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Improving postcryopreservation survival capacity: an embryo-focused approach

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Abstract

The major challenge for a greater dissemination of *in vitro* produced (IVP) bovine embryos is to improve embryonic survival after cryopreservation. The involvement of embryonic lipids on this issue is well documented. However, it has been recognized that not only the amount of lipids that affects embryo cryotolerance, but the embryo survival capacity after cryopreservation is a rather multifactorial event. In this review, some strategies to improve embryonic lipid composition and postcryopreservation survival by modifying the embryos themselves to make them more cryopreservable are overviewed. The use of semi-defined and defined serum-free culture media, the addition of some chemicals in the culture media to modify embryo lipid composition, and the modulation of embryo cell membrane fluidity by cholesterol or unsaturated fatty acids added to the culture media and oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids, were described as alternatives for the improvement of IVP embryo survival after cryopreservation.

Keywords: bovine, cryotolerance, cryosurvival, *in vitro* produced embryos, lipid.

Introduction

The global *in vitro* production of bovine embryos has increased for the 6th consecutive year in 2011. The total number of *in vitro* produced (IVP) embryos transferred worldwide was 373,836. Brazil alone was responsible for 85% of the global market of IVP embryos (Stroud, 2012). This achievement can be attributed to the high number of oocytes recovered by ovum pick-up (OPU) of zebuine breed donors, mainly represented by Nellore (*Bos taurus indicus*) animals, allowing the commercial application of *in vitro* production on large-scale programs (Pontes *et al.*, 2011).

Embryo cryopreservation is an assisted reproductive technology that allows the storage of excess embryos derived from *in vitro* production and embryo transfer programs so they can be commercialized or transferred at the most convenient time. It is considered a strategy to overcome some logistic problems associated with the transfer of large numbers of fresh embryos and mainly for expanding the commercialization of embryos between countries (Sudano *et al*., 2012c).

Despite the very good results associated with the fresh IVP embryo transfer, the use of cryopreserved embryos is extremely limited. The modest results of cryopreserved IVP embryos limit their application at the field conditions as it is successfully done with the semen in the artificial insemination. Even after many advances in embryo research over the past decades, embryo cryopreservation remains one of the most challenging biotechnologies of bovine reproduction, since the cryopreservation results are still inconsistent. This fact reflects directly in the lower number of cryopreserved embryos (Fig. 1) in Brazil (3 to 7%) and worldwide (7 to 8%) over the last years (Stroud, 2010, 2012; Viana *et al.*, 2010; Viana, 2012).

The most common approach to deal with the disappointing results of cryopreservation is to vary the cryopreservation procedures by altering, for example, the concentration and type of cryoprotectants, the time and temperature of the protocol, and the addition of additives (sugars or surfactants). Despite the fact that this approach usually results in improvements, they are often limited, what has led to increasing the efforts on an embryo-focused approach by modifying the embryos themselves to make them more cryopreservable (Seidel, 2006). Therefore, the objective of this review is to present some strategies for improving postcryopreservation survival capacity through an embryo-focused approach in order to produce an embryo more resistant to the cryopreservation.

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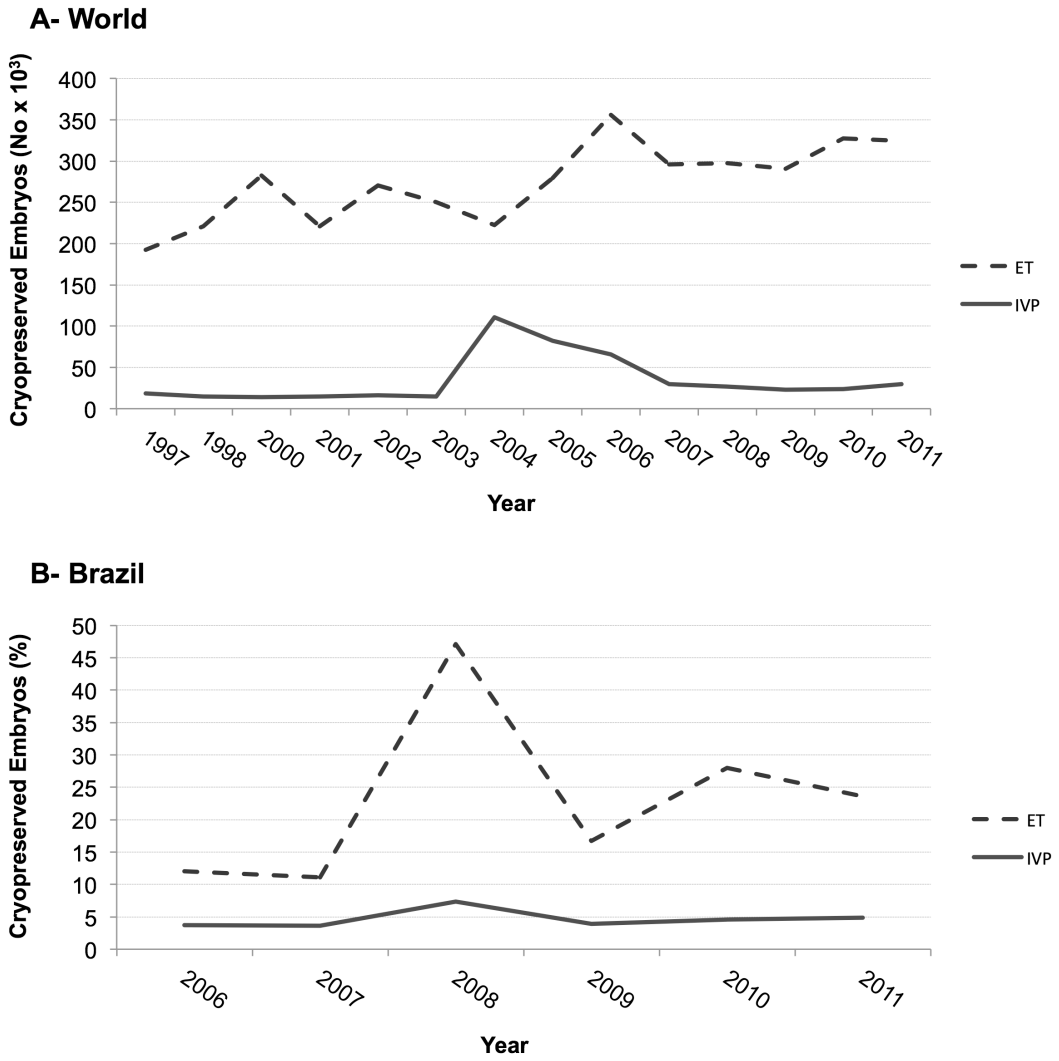

Figure 1. Total number and percentage of cryopreserved *in vivo* (ET) and *in vitro* (IVP) produced bovine embryos in the world (A) and Brazil (B). Data were obtained from IETS annual statistics and data retrieval committee report (http://www.iets.org/comm_data.asp), and Brazilian record data (Viana and Camargo, 2007; Viana, 2009; Viana *et al.*, 2010; Viana, 2012).

Differences between *in vitro*- and *in vivo*-produced embryos and quality control of *in vitro* production systems

Since the first success of the cryopreservation of a mouse embryo (Whittingham *et al.*, 1972), several procedures were developed to cryopreserve embryos. These methods can be basically classified in two major strategies: slow freezing and vitrification. Despite that both of them are considered cryopreservation techniques, they have important conceptual differences. In the slow freezing system, extracellular water crystallizes resulting in increased osmotic gradient that draws water from the intracellular compartment until intracellular vitrification occurs. In the vitrification system, both intra and extracellular compartment vitrify after cellular dehydration has already occurred (Saragusty and Arav, 2011).

When comparing the results of these cryopreservation methods based on the embryo origin, i.e. IVP *versus in vivo* produced (ET) embryos, ET embryos showed similar pregnancy rates (varying from 39 to 59%) for both slow freezing and vitrification procedures (Massip, 1987; van Wagtendonk-de Leeuw *et al.*, 1997; Inaba *et al.*, 2011), while the best results for IVP embryos (varying from 52 to 100% of re-expansion and from 36 to 93% of hatching/hatched rate) were achieved using vitrification (Nedambale *et al.*, 2004; Mucci *et al.*, 2006; Yu *et al.*, 2010; Inaba *et al.*, 2011). It is largely known that IVP embryos have a greater sensitivity to the cryopreservation techniques than ET ones (Leibo and Loskutoff, 1993) based on the fact that comparison of embryos from these two origins has demonstrated that the embryos do not survive equally in the different cryopreservation methods.

There are many morphological and metabolic differences between IVP and ET embryos, such as: very

electron-dense cytoplasm, loose blastomeres, buoyant density, metabolic abnormalities ("crabtree effect" and "unquite metabolism"), gene overexpression, apoptosis rate, lipid content, and postcryopreservation survival (Fair *et al.*, 2001; Abe *et al.*, 2002; Rizos *et al.*, 2002; Corcoran *et al.*, 2006; De La Torre-Sanchez *et al.*, 2006b; Mucci *et al.*, 2006; Leese *et al.*, 2008a; Côté *et al.*, 2011; Sudano *et al.*, 2011). All these alterations observed in IVP embryos can be attributed to the different *in vitro* culture conditions during oocyte maturation and embryo development that modulate the occurrence of these distinctive phenotypes (Lonergan *et al.*, 2003).

In the literature, the large amount of cytoplasmic lipid droplets observed in IVP embryos has been suggested to be the major cause of reduced postcryopreservation survival (Abe *et al.*, 2002; De La Torre-Sanchez *et al.*, 2006b; Mucci *et al.*, 2006; Barceló-Fimbres and Seidel, 2007a, b). Indeed, an increased amount of lipid droplets had a moderate correlaction with the postcryopreservation survival. However, the embryo quality evaluated by the apoptosis rate had a strong correlaction with the embryo survival after cryopreservation (Sudano et al., 2012b), highlighting the importance of embryo quality after cryopreservation and suggesting that embryo cryosurvival capacity is a multifactorial event. Several factors are invoved in the embryo cryotolerance, such as: lipid content, lipid composition, embryo metabolism, apoptosis, and global gene expression pattern (Sudano *et al.*, 2011, 2012a, b, c).

The goal during *in vitro* embryo production is try to mimick as much as possible the *in vivo* enviromental condition to achieve a good quality embryo, which, in turn, could be cryopreserved more efficiently. Several researchers suggest a rigorous quality control during all steps of *in vitro* embryo production to obtain good results (Lane *et al.*, 2008; Hasler, 2010; Saragusty and Arav, 2011).

In this context, we explore in this review the important aspects that could affect IVP embryo quality and cryotolerance, namely: culture media composition (additives, salts, aminoacids, hormones, sugars, antioxidants, pH, and osmolarity), atmosphere (lower or higher oxigen tension), temperature, oocyte donor, semen, sire, and technician (Gardner, 2008; Leese *et al.*, 2008b; Feugang *et al.*, 2009; Hasler, 2010; Hugentobler *et al.*, 2010).

Embryo lipids

There are evidences that at least four classes of lipids affect embryo survival after cryopreservation: triacylglycerides (TAG; mainly stored at the cytoplasmic lipid droplets), free fatty acids (FFA), cholesterol (Chol) and phospholipids (PL; cell membrane lipids).

The reason for the increased number of cytoplasmic lipid droplets in IVP embryos is unknown. However, it is speculated that it is related to fetal calf serum (FCS) supplementation in the culture media. It seems that FCS increases embryo lipid content through: a) the lipoproteins from the serum are absorbed by the embryonic cells (Sata *et al*., 1999); b) the embryo is induced to perform neosynthesis of triacylglycerides due to the presence of FCS (Razek *et al.*, 2000); and c) the FCS changes the function of β-oxidation in the mitochondria (Abe *et al.*, 2002). Another potential reason is that lipid accumulation occurs as an effect of abnormal energetic metabolism. An imbalance in the cellular oxidation-reduction process also occurs, affecting mitochondrial function and impairing metabolism of lipid complexes through β-oxidation (Abe *et al.*, 2002).

The lipid droplets present in the IVP embryos cytoplasm are mainly composed by TAG, the predominant lipid in the cytoplasm of mammalian cells (McKeegan and Sturmey, 2011). These stored lipids constitute an important source of energy for oocytes and embryos (Sturmey *et al.*, 2009). The estimated TAG content remained constant in a serum-free medium during embryo development (33 ng/embryo); however, in a serum-supplemented medium the TAG amount increased from 33 ng in 5-8 cell stage to 62 ng in hatched blastocysts (Ferguson and Leese, 1999). In addition, FCS also increased the total fatty acid amount compared with a serum-free media (74.2 *vs*. 57.2 ng, respectively), mostly represented by an increase in the palmitic (28.9 *vs*. 20.1 ng), stearic (18.0 *vs*. 13.1 ng), oleic (12.1 *vs*. 4%), and palmitoleic (16.3 *vs*. 3.7%), which are saturated (Reis *et al.*, 2003) and monounsaturated (Sata *et al*., 1999) fatty acids.

On the other hand, PL are the most abundant lipid in eukaryotic cell membranes and their role in successful embryo cryopreservation remains poorly understood (van Meer *et al.*, 2008). Phospholipids, particularly phosphatidylcholines (PC) and sphingomyelins (SM), are structural units of functional membranes, and their composition determines the physicochemical properties of cell membranes, including fluidity, permeability, and thermal phase behavior (Edidin, 2003). We have recently reported that the PL profiles of bovine embryos vary between subspecies (*B. taurus indicus vs*. *B. taurus taurus*) and origin (IVP *vs*. ET) and that specific lipid species can potentially be used as biomarkers of embryonic postcryopreservation survival. These results indicate that not only the lipid amount but also the lipid composition accounts for embryo survival after cryopreservation (Sudano *et al.*, 2012c).

Strategies to improve embryo lipid composition and postcryopreservation survival capacity

Fetal calf serum-free media

It is well known that FCS provides energy substrates, amino acids, vitamins, growth factors, and heavy-metal chelators. Although FCS has useful properties, its use has been associated with several

abnormalities, such as cell organelles modification, mitochondrial degeneration, gene expression modification, large offspring syndrome, increased lipid droplets number and reduced postcryopreservation survival (Abe *et al.*, 2002; Lazzari *et al.*, 2002; Rizos *et al.*, 2002, 2003; Sudano *et al.*, 2011). As a result, chemically-defined media (without FCS) have been developed (Keskintepe and Brackett, 1996).

It has already been described that it is possible to produce *in vitro* bovine embryos in defined (Block *et al.* 2010; Momozawa and Fukuda, 2011) or semidefined (Mucci *et al.*, 2006) serum-free media without affecting blastocyst yield and increasing embryo cryosurvival. In addition, a reduction of FCS concentration in the culture media alone was enough to decrease the lipid content and increase the postcryopreservation survival (Sudano *et al.*, 2011). The use of serum-free media has been considered as one of the first actions for the establishment of a successful *in vitro* embryo production system, allowing higher embryo survival after cryopreservation (Rizos *et al.*, 2003; Mucci *et al.*, 2006).

Use of chemical additives

Energetic substrate is promptly metabolized through the glycolytic pathway (Fig. 2). However, the energetic metabolism during early embryo development (pre and post-compaction stage) is abnormal in IVP embryos (De La Torre-Sanchez *et al.*, 2006b). Under *in vitro* conditions, embryos show an increased activity of the glycolytic pathway and a consequently inhibition of oxidative phosphorylation pathway, characterized as "Crabtree effect" (Crabtree, 1929; Seshagiri and Bavister, 1991). A higher metabolic activity through the glycolysis impairs embryo development, because too little energetic substrate is partitioned to the pentose phosphate pathway (PPP) which is part of an important biosynthetic pathway (Wales and Hunter, 1990), by favoring lipid accumulation and rising cellular concentrations of lipid synthesis precursors (Rieger, 1992).

An interesting approach would be the use of phenazine ethosulfate (PES) in order to balance the energetic metabolism and reduce the lipid accumulation, by favoring the enzymatic reactions of PPP (Fig. 2), since this chemical oxidizes NADPH to NADP (De La Torre-Sanchez *et al.*, 2006a; Sudano *et al.*, 2011). The use of PES in the post-compaction period resulted in a reduction of the embryo lipid accumulation and an increase in the postcryopreservation survival (Barceló-Fimbres and Seidel, 2007b; Sudano *et al.*, 2011).

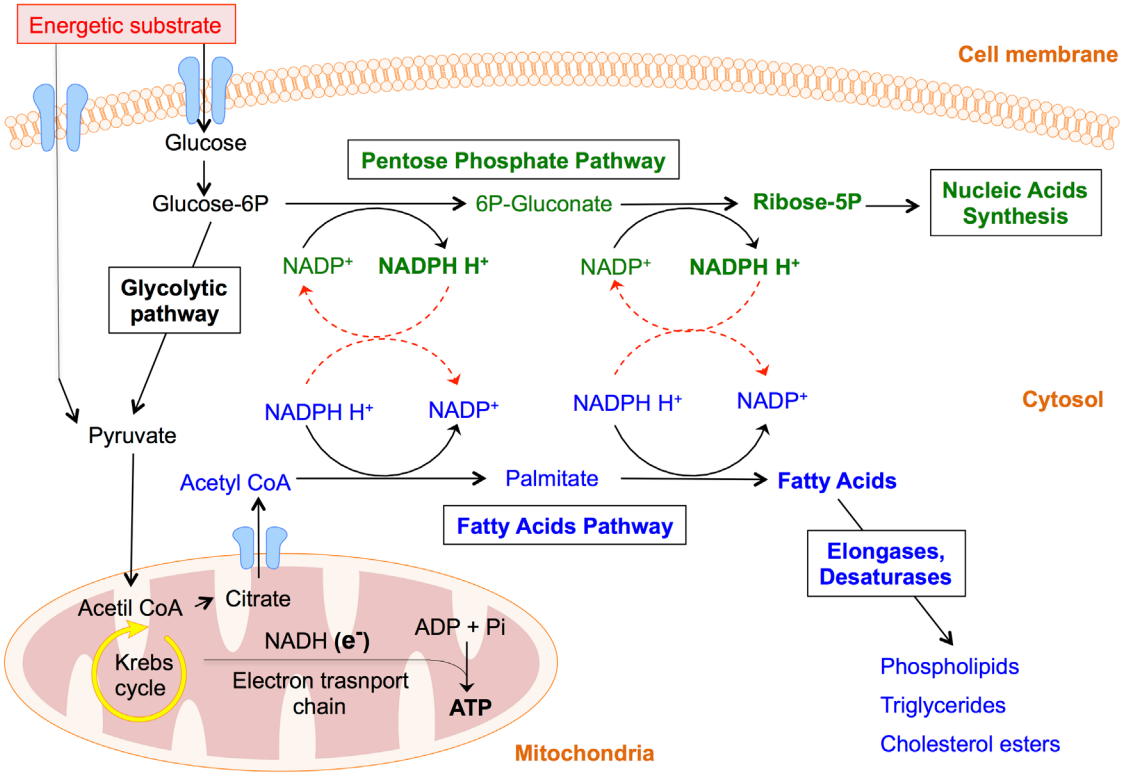

Figure 2. Energetic metabolism and phenazine ethosulfate (PES) mechanism of action in the preimplantation embryo. Energetic substrate is promptly metabolized in the glycolytic pathway (represented in black) through glycolysis within cytoplasm, followed by the krebs cycle and oxidative phosphorylation within mitochondria to produce energy in the form of ATP. PES reduces NADPH to NADP (dashed red arrows) favoring the pentose phosphate pathway (represented in green) with inhibition of the fatty acids pathway (represented in blue). Adapted from Barceló-Fimbres and Seidel (2007a).

Another chemical used to reduce lipid content of IVP embryos is the forskolin (Fig. 3A), a potent adenylate cyclase activator that stimulates the lipase activity through the cAMP/protein kinase pathway (Men *et al.*, 2006). Forskolin supplementation in the culture media reduced lipid content and increased the embryo survival after cryopreservation of bovine and porcine IVP embryos (Men *et al.*, 2006; Paschoal *et al.*, 2012).

More recently, L-carnitine, a small water-soluble molecule and cofactor of β-oxidation, was found to play an important role in the lipid metabolism (Sutton-McDowall *et al.*, 2012; Moawad *et al.*, 2013). This chemical is crucial for fatty acids (in form of acyl-CoA) translocation into the mitochondria (Fig. 3B), where they will be metabolized to acetyl-CoA through β-oxidation, and can be further metabolized in Krebs cycle and oxidative phosphorylation for ATP production (Sutton-McDowall *et al.*, 2012). L-carnitine also has an antioxidant activity protecting the cells from DNA damage (Abdelrazik *et al.*, 2009). Several beneficial effects of L-carnitine supplementation to the culture media have already been reported, including the improvement in the embryo development (Sutton-McDowall *et al.*, 2012), lipid metabolism and cryotolerance of bovine embryos (Takahashi *et al.*, 2012). The unique dual effects of L-carnitine enriching cellular lipid metabolism and providing antioxidative protection make it a chemical candidate for a non-invasive improvement of cryotolerance and developmental competence in IVP embryos (Takahashi *et al.*, 2012).

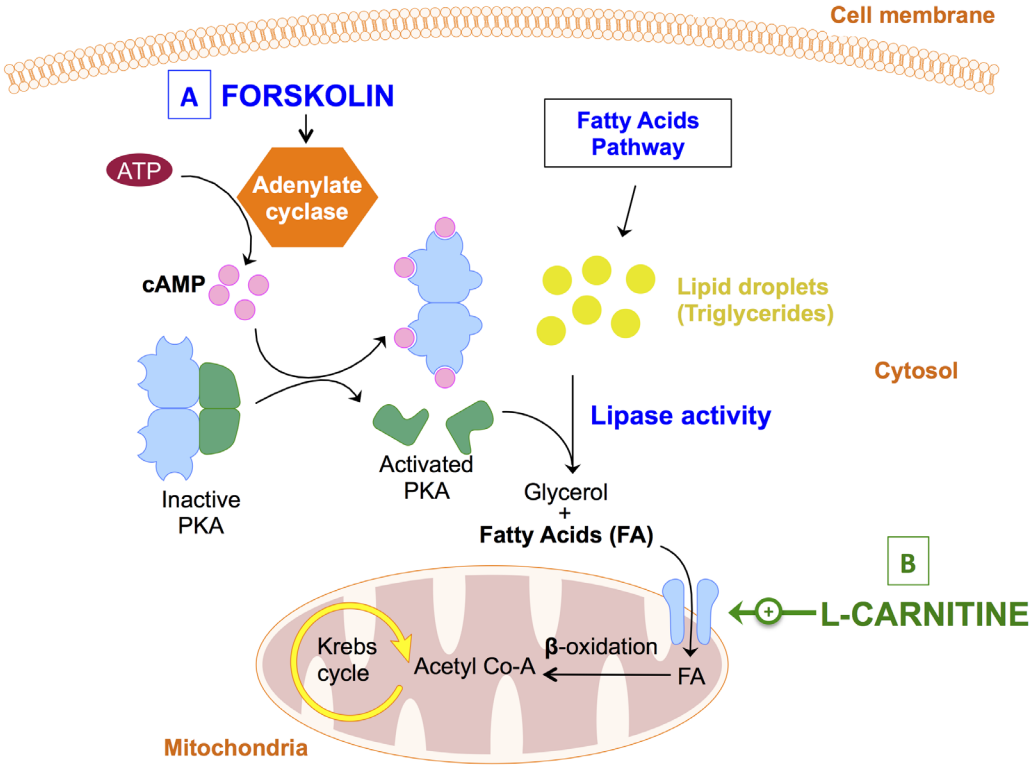

Figure 3. Mechanism of action of forskolin (A), a potent adenylate cyclase activator that stimulates the lipase activity through the cAMP / protein kinase (PKA) pathway, and L-carnitine (B), that favors fatty acid translocation into the mitochondria (in form of acyl-CoA) where they will be metabolized to acetyl-CoA through β-oxidation.

Cell membrane

In 1972, the structure of cell membrane was presented as the fluid mosaic model, which describes a cell membrane composed by a fluid bilayer of phospholipids oriented with the hydrophilic and hydrophobic portion to outside (extracellular and cytosolic compartments) and interior of the membrane, respectively (Singer and Nicolson, 1972).

The PL are composed by a three carbon backbone to which a phospho-head group is attached to an end carbon of the backbone and two fatty acyl chains (fatty acids) are attached to the other carbons. Both the phospho-head groups and the fatty acids can vary in their composition and this will determine the properties of the cell membrane (Edidin, 2003). Indeed, it was reported that embryos with an increased abundance of unsaturated lipid species had greater cryosurvival (Sudano *et al.*, 2012c).

Cholesterol is another molecule present in cell membrane, and its level and the ratio between cholesterol and PL also affects the membrane fluidity (Horvath and

Seidel, 2006). Enriching the embryo cell membrane with unsaturated fatty acids and cholesterol to improve embryo cryotolerance has already been performed by two procedures, as follows: 1) membrane incorporation through its supplementation in the culture media; 2) nutritional management of oocyte/embryo donors and by offering a diet-rich with polyunsaturated fatty acids.

While the addition of cholesterol-loaded methyl-β-cyclodextrin to the cryopreservation media had no effect on cryopreserved IVP bovine blastocysts (Pugh *et al.*, 1998), it seems to have a positive effect on vitrified oocytes as measured by an increase in the cleavage rate and number of eight-cell embryos (Horvath and Seidel, 2006), as well as an improvement in the nuclear maturation (Sprícigo *et al.*, 2012) after warming in comparison with the untreated group.

In addition, the unsaturated fatty acid supplementation (especially linoleic acid) in the culture media improved embryo cryotolerance (Hochi *et al.*, 1999; Pereira *et al.*, 2007) and reduced lipid content (Pereira *et al.*, 2007) of IVP embryos. Likewise, the oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids increased the cryosurvival of ewe oocytes (Zeron *et al.*, 2002) and porcine embryos (Kojima *et al.*, 1996).

Conclusion

The major obstacle for a greater dissemination of the use of *in vitro* produced bovine embryos is their high sensitivity to the cryopreservation process. The involvement of the embryo lipids on this aspect is well documented. However, it has been recognized that not only the amount of cytoplasmic lipids affects embryo cryotolerance. The embryo survival capacity after cryopreservation is a multifactorial event. A rigorous quality control during all steps of *in vitro* embryo production is required to obtain a good quality and cryopreservable embryo. The use of a serum-free media, the addition of chemicals to change lipid metabolism, and the modulation of membrane lipid composition have been described as some alternatives for the improvement of the IVP embryo survival after cryopreservation

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