Biotechnology in Animal Husbandry 36 (3), 251-270 , 2020
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 636.082'62
https://doi.org/10.2298/BAH2003251H

CRYOPRESERVATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS BY VITRIFICATION: IN PURSUIT OF A SIMPLIFIED, STANDARDIZED PROCEDURE THAT IMPROVES PREGNANCY RATES TO PROMOTE CATTLE INDUSTRY USE

Van Huong Do[1], Andrew W. Taylor-Robinson[2]

[1]Genesis International Co. Ltd, Hanoi, Vietnam
[2]School of Health, Medical & Applied Sciences, Central Queensland University, Brisbane, QLD, Australia
Corresponding author: Prof A.W. Taylor-Robinson, a.taylor-robinson@cqu.edu.au
Review Paper

Abstract: The goal of cryopreservation is to retain the original stage of gametes and embryos after they have endured cooling and warming. Slow freezing is a standard method for *in vivo*-derived bovine embryo cryopreservation, three-fifths of such embryos being frozen by this method globally. However, it is evident that slow freezing is not efficient for cryopreserving *in vitro*-produced bovine embryos. Hence, only one-third of *in vitro*-produced bovine embryos are cryopreserved. Vitrification is a preferred method for storage of human embryos; consequently, it has been explored as a novel means to store *in vitro*-produced bovine embryos, for which it shows considerable promise as an alternative to slow freezing. This is due to several reasons: vitrification is often less time-consuming than slow freezing; it does not need expensive slow rate freezing machines; and it has been proven to have comparatively higher survival rates. Yet, in the cattle industry vitrification continues to present shortcomings, such as possible toxicity of vitrification solutions and failure to standardize methods, which pose a challenge for its application to *in vitro*-produced bovine embryos. Therefore, determining the most suitable procedure is crucial to make vitrification more practical in commercial settings.

Keywords: bovine, cryopreservation, cryoprotectant, embryo, slow freezing, vitrification.

Introduction

In commercial breeding of farmed livestock embryo transfer is an important tool to increase the number of elite animals in a herd or for herd replacement. In the cattle industry embryos are generated by means of either *in vivo* or *in vitro* production. With the *in vivo* method, bovine embryos are generated by a superovulation technique in which donors are given exogenous gonadotropin hormones in order to stimulate the activity of their ovaries. As a result, more oocytes are able to develop to maturity, when they are receptive to fertilization by sperm. When oocytes are fertilized in the oviduct of a cow, they migrate to the uterus and develop to the morula and blastocyst stage; hence these are known as *in vivo*-derived embryos. The alternative route of embryo production, *in vitro* to *in vivo*, involves the aspiration of either mature or immature oocytes from donor ovaries, *in vitro* fertilization, usually with frozen sperm, and subsequent *in vitro* culture to the blastocyst stage. This means of embryo production has been established since the 1980s but as late as the early 2000s the great majority of bovine embryos were still generated by the *in vivo* method (*Merton et al., 2009*).

The last decade has witnessed a pronounced shift in the practice of bovine embryo transfer such that the most recently published statistics indicate that globally the number of *in vitro*-produced embryos exceeded that of their *in vivo*-derived counterparts (742,908 to 386,133 recorded transfers, respectively) (*Viana et al., 2019*). This trend particularly reflects the increasing reliance on *in vitro* technologies in North America and South America, the two continents that are together responsible for the majority of the world's bovine assisted reproduction industry. In all regions, *in vitro*-produced embryos were transferred predominantly fresh (overall 73.2%) (*Viana et al., 2019*). Bovine embryos can be transferred into the uterus of recipients on the same day that they are harvested, when they are termed 'fresh embryos'. Typically, remaining embryos that are not transferred immediately are retained by cryopreservation for future use.

Embryo cryopreservation has become an essential aspect of assisted reproductive technology (*Leibo, 2008*). The proportion of frozen *in vitro*-produced bovine embryos transferred in 2018 decreased compared to 2017 (26.8 vs. 33.9%, respectively), breaking an annual rise observed since 2013. On the other hand, more *in vivo*-derived frozen-thawed embryos were transferred (60.1%) compared to *in vivo*-derived fresh embryos (*Viana et al., 2019*). Development of the cryopreservation procedure in cattle, as for all mammals, is facilitated by, and is closed associated with, the success of cryopreservation of sperm, oocytes and embryos (*Vajta and Nagy, 2006*). Slow freezing and vitrification are the two recognized methods for cryopreservation of each of *in vivo*-derived and *in vitro*-produced bovine embryos. Among beef cattle, animals produced from *in vitro*-produced embryos greatly exceed those from *in vivo*-derived embryos. This is due

to the intrinsically large number of follicles in beef breeds such as Nelore (*Pontes et al., 2009*).

It is apparent that understanding the cryopreservation process and identifying a simple but reliable method for *in vitro*-produced bovine embryos is necessary in order to enhance the efficiency of their production and transfer in on-farm contexts. While the published literature covers a broad scope of bovine embryo cryopreservation, including the standard slow freezing procedure, this review focuses primarily on vitrification as a method to cryopreserve *in vitro*-produced bovine embryos. It also examines theoretical aspects in regard to ice crystal formation, cooling/warming rates, and roles of cryoprotectants in protecting cells from the chilling process. Furthermore, the specific demands for storage of *in vitro*-produced bovine embryos are considered. Efforts to raise the efficiency of vitrification through modifications to *in vitro* culture and enhancements in the capacity of *in vitro*-produced embryos are also discussed. Finally, biosafety issues relating to vitrification are addressed.

Different methods of freezing embryos

Of the two methods of bovine embryo cryopreservation slow freezing was developed in the 1970s whereas vitrification was realized a decade later and has seen a rapid period of more recent development (*Hasler, 2014*). The majority of *in vivo*-derived embryos are generated in donors with the aid of exogenous gonadotropin hormones and are then frozen. *van Wagtendonk-de Leeuw et al.* (*1997*) reported that these two methods of cryopreservation led to similar conception rates of recipients. *Hasler* (*2014*) affirmed that on occasion vitrification is recommended for cryopreservation of *in vivo*-derived embryos yet maintains that slow freezing yields similar pregnancy rates among recipients.

A major objective of cryopreservation is to minimize intracellular ice formation. *Kleinhans and Mazur* (*2009*) proposed that during the slow freezing process, survivability of cells is dependent on a cooling rate slow enough for unfrozen intracellular water to dehydrate by osmosis to near the equilibrium level before reaching the temperature at which intracellular nucleation occurs. In contrast, *Kim et al.* (*2012*) and *Lawson et al.* (*2012*) each argued that vitrification negates any risk of intracellular ice crystals forming.

(a) Slow freezing

In North America 70% of superovulated bovine embryos are slow frozen, indicating that if a programmable freezer is available this method is preferred (*Hasler, 2014*). As an example, *Hasler* (*2001*) demonstrated a high overall pregnancy rate of 56.1% for recipients implanted with *in vivo* frozen embryos. Furthermore, *Lopatarova et al.* (*2010*) reported a conception rate of 48.8% for recipients implanted with frozen and biopsied *in vivo* embryos; for recipients of intact and frozen *in vivo* embryos the conception rate was 50.7%. Bovine embryos

are standardly frozen and stored in a straw with 1.8 M ethylene glycol (EG) as cryoprotectant so enabling the possibility upon thawing of direct transfer to a recipient cow (*Dochi et al., 1995; Voelkel and Hu, 1992*). The success of this method for *in vivo*-derived embryos has paved the way for its commercial use on cattle herds worldwide.

Although slow freezing is considered the conventional method of storing *in vivo*-derived embryos, several studies have shown it to be inefficient at cryopreserving *in vitro*-produced bovine embryos (*Do et al., 2017; Enright et al., 2000; Mahmoudzadeh et al., 1994; Mucci et al., 2006; Nedambale et al., 2004; Yu et al., 2010*). Prevention of intracellular ice formation is a key factor in the survival of cryopreserved cells (*Mazur et al., 2005; Mazur et al., 2007*). Ice crystals forming during cooling and warming processes are the main cause of cell damage and death (*Lee et al., 2013; Mazur and Seki, 2011*). *Seki et al.* (*2014*) observed extracellular ice crystal formation initially during the slow freezing process. An additional difficulty of cryopreserving *in vitro*-produced bovine embryos is that they contain more lipid droplets in their cytoplasm than do their *in vivo* counterparts (*Abe et al., 1999; Pryor et al., 2011*), leading to greater susceptibility to the freezing process (*Pereira et al., 2008; Seidel, 2006*).

(b) Vitrification

Characterized by rapid cooling/warming rates and high concentrations of cyoprotectants (*Jin et al., 2008*), vitrification is an alternative procedure to slow freezing for *in vitro*-produced embryo cryopreservation. *Wowk* (*2010*) stated that cells – cryopreserved by slow freezing – survive in glass between ice crystals, while cells – cryopreserved by vitrification – survive in glass as the whole sample is vitrified. In fact, since vitrification has become the main cryopreservation method to store human embryos (*Vajta et al., 2015*), it has also been recommended for cryopreservation of *in vitro*-produced bovine embryos (*Mucci et al., 2006; Hasler, 2014; Taylor-Robinson et al., 2014*).

Vitrification is a technically simple method (*Do et al., 2016a; Rios et al., 2010*), which does not require the use of a programmable freezer (*Hasler, 2014; Sinha, 2009; Vajta and Nagy, 2006*). Laboratory experiments have indicated that *in vitro*-produced bovine embryos cryopreserved by vitrification achieve a better survival rate than do those cryopreserved by slow freezing (*Do et al., 2017; Gómez et al., 2008; Mucci et al., 2006; Yu et al., 2010*). Vitrification has not yet, however, achieved widespread application in commercial cattle production. This is because most embryos are produced by superovulation, and conventional slow freezing has already become a standard method to store *in vivo* embryos, while well over half of the transfers in both Europe and the USA are from frozen embryos (Table 1). In contrast, there is a smaller proportion of embryos produced by *in vitro* fertilization, especially in Europe (Table 1). Most fresh *in vitro*-produced embryos are transferred due to the lack of a reliable cryopreservation method (*Pontes et al.,*

2011), and of an efficient method that allows the directly transfer of those embryos after being thawed (*Sanches et al., 2016*).

Table 1. Commercial bovine embryo transfer activities in Europe and the USA in 2018 ***(the most recent date for which combined data are available)***

Region	Embryo types	Transferable embryos	Frozen embryos	Fresh embryo transfers	Frozen embryo transfers
* Europe	*In vivo*	141,371	N/A	52,344	70,190
	In vitro	36,832	N/A	13,928	13,233
† USA	*In vivo*	418,349	179,810	58,458	99,698
	In vitro	260,193	238,539	180,081	80,112

***** *Association of Embryo Technology in Europe annual data collection 2018*
† *American Embryo Transfer Association statistics committee report 2019 (2018 data)*

Limitations of current vitrification protocols

Cryobiologists have progressively modified vitrification procedures to achieve incremental improvement but shortcomings remain, and, unlike with the slow freezing technique, no standardized method has been agreed (*Leibo, 2008; Seidel, 2006*). There is a profusion of different vitrification protocols utilizing varied timings, temperatures and cryoprotectants, so the outcome is multifactorial. In addition, the operator has a choice of vitrification carriers (for example, plastic straw, electron microscope grid microdrop, open pulled straw, cryotop, cryohook), with drop-size and straw barriers affecting cooling and warming rates. Warming protocols can differ too, which adds further to the complexity of choice for an efficient vitrification method (*Vajta et al., 2015*); this is a particular concern when vitrified samples are exchanged between laboratories. However, perhaps the most influential factor to consider when developing a highly successful vitrification method is the skill of the operator. Although described as a simple method, not requiring slow-rate freezing machines, in our experience vitrification actually requires much more skill and dexterity of the operator compared to slow freezing (*Do et al., 2019*).

While vitrification appears more favourable than slow freezing, further investigation is required to compare its efficacy with the slow freezing method (*Sinha, 2009*). Moreover, although limited data on pregnancy rates are available (*Gutnisky et al., 2013; Kim et al., 2012; Nedambale et al., 2004*), the reported sample sizes were relatively small. For instance, *Gutnisky et al.* (*2013*) transferred vitrified embryos to recipients and obtained conception rates of 46.8%. However, the number of embryos transferred, 96, was modest and thus may not reflect the

potential efficacy of the vitrification method. Of 45 confirmed pregnancies 33 calves were born (34.3%) (*Gutnisky et al., 2013*), which is not a promising outcome under commercial conditions where the ultimate goal of an embryo transfer program is to achieve a high number of healthy calves (*Do et al., 2018a*). Previously, *Enright et al.* (*2000*) reported that abortion rates of surrogate cattle receiving *in vitro*-produced embryos are raised, and that care should be taken with pregnancy interpretation.

Considerable attempts aimed at improving the quality of *in vitro*-produced embryos before cryopreservation have been made in various ways, such as modification of *in vitro* culture systems (*Do et al., 2016b; Sanches et al., 2013; Sudano et al., 2011*), enhancement of the intrinsic capacity of embryos (*Filho et al., 2011*), and other means of pretreatment of embryos before vitrification (*Min et al., 2013*). Nonetheless, a significant advancement has so far proved elusive.

Impact of cooling rates

During slow rate cooling of embryos the temperature drops at a rate of approximately 0.5°C per minute to -30°C or -35°C, at which point they are plunged into liquid nitrogen (*Leibo, 2008*). For vitrification, by comparison, cooling rates of thousands of degrees Celsius are attained, such that a sample containing embryos can reach extremely rapidly a glassy state. Two crucial characteristics of vitrification are extreme cooling rates and high concentration of cryoprotective additives (*Seki and Mazur, 2014*).

Minimum volume vitrification theory pertains to the rate of cooling and vitrification carriers. When plastic straws are used as the principal carrier to vitrify and store embryos, their survival rates of embryos following vitrification process (*Enright et al., 2000*). For example, in all experiments in which 0.25 ml plastic mini-straws were used by *Palasz et al.* (*1997*), only around 20% of vitrified embryos were recovered. Hence, cryobiologists have aimed to establish a carrier that contains the minimum volume of vitrification solution needed to raise cooling rates. *Kuwayama* (*2007*) determined that this strategy prevents breakage of zonae pellucidae.

Vajta et al. (*1998*) pioneered a novel vitrification carrier – open pulled straw – made from a standard plastic straw (typically used in slow freezing of embryos) heat-softened and pulled to reduce its internal diameter so that 1-2 µl vitrification solution containing embryos loads spontaneously due to capillary action. Cooling rates as high as 25,000°C/min were recorded over a temperature range of -25°C to -175°C (*Vajta et al., 1998*). These compare favourably to 2,250°C/min, ten times less, when using a larger diameter plastic straw (5 µl, typically utilized for sperm freezing). More recently, *Malenko et al.* (*2017*) modified this protocol, replacing the plastic straw with a glass capillary tube. The heat-pulled conical tip can be moulded into a protective sheath that may be moved into either a closed or open position. Initial results for survival and hatching rates post-warming were

comparable to those gained using various vitrification carriers. Further research is required to achieve high and stable survival rates with different developmental stages of *in vitro*-produced bovine embryos.

Kuwayama (*2007*) described a purpose-built carrier, named Cryotop®, a narrow and thin strip of film affixed to a hard plastic holder. This simple device enables loading of < 0.1 µl volume of vitrification solution containing the sample. As a result, cooling rates of 69,250°C/min may be achieved (*Seki and Mazur, 2014*). However, *Rios et al.* (*2010*) suggested that despite the contention that minimum volume vitrification enhances thermal conductivity and cooling rates, it does not improve hatching rates of embryos upon vitrifying/warming and subsequent culture *in vitro* (*Rios et al., 2010*).

Impact of warming rates

Recent research has highlighted the significance of warming rates to cryopreservation because recrystallization influences vitrification outcomes (*Zhou et al., 2010*). This starts at -109°C during the warming process (*Lee et al., 2013*), whereupon devitrification can cause cryo-injury to the cell (*Sansinena et al., 2014*). *Hopkins et al.* (*2012*) observed that warming rates are closely associated with cooling rates due to the presence of ice fractions inside vitrified cells. In accord with this, *Seki et al.* (*2014*) reported that during the cooling process, small ice crystals form; this causes recrystallization. Water molecules move from smaller to larger crystal size, ultimately reaching a size that proves lethal to the cell.

Faster cooling rates therefore need more rapid warming rates in order to block recrystallization (*Mazur and Seki, 2011*). *Seki et al.* (*2014*) found that combining moderate cooling rates with high warming rates of 117,500°C/min can avert recrystallization. Although it is apparent that rapid warming allows cells to avoid devitrification, insufficient cryoprotectant concentrations can promote recrystallization during the warming process (*Jin et al., 2008*). In concurrence, *Seki et al.* (*2014*) suggested that the short time, 1-2 minutes, for which embryos are exposed to equilibration solution is not enough for permeable solutes to penetrate cells to replace water molecules (by dehydration).

Morató and Mogas (*2014*) showed that keeping the warming solution of vitrified embryos at 45°C facilitates an improved survival rate compared to at 50°C, 60°C or 70°C. Corroborating this, *Caamaño et al.* (*2015*) considered that 41°C is the most suitable temperature to warm vitrified embryos.

Pros and cons of cryoprotectants

In cryobiology cryoprotectants perform a key function in preventing cryo-damage to cells. Permeating and non-permeating cryoprotectants are used frequently to cryopreserve gametes and embryos. Replacing intracellular water with a permeating compound protects a cell from ice crystals forming; the cell is then rehydrated upon warming (*Palasz and Mapletoft, 1996*). Low molecular

weight, non-electrolyte cryoprotectants penetrate cells at different rates to replace cytoplasmic water (*Leibo, 2008*). Embryos are equilibrated with each of the five most common permeable cryoprotectants – dimethyl sulfoxide (Me_2SO), glycerol, EG, methanol and propylene glycol – at half the strength of the final vitrification solution. For instance, using a Cryotop® embryos are first exposed to an equilibration solution containing 7.5% v/v EG and 7.5% v/v Me_2SO, followed by a vitrification solution (15% v/v EG and 15% v/v Me_2SO).

Permeability of cells varies with the type of cryoprotectant. EG is more permeable to bovine embryos than is glycerol (*Morató and Mogas, 2014*), so EG is typically the cryoprotectant of choice in the field using a one-step dilution (*Dochi et al., 1998*). A high concentration of cryoprotectant is required to prevent intracellular ice crystal formation (*Seki et al., 2014*), and to attain vitrification (*Lawson et al., 2012*). However, this risks causing osmotic shock to cells (*Morató and Mogas, 2014*). *Vanderzwalmen et al.* (*2013*) argued that high concentrations of cryoprotectant intracellularly and extracellularly are necessary to minimize embryo swelling, which is a consequence of water permeation during the warming process. Therefore, non-permeable cryoprotectants such as the sugars sucrose and trehalose are used to reduce osmotic stress. While cryoprotectants preserve the integrity of cellular organelles during cooling and warming (*Dobrinsky, 2002*), their toxic characteristics may be detrimental (*Fahey, 2010; Wu et al., 2013*).

Toxicity is a major drawback of cryopreservation regardless of whether slow freezing or vitrification is used, so lessening the effects on cell viability should be an ongoing priority of cryopreservation methods. In regard to bovine assisted reproduction, it is a commonly held belief that exposure to a high concentration of cryoprotectant during vitrification may be harmful to embryos. However, *Vanderzwalmen et al.* (*2013*) maintained that intracellular levels of cryoprotectant in vitrified murine embryos are significantly lower than those detected within slow frozen embryos. In supporting this perspective, *Do et al.* (*2018b*) indicated that vitrification may not be toxic to bovine embryos since no miscarriage is observed among recipients that are implanted with vitrified *in vitro*-derived embryos.

Impact of cytoplasmic lipid droplets

The presence of cytoplasmic lipid droplets in embryos is suggested to have no apparent effect on their viability after cryopreservation (*Block et al., 2010*); however, it is also argued that the survivability of cryopreserved embryos is related to their lipid contents (*Cagnone and Sirard, 2014; Seidel, 2006*). *Pryor et al.* (*2011*) and *Seidel* (*2006*) identified a correlation between lipid droplets and embryo cryotolerance. Furthermore, *Cagnone and Sirard* (*2014*) noted that *in vitro* culture affects the embryonic lipid content of embryos and their subsequent cryo-survival. Previously, in a preliminary study *Abe et al.* (*1999*) had discovered that the sensitivity of *in vitro* embryos to cryopreservation is due to the substantial cytoplasmic accumulation of lipid droplets. Embryos cultured in serum-free

medium contain predominantly lipid droplets of < 2 μm; in contrast, embryos cultured in serum-supplemented medium have lipid droplets > 62 μm. Such large lipid droplets were frequently observed in morulae and blastocysts cultured in media containing 5% v/v calf serum (*Abe et al., 1999*). The lipid content of *in vitro*-produced embryos cultured in a serum-free medium was similar to their *in vivo*-derived counterparts, but the lipid volume in embryos cultured with serum was almost double that of *in vivo* embryos (*Ferguson and Leese, 1999*).

The abnormal accumulation of lipid in the cytoplasm of *in vitro* embryos resulting from culture together with serum is the main factor that makes them unsuitable for cryopreservation (*Pereira et al., 2008*). *Mucci et al.* (*2006*) contended that raised numbers of lipid droplets in an embryo affect its cellular repair after cryopreservation. Moreover, *Sudano et al.* (*2011*) maintained that high concentration of fetal calf serum in *in vitro* culture media causes a rise in accumulated lipid in blastocysts, and in apoptosis, but reduces expansion of blastocoels after vitrification.

Vitrification of embryos at various developmental stages

Vitrification of embryos at different stages is controversial (*Asgari et al., 2012*). Cryopreservation of the blastocyst stage enables immediate embryo transfer after warming and to skip the repeated *in vitro* culture of embryos; moreover, advanced embryos can overcome the period of genome embryonic transition that occurs at or between the fourth and fifth cell cycle in bovine embryos (*Aono et al., 2013; Garcia-Garcia et al., 2006*). *Asgari et al.* (*2012*) revealed that a large number of 5-8 cell stage bovine embryos were arrested at the morula stage due to the negative effect of cryopreservation on the embryonic activation transition period. *Shirazi et al.* (*2009*) showed that early blastocysts have a greater cryo-tolerance than do expanded ones. However, *Sommerfeld and Niemann* (*1999*) observed that expanded blastocysts survive vitrification better than do non-expanded blastocysts; hatching rates of 42% and 12%, respectively. On the other hand, early stage embryo cryopreservation delays the *in vitro* embryo production procedure. Hence, it is important that after warming these embryos are cultured further *in vitro* to develop into blastocysts at which point they can be used as fresh embryos.

Direct transfer of vitrified *in vitro*-produced bovine embryos

Most vessels used for vitrification are not designed specifically for this purpose so they are ill suited to the direct transfer of embryos with a standard 0.25 mL plastic mini-straw (*Ha et al., 2014; Inaba et al., 2011*). Furthermore, several steps are taken when warming in order to gradually reduce the cryoprotectants left over from placing embryos in equilibrium and vitrification media before vitrification. Simplification of this process through a single warming step is required to enable the direct transfer of vitrified bovine embryos. It is thought that

warming by single or multiple steps achieves comparable results (*Caamaño et al., 2015; Morató and Mogas, 2014*). *Vajta et al.* (*1999*) designed an in-straw dilution procedure whereby a French mini-straw is loaded with holding medium and 0.2 M sucrose; however, this requires a skilled operator (*Vieira et al., 2007*). Another drawback of this modification is embryo loss during warming. *Vieira et al.* (*2007*) considered that unintentional warming in air for a few seconds before an embryo is loaded possibly reduces the warming rate, which is a critical factor in its survival (*Mazur and Seki, 2011*). Although direct transfer has provided promising results (*Caamaño et al., 2015; Morató and Mogas, 2014; Vieira et al., 2007*), the loss of embryos, evaluated by transfer, is limiting to successful outcomes.

Refined techniques to enhance cryopreservation outcomes

Prior to vitrification of *in vitro*-fertilized embryos micro-manipulation could increase their survival rates following cryopreservation. As an example, it is suggested that dehydrating blastocysts sufficiently before vitrification, through artificially collapsing the blastocoel, increases their implantation potential (*Liebermann et al., 2012*). By using this technique *Min et al.* (*2013*) observed improved survival rates of bovine *in vitro* embryos and cloned embryos after post-warming and incubation for a further 24 hours (*Min et al., 2013*). The survival of *in vitro* embryos following blastocoel forced treatments was significantly greater than for untreated controls (81.9% versus 69.8%). However, this technique is complicated due to time constraints and requires a highly skilled embryologist. *Pryor et al.* (*2011*) used a laser-assisted hatching method in which a laser micro-beam drills a hole in the zona pellucida surrounding the embryo, through which lipid droplets can exit the cytoplasm. While technically feasible, this appears a complex method.

Enhancing the intrinsic characteristics of *in vitro*-fertilized bovine embryos is another way to raise their quality prior to cryopreservation. According to *Pribenszky et al.* (*2010*) and to *Pribenszky and Vajta* (*2011*), exposing gametes and embryos to sublethal hydrostatic pressure elevates their intrinsic developmental competence to overcome physiological stress such as experienced during cooling. This improved cell performance may be due to high hydrostatic pressure causing a minimum stressful condition under which cells can synthesize heat shock proteins (*Díez et al., 2012; Pribenszky et al.; 2008*). In support of this, pretreating bovine blastocysts with high hydrostatic pressure, *Filho et al.* (*2011*) reported that re-expansion and hatching rates of treated embryos are significantly higher than those of untreated embryos. However, pregnancy rates were not detailed. This promising approach to enhancing the survivability of embryos requires further investigation.

Cryopreservation-related biosafety considerations

Biosafety is of paramount importance when importing and exporting bovine embryos internationally. Therefore, the threat posed by cryopreserved embryos of

pathogen transmission demands evaluation. The two systems of vitrification, closed and open, present different risks. Theoretically at least, there is a reduced risk of microbial contamination using a closed vitrification system, as for slow freezing in straws, compared to methods that require direct contact of embryos with liquid nitrogen (*Vajta et al., 2015*). It has been argued that it is a false assumption to consider liquid nitrogen or cryopreserved samples to be sterile (*Morris, 2005*). *Mirabet et al.* (*2012*) indicated that some viruses can survive at subzero temperatures without the need for a cryoprotectant or cryopreservation. For instance, *Bielanski et al.* (*2000*) investigated possible contamination from liquid nitrogen of three species of virus; bovine viral diarrhea virus, bovine herpesvirus and bovine immunodeficiency virus. Unsealed carriers tested either positive or negative for these viruses but no sealed source was contaminated. While 32 bacterial and one fungal species were identified in various liquid nitrogen cannisters storing semen and embryos for between 6-35 years, bovine viral diarrhea virus and bovine herpesvirus were not detected in clean semen and embryo straws kept alongside infected straws (*Bielanski et al., 2003*). Nonetheless, it is not easy to maintain long-term cleanliness of samples that are contained in the same liquid nitrogen cannister as unclean ones.

Despite the apparent low risk of microbial contamination of samples through contact with liquid nitrogen, it remains a potential hazard (*Vajta and Kuwayama, 2006*). An open system achieves sufficiently high cooling rates, but a closed system may not; hence, striking a balance between technical and sterility considerations presents a challenge to the operator. For example, *Yu et al.* (*2010*) used closed pulled straw in preference to open pulled straw to negate any threat of contamination. However, the survival rates of vitrified embryos were modest.

Conclusion

It is evident that formation of intracellular ice crystals during cooling and warming processes is the main cause of damage to cryopreserved cells. Slow freezing and vitrification both aim to reduce to a minimum, if not prevent, ice accumulation. While cryoprotectants are necessary to protect cells from chilling they are possibly cytotoxic. Although slow freezing and vitrification are each efficient for *in vivo*-derived bovine embryo cryopreservation, slow freezing may not be suited to cryopreserving *in vitro*-produced bovine embryos. This is perhaps due to the cytoplasmic lipid content of such embryos being greater than that of their *in vivo* counterparts, although improved culture conditions may mitigate this effect. Collective research indicates the advantages of vitrification as the method of choice for storing *in vitro*-produced embryos. A better understanding of cooling and warming processes, cryoprotectants, modifications to *in vitro* culture media, and to treatment of cells prior to cryopreservation is each necessary in order to improve outcomes. Furthermore, embryo biosafety should be safeguarded.

Vitrification appears a superior procedure to slow freezing for cryopreserving *in vitro*-produced bovine embryos, yet non-standardization of methods between laboratories and, consequently, inconsistent results currently hamper its applicability to the international trade in cattle genetics and the management of embryo biobanks (*Mogas, 2018*). Thus, it is important to optimize *in vitro* production of blastocysts with fewer lipid droplets, together with identifying the most efficient means to vitrify *in vitro*-produced bovine embryos. In turn, such technical advancements will both contribute to a greater understanding of bovine cryobiology and facilitate increased use of *in vitro* embryos in commercial livestock settings.

Krioprezervacija in vitro dobijenih goveđih embriona vitrifikacijom: u potrazi za pojednostavljenim, standardizovanim postupkom koji poboljšava stope graviditeta/steonosti radi unapređenja govedarstva

Van Huong Do, Andrew W. Taylor-Robinson

Rezime

Cilj krioprezervacije je zadržati prvobitnu fazu polnih ćelija i embriona nakon što izdrže hlađenje i zagrevanje. Polako zamrzavanje je standardni metod za krioprezervaciju goveđih embriona in vivo, pri čemu se tri petine takvih embriona globalno zamrzava ovom metodom. Međutim, očigledno je da sporo zamrzavanje nije efikasno za krioprezervaciju goveđih embriona proizvedenih in vitro. Dakle, samo jedna trećina goveđih embriona proizvedenih in vitro je krio-konzervirana. Vitrifikacija je poželjna metoda za skladištenje ljudskih embriona; shodno tome, istražena je kao novo sredstvo za čuvanje in vitro proizvedenih goveđih embriona, za koje se pokazala kao značajna alternativa sporom zamrzavanju, i to iz nekoliko razloga: vitrifikacija često zahteva manje vremena nego sporo zamrzavanje, nisu potrebne skupe mašine za zamrzavanje sa sporim tempom, i dokazano je da ima relativno veće stope preživljavanja. Ipak, u stočarstvu/govedarstvu, vitrifikacija i dalje pokazuje nedostatke, kao što su moguća toksičnost rastvora za vitrifikaciju i neuspeh u standardizaciji metoda, što predstavlja izazov za njegovu primenu na in vitro proizvedene embrione goveda. Zbog toga je određivanje najprikladnijeg postupka presudno kako bi se vitrifikacija učinila praktičnijom u komercijalnim uslovima.

Ključne reči: goveda, krioprezervacija, krioprotektant, embrion, sporo zamrzavanje, vitrifikacija.

Acknowledgements

We warmly thank Dr. Sally Catt (Monash University, Clayton, VIC, Australia) and Mr Simon Walton (Australian Reproductive Technologies (Mt Chalmers, QLD, Australia) for fruitful discussions on technical aspects of assisted bovine reproduction. No specific funding was provided for the preparation of this review. Financial support for our published work that is cited herein was provided by Central Queensland University and Australian Reproductive Technologies.

Author Contributions

VHD conceptualized the paper, which was developed further in discussion with AWTR. Both authors collated articles for review, wrote and critically reviewed various drafts, contributed to the preparation of the final version and provided consent for submission.

Conflicts of Interest

The authors declare no conflicts of interest.

References

ABE H., YAMASHITA S., ITOH T., SATOH T., HOSHI H. (1999): Histochemical and ultrastructural evaluations of cytoplasmic lipid droplets in bovine embryos cultured in serum-free and serum-containing media. Theriogenology, 51, 1, 232. [Abstract] doi:10.1016/S0093-691X(99)91791-0

AMERICAN EMBRYO TRANSFER ASSOCIATION STATISTICS COMMITTEE REPORT 2019 (2018 DATA). Available at: https://www.aeta.org/docs/2018_Stats.pdf

AONO A., NAGATOMO H., TAKUMA T., NONAKA R., ONO Y., WADA Y., ABE Y., TAKAHASHI M., WATANABE T., KAWAHARA M. (2013): Dynamics of intracellular phospholipid membrane organization during oocyte maturation and successful vitrification of immature oocytes retrieved by ovum pick-up in cattle. Theriogenology, 79, 8, 1146-1152.

ASGARI V., HOSSEINI S.M., FOROUZANFAR M., HAJIAN M., NASR-ESFAHANI M.H. (2012): Vitrification of *in vitro* produced bovine embryos: effect of embryonic block and developmental kinetics. Cryobiology, 65, 3, 278-283.

ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE ANNUAL DATA COLLECTION 2018. Available at: https://www.aete.eu/publications/statistics/

BIELANSKI A., NADIN-DAVIS S., SAPP T., LUTZE-WALLACE C. (2000): Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology, 40, 2, 110-116.
BIELANSKI A., BERGERON H., LAU P.C.K., DEVENISH J. (2003): Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology, 46, 2, 146-152.
BLOCK J., BONILLA L., HANSEN P.J. (2010): Efficacy of *in vitro* embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium. Journal of Dairy Science, 93, 11, 5234-5242.
CAAMAÑO J.N., GÓMEZ E., TRIGAL B., MUÑOZ M., CARROCERA S., MARTÍN D., DÍEZ C. (2015): Survival of vitrified *in vitro*-produced bovine embryos after a one-step warming in-straw cryoprotectant dilution procedure. Theriogenology, 83, 5, 881-890.
CAGNONE G., SIRARD M.A. (2014): The impact of exposure to serum lipids during *in vitro* culture on the transcriptome of bovine blastocysts. Theriogenology, 81, 5, 712-722.
DÍEZ C., MUÑOZ M., CAAMAÑO J.N., GÓMEZ E. (2012): Cryopreservation of the bovine oocyte: current status and perspectives. Reproduction in Domestic Animals, 47 (3 Suppl.), 76-83.
DO V.H., WALTON S., CATT S., TAYLOR-ROBINSON A.W. (2016a): Requirements for cryopreservation of *in vitro*-produced bovine embryos by a standard method of vitrification. Journal of Veterinary Science & Animal Husbandry, 4, 1, 102.
DO V.H., WALTON S., TAYLOR-ROBINSON A.W. (2016b): Improvements to *in vitro* culture media for use in bovine IVF. Journal of Veterinary Science & Animal Husbandry, 4, 2, 205.
DO V.H., WALTON S., CATT S., TAYLOR-ROBINSON A.W. (2017): A comparative analysis of the efficacy of three cryopreservation protocols on the survival of *in vitro*-derived cattle embryos at pronuclear and blastocyst stages. Cryobiology, 77, 58-63.
DO V.H., CATT S., WALTON S., TAYLOR-ROBINSON A.W. (2018a): Development rate of *in vitro*-derived cattle embryos does not affect their survival or hatching rate following recovery from cryopreservation by vitrification. Animal Production Science, 58, 8, 2502.
DO V.H., CATT S., AMAYA G., BATSIOKIS M., WALTON S., TAYLOR-ROBINSON A.W. (2018b): Comparison of pregnancy when non-vitrified and vitrified *in vitro*-derived embryos are transferred into recipients. Theriogenology, 120, 105-110.
DO V.H., CATT S., KINDER J.E., WALTON S., TAYLOR-ROBINSON A.W. (2019): Vitrification of *in vitro*-derived cattle embryos: targeting enhancement of quality by refining technology and standardizing procedures. Reproduction, Fertility and Development, 31, 5, 837-846.

DOBRINSKY J.R. (2002): Advancements in cryopreservation of domestic animal embryos. Theriogenology, 57, 1, 285-302.
DOCHI O., IMAI K., TAKAKURA H. (1995): Birth of calves after direct transfer of thawed bovine embryos stored frozen in ethylene glycol. Animal Reproduction Science, 38, 3, 179-185.
DOCHI O., YAMAMOTO Y., SAGA H., YOSHIBA N., KANO N., MAEDA J., MIYATA K., YAMAUCHI A., TOMINAGA K., ODA Y., NAKASHIMA T., INOHAE S. (1998): Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. Theriogenology, 49, 5, 1051-1058.
ENRIGHT B.P., LONERGAN P., DINNYES A., FAIR T., WARD F.A., YANG X., BOLAND M.P. (2000): Culture of *in vitro* produced bovine zygotes *in vitro* vs *in vivo:* implications for early embryo development and quality. Theriogenology, 54, 5, 659-673.
FAHY G.M. (2010): Cryoprotectant toxicity neutralization. Cryobiology, 60 (3 Suppl.), S45-53.
FERGUSON E.M., LEESE H.J. (1999): Triglyceride content of bovine oocytes and early embryos. Journal of Reproduction and Fertility, 116, 2, 373-378.
FILHO E.S., CAIXETA E.S., PRIBENSZKY C., MOLNAR M., HORVATH A., HARNOS A., FRANCO M.M., RUMPF R. (2011): Vitrification of bovine blastocysts pretreated with sublethal hydrostatic pressure stress: evaluation of post-thaw *in vitro* development and gene expression. Reproduction, Fertility and Development, 23, 4, 585-590.
GARCIA-GARCIA R.M., GONZALEZ-BULNES A., DOMINGUEZ V., VEIGA-LOPEZ A., COCERO M.J. (2006): Survival of frozen-thawed sheep embryos cryopreserved at cleavage stages. Cryobiology, 52, 1, 108-113.
GÓMEZ E., RODRÍGUEZ A., MUÑOZ M., CAAMAÑO J.N., HIDALGO C.O., MORÁN E., FACAL N., DÍEZ C. (2008): Serum free embryo culture medium improves *in vitro* survival of bovine blastocysts to vitrification. Theriogenology, 69, 8, 1013-1021.
GUTNISKY C., ALVAREZ G.M., CETICA P.D., DALVIT G.C. (2013): Evaluation of the Cryotech Vitrification Kit for bovine embryos. Cryobiology, 67, 3, 391-393.
HA A.N., LEE S.R., JEON J.S., PARK H.S., LEE S.H., JIN J.I., SESSIONS B.R., WANG Z., WHITE K.L., KONG I.K. (2014): Development of a modified straw method for vitrification of *in vitro*-produced bovine blastocysts and various genes expression in between the methods. Cryobiology, 68, 1, 57-64.
HASLER J.F. (2001): Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. Theriogenology, 56, 9, 1401-1415.
HASLER J.F. (2014): Forty years of embryo transfer in cattle: a review focusing on the journal *Theriogenology*, the growth of the industry in North America, and personal reminisces. Theriogenology, 81, 1, 152-169.

HOPKINS J.B., BADEAU R., WARKENTIN M., THORNE R.E. (2012): Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions. Cryobiology, 65, 3, 169-178.
INABA Y., AIKAWA Y., HIRAI T., HASHIYADA Y., YAMANOUCHI T., MISUMI K., OHTAKE M., SOMFAI T., KOBAYASHI S., SAITO N., MATOBA .S, KONISHI K., IMAI K. (2011): In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. Journal of Reproduction and Development, 57, 4, 437-543.
JIN B., KUSANAGI K., UEDA M., SEKI S., VALDEZ D.M. JR, EDASHIGE K., KASAI M. (2008): Formation of extracellular and intracellular ice during warming of vitrified mouse morulae and its effect on embryo survival. Cryobiology, 56, 3, 233-240.
KIM Y.M., UHM S.J., GUPTA M.K., YANG J.S., LIM J.G., DAS Z.C., HEO Y.T., CHUNG H.J., KONG I.K., KIM N.H., LEE H.T., KO D.H. (2012): Successful vitrification of bovine blastocysts on paper container. Theriogenology, 78, 5, 1085-1093.
KLEINHANS F.W., MAZUR P. (2009): Determination of the water permeability (Lp) of mouse oocytes at -25^{o}C and its activation energy at subzero temperatures. Cryobiology, 58, 2, 215-224.
KUWAYAMA M. (2007): Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. Theriogenology, 67, 1, 73-80.
LAWSON A., MUKHERJEE I.N., SAMBANIS A. (2012): Mathematical modeling of cryoprotectant addition of engineered or natural tissues. Cryobiology, 64, 1, 1-11.
LEE K.H., SUN J.C., CHUANG C., GUO S.F., TU C.F., JU J.C. (2013): An efficient and mass reproducible method for vitrifying mouse embryos on a paper in cryotubes. Cryobiology, 66, 3, 311-317.
LEIBO S.P. (2008): Cryopreservation of oocytes and embryos: Optimization by theoretical versus empirical analysis. Theriogenology, 69, 1, 37-47.
LIEBERMANN J., PELTS E.J., MATTHEWS J.M., SANCHEZ S.R, RAPISARDA J., LEDERER K. (2012): Does artificial collapse of human day 6 blastocysts prior to the cooling steps of vitrification improve their probability of increased outcome? Fertility and Sterility, 98, (3 Suppl.), S127. [Abstract]. https://doi.org/10.1016/j.fertnstert.2012.07.469
LOPATAROVA M., CECH S., KRONTORAD P., HOLY L., LALOVA H., DOLEZEL R. (2010): Conception rate after sex determination and cryopreservation of D7 bovine embryos. Veterinarni Medicina, 55, 1, 10-18.
MAHMOUDZADEH A.R., VAN SOOM A., YSEBAERT M.T., DE KRUIF A. (1994): Comparison of two-step vitrification versus controlled freezing on survival of *in vitro* produced cattle embryos. Theriogenology, 42, 8, 1389-1397.
MALENKO G.P., KORNIENKO E.V., NESTEROV I.V., KOSOVSKY G.Y. (2017): A new simple and reliable vitrification device based on Hollow Fiber

Vitrification (HFV) method evaluated using IVP bovine embryos. Animal Reproduction, 14, 2, 392-399.
MAZUR P., SEKI S. (2011): Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to 70,000°C/min and warmed at 610° to 118,000°C/min: A new paradigm for cryopreservation by vitrification. Cryobiology, 62, 1, 1-7.
MAZUR P., SEKI S., PINN I.L., KLEINHANS F.W., EDASHIGE K. (2005): Extra- and intracellular ice formation in mouse oocytes. Cryobiology, 51, 1, 29-53.
MAZUR P., PINN I.L., KLEINHANS F.W. (2007): Intracellular ice formation in mouse oocytes subjected to interrupted rapid cooling. Cryobiology, 55, 2, 158-166.
MERTON J.S., ASK B., ONKUNDI D.C., MULLAART E., COLENBRANDER B., NIELEN M. (2009): Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up *in vitro* production embryo-production program. Theriogenology, 72, 7, 885-893.
MIN S.H., LEE E., SON H.H., YEON J.Y., KOO D.B. (2013): Forced collapse of the blastocoel enhances survival of cryotop vitrified bovine hatching/hatched blastocysts derived from *in vitro* fertilization and somatic cell nuclear transfer. Cryobiology, 66, 2, 195-199.
MIRABET V., ALVAREZ M., SOLVES P., OCETE D., GIMENO C. (2012): Use of liquid nitrogen during storage in a cell and tissue bank: contamination risk and effect on the detectability of potential viral contaminants. Cryobiology, 64, 2, 121-123.
MOGAS T. (2018): Update on the vitrification of bovine oocytes and *in vitro*-produced embryos. Reproduction, Fertility and Development, 31, 1, 105-117.
MORATÓ R., MOGAS T. (2014): New device for the vitrification and in-straw warming of *in vitro* produced embryos. Cryobiology, 68, 2, 288-293.
MORRIS G.J. (2005): The origin, ultrastructure, and microbiology of the sediment accumulating in liquid nitrogen storage vessels. Cryobiology, 50, 3, 231-238.
MUCCI N., ALLER J., KAISER G.G., HOZBOR F., CABODEVILA J., ALBERIO R.H. (2006): Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. Theriogenology, 65, 8, 1551-1562.
NEDAMBALE T.L., DINNYES A., GROEN W., DOBRINSKY J.R., TIAN X.C., YANG X. (2004): Comparison on *in vitro* fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. Theriogenology, 62, 3-4, 437-449.
PALASZ A.T., MAPLETOFT R.J. (1996): Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnology Advances, 14, 2, 127-149.
PALASZ A.T., GUSTAFSSON H., RODRIGUEZ-MARTINEZ H., GUSTA L., LARSSON B., MAPLETOFT R.J. (1997): Vitrification of bovine IVF blastocysts in an ethylene glycol/sucrose and heat-stable plant-extracted proteins. Theriogenology, 47, 4, 865-879.

PEREIRA R.M., CARVALHAIS I., PIMENTA J., BAPTISTA M.C., VASQUES M.I., HORTA A.I., SANTOS I.C., MARQUES M.R., REIS A., PEREIRA M.S., MARQUES C.C. (2008): Biopsied and vitrified bovine embryos viability is improved by trans10 and cis12 conjugated linoleic acid supplementation during *in vitro* embryo culture. Animal Reproduction Science, 106, 3-4, 322-332.

PONTES J.H.F., NONATO-JUNIOR I., SANCHES B.V., ERENO-JUNIOR J.C., UVO S., BARREIROS T.R.R., OLIVEIRA J.A., HASLER J.F., SENEDA M.M. (2009): Comparison of embryo yield and pregnancy rate between *in vivo* and *in vitro* methods in the same Nelore (*Bos indicus*) donor cows. Theriogenology, 71, 4, 690-697.

PONTES J.H.F., MELO STERZA F.A., BASSO A.C., FERREIRA C.R., SANCHES B.V., RUBIN K.C.P., SENEDA M.M. (2011): Ovum pick up, *in vitro* embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. Theriogenology, 75, 9, 1640-1646.

PRIBENSZKY C., VAJTA G. (2011): Cells under pressure: how sublethal hydrostatic pressure treatment increases gametes and embryos performance. Reproduction, Fertility and Development, 23, 1, 48-55.

PRIBENSZKY C.S., DU Y., MOLNÁR M., HARNOS A., VAJTA G. (2008): Increased stress tolerance of matured pig oocytes after high hydrostatic pressure treatment. Animal Reproduction Science, 106, 1-2, 200-207.

PRIBENSZKY C., VAJTA G., MOLNAR M., DU Y., LIN L., BOLUND L., YOVICH J. (2010): Stress for stress tolerance? A fundamentally new approach in mammalian embryology. Biology of Reproduction, 83, 5, 690-697.

PRYOR J.H., LOONEY C.R., ROMO S., KRAEMER D.C., LONG C.R. (2011): Cryopreservation of *in vitro* produced bovine embryos: effects of lipid segregation and post-thaw laser assisted hatching. Theriogenology, 75, 1, 24-33.

RIOS G.L., MUCCI N.C., KAISER G.G., ALBERIO R.H. (2010): Effect of container, vitrification volume and warming solution on cryosurvival of *in vitro*-produced bovine embryos. Animal Reproduction Science, 118, 1, 19-24.

SANCHES B.V., MARINHO L.S.R., FILHO B.D.O., PONTES J.H.F., BASSO A.C., MEIRINHOS M.L.G., SILVA-SANTOS K.C., FERREIRA C.R., SENEDA M.M. (2013): Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forkolin before vitrification. Theriogenology, 80, 4, 372-377.

SANCHES B.V., LUNARDELLI P.A., TANNURA J.H., CARDOSO B.L., PEREIRA M.H.C., GAITKOSKI D., BASSO A.C., ARNOLD D.R., SENEDA M.M. (2016): A new direct transfer protocol for cryopreserved IVF embryos. Theriogenology, 85, 6, 1147-1151.

SANSINENA M., SANTOS M.V., TAMINELLI G., ZARITKY N. (2014): Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos. Theriogenology, 82, 3, 373-378.

SEIDEL G.E. (2006): Modifying oocytes and embryos to improve their cryopreservation. Theriogenology, 65, 1, 228-235.

SEKI S., JIN B., MAZUR P. (2014): Extreme rapid warming yields high functional survivals of vitrified 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to -196^{o}C. Cryobiology, 68, 3, 71-78.

SHIRAZI A., NAZARI H., AHMADI E., HEIDARI B., SHAMS-ESFANDABADI N. (2009): Effect of culture system on survival rate of vitrified bovine embryos produced *in vitro*. Cryobiology, 59(3), 285-290.

SINHA S. (2009): Role of cryopreservation in assisted reproductive technology (ART). Apollo Medicine, 6, 3, 212-221.

SOMMERFELD V., NIEMAN H. (1999): Cryopreservation of bovine *in vitro* produced embryos using Ethylene Glycol in controlled freezing or vitrification. Cryobiology, 38, 2, 95-105.

SUDANO M.J., PASCHOAL D.M., RASCADO T.S., MAGALHAES L.C.O., CROMOCO L.F., LIMA-NETO J.F., LANDIM-ALVARENGA F.C. (2011): Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology, 75, 7, 1211-1220.

TAYLOR-ROBINSON A.W., WALTON S., SWAIN D.L., WALSH K.B., VAJTA G. (2014): The potential for modification in cloning and vitrification technology to enhance genetic progress in beef cattle in Northern Australia. Animal Reproduction Science, 148, 3-4, 91-96.

VAJTA G., KUWAYAMA M. (2006): Improving cryopreservation system. Theriogenology, 65, 1, 236-244.

VAJTA G., NAGY Z.P. (2006): Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reproductive BioMedicine Online, 12, 6, 779-796.

VAJTA G., HOLM P., KUWAYAMA M., BOOTH P.J., JACOBSEN H., GREVE T., CALLESEN H. (1998): Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Molecular Reproduction and Development, 51, 1, 53-58.

VAJTA G., MURPHY C.N., MACHÁTY Z., PRATHER R.S., GREVE T., CALLESEN H. (1999): In-straw dilution of bovine blastocysts after vitrification with the open-pulled straw method. Veterinary Record, 144, 7, 180-181.

VAJTA G., RIENZI L., UBALDI F.M. (2015): Open versus closed systems for vitrification of human oocytes and embryos. Reproductive BioMedicine Online, 30, 4, 325-333.

VANDERZWALMEN P., CONNAN D., GROBET L., WIRLEITNER B., REMY B., VANDERZWALMEN S., ZECH N., ECTORS F.J. (2013): Lower intracellular concentration of cryoprotectant (ICCP) after vitrification (VIT) than slow freezing (SF) despite exposure to high concentration of cryoprotectant solutions (CPS). Human Reproduction, 28, 8, 2101-2110.

VAN WAGTENDONK-DE LEEUW A.M., DEN DAAS J.H.G., RALL W.F. (1997): Field trial to compare pregnancy rates of bovine embryo cryopreservation methods:

vitrification and one-step dilution versus slow freezing and three-step dilution. Theriogenology, 48, 7, 1071-1084.
VIANA J., THE INTERNATIONAL EMBRYO TRANSFER SOCIETY DATA RETRIEVAL COMMITTEE (2019): 2018 statistics of embryo collection and transfer in domestic farm animals. Available at: https://www.iets.org/pdf/comm_data/IETS_Data_Retrieval_Report_2018.pdf
VIEIRA A.D., FORELL F., FELTRIN C., RODRIGUES J.L. (2007): In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes. Animal Reproduction Science, 99, 3-4, 377-383.
VOELKEL S.A., HU X.Y. (1992): Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen-thawed embryos to recipient females. Theriogenology, 37, 3, 687-697.
WOWK B. (2010): Thermodynamic aspects of vitrification. Cryobiology 60, 1: 11-22.
WU G., JIA B., MO X., LIU C., FU X., ZHU S., HOU Y. (2013): Nuclear maturation and embryo development of porcine oocytes vitrified by cryotop: effect of different stages of *in vitro* maturation. Cryobiology, 67, 1, 95-101.
YU X.L., DENG W., LIU F.J., LI Y.H., LI X.X., ZHANG Y.L., ZAN L.S. (2010): Closed pulled straw vitrification of *in vitro*-produced and *in vivo*-produced bovine embryos. Theriogenology, 73, 4, 474-479.
ZHOU X.L., AL NAIB A., SUN D.W., LONERGAN P. (2010): Bovine oocyte vitrification using the Cryotop method: effect of cumulus cells and vitrification protocol on survival and subsequent development. Cryobiology, 61, 1, 66-72.

Received 12 August 2020; accepted for publication 9 September 2020