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Cryopreservation of Intact and Biopsied Buffalo Blastocysts

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ABSTRACT

A practical cryopreservation method of embryos is a key factor in commercial embryo transfer and production technology and offers the opportunity of implementing novel animal breeding and production programmes. Initially, work was done with conventional slow cooling technologies, using cryoprotectants, such as glycerol or ethylene glycol and sucrose. More recently, however, greater advances have been made with vitrification, a procedure that bypasses ice crystal formation and places the embryo in a glass-like state. This review will briefly summarize the current understanding of embryo cryopreservation technologies and we strongly believe that we are now eyewitnesses and participants in the creation of a new era of cryopreservation for *in vitro* produced (IVP) buffalo blastocysts that will be applicable to direct transfer of the post-thaw embryos. Blastocysts were harvested 7 days after IVF and allocated to either an intact or biopsy group. The purpose of the present article is to reflect upon and promote this process.

Key words: Cryopreservation, biopsy, blastocyst, buffalo

INTRODUCTION

The goal of embryo cryopreservation is long term storage and reproducible high survival rates of embryos following warming, leading to the successful establishment of pregnancy and live offspring following embryo transfer. Embryo ryopreservation also provides an option when superovulation yields are greater than recipient availability. Additionally, cryopreservation provides a method to preserve embryos from exotic or endangered species and allows for import and export between countries. Furthermore, cryopreservation of embryos provides a means for reducing animal space requirements and protects valuable animal lines from potential loss due to environmental disasters, genetic drift and infectious diseases (Landel, 2005). This review will summarize the latest information known about conventional freezing, but will focus primarily on the recent advances in vitrification and possible future directions for the field of embryo cryopreservation.

IVP embryos: Buffalo embryos were produced *in vitro* according to a method described previously (Abd-Allah, 2009). Briefly, cumulus-oocyte complexes (COCs) from abattoir-derived ovaries were cultured in tissue cultured medium-199 (TCM-199) supplemented with 5% (v/v) fetal calf serum (FCS), 10 μL mL^{-1} pregnant mare serum gonadotropin (PMSG, Folligon, Cairo) and 10 μL mL^{-1} Human Chroionic gonadotropin (hCG, Pregnyl, Nile company for Pharmacuetical and Chemical Industries, Cairo) for 22 h at 38.5 C in 5% CO 2 in air with a density of 20-35 COCs per 750 μL of medium. A frozen-thawed sperm suspension (5×10^6 sperm mL^{-1}) was prepared in Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) supplemented with 20 mg mL^{-1} BSA (Sigma) and 20 μg mL^{-1} heparin for 20 min at 700× g. For IVF, a 50 μL sperm suspension was added to

20-25 COCs in a 50 μL microdrop of BO/BSA/heparin medium under mineral oil and incubated for 24 h. The presumptive zygotes were cultured for 7 days in TCM-199 supplemented with 10% FCS and 50 μg gentamycin sulphate. The day of IVF was defined as Day-0 (Abd-Allah *et al*., 2010).

Biopsied blastocysts: Blastocysts of code-1 quality (equivalent to excellent and good in the International Embryo Transfer Society grading system (Zheng *et al*., 2005) were harvested 7 days after IVF and biopsied according to the method described by Zheng *et al*. (2005).

Briefly, the blastocysts were placed into 200 μL droplets of BMOC-3 medium supplemented with 20% FCS and 1.0% Solcoseryl® (Solco Basel, Birsfelden, Switzerland) in a 90 mm Petri dish without overlaying mineral oil. We used a metal blade (Feather Safety Razor, Osaka, Japan) attached to a micromanipulator to split approximately 20 to 30% of the total cells from the blastocysts. The biopsied blastocysts were further cultured in the BMOC-3/FCS/Solcoseryl® medium at 38.5 C in 5% CO_2, 5% O_2 and 90% N2 for 1 h and in TCM199/FCS/β-me medium at 38.5 C in 5% CO_2 in air for 2 h until use for freezing experiments.

Normally blastocysts were transferred into drops of HEPES-buffered medium (Earle's) in a Petri dish under mineral oil.

Biopsy was performed using a pair of micromanipulators (Leitz, Germany) in conjunction with an inverted microscope (Leitz). Each embryo was immobilized by suction with a flame-polished holding pipette held in one micromanipulator. The second micromanipulator with a double holder controlled a drilling pipette (internal diameter 10 containing acid Tyrode's solution (pH 2.2) and a sampling pipette (internal diameter 30 containing buffered medium). The drilling pipette was placed in close contact with the zona pellucida and a hole made with a controlled stream of acid Tyrode's solution. Immediately the zona was penetrated this pipette was removed and the sampling pipette was pushed through the hole.

Embryo cryopreservation: There are six steps to successful embryo cryopreservation: exposure to cryoprotectant, cooling to subzero temperatures, storage, thawing or warming, removal of cryoprotectant and return to a physiological environment (Liebermann *et al*., 2003). There are two main methods for cryopreserving embryos, conventional slow cooling/freezing and vitrification. Each of these will be addressed below followed by conditions that should be considered in order to get optimal results for embryo survival.

Conventional slow 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.

Interestingly, *in vitro* produced (IVP) embryos were shown to be more sensitive to cryoinjury (Hasler *et al*., 1995). This delayed the success of the first cryopreserved IVP embryos.

Conventional slow cooling is based on the principle of dehydration, where cooling rates are optimized to remove water from the embryo, preventing cryoinjury from ice crystal formation while minimizing chemical toxicity and osmotic stress from exposure to high concentrations of salts (Campos-Chillon *et al.*, 2006). Slow cooling methods allow solution exchange between the intracellular and extracellular compartments without inducing serious osmotic effects (Vajta and Kuwayama, 2006). The chilling sensitivity of the embryo is dependent on the stage of development and on the culture conditions under which it developed (Pollard and Leibo, 1994). Moreover, the success of slow cooling depends on achieving the optimal equilibrium between the rate at which water can leave the cells and the rate at which it is converted into ice (Visintin *et al.*, 2002). Equilibrium is achieved at low cryoprotectant concentrations and slow cooling rate, allowing dehydration to occur during cooling.

Using the slow freezing method, the dehydration of the embryo is usually achieved by placing the embryo in a solution containing 10 to 11% of a penetrating cryoprotectant. The temperature is then lowered and ice crystal growth is initiated by seeding (Visintin *et al.*, 2002), which is done by touching a column of solution with a supercooled instrument. As the ice crystals grow, the water in the solution is converted to a solid state, increasing the concentration of extracellular solutes, which draw water out of the cells (Visintin *et al.*, 2002). The most common conventional cryopreservation procedure for embryos consists of equilibration in cryoprotectant for 5-10 min at 20-25°C, seeding at -5 to -9°C and cooling at 0.3 to 0.6°C min^{-1} down to -33 to -40°C, followed by plunging into liquid nitrogen (Fair *et al.*, 2001; Visintin *et al.*, 2002; Campos-Chillon *et al.*, 2006; Mucci *et al.*, 2006).

Vitrification: The physical definition of vitrification is the solidification of a solution (glass formation) at low temperatures without ice crystal formation. The phenomenon can be regarded as an extreme increase of viscosity and requires either rapid cooling rates (according to theoretical calculations, at a cooling rate of approximately 107°C sec^{-1} even pure water vitrifies; Rall, 1987) or the use of cryoprotectant solutions, which depress ice crystal formation and increase viscosity at low temperatures. Until recently, the highest cooling rate of common vitrification procedures was limited to that which could be achieved by plunging of a sealed 0.25 mL insemination straw directly into liquid nitrogen, i.e., approximately 2500°C min^{-1} (Palasz and Mapletoft, 1996).

Vitrification is a newer method that eliminates both intracellular and extracellular ice formation, producing instead a glass-like state. At sufficiently low temperatures, solutions become very viscous and solidification occurs without ice crystal formation. It does this through dehydration and the extreme elevation in viscosity caused by ultra rapid cooling rates, from 15,000 to 30,000°C min^{-1} (Liebermann *et al.*, 2003).

The glass transition state is ~-130°C (Kassai and Mukaida, 2004), but varies depending upon components in the vitrification solution. Vitrification of solutions has been known since 1948, but was first used for preserving embryos from the mouse in 1985 (Rall and Fahy, 1985). A year later the first bovine embryos successfully vitrified were reported (Massip and van der Zwalmen, 1984). Considerable efforts have been made since the mid 1980's developing simpler protocols and more stable and less toxic solutions for vitrification.

Improvements have been made by using less toxic and more permeable chemicals, by using a combination of cryoprotectants to reduce toxicity, using a stepwise approach to equilibration and increasing cooling and warming rates (Vajta and Kuwayama, 2006). Factors that determine whether a vitrification solution will remain uncrystalized are the total solute concentration (>40%),

the capacity of the cryoprotectant to form glass and the rate of cooling/warming (Shaw *et al*., 1997). Increasing the cooling/warming rate can also decrease the amount of solute required to form a stable glass.

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 minimize the volume and to submerge the sample quickly into the liquid nitrogen, including electron microscopic grids (Martino *et al*., 1996), Open Pulled Straws (OPS) (Vajta *et al*., 1997), cryoloops (Lane *et al*., 1999) and stepwise vitrification method (Abd-Allah, 2009). 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. An alternative way to avoid vapor formation around the sample was the Solid Surface Vitrification (SSV) method (Dinnyes *et al*., 2000).

The ultra-rapid vitrification procedure has been clinically applied using a special vitrification container (CryoTop, Kitazato Supply Co., Ltd.), which was developed for the clinical application of the MVC method.

A preliminary report on the vitrification of buffalo morulae and blastocysts made by Abd-Allah (2003) showed that both buffalo morulae and blastocysts could be successfully frozen and thawed and their survival rate post-thawing reaches 79.4%.

Abd-Allah an Ali (2005) revealed that the viability of embryos vitrified with EG medium (65.91±4.36 and 50.67±4.27% for re-expansion and hatching rates, respectively) was significantly higher ($p<0.01$) than the corresponding values with glycerol (48.18±2.96 and 25.67±6.52%, respectively) and it was nearly similar to that of the control group (67.78±4.79 and 58.33±4.39%, respectively).

Abd-Allah (2010) revealed a higher percentage of post-thawing recovered embryos for stepwise vitrification (100%) compared to one step method (60%). The rates of blastocyst re-expansion and hatching of stepwise vitrified blastocysts (66 and 55%, respectively) were significantly higher ($P<0.01$) than the corresponding values with one step method (40 and 20%, respectively) and it was nearly similar to that of the control group (68, 58%, respectively). This is the first report on stepwise vitrification of buffalo embryos. Present results suggest that stepwise vitrification supports better *in vitro* survival of frozen thawed buffalo embryos.

Vitrification versus conventional freezing: 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.

Vitrification has a great advantage over conventional freezing due to its simplicity, speed and does not require additional equipment, making it more user friendly especially for on-farm procedures. Slow cooling has been proven effective, but requires more time and use of expensive equipment.

The strategy of vitrification is basically different from that of freezing by slow cooling. A slow rate of cooling attempts to maintain a delicate balance between the various factors, which may result in damage, such as ice crystal formation, osmotic injury, toxic effect of cryoprotectants, concentrated intracellular electrolytes, chilling injury, zona and embryo fracture and alterations of intracellular organelles, cytoskeleton and cell-to-cell contacts (Massip and van der Zwalmen, 1984) whereas vitrification totally eliminates ice crystal formation.

Abd-Allah (2010) revealed a higher percentage of post-thawing recovered embryos for stepwise vitrification (100%) compared to one step method (60%). The same author suggests that stepwise vitrification supports better *in vitro* survival of frozen thawed buffalo embryos.

Cryopreservation of biopsied blastocysts vs. intact blastocysts: The standard embryo cryopreservation method is still less than optimal for biopsied embryos. Non-biopsied blastocysts that initiated hatching or biopsied blastocysts that regained the initial size of the blastocoel were considered to be surviving.

Dehydration of blastocyst- and expanded blastocyst-stage embryos at vitrification may be impaired due to difficultly of cryoprotectants to permeate the blastocoelic cavity, increasing the susceptibility to ice crystal formation. By artificial reduction of blastocoelic fluid, osmotic shock, permeability issues and ice crystal formation can all be reduced. Viability, implantation and pregnancy rates have all been improved in human embryos that have been artificially collapsed by microsuction prior to vitrification (Joris *et al*., 1999). Microsuction of blastocoelic fluid prior to vitrification of mouse blastocysts improved survival post-warming to rates comparable to nonvitrified controls, increasing live young (Jericho *et al*., 2003). Unique attempts to improve dehydration of embryos, such as this may further enhance cryosurvival of embryos from many more species, allowing survival similar to non-cryopreserved controls (Zheng *et al*., 2005).

We have recently reported that IVP and biopsied bovine blastocysts can survive cryopreservation by the conventional freezing procedure of embryos with a programmable freezer and plastic straws is still convenient, especially for technicians working at farms (Zheng *et al*., 2005). The use of cryoprotective solution containing glycerol and/or sucrose at a relatively low concentration (osmolality ranged from 1,086 to 1,260 mOsm kg^{-1}) made it possible for post-thaw embryos to be diluted in a one-step manner without loss of viability. However, the true viability of cryopreserved embryos needs to be investigated by direct transfer into recipients because confirmation of pregnancy or birth of calves is the most rigorous criterion for assessment.

More recently, a study showed the effects of freeze/thaw on the biopsied blastocysts. Zheng *et al*. (2005) evaluated the biopsied blastocysts cryopreserved by slow cooling or vitrification, as compared with controls. the survival rate of biopsied human embryos is significantly lower than non-biopsied embryos when conventional cryopreservation methods are used (Joris *et al*., 1999; Magli *et al*., 1999).

Vitrification has become quite useful for storage of biopsied embryos (Jericho *et al*., 2003) it was shown that a modified cryopreservation method with increased sucrose concentration up to 0.2 mol L^{-1} could significantly improve the survival rate of biopsied embryos compared with standard freezing methods. This may be due to greater access of the cryoprotectant, caused by the rent in the zona pellucida of micromanipulated embryos.

A major challenge for researchers is to establish a universal standardized vitrification method, which can be successfully applied for cryopreservation of intact and biopsied embryos of buffaloes.

In conclusion, in most cases, modifying cryopreservation methods to fit the cells to be cryopreserved is likely to be preferable to modifying cells to fit procedures for cryopreservation. Nevertheless, there are opportunities to do the latter and in conclusion, cryopreservation of intact and biopsied buffalo blastocysts has revolutionized the field of assisted reproduction.

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