*., 2005; in-vitro study
Equine in-vivo-matured oocytes	Maclellan *et al*., 2002
European polecat in-vivo-derived morulae and blastocysts	Piltty *et al*., 2004
Siberian Tiger in-vivo-derived embryos	Crichton *et al*., 2003; in-vitro study
Minke whale immature oocytes for maturation	Iwayama *et al*., 2004; in-vitro study

ICSI = intracytoplasmic sperm injection.

negative effect of zona hardening caused by cortical granule release during cryopreservation was eliminated with the application of the intracytoplasmic sperm injection (Palermo *et al*., 1992; Porcu *et al*., 1997; Wurfel *et al*., 1999).

Consequently, oocytes can be regarded as excellent candidates to prove the value of the new vitrification techniques. However, initial results were rather discouraging. In 1999, Kuleshova *et al*. (1999a) reported a baby born after vitrification of a relatively low number of oocytes in the open pulled straw method; however, according to our knowledge no further babies have been born using identical parameters. Some other vitrification approaches failed to produce considerable improvement in the overall result, either. Apart from the technical problems, the lack of appropriate test objects and in-vitro evaluation methods also hampered the advancement. So far, no appropriate animal model for human oocyte cryopreservation exists. Unfortunately, and in contrast to the expectation, freshly collected human oocytes are more sensitive to cryoinjuries than those incubated for several hours or 1 day. Therefore methods established on oocytes that failed to be fertilized were often inefficient for the freshly collected ones with full developmental potential (Stachecki and Cohen, 2004; Vajta, unpublished). Moreover, the morphological survival that was often used as the single criterion of successful vitrification, has no more value from the point of developmental competence as evaluation of a bovine embryo production system on the basis of two pronuclei formation or cleavage.

Recently, however, a breakthrough has been achieved in this field. With the Cryotop method, 50% of the vitrified oocytes developed to blastocysts after intracytoplasmic sperm injection, and from a total of 29 embryo transfers, 12 pregnancies were obtained (Kuwayama *et al*., 2005a) resulting in the birth of 10 healthy babies (M Kuwayama, Kato Ladies' Clinic, Tokyo, Japan: personal communication). The success may be attributed (apart from the efficient basic embryo production system) to the extremely high cooling and warming rate permitted by the minimal cryoprotectant volume and the tiny carrier system, as well as the carefully selected incubation parameters that minimize the toxic and osmotic effect while providing even protection in the whole ooplasm. Considering the fact that in the past few years approximately 50 babies were born all over the world (USA, Japan, Colombia, Mexico) from Cryotop vitrified oocytes (M Kuwayama, personal communication), and the overall efficiency of the procedure is generally close to that described above, the new technique may contribute significantly in solution in this crucial problem.

Embryos

In humans, the clinical pregnancy rate from embryo transfer after slow-freezing is approximately two-thirds that from the fresh transfer of embryos (Check *et al*., 2001), although new techniques have recently been introduced to restore (cleavage stage) embryo viability (Nagy *et al*., 2005). The theoretical possibility for improvement is supported by the results obtained in cattle, where the difference is no more than 10–15%.

Until recently, the published sporadic results based on relatively low numbers proved only the feasibility and potential competitiveness, but not the superiority of vitrification in this field (Barg *et al*., 1990; Feichtinger *et al*., 1991; Vanderzwalmen *et al*., 1997, 2000, 2002, 2003; Mukaida *et al*., 1998, 2001,

2003; Choi *et al*., 2000; Park *et al*., 2000; Saito *et al*., 2000; Yokota *et al*., 2000, 2001; El-Danasouri and Selman, 2001; Cho *et al*., 2002; Jelinkova *et al*., 2002; Liebermann and Tucker, 2002; Reed *et al*., 2002; Selman and El-Danasouri, 2002; Son *et al*., 2002, 2003; Isachenko V *et al*., 2003a; Hiraoka *et al*., 2004; also reviewed in Kasai and Mukaida, 2004; Liebermann and Tucker, 2004).

In 2005, however, three comparative investigations were published, and all the three concluded that vitrification was a more efficient way for cryopreservation of human embryos than slow-rate freezing. Zheng *et al*. (2005) performed an in-vitro experiment with biopsied non-transferable human embryos. Three versions of slow freezing were compared with vitrification, and survival rates were the highest after vitrification. Stehlik *et al*. (2005) used slow freezing versus Cryotop vitrification for cryopreservation of supernumerary human blastocysts, and observed a significant difference in pregnancy rates after 44 transfers (16.7 versus 50% respectively). The largest comparative investigation between the effect of slow freezing versus vitrification was published by Kuwayama *et al*. (2005b), based on cryopreservation of more than 16,000 human embryos at different stages. Cryotop vitrification was found superior for pronuclear embryo cryopreservation in regard to survival, cleavage, and developmental rates. Survival rates of 4-cell stage and blastocyst stage human embryos were also significantly higher than those after slow freezing. Pregnancy and birth rates after cryopreservation with the two methods were not significantly different. Accordingly, this representative comparison has proved that vitrification is at least as efficient as slow-rate freezing for cryopreservation of human embryos in all developmental stages.

Summarizing the application fields of different vitrification methods, two technologies have obtained the greatest attention: the OPS, predominantly in the animal field, and the Cryotop for human areas. It should be emphasized, however, that the differences do not necessarily mean an unbreakable frontier. The OPS is more robust and is easier to perform even under compromised conditions, while the delicate Cryotop method may be the choice where extremely high cooling rates are the primary objectives. However, if properly applied, both OPS and Cryotop methods seem to be suitable for the given purposes. A good example to support this statement that very healthy piglets were born after OPS vitrification and transfer of the extremely sensitive somatic-cell cloned blastocyst derived from delipated oocytes (Li *et al*., 2006; Rongfen Li, University of Missouri-Columbia, Missouri, Columbia, USA: personal communication)

Conclusion: is there any reason not to replace slow cooling with vitrification?

In the more than 500 reviewed publications, strong concern against the use of vitrification was found in only in one. Even this paper mentioned referred only theoretical dangers that were not supported by experimental or clinical results. Yves Ménézo (2004) stated that the impact of vitrification, especially when ethylene glycol is used, has to be carefully evaluated before its use on a large scale. Ethylene glycol and its metabolites can be toxic at a very low concentration (cited literature: Klug *et al*., 2001). Ménézo also states that 'the balance between permeable and non-permeable cryoprotectants has to be re-evaluated probably in favour of non-permeable ones'.

As described earlier, this suggestion is not based on an entirely new idea, and has partially been realized already. Survival, development, pregnancy and healthy offspring rates have also demonstrated the limits of the non-permeable versus permeable cryoprotectant approach. Regarding the specific toxicity of ethylene glycol, both authors of this paper have spent years in human pathology, and are fully aware of the potential toxic effect of ethylene glycol. However, this effect may depend on the stage of development as according to our knowledge no developmental abnormalities in animals or humans were related to it after cryopreservation even when using concentrations up to 40%. On the other hand, as the result of combined application of different approaches to minimize the toxicity, the concentration of ethylene glycol can be as low as 15% (2.2 mol/l), and is applied for a very short period before and after deep cooling. This concentration is similar to that used at industrial level for traditional slow freezing of cattle embryos, where no increase of developmental abnormalities or other toxic effects have been reported so far.

The rest of the reviewed papers almost unanimously support the application of vitrification by emphasizing its advantages: the simple, inexpensive and rapid procedure leading to higher survival and developmental rates than those achievable with alternative methods. Concerns regarding disease transmission are partially justified, but safer methods are now available to lessen this danger. As mentioned earlier, the main predicament has been the general conservatism and not entirely justified cautiousness. Convincing results like the emerging breakthrough in human oocyte vitrification may help to eliminate these obstacles. Recently achieved results using vitrification seem to convince more and more professionals about the advantage of the technique, reflected by the increasing number of publications and also by the number of introduced (or soon to be introduced) commercial kits for vitrification.

However, in the end, we have to confess that a considerable advancement is also required from the supporters and developers of the new vitrification methods, predominantly in the level of standardization. Among others, Liebermann *et al*. (2002b) have called for a 'universal' vitrification protocol. Based on the fact that human oocytes, zygotes, early cleavage stage embryos, morulae and blastocysts have different structure, metabolism and consequently sensitivity to cryoinjuries, this universal protocol might be of compromised value for some of these stages. A more realistic goal should be to find an appropriate, widely accepted method for a given stage of development. This kind of standardization should not be performed by committees or authorities, but through a spontaneous, although carefully monitored, way (as mentioned earlier, this process has already been started and is proceeding slowly). Openness, wide understanding of the present protocols and determination to improve the existing systems involving embryologists, officials and industry are required for advancement.

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