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Requirements for Cryopreservation of *In Vitro*-Produced Bovine Embryos by a Standard Method of Vitrification

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Abstract

In relation to assisted mammalian reproduction, the goal of cryopreservation is to preserve without significant loss of viability a stock of gametes and/or embryos with a view to thawing those cells for use in *in vitro* reproduction treatments. There are numerous cryopreservation protocols, which vary in terms of cryoprotectant used, storage temperature, freezing and thawing rates, and the particular cells that they are suitable for preserving. Although slow freezing has become a standard method for *in vivo* bovine embryo cryopreservation, it seems not to be efficient for preserving *in vitro*-produced (IVP) bovine embryos. Over the past decade, vitrification, a process of glass-like solidification, has become the cryopreservation method of choice for human oocytes and embryos. This is because it is often less time-consuming than slow freezing, does not require expensive 'slow rate' freezing machines, and has been proven to increase survival rates compared to slow frozen embryos. In contrast to clinical applications, in the cattle industry vitrification presents shortcomings, especially when applied to IVP embryos. A high concentration of cryoprotectant in solution is needed to induce vitrification. However, if left too long with metabolizing embryos, cryoprotectants can become toxic. Failure to standardize vitrification protocols leads to inconsistent results between laboratories, making application less practical in field settings. Therefore, determination of the most suitable vitrification method is important to advance its routine commercial use. Moreover, simplification of the vitrification procedure through development of an in-straw dilution without the use of a microscope may help to expand the use of vitrification methods on the farm.

Keywords: Cryopreservation; Slow freezing; Vitrification; Cryoprotectant; Bovine

Introduction

According to Leibo [1], cryopreservation is an essential component of the embryo transfer industry in cattle that, as Hasler points out [2], is now an established commerce on a global scale. The standard method of bovine embryo cryopreservation, slow freezing, was refined over a period of 40 years of research whereas the alternative vitrification procedure has developed more recently and more rapidly [2].

Pregnancy rates of recipients implanted with *in vivo* frozen embryos are not inferior to those of recipients receiving fresh embryos [3,4]. Nevertheless, several studies have highlighted the inefficiency of this conventional method of cryopreserving *in vitro*-produced (IVP) bovine embryos [4-9]. These possibly contain more lipid droplets in their cytoplasm than do their *in vivo* counterparts [10-12], leading to their greater susceptibility to the freezing process [13,14].

In vitro production of bovine embryos has progressed rapidly and they are now relatively cheap to generate in large quantity [4]. Although oocyte numbers from different donors are variable, the *in vivo* ovum pick-up method coupled with *in vitro* fertilization and culture can produce an average of 50 calves from repeated aspiration of oocytes of a single donor cow per year [15]. While the yield of IVP embryos may be sizeable, a corresponding number of recipients are often not available [16]. Thus, valuable unused embryos are frequently discarded in the laboratory. Since vitrification is now the main cryopreservation method to store human oocytes and embryos [17], this procedure has also been recommended to assist in the provision of stocks of IVP bovine embryos [2,4,18].

Vitrification is technically simple [19], and does not require a programmable freezer [2,20]. Laboratory experiments have shown that vitrified IVP bovine embryos achieve a better survival rate than do those cryopreserved by slow freezing [4,9,21,22]. As a proof of principle, calves were born successfully after transfer to recipients of vitrified IVP embryos [23,24]; moreover, the pregnancy rates of recipients that are implanted with vitrified IVP embryos may be acceptable in commercial conditions [25].

Despite these successful reports, the cattle industry still relies heavily on the transfer of fresh IVP embryos due to the lack of a repeatable cryopreservation technique [16]. There is a plethora of vitrification protocols utilizing different timings, temperatures and cryoprotectants (CPAs). In addition, the user has a choice of vitrification carriers (such as plastic straws, electron microscope grid microdrop, open pulled straw, cryotop, cryohook), with drop-size and straw barriers affecting cooling and warming rates. The warming protocols can differ too, which compounds further the choice of an efficient vitrification method [17]. This is problematic when there is an exchange of vitrified samples between laboratories that adopt different protocols.

Arguably, however, the most important factor to consider in developing a highly successful cryopreservation method is the skill of the operator. Although described as a simple method, without need for slow-rate freezers, in our experience vitrification actually requires more technical dexterity than does slow-freezing.

In parallel with efforts to develop a novel vitrification method, considerable attempts aimed to improve the quality of IVP embryos before cryopreservation have been made. These include modification of *in vitro* culture systems [12,25], enhancing the intrinsic capacity of embryos [26] and other means of pretreatment [27]. Nevertheless, a significant improvement has so far proved elusive.

This review considers several contributing factors to cryopreservation; ice crystal formation, cooling/warming rates, and the role of CPAs in protecting cells from the chilling process. Moreover, the specific challenges of storing *in vitro*-fertilized embryos by either slow freezing or vitrification are examined. In addition, attempts to increase the efficiency of vitrification through modifications to *in vitro* culture and enhancement in the capacity of IVP embryos are discussed. Furthermore, biosafety considerations relevant to vitrification are also taken into account.

Ice Crystal Formation

During cooling, ice crystals that form outside and inside cells have a detrimental effect on the survival of cryopreserved cells. Seki *et al.* [28] note that ice crystals occur initially externally during the slow freezing process, a phenomenon to which they refer as extracellular ice crystal formation. According to Jin *et al.* [29], extracellular ice crystals are harmless to cells, but Mazur *et al.* [30] maintain that external ice causes distortion to mouse oocytes and their zona pellucida. In addition, intracellular ice formation is one of the most important factors affecting survival of cryopreserved cells [30,31]. Ice crystals forming during the cooling and warming processes are the main cause of cell damage and death [32,33].

Hence, a key aim of cryopreservation is to minimize or even eliminate intracellular ice formation. Kleinhans and Mazur [34] point out that during slow freezing, cell survival is dependent on the cooling rate being sufficiently slow for the volume of unfrozen intracellular water to dehydrate by osmosis to near the equilibrium level before the temperature at which intracellular nucleation occurs is reached. In contrast, Kim *et al.* [35] and Lawson *et al.* [36] contend that vitrification eliminates totally intracellular ice crystals. Interestingly, Kobayashi and Kirschvink [37] propose that exposure of cells to electromagnetism reduces ice crystal formation.

Roles of Cooling Rates

In slow rate cooling, embryos are cooled at a rate of around 0.5 °C per minute to -30 °C or -35 °C and are then plunged into liquid nitrogen [1]. In contrast, for vitrification the cooling rates are thousands of degrees Celsius so that each sample containing embryos reaches a glassy state very rapidly. It is thought that two essential elements that vitrification exhibits are high cooling rates and high concentration of cryoprotective additives [28].

Minimum volume vitrification theory relates to the rate of cooling and vitrification carriers. Plastic straws were originally used as the main carrier to vitrify and store oocytes and embryos, but produced poor survival rates of embryos [6]. For example, Palasz *et al.* [38] achieved around 20% survival in all experiments in which plastic straws were used. Therefore, cryobiologists have sought to develop a carrier that contains minimum vitrification solution to increase the cooling rates. Kuwayama [39] noted that minimum volume vitrification avoids breakage of zona pellucida, which often occurs when embryos are vitrified in plastic straws. Moreover, the direct contact of vitrification solution with embryos induces ultra-rapid cooling [40]. Vajta *et al.* [23] reported on a new vitrification carrier, the open-pulled straw (OPS), a 2.5 ml standard plastic straw (used normally for slow freezing embryos) heat-softened and pulled to decrease the diameter of the straw so that, based on capillary effect, 1-2 µl vitrification solution containing oocytes or embryos is spontaneously loaded into the straw. Using the OPS method cooling rates of 25,000 °C/min are measured at temperatures from -25 °C to -175 °C, whereas those with the larger plastic straw (5 µl, typically used for sperm freezing) are 2,250 °C/min, over ten-fold less [23].

Kuwayama [39] described a specially designed carrier, named Cryotop®, comprising a hard plastic holder attached to a narrow and thin film strip, that enables a relatively small volume of vitrification solution containing the sample to be loaded (< 0.1 µl). As a result, this device can achieve a cooling rate of 69,250 °C/min [28]. However, Rios *et al.* [19] argue that despite a belief that minimum volume vitrification enhances thermal conductivity and cooling rates, this does not improve the hatching rates of embryos after vitrifying/warming and following *in vitro* culture. Moreover, Paredes and Mazur [41] noted that vitrification appears not to be influenced by the size of droplets used with a Cryotop®.

Roles of Warming Rates

Recent research has paid attention to the importance of warming rates because recrystallization affects vitrification outcomes [42]. A process of recrystallization occurs during the warming process [32], during which devitrification can cause cryo-injuries to the cell [43]. Sansinena *et al.* [43] found that recrystallization starts at -109 °C. In addition, Hopkins *et al.* [44] note that warming rates are tightly dependent on cooling rates due to the presence of small ice fractions inside vitrified cells. Consistent with this, Seki *et al.* [28] state that during the cooling process, small ice crystals form; consequently, these crystals could cause recrystallization during the warming process. Thus, faster cooling rates require faster warming rates to block recrystallization [33]. Seki *et al.* [28] showed that moderate cooling rates combined with high warming rates of 117,500 °C/min can prevent recrystallization. An ultra-high warming rate of 10,000,000 °C/min can be achieved by using a laser pulse, which is a novel method for ultra-rapid warming [45]. Although it is clear that rapid warming enables cells to pass through the recrystallization process, the concentration of CPAs needs to be taken into account. An inadequate CPA level can cause recrystallization during the warming process [29]. In agreement with this assertion, Seki *et al.* [28] contend that the short time (1-2 minutes) for which embryos are exposed to equilibration solution is not enough for permeable solutes to penetrate cells and replace water molecules (through dehydration). However, recent studies have indicated that if the rate of warming is extremely rapid cooling rates and use of cryoprotectants appear not to be as important to the survival of oocytes and embryos after vitrification as was previously thought [46-48].

Cryoprotectants

CPAs play a central role in protecting cells from cryo-damage. Permeating and non-permeable CPAs are commonly used in gamete and embryo cryopreservation. Permeating compounds can penetrate into cells and replace intracellular water; consequently, these cells rehydrate and are protected from ice crystal formation [49]. Non-electrolyte CPAs of low molecular weight enter cells with different rates of penetration [1]. Frequently used permeable CPAs include dimethyl sulfoxide (Me_2SO), glycerol, ethylene glycol (EG), methanol and propylene glycol. According to Leibo [1], oocytes and embryos are equilibrated with CPAs at half the concentration of the final vitrification solution. For example, in Cryotop® – a typical vitrification – embryos are first exposed to an equilibration solution containing 7.5% v/v EG and 7.5% v/v Me_2SO, then to a vitrification solution of 15% v/v EG and 15% v/v Me_2SO. Permeability of cells is also dependent on type of CPA. Mazur *et al.* [30] found that glycerol does not permeate mouse oocytes. EG is more permeable to embryos than is glycerol, so EG is a preferred CPA in the field with a one-step dilution [50].

In order to avoid intracellular ice crystal formation [28] and to achieve vitrification [36], a high concentration of CPA is required; however, this may cause osmotic stress [51]. Vanderzwalmen *et al.* [52] contend that both a high intracellular and extracellular concentration of CPAs is necessary to minimize excessive swelling of embryos, which is a consequence of water entrance during the warming process. Thus, non-permeable CPAs such as the sugars sucrose and trehalose are used to lessen osmotic stress. Dobrinsky [53] considers that CPAs protect intracellular organelles during the cooling and warming processes; on the other hand, their toxic characteristics can cause osmotic injuries to the cells [22,54].

While Fahy [54] contends that CPA toxicity is a major drawback of cryopreservation by either slow freezing or vitrification, minimizing toxic effects makes this approach a more attractive proposition. It is a common perception that a high concentration of CPAs used in vitrification can be harmful to oocytes and embryos. Interestingly, some recent opposing views attempt to redress this perception. Vanderzwalmen *et al.* [52] argue that intracellular CPA levels in vitrified embryos are significantly lower than those in frozen embryos. In addition, provided that the warming rate reaches 1 x 10^7 °C, even when the permeating CPA concentration in vitrification solution is reduced to one half [46], one third [47] or zero [48], survival rates of mouse oocytes and embryos after vitrification can attain close to 100%. Jin and Mazur [48] maintain that the molarity of permeating CPAs penetrating into oocytes and embryos is not as important for their survival as the volume of cytoplasmic water that has been withdrawn from these cells before vitrification.

Suitability of Both Slow Cooling and Vitrification for *In Vivo*-Derived Embryos

In vivo embryos are generated in donors with assistance of exogenous gonadotropin hormones, and the majority of these embryos are frozen. Although slow freezing has become accepted practice for *in vivo* embryo cryopreservation, vitrification is the method of choice for storing *in vivo* embryos. Van Wagtendonk-de Leeuw *et al.* [55] report that vitrification and slow freezing resulted in similar conception rates in recipients. In North America 70% of superovulated embryos are slow frozen, so this is the apparent method of choice for cryopreserving *in vivo* embryos when a programmable freezer is available [2]. Hasler [3] reports high overall pregnancy rates of recipients implanted with *in vivo* frozen embryos (56.1%). Moreover, Lopatarova *et al.* [56] demonstrated a 48.8% conception rate for recipients implanted with frozen and biopsied *in vivo* embryos, while that for recipients of intact and frozen *in vivo* embryos was 50.7%. While confirming that vitrification is sometimes recommended to cryopreserve *in vivo* embryos, Hasler [2] maintains that slow freezing and vitrification produce similar pregnancy rates among recipients. Since slow freezing has become a standard method for *in vivo* bovine embryo cryopreservation, further research on refining this technique may enable improved outcomes for embryo production [2,57].

In Vitro-Fertilized Embryo Cryopreservation

In contrast to *in vivo* embryo cryopreservation slow freezing may not be efficient for storage of IVP embryos. Seidel [13] recognizes the difficulty when cryopreserving ruminant *in vitro*-derived embryos. Table 1 summarizes a series of experimental studies which demonstrates that survival rates of IVP frozen bovine embryos after thawing and further culture *in vitro* are lower than those of vitrified embryos.

Source	Slow freezing	Vitrification
Mahmoudzadeh *et al.* [5]	43.3	71.3
Enright *et al.* [6]	0	17
Nedambale *et al.* [7]	22	54
Mucci *et al.* [4]	12	43
Yu *et al.* [8]	24.7	34.9

Table 1: Percentage hatching rates of bovine IVP blastocysts following slow freezing or vitrification (listed in chronological order of publication)

Enright *et al.* [6] reported that no *in vitro* embryos survived when recovered from frozen. Similarly, Nedambale *et al.* [7] observed that the number of cells damaged by fragmentation was higher in frozen embryos than in vitrified embryos. For goats, Begin *et al.*[58] and Al Yacoub *et al.* [59] reported that OPS vitrification was utilized successfully for *in vitro*-derived goat embryos.

Although vitrification appears to be preferable to slow freezing, further investigation is required [20]. While several data sets on pregnancy rates are available [7,24,35], the sample sizes in these studies were relatively small. For example, Gutnisky *et al.* [24] transferred vitrified embryos to recipients and obtained 46.8% conception rates. However, as the number of embryos used was modest (n = 96), this may not provide an accurate representation nor reflect the potential efficacy of the vitrification method. Also, a calving rate of only 34.3% does not represent a promising outcome under commercial conditions where the ultimate goal of an embryo transfer program is to achieve a high yield of healthy livestock. Previously, Enright *et al.* [6] reported that the abortion rate of IVP embryos in recipient cows is high and that due care should be taken with pregnancy interpretation.

Challenges Facing Cryopreservation of IVP Bovine Embryos

The cytoplasmic lipid content of oocytes facilitates mitochondrial activity essential to early development of embryos [60]. Additionally, Ferguson and Leese [61] showed that the triglyceride in bovine oocytes is a nutrient that provides energy for oocytes and embryos during the maturation and fertilization processes. The amount of lipid in *in vitro*-derived embryos that were cultured in a serum-free medium was similar to their *in vivo*-derived counterparts, but the lipid content of embryos cultured in the presence of serum was nearly twice as much as those originating from *in vivo* embryos [61].

Block *et al.* [62] indicated that cytoplasmic lipid droplets in embryos have no effect on their viability after cryopreservation; however, others have argued that the ability of cryopreserved embryos to survive is related to the presence of such lipid content [13,63]. Seidel [13] and Pryor *et al.* [11] note that there is a correlation between lipid droplets and embryo cryotolerance. Moreover, Cagnone and Sirard [63] maintain that the *in vitro* culture system affects the lipid content of embryos and influences their subsequent cryo-survival. Previously, Abe *et al.* [10] had found that the sensitivity of *in vitro* embryos to cryopreservation is due to the substantial accumulation of lipid droplets in their cytoplasm. Embryos cultured in serum-free medium contain mostly small lipid droplets (< 2 μm); in contrast, embryos cultured in serum-supplemented medium possess larger lipid droplets. Large droplets (> 62 μm) were often observed in morulae and blastocysts when cultured in media containing 5% v/v calf serum [10]. This abnormal accumulation of lipid in the cytoplasm resulting from culture in the presence of serum is the main reason IVP embryos are considered unsuitable for cryopreservation [14]. Mucci *et al.* [4] point out that elevated levels of lipid droplets in embryos affect cellular repair after cryopreservation. Furthermore, Sudano *et al.* [12] contend that high concentrations of fetal calf serum in *in vitro* culture media lead to increased lipid accumulation in blastocysts, and apoptosis, but reduced expansion of blastocoels after vitrification. In sheep, it is also evident that addition of fetal calf serum to culture media increases lipid droplet abundance [64].

Direct Transfer of Vitrified IVP Embryos

Most vitrification carriers are not designed for direct transfer because they are poorly suited to use with a standard 0.25 mL plastic straw [65]. In addition, vitrification includes several steps in which embryos are placed into equilibrium and vitrification media; thus, warming involves multiple steps to eliminate CPAs. Simplification of the procedure by a single warming step is necessary to make possible the direct transfer of vitrified bovine embryos. It is evident that warming by single or multiple steps achieves similar results [9,51]. Vajta *et al.* [66] developed an in-straw dilution protocol in which a mini French straw is loaded with holding medium and 0.2 M sucrose; however, this procedure requires skilled technicians [67]. Another drawback of this modification is the loss of embryos during warming [9]. Vieira *et al.* [67] state that warming in air for 3-5 seconds before an embryo is loaded possibly reduces the warming rate, which is critical to its survival [33]. Morato and Mogas [51] found that keeping the warming solution at 45 °C facilitates a better survival rate of vitrified embryos than at 50 °C, 60 °C or 70 °C. In accord, Caamano *et al.* [9] record that 41 °C is the most suitable temperature to warm vitrified embryos. Although reports of direct transfer have provided promising results [9,51,67], embryo losses, evaluated through transfer, are limiting.

Modifications to *In Vitro* Culture Systems

If, as the literature suggests, chilling sensitivity of *in vitro*-derived bovine embryos is related to the amount of lipid in the cytoplasm, adapting IVP conditions to reduce the lipid content appears necessary to increase cryo-tolerance. Serum has been shown to increase the amount of lipids; thus, embryologists have attempted to modify *in vitro* culture media by substituting serum-based media for chemical-defined ones. Seidel [13] reported that *in vitro*-fertilized embryos cultured in a chemical-defined medium without blood serum can be more cryotolerant than those cultured in bovine serum albumin (BSA) or a medium with added fetal calf serum (FCS). It is important to note that serum supplementation of *in vitro* culture media plays a crucial role in the development of oocytes and embryos because serum provides nutrients, vitamins, growth factors and antioxidants necessary for the healthy development of *in vitro*-fertilized embryos [63]. In contrast to previous reports, Castaneda *et al.* [68] claim that bovine oocytes with a high number of lipid droplets show greater developmental ability since lipid sources provide energy for functioning intracellular systems.

Studies on modifications to *in vitro* culture systems have sought to keep a sufficient concentration of FCS and to add redox regulator compounds such as phenazine ethosulfate to enhance the viability of embryos after cryopreservation. Sudano *et al.* [12] found that cytoplasmic lipid can be reduced by supplementation of 0.25% v/v FCS. Moreover, in combination with exposure of embryos at day four to phenazine ethosulfate, which restricts fatty acid synthesis, embryo quality and their viability after vitrification was improved. These authors also argue that the cryosurvival of embryos is dependent not only on lipid content but also apoptosis [12]. Consistent with this, Barcelo-Fimbres and Seidel [69] demonstrated that phenazine ethosulfate decreases lipid composition while serum increases lipid accumulation in bovine embryos, yielding expanded blastocysts with few cytoplasmic lipid droplets. It is also recommended that the *in vitro* culture medium is supplemented with other lipolytic substances. For example, Sanches *et al.* [25] showed that addition of forskolin to an *in vitro* culture medium on day five post *in vitro* fertilization leads to satisfactory pregnancy rates of recipients.

Enhancement of Some Specific Features of Embryos

Improving particular characteristics of *in vitro*-fertilized bovine embryos is another means to enhance their quality prior to cryopreservation. Appropriate exposure of gametes and embryos to sub-lethal hydrostatic pressure raises their intrinsic developmental competence to overcome extrinsic stressors such as the cooling process [70,71]. An enhancement in cell performance is possibly due to high hydrostatic pressure (HHP) causing a minimum stressful condition under which cells can produce and accumulate heat shock proteins [72,73]. For example, Pribenszky *et al.* [72] found that HHP treatment of pig oocytes before Cryotop® vitrification increased rates of cleavage and blastocyst formation. Similarly, Filho *et al.* [26], pretreating bovine blastocysts with HHP, noted that re-expansion and hatching rates of treated embryos are significantly higher than those of untreated embryos. However, pregnancy rates were not reported. Evidently, this promising approach to promoting oocyte and embryo survival merits further investigation.

Sophisticated Techniques to Improve Cryopreservation Outcomes

Micro-manipulation of *in vitro*-fertilized embryos prior to vitrification may increase the survival of treated embryos after cryopreservation. For instance, sufficient dehydration of blastocysts before vitrification, achieved by artificially collapsing the blastocoel, improves their implantation potential [74]. Using this technique, Min *et al.* [27] observed increased survival of *in vitro* bovine embryos and cloned embryos post-warming and a further 24 hours culture. Survival rates of *in vitro* embryos following blastocoel forced treatments were significantly higher than in control groups (81.9% versus 69.8%). However, this protocol is complicated by time requirements and the necessity of the embryologist to be highly proficient. Unlike Min *et al.* [27], Pryor *et al.* [11] used a laser-assisted hatching method whereby a laser micro-beam drills a hole in the zona pellucida through which lipid droplets can escape from the cytoplasm. However, this delipidation technique appears to be technically complex.

Embryo Biosafety

Biosafety is a primary consideration for the exportation and importation of bovine embryos between countries. Hence, it is important to recognize the potential for pathogen transmission following transfer of cryopreserved gametes and embryos. Vitrification has two distinct systems, closed and open, which present different risks. Similar to slow freezing in straws, a closed vitrification system has a reduced risk of microbial contamination compared to methods requiring direct contact of embryos with liquid nitrogen. In theory at least, direct contact of the vitrification solution with liquid nitrogen may result in contamination [17]. Morris [75] argues that it is a false assumption to think that liquid nitrogen is sterile and points to the possibility that contamination could originate in cryopreserved samples. Furthermore, Mirabet *et al.* [76] maintain that various viruses are able to survive at subzero temperatures without CPA requirements and cryopreservation methods. Bielanski *et al.* [77] tested the feasibility of contamination from liquid nitrogen of three species of virus – bovine immunodeficiency virus (BIV), bovine viral diarrhoea virus (BVDV) and bovine herpes virus (BHV). While unsealed carriers were either positive or negative for these viruses, sealed ones were all negative. Although identifying 32 bacterial and one fungal species from liquid nitrogen storing semen and embryos over a 35 year period, Bielanski *et al.* [78] did not detect BVDV and BHV in clean semen and embryo straws stored with infected straws. Nevertheless, it is not straightforward to keep samples clean if contained alongside unclean ones in the same liquid nitrogen tank. It is important to note that although cryopreservation can reduce the percentage of embryos infected by BVDV and BHV, this process may not totally remove the risk of infectious pathogens [79].

In spite of low microbial contamination risks relating to contact of samples with liquid nitrogen, it is worth considering these potential hazards [80]. A closed system may not attain high enough cooling rates, but an open system does; hence, compromising technical and sanitary issues is rather difficult. Yu *et al.* [8] modified OPS to closed pulled straw (CPS) to eliminate the chance of contamination. Although these authors claim CPS maintains the advantages of OPS, the survival rates of embryos vitrified by CPS were relatively low.

Conclusion

Intracellular formation of ice crystals during cooling and warming processes is the principal cause of cryo-damage to cells. Cryopreservation by slow freezing and vitrification are aimed to minimize or, ideally, to totally eliminate this accumulation. CPAs are indispensable for protection from chilling but they are possibly toxic to the cell. While both slow freezing and vitrification are efficient methods of cryopreserving *in vivo*-derived bovine embryos, slow freezing may not be suitable for *in vitro*-fertilized embryos. This may be due to the fact that *in vitro*-fertilized bovine embryos contain more cytoplasmic lipid droplets than do their *in vivo* counterparts, although improved culture conditions may ameliorate this. Considerable research has indicated the advantages of vitrification to become the method of choice for storing *in vitro*-derived embryos. In order to further improve outcomes a greater understanding is required of cooling and warming, the role of CPAs, modifications to *in vitro* culture media and to treatment of cells prior to cryopreservation. Moreover, biosafety of embryos is an important factor that warrants investigation. Although vitrification is apparently superior to slow freezing to cryopreserve *in vitro*-fertilized bovine embryos, non-standardization of techniques and an inconsistency of results between laboratories may hamper widespread adoption of vitrification. Hence, it is necessary to identify the most efficient way to generate IVP blastocysts with a reduced lipid content as well as optimizing vitrification methods for *in vitro*-fertilized bovine embryos. In turn, this will enhance theoretical understanding of bovine cryobiology while, from a practical perspective, facilitating increased use of *in vitro* embryos in the field.

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