Vitrification in ART – getting closer?

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ABSTRACT

Background: Twenty five years after the first successful application in mammalian embryology vitrification seems to emerge now from the “new”, “experimental”, “immature” category and has become a method of choice for routine use both in domestic animals and humans.

Review: The aim of this review is to summarize major steps of this regretfully slow development, outline the actual possibilities and limitations, highlight some promising perspectives, and deal also with factors that have hampered and still hamper the widespread application. The unique feature of vitrification in cryobiology is the total elimination of ice formation both in the sample and the entire solution surrounding it. This radical approach requires some drastic measures including high cryoprotectant concentration and high cooling/warming rates, although theoretically none of these factors are absolute requirements for vitrification. Initial efforts to improve vitrification results were focused on decreasing the toxic and osmotic effect of cryoprotectants. In the second period, the increase of cooling and warming rates has become the major goal. Subsequent changes in the equilibration parameters have improved the protection of the whole sample and allowed efficient cryopreservation of challenging structures including mature and immature oocytes, early stage domestic animal embryos and human blastocysts. In spite of proven superiority over other cryopreservation approaches, vitrification is still surrounded by a suspicious atmosphere, as technical problems, financial limitations and legal restrictions compromise its well-deserved acknowledgment. Ongoing debate about liquid nitrogen-mediated disease transfer issues illustrates this controversial situation well: although the danger is negligible, never proven, and there are established solutions to eliminate even the theoretical possibility, the incipient legislative ban of all open systems may compromise efficiency and reduce benefits of vitrification in the most important fields including bovine blastocyst and human oocyte cryopreservation. On the other hand, some supplementary techniques including low pressure treatment of liquid nitrogen or high pressure treatment of samples may further improve the efficiency of vitrification, and eliminate the — already slight — differences between the developmental competence of fresh and cryopreserved oocytes and embryos.

Conclusion: During the past decade vitrification has become the method of choice for cryopreservation of oocytes and embryos in all mammalian species. It may offer special benefits for samples that are difficult to cryopreserve with other methods including porcine embryos, domestic animal and human oocytes, and human blastocysts. Full exploitation of these possibilities may considerably increase the overall efficiency and broaden the application fields of in vitro procedures in ART; however, its acceptance requires an open-minded approach from both specialists and authorities.

Keywords: vitrification, cryopreservation, oocyte, embryo, biosafety.
I. INTRODUCTION – BACKGROUND AND HISTORY

The physical phenomenon of vitrification, i.e. crystal-free amorph solidification of solutions is common both in nature and industry. It can also be defined as an extreme increase of viscosity at temperatures below the melting point. Typical examples of vitrification include solidification of silicon dioxide to glass versus the crystallized form, sand; or solidification of sugar to cotton candy versus rock candy. A very important application area of vitrification is transformation of radioactive waste into a stable state for storage.

Vitrification of solutions including water usually occurs under special conditions. Factors including rapid cooling, high pressure, small volume, and special additive substances that may prevent crystallization. Vitrification of water is not typical in nature, but may be part of the survival strategy of some lower animals and plants at polar temperatures by extreme dehydration that may allow sufficient concentration of the intracellular solution, by accumulation of endogenous cryoprotectants including glycerol and by using a sophisticated defense mechanism to minimize intracellular ice nucleation [8].

Vitrification of water was outlined as early as in the middle of the 19th century, but suggested for cryopreservation of living creatures only in 1930 by Stiles, and attempted later by Luyet [1937] by using rapid cooling and warming rates, although the achievements were later questioned even by the author. Eventually application of high cryoprotectant concentration was (and still is) the key factor to achieve and maintain the vitrified state in water-based solutions, allowing cryopreservation of mammalian cells and tissues [9], although the balance between the toxic and cryoprotective effect was (and still is) a crucial problem of all vitrification procedures. The first successful application of vitrification in embryology was the result of an efficient cryoprotectant solution and equilibration strategy that allowed survival even with a rather moderate cooling rate, if combined with rapid warming to prevent de-vitrification [23].

II. MATURATION OF A TECHNOLOGY

The initial success has demonstrated some unquestionable benefits of the new approach compared to slow-rate freezing. There was no need to purchase an expensive and sophisticated controlled-rate freezer, vitrification could be performed in a simple foam box. No tiresome search for optimization of equilibration and cooling rates for each biological sample was needed. The time required to carry out the vitrification was fraction of the time needed for traditional freezing. Although the latter benefit is now questioned with the recent change of equilibration times and the need of many repeats when mass vitrification of oocytes or embryos is performed, the practical advantages of vitrification are still evident. However, embryologists are basically conservative, and prefer to use proven techniques that work appropriately in their hand. They need clear evidence regarding the superiority of a new approach, in terms of the overall efficiency, including in vitro and in vivo survivals, calves on the ground, take home babies, and also such technical details as the possibility for direct transfer in cattle industry. Vitrification, as established in the eighties of the last century, failed to prove its extra value for cryopreservation of bovine, porcine, ovine and human embryos. To qualify the approach for routine application, considerable efforts to improve efficiency were required.

During the first phase efforts were focused on decreasing the toxic and osmotic effect of cryoprotectants. The double or triple amount of cryoprotectants required for vitrification compared to traditional freezing was shocking...
for most cryobiologists, although retrospectively the concerns – especially regarding the long-term, in vivo consequences – were not entirely justified.

Elements of this approach included application of the most appropriate permeable cryoprotectants. Eventually glycerol has been omitted as ethylene glycol, propylene glycol and dimethylsulphoxide have proven their superiority for the purpose. Although the latter compound had and still has a very bad reputation among cryobiologists, its toxic, mutagenic or carcinogenic effect has not been clearly proven. In some countries dimethylsulphoxide is an approved drug for human medical application, and is regarded as one of the least toxic organic solutions. Sugars including sucrose and trehalose are the most common non-permeable compounds, while the use of high molecular weight polymers (dextran, ficoll, polyethylene glycol) has largely been abandoned because of the low overall efficiency.

To decrease the specific toxicity, mixtures of two or three cryoprotectants were applied. At least one of these compound was permeable, and the preferred choice for this primary component was ethylene glycol.

Stepwise addition of cryoprotectants was the next approach. Two, three or more steps were applied. During the first decade, the suggested exposition to the diluted cryoprotectant mixture was relatively short (approx. 3 min), accordingly no full equilibration was achieved before plunging samples into the concentrated solution for an even shorter period (45 to 90 s). The theoretical background of these short exposition was to minimize the toxic effect. As discussed later, this theory was not entirely justified by the practice, as the short exposition failed to protect all parts of the embryos and oocytes from cryoinjuries.

In the middle of 90s, attention was focused to the supposed damage of the cytoskeleton as the possible consequence of misshape that has occurred during the equilibration process. Cytoskeleton relaxant (cytochalasin B) treatment was applied to minimize the damage [6]. Although some improvements in the in vitro outcome were detected, the approach did not result in a breakthrough in the in vivo efficiency, and as later mostly abandoned. On the basis of the same principle, Taxol was also used recently and results seem to be promising [34]. However, Taxol is a strong cytostatic substance, accordingly its application in human, but also in domestic animal embryology may create major concerns.

Finally, an early and successful method to decrease toxicity was to cool the final cryoprotectant solution to temperatures close to 0°C. As the consequence, excellent survival and developmental rates were reported in several species and developmental stages. However, the increased chilling damage has counterbalanced the benefits in other embryos and oocytes, and eventually the practice has been abandoned. On the other hand, room temperature equilibration (in contrast to incubation at the core temperature) is now a routine approach in both domestic animals and humans, and may contribute significantly in the improvements in survival and developmental rates that has been achieved in the past few years.

Radical increase of the cooling and warming rates was one of the early suggestions of Luyet. However, since the first embryos were vitrified successfully in normal straws, and with the applied cryoprotectant concentration no significant effect of the cooling rate on survival was detected, efforts were largely abandoned to use this – theoretically – fascinating perspective. Sporadic efforts were made with the minimum drop size (MDS) vitrification [1], but most scientist continued the 0.25 ml standard insemination straws or cryovials that were intrinsically inappropriate for the purpose, with a maximum achievable cooling rate of 2000°C/min for straws and even lower for cryovials. Direct dropping of the sample-containing solution into the liquid nitrogen was seemingly a feasible solution, but in practice it failed to fulfill the expectations. To form a drop, a considerable amount of solution is required, and – as the result of evaporation of liquid nitrogen, this drop floats for relatively long (5 to 15 s) on the surface of the nitrogen before plunging. Both facts compromise seriously the achievable cooling rate.

The first carrier tool applied for vitrification was a simple electron microscopic grid [25,17], followed by the first purpose-developed tool, the Open Pulled Straw, OPS [28]. The narrow tube idea has fascinated many scientists, and at least a dozen of clones of this approach were subsequently published, with no convincing benefits over the initial model. These variations included the so-called "safe" closed versions with a rather misleading claims, as during the application either safety or efficiency had to be sacrificed (see discussed later). On the other hand, due to the possibility of really safe wrapping in a second straw as well as semi-direct transfer [29,30], the original OPS has survived its first decade and is applied today in domestic animals, experimental purposes, and in some laboratories for cryopreservation of human embryos and oocytes, as well.

The aim to increase the cooling and warming rates further has lead to the introduction of the Cryotop [12], a combination of the minimum volume vitrification with the simplicity of the OPS. Although in contrast to the OPS,
loading and also warming of the Cryotop requires some practice, it is now routinely used in many human and experimental laboratories worldwide due to the consistent results and high overall efficiency [13,4]. Similar techniques have been developed by other scientists, such as Hemistraws, Cryoleafs, Cryolocks, and Vitri-Inga.

Another tool, the Cryoloop (developed originally to visualize cryopreserved protein crystals) was also successfully used in experimental and human embryology [15,18].

Although very few comparative experiments have been performed, various results published with the tools mentioned above suggest that with proper application in skilled hands, all of them are suitable to achieve good survival and developmental rates even with such fragile and sensitive samples as porcine blastocysts created by somatic cell nuclear transfer [16,7].

A kind of byproduct of the rapid cooling and warming rates was the possibility to decrease radically chilling injuries. This damage that occurs at a relatively high temperature zone (between +15 or even 20 and -5°C) is very difficult to eliminate with any method except for the radical decrease of time embryos or oocytes spend between these temperature limit. In contrast to traditional freezing, new vitrification methods were intrinsically suitable to achieve this goal.

A relative recent approach to improve vitrification results, i.e. to provide appropriate protection has been made on the basis of realization that the benefits of decreased toxicity with short exposition to cryoprotectants may be less important than the damage that occurs as the consequence of insufficient saturation of certain part of oocytes or embryos. The initial success by using a long-term first equilibration has been achieved with a very diluted cryoprotectant mixture [19], however, later it has been discovered that a 10 to 15 min equilibration in the 50% diluted solution of the final permeable cryoprotectant medium at room temperature may still be relatively harmless for sensitive structures including human oocytes, while it increases considerably their survival rate and developmental competence [13].

III. ACCOMPLISHED TASK?

As the result of these crucial improvements, i.e. the application of the appropriate cryoprotectant mixture; the increase of cooling and warming rates to decrease the toxicity of cryoprotectants and to minimize chilling injury; and the use of extended equilibration times to provide protection for the whole sample, the efficiency of vitrification has been increased substantially. Accordingly, the vast majority of the approx. 800 publications dealing with this area finds vitrification more efficient than traditional slow-rate freezing. Vitrification has proved its superiority even in areas where traditional slow-rate freezing was successful, i.e. cryopreservation of bovine blastocysts and morulae, or early cleavage stage human embryos; and has resulted in impressing breakthroughs in cryopreservation of early stage bovine embryos, bovine mature and immature oocytes [28,32], cloned domestic animal embryos [10,16], human blastocysts and human oocytes [13,4], human embryonic stem cells [24] and many other areas where traditional freezing was insufficient to provide acceptable results.

One may regard this achievement as a great success story; however, being part of the process, many of us find the past advancement painfully slow, full of capital errors, dead ends, lack of support and interest, and extremely long waiting periods between the proven idea and the commercially available method, tools and solutions. Even a close look to the seemingly inventive area, the production of various vitrification tools generates a bitter taste: with all our efforts, all our creativity these tools are ridiculously primitive – none of them may compete with the complexity of a cheap ballpen, although their price is shamelessly high. Even with the rapidly increased interest, vitrification has failed to meet the basic requirement of a medical technology including standardization, complexity, practicality, legal approval and widespread public acceptance. A good example is the foam box for the liquid nitrogen: it is usually recovered from the garbage, was a worthless packaging material of a cooled chemical or biomaterial, varies in size and shape from lab to lab causing unnoticed inconsistencies, and in most cases was never subjected to a professional cleaning procedure, not speaking about the highly advisable routine decontamination between patients. In a broader sense, this handicap is typical for the whole mammalian embryology: our tools and approaches do not differ much from those applied 25 to 30 years ago, the rate of advancement is simply miserable if we compare it with the skyrocket development of other branches in biology including applied genetics and molecular biology.

To meet the requirement of the second decade of our millennium, we have to eliminate quickly these formal errors while maintaining or rather increasing the great value of vitrification, the high overall efficiency.
IV. BIOSAFETY

A crucial question for the future of vitrification is not purely a biological but rather a legal problem. Since the earliest application of open vitrification methods, overwhelming concerns arose regarding the potential danger of disease transmission. These concerns were based on a single clinical report describing liquid nitrogen mediated HBV infection when two blood samples were stored in large leaky containers in the same Dewar [27], a situation that is hard to compare with that in any embryology unit. Another report describing viral transmission between two OPS samples [2] has further increased fears, although the viral concentration of the first sample was far above the level that can occur in a real situation, and there was no evidence that the amount transferred to the second straw may ever create a human infection after transfer to the uterus.

When evaluating these data it is worth to consider the real probability of such an infection. During the past decades a quarter of billion bovine and tens of millions human sperm samples were cryopreserved all over the world. Additionally, millions of human embryos were transferred after cryopreservation either at the oocyte or embryo stage. Compared to that, the cumulative number of reported infections as the result of ART procedures both in animals and humans is less than a dozen, and none of them was ever suspected to be the result of liquid nitrogen mediated disease transmission.

The rarity of this type of infections is quite surprising, as the liquid nitrogen dewars are potential sources of infections. Collection of the semen is not a sterile procedure, and oocytes at collection are also contaminated by blood. Many containers (straws, cryovials) are inappropriately sealed or leaky, their outer surface is basically contaminated. Storage tools (canisters, holders, dewars) are not sterilized routinely; but even after a possible sterilization, liquid nitrogen may get infected at each opening from the external air. Factory derived liquid nitrogen is not sterile, either. On the other hand, many pathogens survive storage in -196°C, and cryoprotectants used to protect embryos also protect pathogens.

Moreover, the theoretically safe closed systems of cryopreservation are not always safe and not always closed. Sealing with powder, a common practice in ART is not safe at all [3]. The material of common straws may not be as compact and safe as generally supposed, otherwise IMV would not advertise its special CBS straws as “impermeable for pathogen agents”. Professional heat sealers suggest homemade tests as no generally applicable parameters are available, this allows a lot of space for inconsistency. Cryovials are not safe at all, producers strongly advise storage in vapor instead of submerging, and to use a second skin for safety, both suggestions are in sharp contrast with the everyday practice. Finally, thawing is frequently performed under circumstances that allow wide possibility for infection.

The fact that infection is so extremely rare cannot be just explained with a pure luck. Several factors may contribute in it, including the extreme low quantity of samples at embryo transfer (but not at insemination), the fact that several pathogens do not attach the zone (however, others do). Zona may also mean an efficient barrier for most pathogens, even after ICSI, although the spontaneous healing of a cell-free extracellular structure may be a pure fantasy and a consequence of an inappropriate investigation method. Sequential washing of embryos is a common and acknowledged procedure in domestic animal embryology, and may also occur spontaneously in the (curiously less organized) human practice during warming and dilution of cryopreserved samples. Finally, the endometrium may have a special strong defense to eliminate minor quantities of infective agents. Although we still need more studies for explanation, the fact that liquid nitrogen mediated disease transfer has never occurred in reproductive biology, so far, although the theoretical possibility existed far before the introduction of open systems cannot be disregarded.

Nevertheless, open vitrification systems may increase the danger, and we have to establish reasonable measures to eliminate it. One possibility is the full ban of all open systems. The legal frames for such a ban have been available since years, and authorities insist to apply them in embryology practice. It is quite evident, that embryologists have to create a completely safe closed system as soon as possible. However, efforts to establish a closed system with the same efficiency as open systems have been performed since the occurrence of the first open systems, and the results were far from satisfying. Although in the case of some important samples including human blastocysts the differences between the results achieved with open vs. closed systems were reported to be minimal, for of human oocytes closed systems never reached the efficiency of the open systems.
It should be considered that the required warming rates are 10 to 1000 times higher than cooling rates [8]. Samples sealed in narrow tubes (Cryopette, Cryotip, sealed straws, etc.) may fail to achieve this high cooling rate when immersed in warm water. Moreover, after warming, the outer walls of the tube require decontamination and the tube should be cut at the proper level before the sample can be expelled into the proper medium. Accordingly, dilution of the toxic cryoprotectants is considerably delayed. Theoretically such a system can only be successful if the structure allows extremely high cooling and warming rates to decrease considerably the required concentration of cryoprotectant solutions. There are some approaches to realize this possibility, but the ultimate evidence, a multicenter study confirming the efficiency in various embryos and oocytes is still missing.

Another approach to establish a closed system is to put the carrier tool into a hermetically sealed second straw before cooling. In these methods warming rates do not cause a problem, as warming is performed by cutting the end of the container straw just above the level of the liquid nitrogen while the part containing the sample is still submerged. However, at cooling the double isolation may eliminate all the benefits that has been achieved during the past 15 years with the cooling rates, and may require the use of high cryoprotectant concentrations with high toxic and osmotic effect.

Alternative attempts to make vitrification on a metal surface also failed to produce satisfactory survival and developmental rates with human oocytes. As the result of further efforts, a really efficient closed system may be introduced anytime in the future. However, we need a safe and highly efficient method today, right now. A good way is to slice the Gordian Knot in half, by separating the cooling and storage phase. Cooling may be performed in a small amount of liquid nitrogen that is sterilized before vitrification, then the carrier tool may be sealed into a pre-cooled straw and stored safely in a common container. At warming, there is no need for sterile liquid nitrogen, as the container straw can be cut while still half-immersed into liquid nitrogen, then the carrier tool can be removed and inserted into the warming medium [29].

Sterilization of a small amount of liquid nitrogen can be performed relatively quickly and easily by filtration [29], or by UV illumination [21]. The method has been tested with both OPS and a Cryotop analogue, the efficiency was not compromised [29; Lin et al., unpublished], and the safety has been confirmed by an independent expert [3]. An alternative solution is the storage in liquid nitrogen vapor without the sealed container [5], however, vapor may also function as a carrier of infective agents [11], so authorities may not support this latter solution.

At present the question is if authorities will support any open system – even if the danger for disease transmission is even less than that in traditional freezing. An open-minded sincere discussion may help to find a reasonable compromise and to save the achievements of the past decade.

V. FURTHER IMPROVEMENTS

As vitrification is now in the focus of research in domestic animal and human embryology, new approaches and ideas are published almost every week to increase efficiency, eliminate setbacks and simplify work. Unfortunately very few of these suggestions survive the subsequent months, and the number of really valuable ideas is rather low, maybe less than one per year. Only two of such innovations are listed here with common features including the radical approach, far-reaching impact, and (curiously) the strong relation to hydrostatic pressure.

The -196°C liquid nitrogen in the laboratory is at the boiling point, just like water at 100°C. Anything that is warmer and immersed into it induces and extensive evaporation, and the emerging gas coat slows the cooling process down considerably. A simple evidence can be obtained by immersing-removing rapidly a finger into the liquid nitrogen: if the action is quick enough, even the change of temperature is imperceptible (this action is of course strongly discouraged but frequently performed by professionals to impress students). This vapor coat also develops at vitrification around the sample, and slows down the cooling rate at the most critical initial phase, where both chilling injury and ice formation may develop.

A feasible possibility to avoid this vapor coat to make vitrification on a pre-cooled metal surface – however, it has failed to produce satisfactory survival and developmental rates with several sensitive samples including human oocytes.

Another possibility is to decrease the temperature of the liquid nitrogen. This seemingly difficult task can be performed by using a moderate vacuum for several minutes. Part of the liquid nitrogen will evaporate, while the temperature of the rest will decrease to temperatures 5 to 10°C below the boiling point. This seemingly negligible
difference will substantially decrease the vapor formation around the sample, and a linear cooling can be achieved; accordingly lower cryoprotectant concentrations can be applied and the chilling damage will be minimized or avoided. An automated equipment to produce liquid nitrogen slush for vitrification purposes has been created by Arav et al. (pers. comm.) as early as ten years ago, and an increasing number of papers have been published about the positive effect, even by using closed systems for vitrification [33,20]. Eventually this approach may help to overcome the closed versus open system dilemma.

In contrast to the vacuum that is needed for supercooling liquid nitrogen, high hydrostatic pressure may also contribute in improvement of vitrification results. Although the main principle of laboratory embryology is to save gametes and embryos from all unnecessary stresses, it has been revealed that a properly adjusted and well controlled stress may induce a kind of general adaptation syndrome (similar to that in humans and other prokaryotic and eukaryotic cells), and may increase the survival and developmental competence after various in vitro procedures. The initial stress may be induced by heat shock, ethanol treatment, high hydrostatic pressure, osmotic shock or even by toxic chemicals [31,22].

The molecular mechanism of stress induced stress tolerance experienced and later purposefully induced in oocytes is only partially understood in eukaryotes and prokaryotes, and almost entirely unidentified in mammalian gametes and embryos. As these cells have either a very modest metabolic activity or their regulatory mechanism is changing radically, the experienced flexibility and adaptability is rather surprising. Chaperones, i.e. intracellular housekeeping proteins may play an important role in the increased defense. Mechanisms may include those that are conserved in all cells and participate in cellular functions including protein, DNA and chromatin stabilization and repair, cell cycle control, redox regulation, energy metabolism, fatty acid/lipid metabolism and elimination of damaged proteins [14].

High hydrostatic pressure seems to be the most appropriate one among these treatments as it does not include any toxic components, the effect can be rapidly induced and suddenly terminated; moreover, it is extremely accurate, consistent and repeatable. Stress tolerance induced by high hydrostatic pressure increased survival after vitrification of oocytes and embryos in various species and developmental stages [31]. If further systematic in vivo studies confirm the harmlessness of this intervention in vivo over several generations, it may be a promising way to improve the efficiency of cryopreservation both in domestic animals and humans.

VI. CONCLUSIONS

Application of vitrification for oocytes and embryos in domestic mammals and humans has opened new perspectives and resulted in a breakthrough in key issues. In spite of the impressing achievements, improved methods, standardized tools and equipment as well as elimination of risk factors related to disease transmission are required to exploit fully the possibilities of this promising approach. Involvement of existing novel methods may further increase the overall efficiency and widen the application fields of vitrification.

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