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MR. LUIS B FERRÉ (Orcid ID : 0000-0003-2368-7039)

DR FERNANDO CAMPOS-CHILLON (Orcid ID : 0000-0003-3223-8059)

DR PABLO J ROSS (Orcid ID : 0000-0002-3972-3754)

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L.B. Ferré, M.E. Kjelland, A.M. Taiyeb, L.F. Campos-Chillon and P.J. Ross analysed the data and drafted the paper.

# Recent Progress in Bovine *In Vitro*-Derived Embryo Cryotolerance: Impact of *In Vitro* Culture Systems, Advances in Cryopreservation and Future Considerations

L.B. Ferré[1*], M.E. Kjelland[2, 3], A.M. Taiyeb[2, 4], L.F. Campos-Chillon[5] and P.J. Ross[6**]

[1]National Institute of Agricultural Technology (INTA), Ruta Nacional 3, Km 488, Tres Arroyos (7500), Buenos Aires, Argentina.

[2]Conservation, Genetics & Biotech, LLC, Vicksburg, MS 39183, USA

[3]Mayville State University, Mayville, ND 58257, USA

[4]Barz IVF Center for Embryo Research and Infertility Treatment, Koya Street, Brayate, Erbil 44001, Kurdistan, Iraq

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[5]Animal Science Department, California Polytechnic State University, San Luis Obispo, CA 93407, USA

[6]Department of Animal Science, One Shields Avenue, University of California, Davis, CA 95616, USA.

[*, **]Corresponding authors. Email: ferre.luis@inta.gob.ar; pross@ucdavis.edu

**Summary**

Cryopreservation of *in vitro*-derived bovine embryos is a crucial step for the widespread reproduction and conservation of valuable high merit animals. Given the current popularity of bovine *in vitro* embryo production (IVP), there is a demand for a highly efficient ultra-low temperature storage method in order to maximize donor ovum pick-up (OPU) turn-over, recipient availability/utilization and domestic/overseas commercial trading opportunities. However, IVP bovine embryos are still very sensitive to chilling and cryopreservation and despite recent progress, a convenient (simple and robust) protocol has not yet been developed. At the moment, there are two methods for bovine IVP embryo cryopreservation: slow programmable freezing and vitrification. Both of the aforementioned

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techniques have pros and cons. While controlled-rate slow cooling can easily be adapted for direct transfer (DT), ice crystal formation remains an issue. On the other hand, vitrification solved this problem but the possibility of successful DT commercial incorporation remains to be determined. Moreover, simplification of the vitrification protocol (including warming) through the use of an in-straw dilution without the use of a microscope is a prerequisite for its use under farm conditions. This review summarizes the bovine IVP embryo cryopreservation achievements, strengths and limitations of both freezing systems and prospective improvements to enhance cryosurvival, as well as perspectives on future directions of this assisted reproductive technology.

Keywords: Bovine; Culture environment; Direct transfer; Embryo; *In vitro* fertilization; Slow freezing; Vitrification

**IVP embryo cryotolerance**

According to the annual International Embryo Technology Society (IETS) statistics (1997-2017), in the last five years, an average of almost 470,000 bovine embryos produced *in vitro* were transferred worldwide annually. From the total number of transferred embryos, about 80% were transferred fresh (non-cryopreserved) (see Table 1). This reflects the fact that embryos produced *in vitro* have less cryotolerance than *in vivo* counterparts. Previously, (i.e., before 2004), the number of frozen embryos transferred in the commercial *in vitro* embryo production (IVP) setting was virtually similar to fresh embryos. A possible explanation for this large number of frozen embryo transfers could be based on the use of somatic cell co-culture systems. Under this culture system, the embryos were suitable for freezing, including slow-freezing for direct transfer (DT) (Hasler et al. 1995). The co-culture system

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requires serum supplementation to keep cells in a growing phase. This serum was linked to large offspring syndrome-LOS (discussed below) and due to inherent complications, removed or reduced from culture media (Farin et al. 2001). From a commercial IVP company standpoint, the loss of the mainstream embryo freezing capacity (especially the DT form) had a great impact. The ability to freeze embryos and directly transfer those after a simple thawing protocol to previously synchronized recipients females facilitates the large scale implementation of this ART, especially for on-site farm conditions (i.e., sterile lab conditions not available), while stimulating the marketing, commerce and domestic and international trade of elite genetics in a safe way. The major advantage of a DT method over step-wise removal of the cryoprotectant after thawing is based on increasing the productivity due to implanting large numbers of embryos in a short period of time. Non-direct transfer methods require equipment set-up, appropriated lab area and it is generally more time consuming. In some instances, recipients are available for a limited time (i.e. milking dairy cows) and because DT is a quicker process, more transfers can be performed without interfering with a commercial daily operation (Voelkel and Hu 1992, Dochi 2019).

Current IVP popularity is based on its flexibility to collect oocytes from multiple donor types (open, pregnant, juvenile calf, heifer, adult cow), greater frequency of oocyte retrievals (i.e., ovum pick-up (OPU)) so that more embryos and pregnancies can be produced per unit of time, requiring a smaller number of sperm (expensive/limited semen straws, sexed semen and reverse sorting) and most recently, combining it with genomic selection. Nevertheless, a primitive form of co-culture is still in use as a consequence of leaving cumulus cells attached to zygotes on purpose or due to an inefficient denudation procedure combined with 2-3% v/v of serum in the culture media (Sanches et al. 2016). It should be noted that leaving cells attached to the zona pellucida can cause an issue in a commercial situation as regulations can require the removal of such cells before sale. Further, this common practice may not be called a real co-culture system in comparison with traditional Buffalo Rat Liver (BRL) or Vero cell co-culture with 10-20% Fetal Calf Serum (FCS). One reason for the increase in the transfer of fresh embryos in recent years is likely due to transport logistics, improved shipping incubator technologies and media used, which all make it more cost efficient. The increased pregnancy rates of fresh embryos compared to frozen embryos offset the costs under some circumstances. Lately (2014-2017), an increase of frozen embryo transfers was noticed again,

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possibly due to the use of defined (or semi-defined) culture medium supplemented with specific additives, embryo quality promoters, recombinant albumin and hyaluronan (Lane et al. 2003b), synthetic serum substitutes (Duque et al. 2003), as well as oviductal and uterine fluids (Lopera-Vasquez et al. 2015). The differences between IVP and *in vivo* produced embryos could explain the low cryosurvival performance of IVP. Among the most important factors affecting cryosurvivability of embryos produced by IVP technology are: (i) culture system (semi-defined or defined media formulation, presence of growth factors, natural and/or synthetic additives, presence of monolayer of heterologous or autologous somatic cells), and (ii) culture environment (embryo density, atmosphere conditions, media change frequency). Sub-optimal *in vitro* culture conditions not only affect the oocyte and the embryo morphologically, but also physiologically. Furthermore, embryo metabolism, cytoplasm lipid content, cell division speed, morula compaction, gene expression, among others, also contribute to define embryo quality, developmental competence and cryotolerance.

Cryopreservation is an essential component of the cattle embryo transfer industry, especially in our current global commercial scale (Hasler 2014). The first successful bovine calf born derived from a frozen (slow freezing) *in vitro* embryo (Fukuda et al. 1990) was not performed using an IVP embryo technique adaptation, e.g., embryo direct transfer. On the other hand, the first successful vitrified bovine embryos were reported by the mid-1980s (Massip et al. 1986) and since then, progress has been made in developing simpler protocols, more stable and less toxic solutions, different embryo carriers and warming procedures to achieve on-farm direct transfer (Vajta and Kuwayama 2006).

Despite encouraging results published recently, there is still great variability among the reported data concerning frozen embryo survival and pregnancy success (Table 2). Although there is a large consensus concerning slow freezing inefficiency with the cryopreservation of IVP bovine embryos (Saragusty and Arav 2011) and pregnancy rates with vitrified IVP embryos, it may be acceptable for ovum pick up (OPU-IVP) commercial laboratories (Marinho et al. 2012). Nonetheless, embryo transfer (ET) practitioners and seed stock producers still rely heavily on the transfer of fresh IVP embryos due to the lack of a repeatable post-thawing/warming implantation performance (Morotti et al. 2014, Pontes et al. 2011).

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Bovine embryo transfer has become an important tool for genetic improvement and breeding programs, in which the status of the embryos (fresh or frozen) is crucial in terms of its applicability. Under farm conditions, DT embryos offer a great advantage over post-thawed dilution protocols. However, only IETS grade 1 (high quality) *in vitro*-derived embryos are suitable for DT cryopreservation methods in order to achieve acceptable pregnancy rates (PR). The PR gap between *in vivo* fresh and frozen embryos is very small, not so with *in vitro*-derived embryos. Currently, the most popular cryopreservation method for IVP embryos is still vitrification (Vajta 2000), although slow freezing is earning more followers (Bruyere et al. 2012, Gómez et al. 2020). Vitrification has based its strengths on an adequate supply of more suitable embryos to freeze and a lower cost of equipment (Vajta and Kuwayama 2006) and may be more suitable for IVP higher lipid content embryos than the slow freezing technique; however, current warming procedures and carrier design are not compatible for DT. In addition, vitrification includes several equilibration steps prior to the nitrogen plunge; which makes vitrification of a high volume of embryos problematic (Figure 1). Attempts to simplify the warming procedure by an in-straw dilution have been achieved (Caamaño et al. 2015, Morató and Mogas 2014, Inaba et al. 2011), although its on-farm application is limited. There are a couple of reasons that the low in-straw dilution procedure is not being widely implemented by field practitioners. First, the in-straw dilution procedure method requires experienced technicians with a good level of dexterity to manage high volumes of embryos in a timely manner. Second, there is lack of large field studies demonstrating that in-straw dilution of vitrified embryos is practical and leads to acceptable pregnancy rates compared to conventional ET. In essence there needs to be a well demonstrated advantage to learn and apply this deemed to be new technique for commercial practitioners to make the change.

The compromised cryotolerance of embryos resulting from the IVP procedures may have its origin in aberrant oocyte and embryo metabolism during culture leading to high cytoplasmic lipid content with different lipid droplet pattern arrangements (Seidel 2006, Massip et al. 1995, Abe et al. 2002) and relatively smaller inner cell mass (Iwasaki et al. 1990). In IVP, the lipid amount in embryos that were cultured in serum-free conditions was similar to *in vivo*-derived counterparts, contrary to the lipid content of embryos cultured in medium supplemented with serum where lipid content was nearly double compared to *in vivo* embryos (Abe et al. 2002). The main sources of cryodamage potentially

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occur during the lipid phase transition and lipid peroxidation at freezing and thawing (Tarín and Trounson 1993, Quinn 1985). At physiological temperatures, lipid droplets are generally in a fluid phase providing elasticity to membranes and intracellular structures. However, upon cooling and below the main lipid phase transition, the diffusion rate of hydrophobic molecules and permeability of membranes are decreased leading to rigidity of membranes, redistribution of intracellular lipids and potentially disruption of organelles associated with lipid droplets (Quinn 1985, Sudano et al. 2011). Furthermore, the degree of lipid saturation varies among species (Ordonez-Leon et al. 2014), breeds and embryological stages (Romek et al. 2011, Baldoceda et al. 2016). One report indicated otherwise (Block et al. 2010), arguing that cytoplasmic lipid droplets in bovine embryos have no effect on their viability after cryopreservation by vitrification; however, lipid droplet pattern and quantification were not reported.

Several strategies have been applied to reduce the chilling sensitivity of *in vitro* produced bovine embryos by adapting culture conditions to reduce the lipid content in the cytoplasm (Braga et al. 2019). Serum reduction [≤5% v/v] (Holm et al. 1999, Sudano et al. 2011) or complete substitution has been a main target of such research (Rizos et al. 2003, Gómez et al. 2020). By keeping a sufficient concentration of serum and adding lipolytic substances, which restrict fatty acid synthesis, one may enhance embryo quality and viability after cryopreservation (Barcelo-Fimbres and Seidel 2007, Sudano et al. 2011, Paschoal et al. 2014). Embryo cryosurvival may be dependent not only on cytoplasm lipid content, but also related to cell apoptosis rate (Sudano et al. 2014). Enriching the embryo cell membrane with unsaturated fatty acids and cholesterol to improve embryo cryotolerance has already been performed by means of two pathways: 1) membrane incorporation through its supplementation in the culture media and 2) nutritional management of oocyte/embryo donors and by offering a diet-rich with polyunsaturated fatty acids. Unsaturated fatty acid supplementation (linoleic acid) in the culture media improved embryo cryotolerance (Hochi et al. 1999) by reducing lipid content. The addition of cholesterol or liposomes is an attempt to modify culture media to restore or strengthen membrane lipid composition and stabilization (Pugh et al. 1998). Because the freezing process could disrupt the embryonic cytoskeleton, cytoskeletal stabilizers were proposed to reversibly depolymerize filamentous actin microfilaments prior to cryopreservation so they repolymerize normally after thawing/rehydration (Dobrinsky 2002). Treatment of cells with cytochalasins makes

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the plasma membrane less rigid and more elastic so microfilaments are not disrupted during cryoprotectant exposure. More recently, L-carnitine, was found to play an important role in the lipid metabolism (Sutton-McDowall et al. 2012) and as antioxidant protecting the cells from DNA damage (Phongnimitr et al. 2013). This dual effect of L-carnitine enriching cellular lipid metabolism and providing antioxidative protection has been associated with an improvement of cryotolerance and developmental competence in IVP embryos (Takahashi et al. 2013). Notably, a decrease in embryo lipid content has also been obtained without the addition of lypolitic substances (Murillo et al. 2017). For example, Murillo-Ríos et al. (2017) found that bovine *in vitro* embryo culture in protein-free medium from Day-6 to Day-7 led to blastocysts with improved pregnancy and birth rates after cryopreservation.

Another approach to assist IVP embryos in tolerating cryopreservation was to enhance specific defense mechanisms of embryos by exposing them to sub-lethal hydrostatic pressure forces which could raise intrinsic developmental competence attributes to overcome extrinsic stressors such as the cooling/freezing process (Pribenszky and Vajta 2011). Polarization of lipid droplets, as a result of centrifugation, has been proposed as an alternative method to mechanically remove excess lipids (Leibo et al. 1995). Although delipidation by means of centrifugation may slightly improve embryo cryosurvival, it could be used before vitrification to separate monospermic zygotes from polyspermic ones, which may increase cryopreservation efficiency (Han et al. 1999). Micro-manipulation of IVP embryos prior to freezing by artificially collapsing their blastocoel may also increase the post-thaw survival (Diez et al. 2001, Min et al. 2014). It is worth mentioning that this kind of procedure is time consuming, requires a micromanipulator and/or a highly proficient embryologist in order to perform a handmade blastocoel needle puncture. Another alternative to mechanically collapsing the IVP embryo blastocoel includes a brief exposure of embryos to a non-permeating agent (e.g., galactose or sucrose) prior to equilibration with highly permeable cell membrane cryoprotectants (such as glycerol or ethylene glycol) in order to reduce blastocoel volume and facilitate a more rapid equilibration (Barfield et al. 2009, Owen et al. 2017).

**Improving IVP embryo cryotolerance through oocyte developmental competence**

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Recent statistics from the IETS indicate a sustained OPU-IVP growth. The rediscovery of OPU-IVP is due to progresses in sexed semen and genomic selection to dairy nucleus herd improvement programs (Schaeffer 2006). The success of a breeding program depends on the number of progeny obtained from selected superior donors. Interestingly, the OPU-IVP technology has become highly desirable because it maximizes the number of offspring per donor and unit of time. Improvements in efficiency of OPU-IVP program are important to satisfy the rising demand. The success of an OPU-IVP program depends greatly on the quantity and quality of retrieved cumulus oocyte complexes (COCs) (Merton et al. 2003), as well as donor age, breed, physiological status (open, pregnant, days port-partum), nutrition, body condition score, expertise of OPU personnel, ultrasound screen definition, aspiration pump regulation/stability, OPU session interval, hormonal (pre) treatment, among many others (Merton et al. 2003). Oocyte origin, follicle microenvironment (status and size) along with many other factors such as donor nutrition, calf raising history, environment, stress level, health, among many other factors, could have a great impact on the oocyte and embryo competence (Borges and Vireque 2019, Lonergan et al. 1994, Len et al. 2019). These factors can alter oocyte morphology, lipid composition and transcriptome, and therefore, affect blastocyst yield. Despite the fact that embryo culture conditions can determine embryo quality and cryotolerance (Rizos et al. 2002b), blastocysts derived from impaired oocytes may also have a negative impact on developmental ability and quality, which could compromise post-thaw cryosurvival and pregnancy establishment (Sirard et al. 2006, Krisher 2004). The majority of commercial IVP labs assess embryo quality using a morphological stereomicroscope evaluation to determine if the embryo scores as transferable or not. This subjective assessment may not concur with the embryo gene expression pattern (Badr et al. 2007). Also, it is known that the inner cell mass to trophectoderm cell number and allocation (Van Soom et al. 1997b), metabolism (Khurana and Niemann 2000, Donnay et al. 1999), oxygen consumption (Lopes et al. 2007), cryotolerance (Rizos et al. 2008) and the ability to establish a pregnancy (Schmidt et al. 1996, Hasler et al. 1995) can differ among embryos within a morphologically equal population (Rødgaard et al. 2015, Merton 2002).

In order to improve pregnancy rates after IVP-ET, more attention should be given to oocyte maturation *in vitro*. Oocyte maturation involves meiotic (i.e. nuclear) and cytoplasmic maturation. Interestingly, oocyte cytoplasmic maturation at follicle phase was found to cease prematurely when

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COCs are isolated from antral follicles because such oocytes can initiate *in vitro* spontaneous meiotic maturation even though their cytoplasmic maturation is not yet completed (Conti and Franciosi 2018). On the other hand, the second phase of oocyte cytoplasmic maturation, which occurs during meiotic maturation, is also believed to fall behind meiotic maturation when COCs are isolated from antral follicles (Taiyeb et al. 2017). Therefore, the lack of synchronized nuclear and cytoplasmic maturation can result in oocytes with heterogeneous cellular development, and subsequent low IVP rates and cryotolerance capacity. As a result, previous studies were conducted to synchronize oocyte meiotic and cytoplasm *in vitro* maturation using a strategy based on oocyte pre-maturation. This type of temporal arrest of oocyte meiotic maturation *in vitro* was found to synchronize both cytoplasmic and meiotic maturation and result in oocytes of high quality and competence (Mermillod et al. 2000, Adona et al. 2008, Albuz et al. 2010, Bilodeau-Goeseels 2012). Simply, isolated immature COCs can be incubated with compounds that maintain intra-oocyte cAMP elevated, such as phosphodiesterase 3A (PDE3A) inhibitors, for a period of time before *in vitro* maturation. Recently, oral administration of cilostazol, which is an FDA approved PDE3A inhibitor, in superovulated mice at doses extrapolated from the therapeutic doses of cilostazol prescribed in humans (Taiyeb et al. 2014) resulted in high rates of oocytes with synchronized meiotic and cytoplasmic maturation, improved competence levels, and increased litter size (Taiyeb et al. 2017). Although this technique has resulted in improved blastocyst rates in immature oocytes matured *in vitro*, the observed improved blastocyst rates never significantly exceeded those noted with oocytes matured *in vivo*, at least in bovine at the present time (Albuz et al. 2010, Mermillod et al. 2000, Thomas et al. 2004). In addition, the actual effectiveness of these protocols has been questioned due to prolonged incubation periods, additional labor, and the lack of studies involving transfer of cryopreserved embryos. Further research will be required to define the merit of these protocols during cryopreservation of immature COCs.

Embryo quality and the ability to establish a pregnancy are largely determined by culture conditions and medium composition (Thompson et al. 2007, Enright et al. 2000). *In vitro* oocyte maturation, especially from slaughterhouse ovaries, differs greatly from the *in vivo* alternative for numerous reasons including the following: selected oocytes come from a heterogeneous population of 2 to 8 mm follicles, unknown females in most cases (physiologic stage, nutrition conditions, breed, genetic background, etc.), exposed to high concentration of exogenous hormones (FSH in particular),

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unsatisfactory maturation time (short for some oocytes and prolonged for others), and suboptimal medium formulation (Korhonen et al. 2010). Consequently, a limited number of oocytes reach the blastocyst stage (<30%) and/or achieve an acceptable quality for transfer or freezing (Lonergan et al. 2006). Thus, in order to improve pregnancy rates after IVP and embryo transfer, the focus should be given to COCs culture conditions. Serum substitution using Bovine Serum Albumin (BSA) has been tried in order to shift to a more defined *in vitro* maturation (IVM) formulation to study the effects of single molecules and reduce the variation between IVP sessions (Ali and Sirard 2002). Serum-free IVM systems have been challenged to perform similarly to traditional media. Cumulus cell expansion is reduced under serum-free IVM conditions (Cetica et al. 1999), however, nuclear maturation and fertilization seem unaffected (Korhonen et al. 2010) and high blastocyst formation can be produced (Ali and Sirard 2002, Keskintepe and Brackett 1996, Keskintepe et al. 1995). Cryosurvival of blastocysts derived from serum-free IVM matured oocytes have shown similar results compared to conventional IVM embryos (Korhonen et al. 2010, Cho et al. 2002, Raty et al. 2011, George et al. 2008).

Other publications put more emphasis on the oocyte origin and its influence on blastocyst production and embryo quality (Sirard et al. 2006, Krisher 2004). As a concept, oocyte maturation and competence were reported to have a positive correlation with oocyte size in different animal species (Lonergan et al. 1994, Hirao et al. 1993, Nogueira et al. 2006, Comizzoli et al. 2011, Otoi et al. 2000, Raghu et al. 2002). The high developmental capacity observed in large oocytes in comparison to small oocytes in many species suggests more accumulation of nutrients and transcripts, and consequently larger oocyte sizes that efficiently support early embryonic development (Marchal et al. 2002, Otoi et al. 2000, Raghu et al. 2002, Schramm et al. 1993). Others have observed that by suppressing the dominance phase of an unstimulated ovary, the dominant follicle cannot interfere with the acquisition of developmental competence of subordinate follicles (Hagemann 1999).

**Improving IVP embryo cryotolerance through *in vitro* culture and cryopreservation methods**

During the first "cleavage phase" of sequential culture systems, media formulations contain primarily nonessential amino acids, ethylenediaminetetraacetic acid (EDTA), pyruvate, special amino acids (taurine), and reduced concentration of glucose/fructose, whereas in the second "blastocyst phase"

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EDTA and taurine are removed, essential amino acids are added, pyruvate concentration decreases and the concentration of glucose/fructose increases to support embryo development after compaction (Gardner 2008, Thompson et al. 2007). Single monophasic formulations are being optimized (Biggers and Summers 2008), but these may require partial/total media renewal every 48 h in order to avoid toxic waste accumulation or embryo density adjustment to benefit from autocrine/paracrine factors. In a bovine commercial setting, media system/formulation that increases the number of freezable embryos produced for direct transfer, and without sacrificing post-thaw viability, will introduce the highest impact.

Developmental arrest in cattle embryos occurs at the 8- to 16-cell stage around days 2-3 of culture, called the "8-cell block" (Barnes and Eyestone 1990, Eyestone and First 1991). Different strategies have been reported to overcome the 8-cell block including the following: co-culture with granulosa cells (Fukuda et al. 1990), oviduct epithelial cells (Xu et al. 1992), buffalo rat liver/hepatocytes (Vansteenbrugge et al. 1994), Vero cells (Menck et al. 1997), or trophoblastic vesicles (Camous et al. 1984); addition of growth factors (Larson et al. 1992); glucose deprivation (Takahashi and First 1993); culture under low oxygen concentration (Thompson et al. 1990); addition of reducing agents (Gardner et al. 2000); cytokines (Hernandez-Ledezma et al. 1996); and adding embryo culture-derived conditioned medium (Fujita et al. 2006). All of these strategies used to overcome the 8-cell blockade also served to improve embryo quality. Although the IVP system has progressed, cryotolerance and pregnancy rates are still lower than *in vivo* counterparts (Greve et al. 1993). Embryo cryotolerance can be improved by 1) adding beneficial substances, 2) subtracting unnecessary and counterproductive additives and 3) a combination of both 1 and 2. Serum and somatic cell co-culture have been used to provide growth factors, hormones, antioxidant compounds, and chelators of heavy metals (Gardner 1994). However, sera/cell co-culture also brings undesirable effects such as inhibition of the first cleavage division (Pinyopummintr and Bavister 1991), lipid accumulation (Abe et al. 2002), affects gene expression (Niemann and Wrenzycki 2000) and contributes to the large offspring syndrome-LOS (Farin et al. 2001). Additionally, serum has shown a biphasic effect, in that - it inhibits the initial cleavage but later stimulates blastocyst formation (Van Langendonckt et al. 1997) and may also result in LOS (asynchronous embryo age and uterine environment, dietary excesses of urea) (Young et al. 1998).

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Some strategies to increase embryo quality (and potentially cryotolerance) include the reconditioning of the *in vitro* culture environment, in an attempt to mimic the *in vivo* environment, considering that when *in vitro*-derived embryos are cultured under *in vivo* conditions (i.e. ewe oviduct) the cryotolerance increases significantly (Enright et al. 2000). Different assessment parameters have been explored in order to predict bovine *in vitro*-derived embryo quality and cryotolerance. Timing of blastocyst formation, expressed as fast-cleaving and early cavitating embryos showed higher cryosurvival (Mahmoudzadeh et al. 1995). Although total cell number and allocation of inner cell mass and trophectoderm cells have been associated with embryo quality (Van Soom et al. 1997a), sheep *in vivo* culture of IVM-IVF presumptive zygotes showed higher cleavage, blastocyst formation, pregnancy rate of frozen-thawed embryos and fewer cells/embryo, in comparison with SOF-BSA and SOF-FCS (Lazzari et al. 2002, Galli et al. 2001). Observations by the Galli group that *in vivo* phase culture embryos can produce more pregnancies with less cells compared to full *in vitro* culture embryos may indicate that embryo developmental capacity, quality, cryosurvival ability, pregnancy establishment and normal calf birth are affected by multifactorial events during folliculogenesis, *in vitro* production conditions and oocyte/embryo handling procedures (Marsico et al. 2019). Acceptable commercial pregnancy rates can be accomplished using BSA up to day 6 of the culture period with no-protein source for an additional 24h before being subjected to the slow freezing procedure. This aforementioned methodology combined with protein free freezing media may favor embryo implantation performance, lower miscarriage rates and produce normal birth weights (Gómez et al. 2020). Previous studies using bovine oviduct epithelial cells revealed a high interaction between gametes during fertilization and during early embryo development (Rottmayer et al. 2006). Important factors involved with the aforementioned interaction included the secretion of embryotrophic factors (Ferraz et al. 2017), the dynamic exchange of nutrients, metabolites and other cell secretions (Edwards et al. 1997) and a modification of gene expression (Schmaltz-Panneau et al. 2014) which produced a positive effect on embryo cryotolerance and survival success after vitrification and warming (Lopera-Vasquez et al. 2016). Similarly, the addition of embryotrophic factors that support early embryo development (Fujita et al. 2006) and group culture promotes embryo development compared to single-culture or co-culture with small numbers of embryos (Keefer et al. 1994, O'Doherty et al. 1997, Reed et al. 2011). Blastocyst formation is promoted by increasing

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embryo density in culture drops (Reed 2012, Tao et al. 2013, Wydooghe et al. 2014, Sugimura et al. 2013). This phenomenon suggests that specific factor(s) are secreted from the embryos to influence embryo growth and development in an autocrine or paracrine manner (Wydooghe et al. 2017).

In the non-co-culture system, embryo development can be promoted by single formulation (also known as one-step, monoculture, monophasic) or sequential (also known as two-steps, biphasic) culture media (Biggers 2002). Functionally, the concept behind single media is based on "let the embryo choose" which nutrients are preferred (Machtinger and Racowsky 2012) whereas sequential formulation tries to mimic *in vivo* conditions ("back to nature" approach) (Gardner and Lane 2002, Leese 1998). Monophasic medium incorporates all the necessary ingredients for the culture to blastocyst stage in a constant concentration while sequential media was designed to cover embryo nutrient demands as the embryo metabolism changes (Leese 1998, Quinn 2012). Initially, bovine embryo culture attempts used monophasic formulations, followed by an increase in the use of sequential culture systems and ending in a balanced mix of users of each system. Commercial laboratories tend to be biased to the use of one-step media formulations due to prevalent labor shortages, simplicity, and a general preference of not disturbing the embryos during the culture period.

**Future considerations**

The IVP tool has significantly increased its market penetration in the dairy and beef cattle sector in recent years, and as a consequence for the first time, the number of *in vitro*-derived embryos surpassed the number of *in vivo* produced embryos, globally (International Embryo Technology Society-IETS, https://www.iets.org/comm_data.asp). This remarkable achievement is based on the fact that the IVP system can create more pregnancies per unit of time than conventional MOET. Even though an important advancement in terms of embryo yield performance has been achieved in the last couple of decades, or at least more consistent blastocyst development results, there is still room for improvement (Sirard 2018). There are several key variables that affect IVP global efficiency and performance including: breed, donor physiological status, synchronization protocols and ovarian stimulation, oocyte retrieval scheme/procedure, semen (conventional or sexed), media (maturation, fertilization, and culture) and general lab production protocols. The current challenges for the IVP

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system are to 1) continue increasing the number of transferable embryos and 2) increase the embryo quality and cryotolerance in order to enhance the number of freezable embryos resulting from each OPU session.

Notably, embryo selection is still based on a morphological evaluation (Lindner and Wright 1983) but few tools have been developed to evaluate cell function and overall embryo competence to overcome the cryopreservation process, to establish a pregnancy and maintain it to full term, and deliver a healthy calf (Van Soom et al. 2003, Rødgaard et al. 2015, Rocha et al. 2016). Many factors can affect embryo competence and cryosurvival (donor status, follicular microenvironment, oocyte quality, IVP media and production conditions, among others) and attention to details is required in order to increase productivity and consistent embryo yields. High lipid content and accumulation of lipid droplets and the high levels of reactive oxygen species (ROS) produced during the culture period are the most damaging factors that undermine IVP efficiency in terms of embryo development yield and cryotolerance (Abe et al. 2002, Len et al. 2019, Bradley and Swann 2019). Therefore, managing the proper lipid content represents a major challenge since metabolic function needs and cell energy source demands must be balanced with embryo cryotolerance integrity (Dunning et al. 2014).

It is known that cryosurvival and gene expression can be altered by culture conditions regardless of the origin of the oocyte (Lonergan et al. 2003). As such, emerging novel culture platforms have been developed based on ultramicrodroplet volumes from 1.5-2 μl (Ali 2004) and microchannel approaches using microfluidic technology (Beebe et al. 2002) offer several advantages: 1) the possibility of controlling fluid flow, 2) automated renewing of media and/or addition of selected ingredients, and 3) maintaining a high concentration of embryotrophic factors, while also providing full time monitoring assessment surveillance through time-lapse cinematography morphokinetic analysis (Wheeler and Rubessa 2017). In addition to microfluidic, shifting from static to continuous or periodic dynamic culture (shaking/rotation, tilting, vibration) could also have an impact on IVP performance (Swain 2014). It is likely that the future generation of culture platforms may not depend on current Petri dishes and large gas-filled incubators. Rather, these emerging platforms could utilize stand-alone culture devices, without the need for large benchtop incubators, with cultured embryos producing their own atmosphere via chemical reaction (Avery et al. 2000, Suzuki et al. 1999, Swain and Smith 2011). The aforementioned embryo culture platform could

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consist of a combination of a perfusion system, three dimensional culture matrix, specialized surface coatings (where embryos generate their own atmosphere), nanosensor monitoring, integrated software for real-time analysis of embryo development, and non-invasive quality/viability assessment to determine potential embryo freezing/implantation success (Thompson 2007).

Utilization of gold nanorods (GNRs) is another interesting, recent technology that has been successfully implemented in the vitrification and ultra-rapid thawing of zebrafish embryos and coral larvae. The new vitrification method uses GNRs to assist with the warming process. Khosla and co-workers microinjected propylene glycol into zebrafish embryos along with GNRs, followed by vitrification in liquid nitrogen. They demonstrated the ability to thaw the zebrafish embryos rapidly ($1.4 \times 10^7$ C/min) by irradiating the sample with a 1064 nm laser pulse for 1 ms (Khosla et al. 2017). Recently, a similar procedure was able to result in the first successful vitrification and warming survival of coral larvae (Daly et al. 2018). To our knowledge, this type of vitrification approach has not yet been applied to bovine oocytes and embryos. It is possible that this technique may hold promises under certain circumstances, such as in the case of vitrification of immature oocytes of wild species or cloned embryos that are difficult to cryopreserve.

## Conclusions

The bovine IVP system presents clear advantages/strengths but also shows some challenges/difficulties that must be overcome (see Table 3). Field practitioners and seedstock producers need DT frozen packaged embryo due to uncertainty of recipient availability and the difficulties in commercializing genetics (domestic and international) when the germplasm is not frozen. Current cryopreservation methods (slow freezing and vitrification) need refinements in order to be considered the preferred method by end users. The differences between both freezing systems (see Table 4) require more lab research and field trials with *in vivo* transfers in order to obtain pregnancy data. In recent years, bovine IVP technology has contributed to accelerated genetic gain in dairy breeds, primarily because of sexed semen and genomics. In several markets, IVP is also important for expanding and improving the beef cattle sector. Applied research and recent innovations have expanded the radius of action and facilitated the possibility of reaching more farmers and seedstock producers.

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Important key improvements have been made lately in IVP by improving oocyte and embryo quality and drastically reducing the number of abnormally heavy calves (Bruyere et al. 2012, Krisher 2004, Lim et al. 2008, Nivet et al. 2012, Vajta 2000). Although some feel that large offspring syndrome may be relegated to the past, for several reasons including: 1) a drastic reduction of bovine serum albumin during culture (0% to 3% max added after day 4), 2) use of sexed semen (females are smaller), 3) determination that *Bos taurus* more susceptible than *Bos indicus* (narrower ribcage and hips), 4) use of proper bulls (low birth weight calves) and 5) use of cows instead of heifers as recipients. But today, we can still see IVP calves sometimes being up to 20% heavier. Currently, in most instances that this problem still occurs, it is associated with a prolongation of gestation.

Current donor follicle stimulation protocols require multiple daily injections due to the relative short half-life FSH preparation in the female blood circulation. Current strategies to overcome this labor intensive situation are based on using long-acting FSH analogs through a slow release formulation (Kimura 2016), or even more desirable, replacing pituitary-derived preparations with recombinant bovine FSH (Carvalho et al. 2014). Promising research developments in the near future will likely be associated with bovine recombinant FSH for donor stimulation (Hesser et al. 2011). Encouraging results are also coming from cryopreserved IVP embryos for direct transfer (Sanches et al. 2016). Other areas for potential progress would be linked to the development of new incubation platforms mimicking natural culture environmental conditions (Smith et al. 2012). The demand for bovine IVP is not only being sustained but increases annually. The current focus by livestock researchers and OPU/IVF practitioners is to improve oocyte competence through donor follicle synchronization and stimulation to achieve partial *in vivo* maturation and to optimize embryo culture conditions to maximize embryo development, quality, viability, cryotolerance and pregnancy rate.

**Conflict of Interest Statement**

The authors declare no conflicts of interest.

**Data Availability Statement**

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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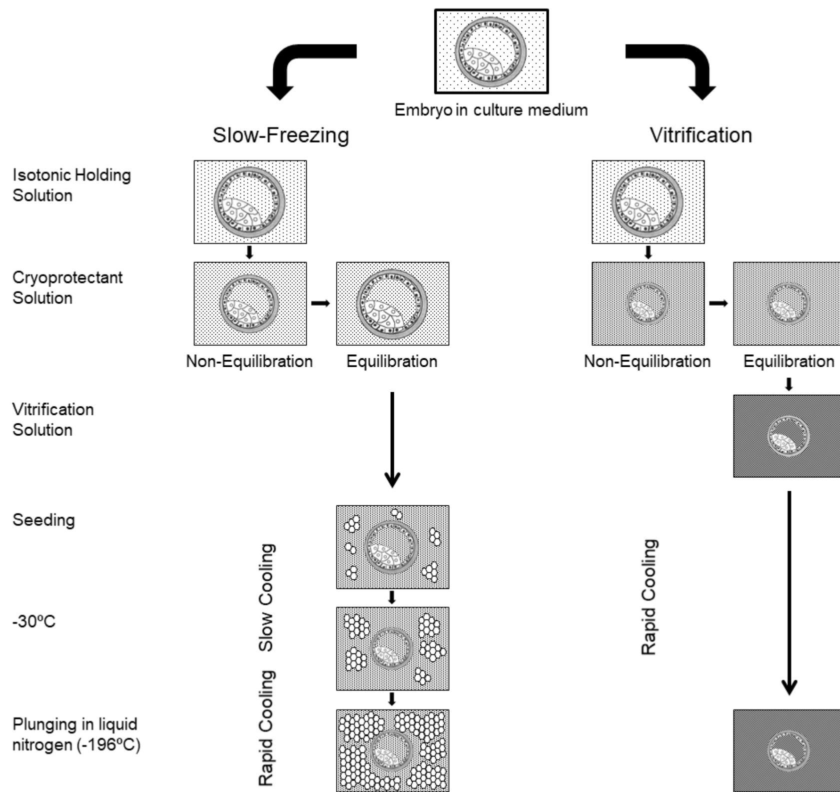


Figure 1. Schematic representation of an embryo during slow-freezing and vitrification steps. Darker shading represents a higher osmolality. Embryo size changes represent shrinking and re-expansion in response to solution osmolality and cryoprotectant concentrations. Hexagons show ice crystal formation.

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Table 1: Transfers of IVP (fresh and frozen) embryos registered by the International Embryo Transfer Society worldwide (IETS, http://www.iets.org/comm_data.asp)

| Year | Embryos | Total Transfers | Fresh Transfers | Frozen Transfers | Fresh:Frozen Ratio |
|---|---|---|---|---|---|
| 1997 | 41,632 | 30,569 | 12,018 | 18,551 | 39% |
| 1998 | 85,026 | 31,327 | 16,269 | 15,058 | 52% |
| 2000 | 168,169 | 168,169 | 140,272 | 27,897 | 83% |
| 2001 | 109,205 | 30,260 | 15,379 | 14,881 | 51% |
| 2002 | 160,695 | 83,329 | 66,951 | 16,378 | 80% |
| 2003 | 341,748 | 106,220 | 91,372 | 14,848 | 86% |
| 2004 | 319,086 | 239,823 | 128,961 | 110,862 | 54% |
| 2005 | 330,647 | 265,991 | 183,477 | 82,514 | 69% |
| 2006 | 441,364 | 291,845 | 226,077 | 65,768 | 77% |
| 2007 | 434,581 | 245,257 | 215,512 | 29,745 | 88% |
| 2008 | 330,953 | 254,714 | 227,800 | 26,914 | 89% |
| 2009 | 376,576 | 305,949 | 283,188 | 22,761 | 93% |
| 2010 | 450,149 | 339,685 | 315,715 | 23,970 | 93% |
| 2011 | 453,471 | 373,869 | 343,927 | 29,942 | 92% |
| 2012 | 443,533 | 385,471 | 348,868 | 36,603 | 91% |
| 2013 | 517,587 | 393,625 | 358,440 | 35,185 | 91% |
| 2014 | 590,359 | 364,727 | 296,666 | 68,061 | 81% |
| 2015 | 612,709 | 404,173 | 304,946 | 99,227 | 75% |
| 2016 | 666,215 | 448,113 | 326,623 | 121,490 | 73% |
| 2017 | 1,018,163 | 738,202 | 541,615 | 196,587 | 73% |
| **Total** | **7,891,868** | **5,501,318** | **4,444,076** | **1,057,242** | **81%** |

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Table 2: Pregnancy rate comparison between cryopreserved (slow freezing / vitrification) and fresh IVP embryos derived from different culture conditions

| Freezing | Co-culture | Serum | Direct ET | # Transfers | Preg Diagnosis Day | Pregnancy Rate | Reference |
|---|---|---|---|---|---|---|---|
| Slow Freezing | + | + | - | 866 | Not specified | 37.8% | (Kajihara et al. 1992) |
| Fresh | + | + | + | 121 | 32 | 42.0% | (Wurth et al. 1994) |
| Slow Freezing | + | + | - | 35 | | 14.0% | |
| Vitrification | + | + | - | 85 | | 24.0% | |
| Fresh | + | + | - | 1884 | 50 | 56.0%[a] | (Hasler et al. 1995) |
| Slow Freezing | + | + | - | 67 | | 42.0%[b] | |
| Fresh | - | + | + | 58 | 35 | 51.7% | (Massip et al. 1996) |
| Fresh | - | - | + | 40 | | 35.0% | |
| Fresh | + | - | + | 427 | | 32.3% | |
| Fresh | + | + | + | 47 | | 14.9% | |
| Slow Freezing | - | + | + | 8 | | 12.5% | |
| Slow Freezing | - | - | + | 53 | | 24.5% | |
| Slow Freezing | + | - | - | 65 | | 43.1% | |
| Slow Freezing | + | + | + | 59 | | 23.7% | |
| Fresh | - | - | + | 14 | 30 | 42.8% | (Martinez et al. 1998) |
| Vitrification | - | - | - | 33 | | 42.4% | |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Fresh | - | - | + | 75 | 40 | 73.3%[a] | (Agca et al. 1998) |
| Slow Freezing | - | - | - | 17 | | 41.0%[b] | |
| Vitrification | - | - | - | 16 | | 63.0%[a] | |
| Vitrification | - | - | - | 20 | 35 | 40.0% | (Lewis et al. 1999) |
| Vitrification | - | - | + | 22 | | 64.0% | |
| Fresh | - | - | + | 6 | 35 | 50.0% | (Pugh et al. 2000) |
| Vitrification | - | - | - | 20 | | 60.0% | |
| Slow Freezing | - | - | - | 20 | 35 | 35.0% | (Hernandez-Fonseca et al. 2002) |
| Slow Freezing | + | + | - | 372 | 90 | 34.7%[a] | (Říha et al. 2002) |
| Slow Freezing | + | + | + | 25 | | 72.0%[b] | |
| Vitrification | + | + | - | 24 | | 50.0%[ab] | |
| Slow Freezing | - | - | + | 154 | 50 | 39.6% | (Hoshi 2003) |
| Slow Freezing | - | + | + | 67 | | 32.8% | |
| Slow Freezing | - | - | + | 40 | 55 | 47.5% | (Lane et al. 2003a) |
| Slow Freezing | + | + | + | 67 | | 42.0% | |
| Vitrification | - | - | + | 10 | 75 | 30.0% | (Nedambale et al. 2004) |
| Vitrification | + | + | - | 3627 | 70 | 40.9% | (Xu et al. 2006) |
| Vitrification | | + | + | 41 | 60 | 19.5% | (Vieira et al. 2007) |
| Slow Freezing | - | - | + | 605 | 35 | 32.0% | (Lim et al. 2008) |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Slow Freezing | + | + | - | 41 | 90 | 48.8% | (Machatkova et al. 2008) |
| Fresh | - | + | + | 20 | 30 | 49.1% | (Inaba et al. 2011) |
| Vitrification | - | + | + | 18 | | 46.7% | |
| Fresh | - | - | + | 12 | 60 | 41.6% | (Caamaño et al. 2015) |
| Vitrification | - | - | + | 12 | | 41.6% | |
| Vitrification | - | - | - | 3392 | 60 | 36.6% | (Marinho et al. 2015) |
| Fresh | - | - | + | 51 | 60 | 58.8% | (Zhang et al. 2015) |
| Vitrification | - | - | + | 46 | | 56.5% | |
| Fresh | + | + | + | 259 | 30 | 51.3%[a] | (Sanches et al. 2016) |
| Slow Freezing | + | - | + | 311 | | 40.2%[b] | |
| Vitrification | + | + | - | 234 | | 35.9%[b] | |
| Fresh | - | + | - | 109 | 35 | 41.3% | (Do et al. 2018) |
| Vitrification | - | + | - | 85 | | 40.0% | |
| Fresh | - | - | + | 30 | 40 | 63.3% | (Gómez et al. 2020) |
| Fresh | - | + | + | 17 | | 64.7% | |
| Slow Freezing | - | - | + | 40 | | 55.0% | |
| Slow Freezing | - | + | + | 14 | | 64.3% | |
| Vitrification | - | - | - | 47 | | 61.7% | |
| Vitrification | - | + | - | 11 | | 45.4% | |
| Fresh | - | - | + | 58 | 35 | 51.7% | (Gómez et al. 2020) |
| Slow Freezing | - | - | + | 80 | | 50.0% | |

Values with different superscripts in the same column and author differ significantly ($p < 0.05$).

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Table 3: Advantages and disadvantages of IVP embryos compared to *in vivo* embryos derived from multiple ovulation and embryo transfer (MOET)

**Advantages/Strengths**

1) Increased embryo production per unit of time due to frequent follicular aspirations (Kruip et al. 1994, Merton et al. 2003).
2) Production of embryos from females in different physiological conditions (Galli et al. 2001).
3) Greater genetic progress as a consequence of combining higher genetic merit (genomics) dams and sires at early ages (Kasinathan et al. 2015, Smidt and Niemann 1999, Shojaei Saadi et al. 2014).
4) Reduced or lack of gonadotropins is necessary to superovulate donors (Bols 2005, Kruip et al. 1991, Pieterse et al. 1991, Gordon 2003b), and because of that, less labor and donor chute interventions.
5) High straw cost dilution among multiple donors due to micro-environment fertilization (Blondin et al. 2009, Gordon 2003a, Wheeler et al. 2006).
6) More females (90%:10% vs. 50%:50%) with sexed semen (Johnson et al. 1999)
7) Transfer of female embryos into lactating cows to increase pregnancy rates especially under high temperature/heat stress conditions (Al-Katanani et al. 2002, Ambrose et al. 1999, Hansen 2011, Jordan 2003, Rensis and Scaramuzzi 2003, Rutledge 2001, Stewart et al. 2011, Block et al. 2010).
8) Secures replacement needs (Rath and Johnson 2008, Wheeler et al. 2006).
9) Facilitates the eradication of diseases from the farm because of a female surplus.
10) First generation crossbred (F1) continues crossbreed female embryo program to maximize heterosis.

**Disadvantages/Weaknesses**

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1) IVP embryo yield efficiency continues to be poor. Only between 20 to 40% of the MII oocytes will reach the blastocyst stage and be suitable for transfer (Lonergan and Fair 2008, Rizos et al. 2008, Rizos et al. 2002b, Thompson and Peterson 2000).
2) IVP embryos show lower quality than *in vivo* counterparts (Lonergan et al. 2001, Leibfried-Rutledge 1999, Merton et al. 2003).
3) IVP embryos are darker and have a lower density because of their higher cytoplasmic lipid content (Enright et al. 2000, Massip et al. 1995, Wright and Ellington 1995, Ferreira et al. 2010), lower number of blastomeres, especially at inner cell mass (Rizos et al. 2002a, Holm and Callesen 1998, Iwasaki et al. 1990), fragile zona pellucida (Duby et al. 1997, Gordon 2004, Abe et al. 1999), reduced perivitelline space (Rizos et al. 2002b), slower developmental speed (Holm et al. 2002), and compaction at morula stage (Van Soom et al. 1997b, Van Soom et al. 1992).
4) High proportion of IVP embryos presents some degree of myxoploidy (Viuff et al. 1999). Among polyploid cells, the most frequent anomalies are triploid and tetraploid (Hyttel et al. 2000, Lonergan et al. 2004, Viuff et al. 2000, Viuff et al. 2002).
5) Functional alterations at the intercellular communication level resulting in compromised gap unions) (Boni et al. 1999, Wrenzycki et al. 1996).
6) Pronounced metabolic differences in oxygen, amino acids, glucose, pyruvate and lactate intake (Gardner 1998, Khurana and Niemann 2000, Rieger 1992, Thompson 1996, Thompson 1997).
7) Gene expression alterations (Lonergan et al. 2003, Badr et al. 2007, Niemann and Wrenzycki 2000, Urrego et al. 2014, Wrenzycki et al. 2004).
8) Increased incidence of apoptosis (Pomar et al. 2005).
9) Increased embryonic mortality, abortions, gestational problems such as severe late-gestation abnormalities, placentome malformation, hydroallantois, and lengthening of gestation and calving difficulties (Agca et al. 1998, Farin et al. 2001, Hasler

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2000, Numabe et al. 2000).

10) Large offspring syndrome (Farin et al. 2004, McEvoy et al. 2000, van Wagtendonk-de Leeuw et al. 1998, Young et al. 1998, Kruip and den Daas 1997).
11) IVP embryos show a reduced cryotolerance and non-satisfactory post-thawing results (Dobrinsky 2002, Massip 2001, Leibo and Loskutoff 1993) due to their structure, composition and responsiveness to ice formation and cryoprotective agents (Vajta 2000, Vajta and Kuwayama 2006).
12) IVP system shows suboptimal maturation, fertilization, cleavage, 8-cell block overcome, blastocyst, hatching, implantation, pregnancy, gestation, and calving survival rates in comparison to *in vivo*-derived embryos (Bavister 2002, Betteridge 2006, Faber et al. 2003, Galli et al. 2003, Gardner and Lane 2014, Gordon 2004, Greve and Callesen 2005, Hansen 2014, Lonergan 2007).

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Table 4: Comparison between vitrification and slow-cooling cryopreservation methods (Vajta and Nagy 2006, Moore and Bonilla 2007, Almiñana and Cuello 2015, Sanches et al. 2017, Mullen and Critser 2008, Assumpção et al. 2008, Massip and Leibo 2002, Massip 2001)

| **Characteristic** | **Procedure** | |
|---|---|---|
| | **Vitrification** | **Slow Cooling** |
| Computer freezer needed | No | Yes |
| Equipment and upfront investments | Low | High |
| Cost of embryo carrier devices | Very high in some cases | Low |
| Cost of solutions | Elevated | Moderate |
| Duration of procedure | Short | Long |
| Cooling rates | 15,000-30,000°C/min | 0.3-0.6°C/min |
| Direct exposure to $LN_2$ | Yes | No |
| Embryo cryo-solution volume | Extremely small (1-2 µl) | Large (100-250 µl) |
| Most applicable embryo stages | Late stages | Early stages |
| Concentration of CPA | High | Low |
| Risk of freeze injury, including ice crystal formation | Low | High |
| Full verification of solute penetration* | Yes | No |
| Full verification of dehydration rate* | Yes | No |
| Mechanical damage | Less | More |
| Fracture of ZP | No | Possible |

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| | | |
|---|---|---|
| Chemical damage | More | Less |
| Risk of toxicity of CPA | High | Low |
| Risk of potential contamination with pathogenic agents | High | Low |
| Post-thaw viability | High | Moderate |
| Protocol standardization/consolidation | Still variable | Fairly standardized |
| Operator technical experience/expertise | Very High | High |
| Manipulation skills/handling difficulty degree | Difficult | Easy |
| Direct embryo transfer | Possible (not standardized) | Yes |

*The term "full verification of solute penetration and dehydration rate" refers to the possibility of following these events under the stereo-microscope to the full extent (and in the case of slow freezing until "seeding") and verifying/confirming the cryo steps through embryo shrinking and expansion.

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