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BOVINE OOCYTE *IN VITRO* MATURATION AND CRYOPRESERVATION: MIRAGE OR REALITY

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ABSTRACT

Selected historical and current aspects of bovine oocyte maturation *in vitro* and cryopreservation were described and discussed in this paper. We have been working on both subjects for years with a rather moderate success, having however good opportunity to observe slow but constant progress being achieved by many research teams worldwide.

Key words: bovine oocyte; oocyte maturation; *in vitro* maturation; cryopreservation; vitrification

INTRODUCTION

An efficient using of oocytes in bovine reproductive biotechnology requires high quality *in vitro* maturation procedures and effective, reliable cryopreservation techniques. Problems related to bovine oocyte *in vitro* maturation and cryopreservation have been focusing attention of researchers for years, and still is difficult to say that all these problems have already been solved. We discuss here some key factors limiting developmental competence of *in vitro* matured oocytes and factors affecting success level of current vitrification methods.

Complex problems with cryopreservation

An efficient, innocuous method of bovine oocyte cryopreservation has been an ambition of cryobiologists for years. Neither numerous experiments on traditional controlled slow freezing, nor more promising vitrification approach was satisfactory. Initially, only a low ratio of blastocyst development (2 to 10 %) was achieved and 2 or 3 calves were born in few occasions, but a final efficiency of those methods was too low to become applicable for practical use. First analyses of biological background of observed vulnerability of bovine oocytes indicated particular sensitivity of certain cellular structures of these cells (cytoskeleton, cortical granules, lipid droplets) on destruction resulting from low temperature and/or cryoprotective agents exposition (Agca *et al*., 1998; Hyttel *et al*., 2000; Rho *et al*., 2002; for review see: Gajda and Smorag, 2009; Prentice and Anzar, 2011; Saragusty and Arav, 2011). Attention was paid also to a spatial mitochondria distribution in cells and on possible compromising a mitochondrial membrane potential (Rho *et al*., 2002; Jones *et al*., 2003)

In 1996 Martino *et al*. presented their results of vitrification, which were through-breaking due to a novel approach. Very small volume of vitrification solutions (few microliters) containing a number

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of bovine cumulus-oocyte complexes (COCs) were mounted on a small electron microscope grid and plunged into liquid nitrogen. As a result, a significant acceleration of cooling rate was achieved, and due to this - much better survival rate of vitrified oocytes enabling development *in vitro* of higher than before ratio of blastocysts (15 %). Idea of such an approach derived from 5 years earlier experiments on vitrification of fruit fly embryos was referred to as Minimum Sample Size (MSS) vitrification system. In 1998 Vajta and his colleagues described even higher blastocysts ratio (25 %) obtained after *in vitro* fertilization (IVF) of oocytes vitrified according to MSS approach. They vitrified oocytes loaded into the tip of narrow plastic capillary made of warmed up and pulled off plastic insemination straws. This method was referred to as an open pulled straw (OPS) vitrification. Many similar methods employing different embryo holders, as for example glass capillaries (Hochi *et al*., 2000), nylon loops (cryoloop) (Lane *et al*., 1999; Begin *et al*., 2003) and many others (Dinnyes *et al*., 2000; Kuwayama *et al*., 2005) were described after that.

In all these MSS vitrification experiments improvement of efficiency was originally attributed mainly to an increase in the cooing rate due to extreme limitation of volume of vitrified sample. Instead of 2,000 to 2,500 ºC/min cooling rate obtainable for insemination straw, such microvolumetric conditions allow to achieve cooling rates of 10,000 ºC to even about 100,000 ºC/min (Martino *et al*., 1996; Arav and Zeron, 1997; Vajta *et al*., 1998; Vajta *et al*., 2000). Such cooling acceleration provides substantial shortening of the time period required for transfer of cells through deleterious range of temperatures, between +20 and -20 ºC. Transition of cells across this dangerous range of temperatures may lead to changes in oolemma bi-layer structure and functionality, dependent on phase transition temperature. This particular temperature point differs depending on maturation stage of oocytes affecting fluidity of membranes (Arav *et al*., 1996). Extremely fast transition across mentioned temperature range should be necessarily considered if innocuous cryopreservation is taken into account. In response to this requirement, microvolumetric vitrification devices (such as CryoTop, CryoLeaf etc.) have been designed, produced and commercially offered to practitioners and researchers.

More recently a significance of high warming rate, easily obtainable in majority of MSS vitrification systems was indicated (Seki and Mazur, 2009). The majority of currently developed vitrification devices allow an obtaining of very high warming rates, enabling high survival rate of eggs or embryos, to some extent independently of their individual cooling rate. This is particularly important in closed vitrification systems recommended from sanitary reasons in human assisted reproduction. On the other hand, microvolumetric vitrification allowed considering a possible decrease in cryoprotectant(s) (CPs) concentration (Arav and Zeron, 1997; Dinnyes *et al*., 2000; Criado *et al*., 2011). In 2005, Kuwayama *et al*. demonstrated a very efficient vitrification device (CryoTop) enabling use of a lower concentration (30 %) of membrane permeating CPs, ethylene glycol and dimethylsulphoxide (DMSO), instead of 40 % used in OPS system. Till now several reports have been published on using MSS vitrification approach for cryopreservation of oocytes and early embryos from species that were extremely difficult to cryopreserve using former methods e.g. bovine oocytes (Dinnyes *et al*., 2000; Hochi *et al*., 2000; Li *et al*., 2002; Chian *et al*., 2004; Anchamparuthy *et al*., 2009), oocytes and cleavage stage embryos of goat (Begin *et al*., 2003) pig (Berthelot *et al*., 2000; Missumi *et al*., 2003), and of many other species (see for reviews Gajda and Smorag, 2009; Saragusty and Arav, 2011; Mullen and Fahy 2012). Due to its efficiency, this approach was also employed in human oocyte and embryo cryopreservation (Liebermann *et al*., 2003; Cobo *et al*., 2008; Kuwayama *et al*., 2005; Papis *et al*., 2011).

Unfortunately, in spite of several attempts of many researchers to improve bovine oocyte vitrification performed during last decade (Hochi *et al*., 2000; Diez *et al*., 2005; Horvath and Seidel, 2008; Magnusson *et al*., 2008; Yang *et al*., 2008; Sripunya *et al*., 2010; Zhou *et al*., 2010), the obtained results (Checura and Seidel, 2007; Chankitisakul *et al*., 2013) only exceptionally approached the level of efficiency, described more than 15 years earlier (Vajta *et al*., 1998; Papis *et al*., 2000; Dinnyes *et al*., 2000).

The real reason of permanent, still existing problems with bovine oocyte cryopreservation is not fully elucidated yet. Successful oocyte cryopreservation requires not only “physical” survival, but also undisturbed further performance of this very complex germ cell. One of important oocyte function affected by cryopreservation, regardless of the method used, is fertilization process, which, if improper, decreases significantly a general efficiency of a whole procedure. Difficult beginnings of human oocyte cryopreservation due to the low efficiency of fertilization were finally overcame by the introducing ISCI - intracytoplasmic sperm injection, which allows to by-pass side effects of cryopreservation, such as cortical granule premature release and/or *zona pellucida* hardening. Unfortunately, in case of bovine cryopreserved oocytes, ICSI procedure is usually much less efficient (Rho *et al*., 2004; Liang *et al*., 2011), even if additional activation is applied.

As mentioned above, low efficiency of embryonic development after IVF of cryopreserved oocytes can

be caused either by problems with successful sperm-egg fusion, or by a hindered pre-term activation that normally occurs after fertilization. Sperm-egg fusion requires a proper cytoskeleton and microvilli structure, as well as expression of proteins that will bind to sperm and mediate the fusion (Le Naour *et al.*, 2000; Saunders *et al.*, 2002; Stein *et al.*, 2004; Runge *et al.*, 2007). Proper oocyte activation depends on Ca^{2+} oscillations induced by a fertilizing sperm, which lead to the degradation of cyclin B and inactivation of MPF complex (reviewed in: Motlik *et al.*, 1998; Ducibella and Fissore, 2008). Cyclin B degradation requires also a proper alignment of the chromosomes in the metaphase II spindle (Stein *et al.*, 2004). Ability of the oocyte to produce Ca^{2+} oscillations has been previously shown to be affected by prolonged *in vitro* culture (aging) and oxidative stress (Jones and Whittingham, 1996; Igarashi *et al.*, 1997; Takahashi *et al.*, 2003). Therefore, it is likely, that this process is perturbed in *in vitro* matured and vitrified oocytes as well.

Key feature of successful fertilization of mammalian eggs seems to be an intracellular Ca^{2+} wave oscillations triggered by sperm cytosolic factor(s) such as PLC zeta, leading to an appropriate activation of the oocyte and several downstream effects triggering subsequent developmental features (Ajduk *et al.*, 2008). On the other hand, wave of calcium ions released from endoplasmic reticulum causes a cortical reaction of the oocyte, resulting in CG exocytose and subsequent changes caused mainly by ovastacin in the *zona pellucida* ZP2 glycoprotein structure conformation (Ducibella and Fissore, 2008; Machaty, 2013; Burkart *et al.*, 2014). In this way, subzonal entry of additional number of sperm is blocked, being at least one of two mechanisms preventing polyspermic fertilization of mammalian oocytes. From papers describing such mechanisms in mouse, rat, sheep, pig and human oocytes it is quite obvious that cryoprotective agents, such as ethylene glycol (EG) or dimethylsulphoxide (DMSO), typically used in slow freezing or vitrification solution formulations, may adversely affect basic mechanisms underlying normal fertilization processes, such as a sperm-egg fusion, exocytose of cortical granules, generation of intracellular calcium oscillations, etc. (Ruppert-Lingham *et al.*, 2003; Takahashi *et al.*, 2004; Tian *et al.*, 2007; Fujiwara *et al.*, 2010; Gualtieri *et al.*, 2011). Similar adverse effects may be also exerted by freezing procedures *per se*.

Taken all above considerations together, it seems reasonable to limit time of exposure and/or concentration of vitrification solution used for vitrification of particularly sensitive bovine oocytes. In 1999 we presented the first effects of a microdroplet vitrification system (Papis *et al.*, 1999a; Papis *et al.*, 1999b), technically based on earlier methods (Landa and Tepla, 1990; Riha *et al.*, (1992). For some variants of equilibration, the generation of 29.6 % blastocyst from mature oocytes subjected to IVF after vitrification was described in these papers. Such a high ratio of blastocyst stage embryos, two pregnancies after transfer of 4 embryos to 4 recipients and a healthy calf delivered in February 1999 occurred possibly in part due to the microvolumetric vitrification, but mainly due to a gentle pre-equilibration of oocytes in diluted (3 %) solution of ethylene glycol. In this approach, necessary intracellular concentration of cryoprotective agent was achieved mainly due to a transient dehydration of cells after short (30 s) equilibration in solution of 5.5 M ethylene glycol and 1.0 M sucrose - main components of vitrification medium. Described here pre-equilibration system turned out to be very efficient for bovine Day 3 embryo vitrification (Papis *et al.*, 1999b) and for Day 2 embryos as well (Papis, unpublished) elevating significantly an efficacy of such embryo vitrification according to MSSV approach comparing with OPS method (Vajta *et al.*, 1998; Vajta, 2000). Unfortunately, the same method of vitrification of immature bovine germinal vesicle stage oocytes was less successful (Papis *et al.*, 2013). The whole procedure has been patented in Japan (Method of Cryopreservation of Cells; no. 3044323) in March 2000.

Other authors attempted to employ the pre-equilibration system with different level of success for vitrification of oocytes intended for enucleation and cloning (Dinnyes *et al.*, 2000; Chang *et al.*, 2004; Yang *et al.*, 2008), for banking of oocytes obtained from endangered bovine breeds (Li *et al.*, 2002), buffalo (Liang *et al.*, 2011) or for vitrification of goat oocytes and cleaving embryos (Begin *et al.*, 2003). Compromised results reported by those authors may be caused by methodological modifications applied, as for instance different components of vitrification media and/or higher concentration of pre-equilibration solution resulting in the need of using a sucrose solution during warming leading to undesirable, in our opinion, shrinkage of warmed cells.

From the above-shown data we can conclude that our best results of bovine oocyte vitrification, presented 15 years ago, might have been obtained due to an accidental beneficial interaction between efficient vitrification procedure and sperm source and/or preparation method (Papis *et al.*, 1999a; Papis *et al.*, 2000). Our own attempts to adapt the method developed in Japan to local laboratory conditions in Poland, failed to be equally successful, as we were able to get less than a half of the previously reported blastocyst ratio (Papis *et al.,* 2003, unpublished). The other results show that development of embryos obtained after chemical activation and nuclear transfer is usually much better than after standard

IVF procedure (Dinnyes *et al*., 2000; Yang *et al*., 2008), which supports the notion that cryopreserved bovine oocytes have a decreased capability of fertilization.

Nevertheless, despite of using extremely fast procedures, the recent success of bovine oocyte vitrification usually remains disappointing (Hochi *et al*., 2000; Diez *et al*., 2005; Yang *et al*., 2008; Sripunya *et al*., 2010; Zhou *et al*., 2010). The serious hope recently came from Tamas Samfai laboratory in Japan, where after many years of intense research on lipid droplet destructive influence on cryopreservation effects (Nagashima *et al*., 1999; Romek *et al*., 2009; Fu *et al*., 2011) a positive effects of L-carnitine supplemented maturation media was described (Chankitisakul *et al*., 2013). L-carnitine, which increases metabolism of lipids in cells, is capable of decreasing lipid contents in oocytes. Lipid droplets abundant in certain cells, such as bovine and pig oocytes or embryos, had been recognized as the other key factor decreasing the efficiency cryopreservation of these cells. It remains unclear, how decreasing of lipid content in the oocyte may overcome all above mentioned problems with the proper fertilization mechanisms after vitrification. However, from practical point of view, it would be significant that smart combination of metabolic digestion of lipids with precisely tailored vitrification procedures may give soon an efficient and reliable effects allowing for wider practical use of bovine oocyte cryopreservation.

In vitro maturation

The other, extremely important molecular/cellular factor possibly affecting cryoresistance of oocytes and further developmental capability of embryos is lower level of developmental competence of oocytes obtained from ovarian follicles and subjected to *in vitro* maturation (IVM). Comparison of the effects of IVM, in terms of good quality embryo development and offspring health, both in animal and human reproduction revealed several impairments of *in vitro* mature oocytes in comparison with *in vivo* maturation (Nagai, 2001; Rizos *et al*., 2002; Eppig *et al*., 2009). Bovine oocytes acquire the developmental competence during follicle growth and become fully competent in a dominant follicle, having a final diameter of about 115 μm (Hyttel *et al*., 1997; Hendricksen *et al*., 2000). The process of acquisition of optimum developmental competence, allowing oocyte for a proper maturation, fertilization and early embryonic development, is known as capacitation (Hyttel *et al*., 1997). It is believed that one of crucial aspects of oocyte capacitation is a finalizing of transcription and completing full amount of maternal RNA, which will decide on proper development of an early embryo, until the activation of the embryonic genome. Other factors, as for example a proper level of heterogenous RNA polyadenylation (Brevini-Gandolfi and Gandolfi, 2001) and accumulation of cAMP (Luciano *et al*., 1999), were also considered as important aspects of oocyte capacitation. It was noticed that prolonged (up to 4 h) storage of ovaries *post-mortem* (Sirard and Blondin, 1996; Blondin *et al*., 1997) or inhibition of the maturation of oocytes released from follicles with specific cell cycle inhibitors may be beneficial for achieving higher level of the oocyte competence. Whilst mechanisms of the first effect remain unclear, it was assumed that temporal inhibition of maturation progression enables oocytes to finalize maternal RNA synthesis, stopped otherwise prematurely by an increase in the maturation promoting factor (MPF) activity (Lonergan *et al*., 1997; Ponderato *et al*., 2001; Hashimoto *et al*., 2002).

So far, only a number of cell cycle inhibitors are known. Some of them are able to prevent resumption of meiosis by protein synthesis inhibition (cycloheximide) (Lonergan *et al*., 1997) or by protein phosphorylation, thus maintaining inactive form of p34cdc2/cyclin B complex (MPF) (6-dimethylaminopurine) (Avery *et al*., 1998). However, efficiency of cycle inhibition mediated via these drugs was not complete and/or full reversibility was questionable. More recently, an effective using of more specific and apparently harmless inhibitors was described. Roscovitine (Mermillod *et al*., 2000; Ponderato *et al*., 2001) or butyrolactone I (Lonergan *et al*., 2000; Ponderato *et al*., 2001) are specific cdc2- and cdk2-kinase inhibitors, able to arrest the cell cycle in the transition between G1/S or G2/M stages. Mermillod *et al*. (2000) reported 80 % of bovine oocytes arrested at the GV stage after 24 h incubation with roscovitine and 89 % of oocytes that progressed to metaphase II after additional 24 h maturation without this drug. Finally almost 40 % of these oocytes developed to the blastocysts after IVF.

Our own recent attempts to improve oocyte quality using roscovitine cycle inhibition brought moderate success. In the best experimental variants blastocyst ratio obtained from inhibited oocytes was comparable (but not higher) to those obtained after classical IVM. However, our subsequent experiments using type 3-specific phosphodiesterase (PDE3) inhibitor - cilostamide combined with roscovitine, gave slightly higher blastocyst yield (Stachowiak, *et al*., 2013) but did not solve the problem. From this point of view a new concept based on more detailed insights into oocyte-follicle interactions seems very promising. Oocyte-secreted factors (OFS), such as growth differentiation factor 9 (GDF9), to some extent bone morphogenetic protein 15 (BMP15) and perhaps many others, were found out to be essential for folliculogenesis and female fertility (Gilchrist, 2011). These factors function in a paracrine manner on granulosa and cumulus cells, mainly during the antral phase of follicular

growth, inducing, probably through the morphogenic gradient derived from the oocyte (Hussein *et al*., 2005; Gilchrist *et al*., 2008). Although numerous functions of OFS signaling still remain under investigation, it seems clear that capability of the oocyte to control some functions of the cumulus cells may be crucial for acquisition of its own developmental competence (Gilchrist *et al*., 2008). To take a practical advantage of these recent findings, a new system of oocyte IVM called Simulated Physiological Oocyte Maturation (SPOM) has been recently proposed (Albuz *et al*., 2010; Gilchrist, 2011). SPOM is an integrated IVM system that includes a short pre-IVM phase (1-2 h) and an extended IVM phase that synergize to generate high embryo and fetal yields following embryo transfer. Forskolin and IBMX, a cAMP- modulating agents, included to the oocyte pick-up medium generate a rapid and large increase in cAMP level, which resembles the increase occurring in COCs after the pre-ovulatory gonadotropin surge *in vivo* (Albuz *et al.*, 2010). An increase in both COC and oocyte cAMP levels prevents rapid loss of oocyte-cumulus gap-junctional communication at the pick-up time (Hussein *et al*., 2005; Albuz *et al*., 2010) and simultaneously loads the oocyte with cAMP, preventing precocious spontaneous resumption of meiosis. The extended IVM phase of SPOM slows down meiotic resumption, which is, however, overridden or induced by FSH. Oocytes subjected to SPOM system are exposed throughout maturation to a low concentration of a type 3-specific PDE3 inhibitor. The PDE3 inhibitor (cilostamide) concentration is too low to completely inhibit meiosis, but sufficient to impair meiotic maturation in the absence of the meiosis-inducing hormone. In SPOM, a relatively high concentration of FSH (100 mIU/mL) is needed to induce oocyte maturation in the presence of the PDE3 inhibitor. Taken together, SPOM method mimics some of important newly described molecular mechanisms that occur during oocyte maturation *in vivo*. Its application should lead to an increase in IVM efficiency, oocyte developmental competence and embryo *in vitro* production.

We assume that it may also affect cryoresistance, fertilization susceptibility and subsequent embryo development of bovine oocytes subjected to vitrification. Our preliminary trials using SPOM of bovine oocytes resulted however in decrease of the oocyte cleaving ratio. Interestingly, a good quality blastocyst rate developed in those cleaved embryos exceeded ratio obtained in control, traditionally matured oocytes (Duda, 2013). This result needs confirmation as it may eventually offer a method for increasing an efficiency of bovine *in vitro* embryo production. From the research performed very recently in Germany, it seems obvious that SPOM may interfere with proper embryo development on the oocyte DNA/histon methylation or acetylation level, affecting epigenetic characteristics of an embryo (Heiligentag *et al.*, 2015). Unfortunately, we found quite difficult to get financial support for this kind of research in Poland, and therefore continuation of our research is problematic. Nevertheless, it seems desirable to continue a work on SPOM and related maturation methods (including the use of active recombinant OSFs) to increase quantity and quality of bovine oocytes available for reproduction or cloning.

CONCLUSION

Reviewing historical and contemporary aspects of bovine oocyte cryopreservation and *in vitro* maturation, we found quite obvious that long lasting efforts of many distinguished researchers from many countries gave much worse effects than it was expected by scientific community and practitioners. There are, however, some evidence leading to the conclusion that recently a significant progress has though been made. Taken together, an answer to the title question seems now to be much closer to “reality” than ever.

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