Improving cryopreservation systems

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Abstract

Cryopreservation of oocytes and embryos is a crucial step for widespread practical application of other techniques in domestic animal embryology. However, in contrast to the rapid development of procedures in the eighties and early nineties of the last century, new advancements with profound practical consequences have only been recently achieved. As a result of a long and controversial development, an alternative group of methods, vitrification, has proved its efficiency and practicality in the past few years. The aim of this short review is to characterize strategies to prevent cryoinjuries, summarize the development of vitrification, overview its recent achievements, and provide a perspective about possible application. Authors strongly believe that the future of mammalian oocyte and embryo cryopreservation will be mainly based on vitrification, and the rate of advancement will be determined by the rate by which embryologists learn and acknowledge this new approach.

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1. Introduction: cryopreservation in reproductive biology

Cryopreservation is a limited and not quite fascinating area of reproductive biology. The application of industrially-produced standard media, automated freezing machines and precisely-determined parameters have degraded most traditional freezing procedures to a routine standard step that can be performed after a short technical education and without any understanding of the underlying processes. On the other hand, the numerous alternative
methods that have been developed in the past 15–20 years in laboratories have long failed to produce results convincing enough to break through the level of experimentation and achieve widespread application in general practice. The lack of success has diminished financial support, and as the result of this vicious circle, advancement in the area of oocyte and embryo cryopreservation has become chilled.

However, although many scientists have left the area to deal with more fashionable and productive fields, some of them have continued the work or returned from time to time to cryopreservation. In the past few years, previously sporadic and inconsistent results have been confirmed and improved in some “dark areas”. Companies that have for long neglected anything but traditional freezing protocols have also realized the commercial potential of new technologies and have started to produce tools and equipment according to the requirements of these alternative techniques. In parallel, advanced versions of these new methods have been developed to meet the requirements of authorities regarding safety issues.

We strongly believe, that we are now eyewitnesses and participants in the creation of a new era of mammalian oocyte and embryo cryopreservation. The purpose of the present article is to reflect upon and promote this process.

2. Damage and its prevention during cryopreservation

All oocytes and embryos suffer considerable morphological and functional damage during cryopreservation. The extent of the injury depends on factors including the size and shape of the cells, the permeability of the membranes, and the quality and sensitivity of the oocytes and embryos. All these factors may be highly variable depending on the species, developmental stage and origin (for example, in vitro produced or in vivo derived). However, oocytes and embryos also have a remarkable, sometimes surprising ability to repair this damage fully or partially, and, for optimal cases, to continue normal development. The purpose of cryopreservation procedures is to minimize the damage and help cells to regenerate.

Almost all cryopreservation strategies are based on two main factors: cryoprotectants and cooling–warming rates. Since the first successes of mammalian embryo cryopreservation in the sixties of the last century, two major groups of methods can be delineated: traditional slow-rate 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.

Traditional slow-rate freezing was introduced first, and for the majority of domestic animal and human embryologists this remains the only acceptable approach. Over time, methods have become highly standardized with a considerable industrial and commercial background. Traditional slow-rate freezing can be interpreted as an attempt to create a delicate balance between various factors causing damage including ice crystal formation, fracture, toxic and osmotic damage. The controlled cooling rate 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). Although the concentrations of cryoprotectants seem to be dangerously
high at the final phases, it happens at low temperatures, where the real toxic effect is minimal. Eventually at solidification the intracellular ice formation is decreased to an acceptable level or is almost entirely eliminated.

This latter phenomenon, the solidification of water without ice crystal formation, is also called vitrification. However, the term “vitrification” in cryobiology refers to the other group of cryopreservation methods, where the main purpose is to ensure ice free solidification of the whole solution containing the sample. The process itself can be described as an extreme increase in viscosity of solutions. The benefits are unquestionable: ice crystal formation, one of the most dangerous causes of cryoinjury, is entirely eliminated except for a transitional and very short freezing of the solutions during warming that is generally regarded harmless to the oocytes and embryos.

Unfortunately we have to pay the price of this benefit. Vitrification can only be induced in exceptional situations: with dangerously-high concentrations of cryoprotectants and/or with extreme increase of the cooling and warming rates. One characteristic of vitrification is that as the cooling rate chosen increases, the cryoprotectant concentration can be lowered and vice versa. Extensive research in the past 20 years has resulted in new, sometimes radically-new approaches and created an acceptable compromise between the positive and negative by decreasing cryoprotectant toxicity and increasing cooling rates. As a result, vitrification has become a competitive alternative to slow-rate freezing. Moreover, the high cooling and warming rates applied at vitrification provide an unique benefit compared to the traditional freezing: the possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop.

Nonetheless, application of vitrification was long restricted to experimental laboratories. Many factors participated in the creation of this situation (see review in [1]). Additionally, vitrification suffered two drawbacks for a long time: the lack of possibility for direct transfer and the concerns regarding possible disease transmission during storage. Eventually, however, practical solutions were found for both problems that required commercial manufacture of particular tools; consequently, the whole idea of vitrification has become more attractive in the commercial arena.

In the past decade, reviews dealing with cryopreservation in embryology have involved considerable efforts to maintain a superficial balance between the values of slow-rate freezing and vitrification. However, we do not see any area in embryology where slow-rate freezing offers significant advantages compared to vitrification. Consequently, although the traditional method had a significant role in the history of cryopreservation, and its present impact should not be underestimated, we expect slow-rate freezing to sooner or later be replaced entirely by the new vitrification techniques in all relevant areas of embryology. To support our opinion, we will shortly review general features of development in vitrification techniques, and detail the main areas where vitrification has convincingly proved its superiority.

3. Efforts to improve vitrification in embryology

Vitrification was first introduced in embryology for cryopreservation of mouse embryos [2]. In the subsequent decade, a moderate number of publications dealing with successes in
cryopreservation of oocytes and embryos from several domestic animal species were published. Meanwhile, considerable efforts were made to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals (first of all ethylene glycol), using two or three cryoprotectants to decrease the specific toxic effect of each, to make stepwise addition, and to cool solutions to temperatures close to 0 °C when the final high concentration was applied.

Approximately 10 years ago, the development of vitrification seemed to reach a plateau phase. The possibilities for modifying cryoprotectant combinations and addition and removal protocols were almost fully exploited. In most species and developmental phases, vitrification enabled researchers to achieve similar survival and developmental rates to traditional slow-rate freezing. However, vitrification did not offer real benefits compared to the earlier methods. To achieve that, a considerable increase in cooling and warming rates was required.

After promising results under experimental conditions [3], the first attempt to use ultrarapid cooling in everyday practice was based on the very simple idea of dropping the embryo containing solution directly into the liquid nitrogen [4]. Theoretical advantages in maximizing cooling rate by doing so were limited at first by the relatively large volume of the drop as well as the delay before the drop floating on the surface of liquid nitrogen sank. Different carrier tools were applied to minimise the volume and to submerge the sample quickly into the liquid nitrogen, including electron microscopic grids [5], open pulled straws (OPS) [6] and cryoloops [7]. The publication of these methods has stimulated the imagination of researchers both in the domestic animal and human field and resulted in a flood of new tools based on the same principles with little or no advantage compared to their precedents.

In parallel, considerable effort has been invested to develop alternative methods to increase cooling and warming rates by minimising the vapour formation around the sample at cooling. Cooling the liquid nitrogen slightly below the boiling point (−196 °C), say from −200 to −205 °C, may minimize the boiling around the submerged sample and increase cooling rates considerably. Although the approach was sporadically used earlier, it was widely available only after the construction of the VitMaster (IMT, Israel), a device producing vacuum in the reinforced liquid nitrogen container [8]. A considerable part of liquid nitrogen evaporates, but the remaining solution cools down and even starts to solidify to form liquid nitrogen slush. Straws, preferably thin straws, even in sealed form, can be cooled safely with an increased cooling rate in this mixture of nitrogen slush and cooled liquid nitrogen (A. Arav, unpublished). An alternative way to avoid vapour formation around the sample was the solid surface vitrification (SSV) method [9]. This approach makes a shortcut to exclude entirely the vapour formation by cooling metal surfaces in liquid nitrogen, and placing the solution containing the biological sample on the surface of metal. Reports have proved that in skilled hands this technique may provide almost as high survival and developmental rates as achieved with the OPS or cryoloop devices.

The latest development in the practical application of ultrarapid vitrification methods was based on the concept of minimum volume cooling (MVC) [10]. In the original procedure, extremely small sample volumes are loaded to the inner wall of a 0.25 ml standard insemination straw. After sealing, the straw is immersed directly into the liquid nitrogen, and can be subsequently used for in-straw dilution, and direct transfer. To avoid the very delicate loading step and to increase further cooling and warming rates, a simple and very efficient method has been developed for ultrarapid cooling. In the Cryotop
procedure [11], a thin plastic film strip attached to a plastic handle is the carrier tool. Loading is performed with the use of a glass capillary under the control of a stereomicroscope. Before cooling, almost all medium is removed, so the embryos and oocytes are only covered with a very thin solution layer. Submersion of the Cryotop into the liquid nitrogen or into the rehydrating solution after vitrification results in extremely high cooling and warming rates, respectively, permitting further decrease in concentration of cryoprotectants. During storage, a protective cap is applied to prevent mechanical damage of the film and the vitrified sample. According to our experience as well as data published by other research groups, the Cryotop technique is at present the most efficient method for cryopreservation of sensitive samples including human oocytes ([12] see discussed later), and pieces of mouse ovarian tissue (Kagawa, personal communication).

With a few exceptions, ultrarapid vitrification procedures face an additional challenge that has hampered their practical application caused by the fact that most procedures depend on direct contact of the sample containing solution with the liquid nitrogen for cooling and on subsequent storage without hermetical isolation. Relevant organizations both in domestic animal and human embryology (International Embryo Transfer Society, Food and Drug Administration, other international organizations and domestic authorities dealing with disease transmission issues) have clearly emphasized that sterile and potentially contaminated samples should be safely isolated from each other in all phases of embryology work. Although the possibility of cross-contamination through liquid nitrogen in embryology is very low, it cannot be neglected. Some ultrarapid vitrification methods including SSV and the original MVC, as well as the modified form of the Cryoloop technique [13], do not require direct contact between the sample and liquid nitrogen during cooling. Others, including the VitSet technique (Minitüb, Germany) offer solutions to the problem by performing the cooling in factory derived, and/or filtered or UV-sterilized liquid nitrogen followed by wrapping the sample and the carrier tool into a sealable container before storage [14]. A recent, alternative solution for the problem is the CryoTip (Irvin Scientific, CA, USA) technique [15]. The possibility was outlined several years ago [1], and has been realized recently in a rather sophisticated and safe way. The samples are loaded into a plastic capillary with ultrathin walls, both ends are sealed, and the capillary is submerged into the liquid nitrogen. The CryoTip also includes a protective tube preventing mechanical damage during handling and storage. For warming, the procedure is identical to that used for 0.25 ml straws after traditional freezing. The capillary is submerged into warm water, then decontaminated with ethanol, cut and the sample is expelled into the rehydrating solution. Although the cooling rates are slightly lower than those achievable by the Cryotop method, the CryoTip was recently applied in human embryo cryopreservation with the same efficiency as the Cryotop [15]. Another solution for the sterility problem was published recently by Isachenko et al. [16]. In their method, the sample is first loaded into the ultrathin capillary (super open pulled straw; SOPS). Instead of ultrarapid cooling, however, the capillary is first wrapped hermetically into a large straw before cooling. For warming, an ultrarapid procedure is applied by removing the SOPS from the straw at $-196 \, ^\circ C$ followed by transfer directly into the warm dehydration solution. Authors reported high survival rates of pronuclear state human oocytes (i.e. zygotes) by using this method and concluded that, in contrast to the situation for ultrarapid warming, ultrarapid cooling is not required for successful vitrification. As the applied cryoprotectant concentration is relatively low, one may have concerns
regarding the theoretical and practical possibility to obtain vitrification (i.e. ice-free cryopreservation of the whole solution) under the obtainable slow cooling rates. Further independent investigations will answer this question, and test the repeatability of the developmental results described in the first publication.

4. Examples of significant achievements obtained by vitrification in mammalian embryology

In cattle, cryopreservation of in vivo-produced, transferable-stage embryos has been successfully resolved by the traditional slow-rate freezing. Consequently, vitrification has been unable to produce additional breakthroughs in this field. Here, alongside a possible slight increase in calving rates, the real benefit of vitrification would be the rapidity, simplicity, and inexpensiveness of the procedure. In contrast, considerable success has been reported for cryopreservation of in vitro-produced embryos from one cell to the hatched blastocyst stage [17]. Vitrified day 3 embryos [18] and cytoplasts [19,20] could be used as donors for nuclear transfer, and somatic cell cloned blastocysts were also successfully vitrified resulting in healthy offspring [21]. Vitrification of bovine immature [22] and in vitro matured oocytes [6] has resulted in healthy offspring after in vitro fertilization and embryo culture. According to the relevant publications, similar results can also be achieved in vitrification of ovine and caprine embryos [23,24].

Cryopreservation has always been one of the greatest challenges hampering practical application of embryo transfer in the pig. Removal of cytoplasmic lipid has improved survival [25], but made the process technically very demanding. After the first initial successes with centrifugation without micromanipulation [26], or application of cytoskeleton relaxants [27], the ultrarapid vitrification techniques provided solution for the problem, first regarding in vitro survival [28], and then in vivo development resulting in large number of healthy offspring [29,30], and making large scale application a realistic perspective. The next challenge may be vitrification of somatic cell cloned embryos as well as oocytes. Promising results after in vitro fertilization [31] and activation [32] and initial successes in oocyte vitrification [33,34] suggest that these goals can be achieved in the near future.

Vitrification of equine oocytes [35] and embryos [36] has shown significant advancement in the past few years. Successes in cryopreservation of embryos of wild species [37,38] prove that vitrification may be a useful tool for preservation of endangered species, as well.

In the human field, the greatest challenge is oocyte cryopreservation. In spite of the fact that the first baby after traditional freezing of oocytes followed by in vitro fertilization was born 19 years ago [39], the efficiency of the procedure has remained disappointingly low, even with application of intracytoplasmic sperm injection for fertilization [40]. Until recently, only about 100 women worldwide are thought to have become mothers this way [40]. The need for an efficient technique is rising rapidly. Cancer therapy is successful in increasing number of young female patients capable of a normal life but lifelong infertility remains an issue. Cryopreservation of oocytes before the start of the therapy would offer a chance to resolve the problem. Additionally, an increasing number of women wish to delay maternity because of career demands despite the decline in oocyte quality with age. Recently, the Cryotop technique has resulted in a breakthrough, with 91% survival rate.
after vitrification, 50% development to the blastocyst stage and 10 babies born after 29 embryo transfers ([12], Kuwayama, unpublished). Another strong argument for the applicability of vitrification in the human field is the fact that the new ultrarapid strategies were found to be the most efficient approach for embryonic stem cell cryopreservation [41].

5. Conclusion

Recent advancement in cryopreservation of mammalian oocytes and embryos has almost exclusively been achieved with the new vitrification techniques. In the foreseeable future, technically improved and standardized vitrification methods may replace traditional slow-rate freezing in everyday practice and may offer new perspectives to commercial embryology.

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