Cryopreservation of female germplasm in pigs

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Cryopreservation of female germplasm has basic importance in preservation and distribution of genetic lines in farm animals. Although vitrification technology has been applied for the cryopreservation of porcine oocytes and ovarian tissues, reduced developmental competence of preserved oocytes and the lack of offspring produced from them underlines the importance for further developments in cryopreservation protocols for this purpose. This review discusses the problems of female germplasm cryopreservation in pigs and the possible strategies to overcome them and gives an update on the present status of cryopreservation of porcine oocytes and ovarian tissues.

Introduction

Cryopreservation of female germplasm has basic importance in preservation and distribution of genetic lines in farm animals. Recent revival of some indigenous pig breeds in the pork market underlines the importance of the preservation of genetic diversity in pigs (Ratky *et al*. 2007; Daza *et al*. 2008). For decades, development of cryopreservation methods of porcine embryos and oocytes was far behind those of other domestic species. This was caused by the high sensitivity of porcine embryos and oocytes to low temperatures, the delayed establishment of *in vitro* embryo production (IVP) systems and the low quality of resultant oocytes and embryos. Although several research groups have established cryopreservation protocols for porcine oocytes in the last decades, embryo development remained low and there has been no report on piglets produced from cryopreserved oocytes to date. Therefore, oocyte cryopreservation in pigs still represents a challenge to be solved. In the meantime, new technologies for the cryopreservation of ovarian tissues are emerging which provide future possibilities for the cryopreservation of female germline. In the chapters below, we will discuss the problems of the germline cryopreservation in pigs and possible strategies to overcome them and give an update on the present status of cryopreservation of porcine oocytes and ovarian tissues.

Cryopreservation-related damages in porcine oocytes and embryos

The unique characteristics of porcine oocytes and embryos fundamentally determine their sensitivity to low temperatures and their feasibility to cryopreservation techniques. Porcine oocytes contain 156 ng lipid (McEvoy *et al*. 2000), which is extremely high, even compared to those of cows (89 ng/oocyte) (Ferguson & Leese, 1999). In porcine oocytes and embryonic cells, lipid droplets are usually form complexes with cytoskeletal elements, membranes and cytoplasmic organelles and play an important role in metabolism as energy source during

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maturation, fertilization and early embryonic development (Sturmey *et al.* 2006; Sturmey *et al.* 2008). When oocytes and embryo cells with high lipid content (such as those of pigs) are cooled below a critical temperature of 15 °C, phase separation of the membrane lipids occurs, causing irreversible damage in membrane structure (Edidin & Petit 1977; Didion *et al.* 1990; Gerelchimeg *et al.* 2009). This phenomenon is often referred as "chilling injury". Furthermore, in those cells which maintain membrane integrity after cooling, sub-lethal damages such as disorganization of the functional units of lipid, mitochondria and endoplasmic reticulum (ER), and altered functions of these cytoplasmic organelles may further reduce their viability and competence (Gerelchimeg *et al.* 2009). A good example for altered organelle function is the cooling-related release of Ca^{2+} in the cytoplasm of porcine oocytes from intracellular stores triggering apoptotic cell death (Mattioli *et al.* 2003). Also, meiotic spindles and cytoskeletal components such as microtubules and microfilaments - which play important roles in distribution and anchoring of cytoskeletal organelles - suffer extensive damage during cooling (Liu *et al.* 2003a; Yang *et al.* 2010). Low temperature at the immature stage greatly impairs the cytoplasmic maturation of porcine oocytes during *in vitro* maturation (IVM) (Liu *et al.* 2003b). Similarly to cooling, cytoskeleton and mitochondria suffer primal damages during cryopreservation of porcine embryos and oocytes (Dobrinsky *et al.* 2000; Rojas *et al.* 2004; Wu *et al.* 2006; Shi *et al.* 2007). Accordingly, cytoplasmic functions which require mitochondrial contributions such as regulation of reactive oxygen species (ROS) and calcium levels in cytosol are also compromised in cryopreserved oocytes (Somfai *et al.* 2007; Gupta *et al.* 2009; Kim *et al.* 2011). As a result, apoptosis is frequently observed in cryopreserved porcine embryos and oocytes (Fabian *et al.* 2005; Cuello *et al.* 2007a; Vallorani *et al.* 2012). These examples demonstrate the complexity and interactions among different types of cryopreservation-induced alterations in cryopreserved cells.

Embryo cryopreservation – lessons for oocyte cryopreservation

In pigs, as in any other mammalian species, development of cryopreservation technologies for embryos was (at least) a step ahead of that of oocytes. The experiences of embryo cryopreservation provide important knowledge for the development of cryopreservation protocols for female germplasm.

Soon after the development of embryo freezing methods it became clear that compared with other farm animals, high susceptibility to chilling injury makes porcine embryos very difficult to preserve by the traditional slow-freezing methods (Wilmut 1972; Polge *et al.* 1974). Nevertheless, to some extent, traditional slow-freezing could be successfully applied for *in vivo* produced embryos (Hayashi *et al.* 1989; Kashiwazaki *et al.* 1991; Fujino *et al.* 2007). A major breakthrough in porcine embryo cryopreservation was achieved by Dr. Hiroshi Nagashima and colleagues, who - realizing the contribution of high lipid content to low cryo-tolerance - improved chilling tolerance of embryos by removing intracellular lipid droplets mechanically from cleavage stage embryos after polarization of lipids by centrifugation (a technique often referred as "delipation" or "delipidation") (Nagashima *et al.* 1994). This method combined with slow freezing has led to the production of piglets from cryopreserved porcine embryos for the first time (Nagashima *et al.* 1995).

As conventional slow freezing of porcine embryos gained limited success, more expectations were placed on the applications of vitrification techniques for embryo cryopreservation in pigs. Vitrification prevents ice crystal formation (Rall & Fahy 1985) and its rapid cooling and warming rates of vitrification were expected to overcome the problems of membrane injuries caused by chilling (Dobrinsky 2001). Since the early 1990's, different vitrification techniques

and carriers have been applied on porcine embryos including "open pulled straw" (OPS) (Vajta *et al.* 1997), "solid surface" (SSV) (Dinnyes *et al.* 2003), "Cryotop" (= minimum volume cooling, MVC) (Esaki *et al.* 2004) and "Cryoloop" (Kawakami *et al.* 2008). As permeable cryoprotectants (CPA), ethylene glycol has been most frequently used alone or combined with dimethyl sulfoxide (DMSO) with approximately similar efficacies when the total CPA concentration was 32-40 % (v/v) (Dinnyes *et al.* 2003; Cuello *et al.* 2008). The first piglets from vitrified embryos have been obtained from *in vivo* derived blastocysts (Dobrinsky *et al.* 2000; Berthelot *et al.* 2000; Beebe *et al.* 2002). Cryopreservation of *in vitro* produced porcine embryos was a step behind that of their *in vivo* derived counterparts because of the relatively late development of reliable *in vitro* fertilization (IVF) and embryo culture systems and the compromised competence of their derivative embryos in this species. Porcine embryos produced by IVF and embryo culture are considered to have lower ability to survive cryopreservation than *in vivo* produced embryos because the high incidences of polyspermy and the stresses during culture reduce their competence (IVP) embryos (Nagai *et al.* 2006). In accordance, IVF embryos transferred at the 1-cell stage show higher pregnancy rates compared with those transferred at 2-4 cell after culture (Kikuchi *et al.* 1999). Suggesting that vitrification and transfer of *in vitro* produced (IVP) embryos at the early stages may be beneficial by avoiding stresses caused by the culture system we have applied the SSV technique for IVP porcine zygotes which resulted in high survival rates (over 93.4%), 60% pregnancy after transfer and the production of piglets from IVP porcine embryos without lipid removal for the first time (Somfai *et al.*, 2009). After IVF, oocytes were centrifuged - which itself does not affect the competence and cryo-tolerance of zygotes (Somfai *et al.* 2008) - to make the cytoplasm transparent and zygotes were selected for vitrification according to the presence and number of pronuclei omitting highly polyspermic oocytes.

Several strategies have been applied to modify embryo characteristics in order to enhance survival of embryos during vitrification. Dobrinsky *et al.* (2000) improved survival of porcine expanded blastocyst embryos by depolimerizing microfilaments with cytochalasin B (CB) prior to vitrification. Such treatment is ought to make cell membranes elastic and therefore less susceptible to vitrification-related damages. Combination of delipation at early stages with vitrification for blastocysts also improved survival rates (Ushijima *et al.* 2004; Esaki *et al.* 2004) and litter sizes after transfer (Beebe *et al.* 2005). The first piglets obtained from cryopreserved fertilization-derived IVP porcine embryos were also achieved by delipation of ICSI-derived embryos at the early stages followed by vitrification at the blastocysts stage (Nagashima *et al.* 2007). On the other hand, mechanical delipation by micromanipulation raises safety concerns since this technique compromises the integrity of zona pellucida clearing the way to pathogen transmissions. To address this point, Esaki *et al.* (2004) have developed a mechanical delipidation protocol without micromanipulation. Another approach was reported by Men *et al.* (2006) who improved cryotolerance of IVP blastocysts by chemical enhancement of lipolysis using Forskolin. A fundamentally novel innovation to improve cryotolerance of porcine embryos was introduced by Pribenszky *et al.* (reviewed in Pribenszky *et al.* 2010) who induced stress resistance of embryos by applying a non-lethal stress (such as with high hydrostatic pressure) to oocytes. This technology was used for oocytes before nuclear transfer and improved the cryo-tolerance of resultant embryos (Du *et al.* 2008a) presumably by inducing the production of stress-proteins. These experiences on embryo cryopreservation basically determined the pathway for the establishment of cryopreservation systems for oocytes and several techniques which led to success with embryos have been tested for oocytes in order to improve cryopreservation efficacies.

Regarding embryo vitrification, recent results have demonstrated that vitrification of *in vivo*-derived porcine blastocysts does not require pretreatment with cytochalasin B and/or

centrifugation (Cuello *et al*. 2010). Current vitrification protocols can provide excellent results with *in vivo*-derived blastocysts without pretreatments, which are time consuming and might be detrimental for embryo quality. Accordingly, *in vitro* embryo survival similar to those of fresh embryos was reported after vitrification of porcine blastocysts by the superfine OPS (SOPS) method without pretreatments (Sanchez-Osotio *et al*. 2010).

Cryopreservation of porcine oocytes – why so difficult?

Similarly to embryos, the large intracellular lipid content in porcine oocytes can be accused for their low cooling tolerance (Ogawa *et al*., 2010). Nevertheless, mammalian oocytes are generally considered to be more difficult to be cryopreserved compared with embryos (Vajta 2000). Indeed, early works have demonstrated membrane damage in almost all of porcine oocytes subjected to slow freezing (Didion *et al*. 1990). Although a significant proportion of oocytes can survive vitrification, their developmental competence is always compromised (reviewed by Zhou & Li, 2009). To date, there is no report on the production of live piglets from cryopreserved oocytes. Compared with multiple-cell embryos, a single-cell oocyte has the obvious problem of a limited ability to recover from vitrification-induced damages and develop into normal embryos - especially, when the IVF/embryo culture system used is stricken with high polyspermy rates and culture stresses, such as in case of pigs. Also, the large size of mammalian oocytes has frequently been accused for making their cryopreservation difficult compared with embryos and other cell types. Nevertheless, high survival rates for zygotes and low survival for oocytes vitrified by the same system dispute if large size of oocytes would be the primarily limiting factor for cryopreservation (Somfai *et al*. 2008b; Somfai *et al*. 2012a). Furthermore, despite of their similar size, immature and *in vitro* matured porcine oocytes show different tolerance for cryopreservation; survival rates after vitrification gradually increase during oocyte maturation and fertilization (Rojas *et al*. 2004; Somfai *et al*. 2012a). Ultra-structural changes during oocyte maturation and fertilization are most likely to cause these differences. In pigs, maturation and fertilization is associated with a decrease of lipid content in oocytes (Sturmey & Leese 2003, Romek *et al*. 2011) and the translocation of organelles such as mitochondria and ER (Cran 1985; Sun *et al*. 2002). Also, changes in membrane structure during nuclear progression from the germinal vesicle (GV) stage to the metaphase-II and pronuclear stages in mammalian oocytes are suggested to affect cryo-tolerance by decreasing phase-transition temperatures (Ghetler *et al*. 2005) and increasing permeability of oocyte membranes to cryoprotective agents (CPAs) (Le Gal *et al*. 1994; Agca *et al*. 1998). These suggest that the optimimum protocol for the cryopreservation of porcine oocytes (i.e. cooling and warming rates and CPA treatment) may be dependent on the actual maturation stage.

Cryopreservation of matured oocytes

Although vitrification was first applied on porcine oocytes at the immature stage (Isachenko *et al*. 1998), the most effort to preserve porcine oocytes was put in those matured *in vitro* so far (Zhou & Li 2009). To date, several vitrification methods have been applied for the cryopreservation of *in vitro* matured porcine oocytes such as OPS (Rojas *et al*. 2004, Shi *et al*. 2006), Cryotop (Fujihira *et al*. 2004; Ogawa *et al*. 2010, Galeati *et al*. 2011) or SSV (Somfai *et al*. 2006; Gupta *et al*. 2007). All of these methods have provided surviving rates over 70 %; however, developmental competence is always compromised compared with non-vitrified counterparts. The number of comparative analysis between these methods in porcine oocytes is limited; some results suggest the superiority of Cryotop device compared with OPS method (Liu *et al*. 2008). To date, the highest survival and developmental rates were achieved by the

Cryotop method combined with lipid-removal and microtubule stabilization (Ogawa *et al.* 2010). Vitrification protocols can differ from each other in CPA treatment and vitrification carrier. Among penetrating CPAs, dimethyl sulfoxide (DMSO) and ethylene glycol (EG) have been often used for the vitrification of metaphase-II stage oocytes. Comparing their effects on oocytes, similar survival rates of porcine oocytes after vitrification were reported using these two CPAs (Gupta *et al.* 2007); however, EG was found to be less toxic than DMSO and therefore appeared more feasible for the purpose (Gupta *et al.* 2007; Taniguchi *et al.* 2011). Also, our results have confirmed that treatment of porcine oocytes with EG-based vitrification solution does not affect the developmental ability of oocytes (Somfai *et al.* 2006). On the other hand, a treatment with a combination of EG with DMSO seems to exert some detrimental effects on matured oocytes (Shi *et al.* 2006, Shi *et al.* 2007). Regarding the vitrification carriers, Cryotop and Cryoloop have been found to provide the highest cooling/warming rates in solutions with minimum CPA volume (Sansinena *et al.* 2011) and therefore may result in high survival rates for porcine oocytes as well. On the other hand, the small capacity (a maximum of 5-10 oocytes per carrier) of these methods (i.e. the number of oocytes preserved at one setting) greatly limits their feasibility for the preservation of porcine oocytes especially considering the relatively low efficacy of IVF or intracytoplasmic sperm injection (ICSI)/embryo culture techniques in pigs. Regarding this problem we have applied a SSV protocol using an EG-based CPA solution as a basic method for the preservation of porcine oocytes (Somfai *et al.* 2007) modified from the original protocols of Dinnyes *et al* (2000) and Gupta *et al.*, (2007). This approach allows the vitrification of up to 100 oocytes at one setting in approximately 20 minutes (Fig. 1). After

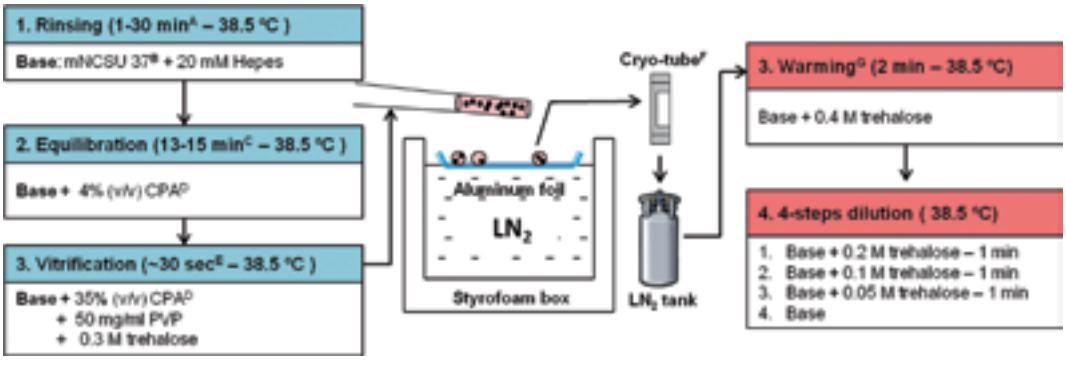

Fig. 1. The schematic diagram of a solid surface vitrification protocol modified for the preservation of porcine oocytes in large quantities.

This protocol under constant improvement.

[A] Up to 100 oocytes can be processed as one setting. In case of immature oocytes rinsing is performed for 30 min in base medium supplemented with 7.5 μg/mL Cytochalasin B according to Fujihira *et al.* (2004).

[B] Glucose-free NCSU 37 supplemented with pyruvate and lactate (Kikuchi *et al.*, 2002)

[C] Up to 100 oocytes can be processed as one setting. In case of immature oocytes rinsing is performed in equilibration medium supplemented with 7.5 μg/mL Cytochalasin B.

[D] Permeable cryoprotectant (CPA) can be either ethylene glycol or the combination of ethylene glycol and propylene glycol.

[E] During vitrification, groups of 30-50 oocytes are washed 3 times in 20 μl of vitrification solution then dropped on the sold surface in 2-3 microdrops (~ 2 μl each, containing 10-25 oocytes).

[F] Microdrops prepared at one setting are placed together in cryo-tubes with a pre-cooled forceps.

[G] Microdrops prepared at one setting are placed together in 2.5 ml warming solution with a pre-cooled forceps.

LN_2 = liquid nitrogen

warming of vitrified oocytes, live ones can be selected either by morphological observation under a stereo microscope or by staining with fluorescein diacetate which stains live oocytes green under UV light without any harmful effect on their competence (Shi *et al*., 2006).

Despite of the reasonable survival rates for porcine oocytes vitrified at the mature stage, all papers reported their reduced ability to undergo cleavage and to develop to the blastocyst stage and reduced quality of blastocysts in terms of cell numbers (Zhou & Li 2009). Previous reports have revealed that, during vitrification, matured porcine oocytes suffer various types of cryoinjuries such as the disruption of cytoskeleton (Rojas *et al*. 2004; Shi *et al*. 2007; Galeati *et al*. 2011) and the meiotic spindle (Rojas *et al*. 2004; Wu *et al*. 2006; Shi et *et al*. 2007), mitochondrial damage (Shi *et al*. 2007) or the premature release of cortical granules (Marco-Jimenez *et al*. 2012). As a result, vitrification is associated with the compromised ability of oocytes to neutralize the intracellular accumulation of reactive oxygen species (Somfai *et al*. 2007; Gupta *et al*. 2010), parthenogenetic activation of oocytes (Somfai *et al*. 2007), reduced penetration rates after IVF (Somfai *et al*. 2007; Galeati *et al*. 2011) and the failure of male pronuclear formation after penetration (Somfai *et al*. 2007). Several strategies have been developed so far to reduce the detrimental effects of vitrification on porcine metaphase-II oocytes. Mechanical delipation before vitrification significantly improved the developmental competence of cryopreserved oocytes providing excellent developmental rates after parthenogenetic activation (Ogawa *et al*. 2010); however, this approach has not yet been tested for IVF. Chemical delipation by forskolin treatment during IVM has been reported to result in some improvement on the cryotolerance of porcine oocytes (Fu *et al*. 2011) although this treatment exerts negative side effects on oocyte competence (Fu *et al*. 2011; Prates *et al*. 2012). Unlike in embryos, treatment with CB before vitrification of metaphase-II stage oocytes does not seem to improve survival rates (Somfai *et al*. 2006; Gupta *et al*. 2007; Marco-Jimenez *et al*. 2012). On the other hand, several studies have confirmed the positive effect of microtubule stabilization by taxol on the competence of vitrified metaphase-II stage oocytes (Fujihira *et al*. 2005; Shi *et al*. 2006; Fu *et al*. 2009; Ogawa *et al*. 2010). Since microtubules play an important role in the distribution and anchoring of mitochondria in porcine oocytes, Taxol treatment is also advantageous for the preservation of mitochondrial compartment in vitrified oocytes (Fu *et al*. 2009). Induced stress resistance either by high hydrostatic pressure or osmotic stress treatment has been reported to improve the post-vitrification developmental ability of porcine metaphase-II stage oocytes after parthenogenetic activation (Pribenszky *et al*. 2008; Du *et al*. 2008b; Lin *et al*., 2009); however, this approach has not yet been tested with IVF or ICSI.

Cryopreservation of immature oocytes

Cryopreservation of oocytes at the immature stages may be inevitable when germ cell preservation for gene banking must be performed under conditions without being able to perform IVM. Also, oocyte cryopreservation at the immature stages, before the formation of meiotic spindle can be advantageous to circumvent the spindle damages and resultant digyny caused by the failure of second polar body extrusion after cryopreservation at the metaphase-II stage (Eroglu *et al*. 1998). Our results have revealed that, despite of lower survival rates compared with *in vitro* matured counterparts, porcine cumulus-enclosed oocytes vitrified at the immature GV stage can maintain/restore competence during IVM to undergo maturation and fertilization in a normal manner and the resultant blastocysts appear similar to those obtained from non-vitrified oocytes in terms of cell numbers (Somfai *et al*. 2010). The obvious disadvantage for the vitrification of immature oocytes seems to be their limited survival rate. A plausible reason for this problem may be the low permeability of oocytes to CPA at the immature stage which has been verified in cattle and goats. This suggestion is supported by our recent results; replacement

of EG with the more permeable propylene glycol (Pedro *et al*. 2005) dramatically improved survival rates after vitrification of immature porcine oocytes from 25% to over 70 % (Somfai *et al*. 2012b, Fig. 2). Nevertheless, despite of the high survival and normal maturation rates, oocytes vitrified in PG showed very low embryo development due to the toxic side effects of PG (Somfai *et al*. 2012b). To reduce the toxic effects of PG and to increase survival rates of EG, the combination of EG with PG was proven to be effective, resulting in approximately 40% survival after vitrification and 10% blastocyst development after IVF of the surviving oocytes. These results demonstrate that the nature of permeating CPAs fundamentally determines the success of oocyte vitrification and that the optimum CPA treatment may depend on the maturation stage. Survival rates and the competence after vitrification of immature oocytes is affected by several other factors as well. Interestingly, the survival rate of immature oocytes after vitrification seems to be primarily affected by the actual developmental competence of the oocyte population. Our investigations have revealed a strong positive correlation between vitrification-survival at the GV stage and the blastocyst development ratio of non-vitrified oocytes of the same batch (Fig. 3). With other words, survival rate after vitrification reflects the actual developmental competence of an oocyte batch. Similar to these results, the batch-dependence of survival after slow-freezing has also been reported in porcine embryos (Fujino *et al*. 2007). The timing of vitrification during the rather long period of the GV stage affects the competence of oocytes as well. Our results have revealed that oocytes vitrified after 20 h culture with the meiosis-inhibitor dbcAMP lose their ability to proceed properly to the MII stage during IVM (Somfai *et al*. 2012a). In this respect, pre-maturation before vitrification is not recommended.

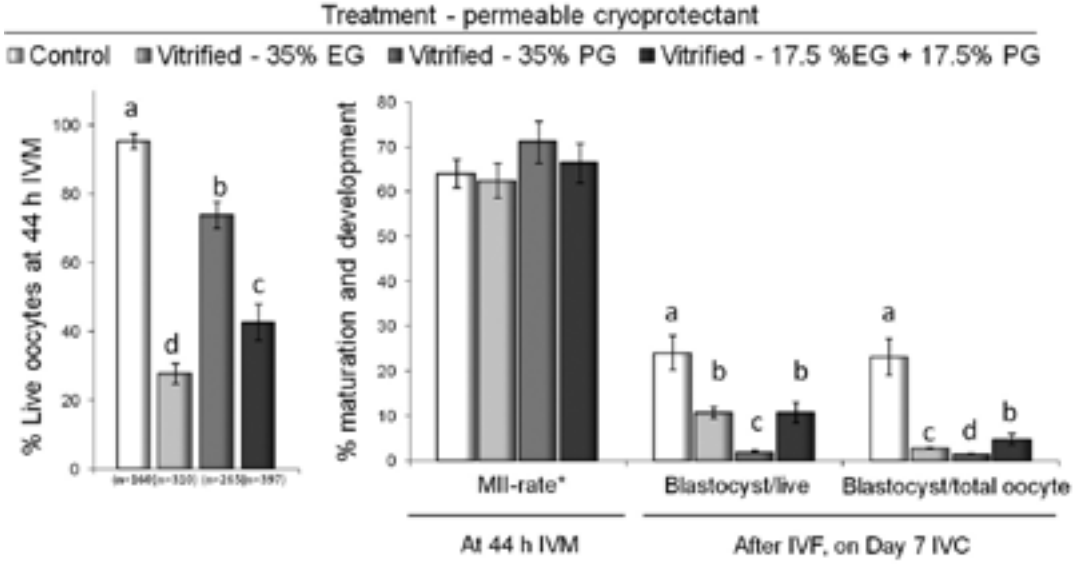

Fig. 2. Survival, maturation and development of immature porcine oocytes vitrified by the SSV method using different CPA combinations.

*MII rate was evaluated by the presence of the first polar body. Oocytes were then subjected to IVF/IVC.

Five replications were performed. Data are presented as mean ± SEM. Different superscripts denote significant difference at $P < 0.05$.

EG = ethylene glycol

PG = propylene glycol

M-II = metaphase-II

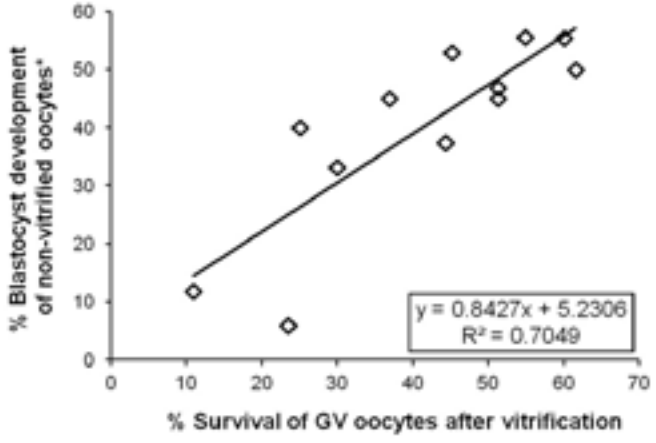

Fig. 3. Correlation between the developmental competence of porcine oocytes and their ability to survive vitrification at the GV stage.

Different batches of oocytes collected on different days were evaluated. Immature cumulus-oocytes complexes were either subjected to IVM/IVF/IVC without cryopreservation or vitrified by SSV method in the combination of EG and PG.

*Blastocyst development was calculated from cleaved (thus presumably fertilized) oocytes after IVF using the same batch of oocytes without vitrification.

From the different methods developed to improve cryo-tolerance of porcine embryos delipation has been proven to improve survival rates after vitrification of immature oocytes; however, this may not be an expedient approach since it reduces meiotic competence of oocytes (Park *et al.* 2005). On the other hand, elastication of the membrane with CB before vitrification – which does not seem to work well for matured oocytes – has been reported to improve survival rates after vitrification of immature porcine oocytes (Ishachenko *et al.* 1998; Fujihira *et al.* 2004).

The normal ability of vitrified cumulus enclosed GV-stage oocytes to undergo maturation and fertilization and the high quality of the resultant blastocysts suggest the existence of a recovery mechanism(s) in vitrified immature oocytes during IVM which requires further clarification. Our recent observations indicate that the base medium itself and the optional use of porcine follicular fluid (pFF) as a supplement during IVM greatly affect the ability of vitrified oocytes to undergo nuclear maturation. In NCSU 37 medium defined chemically with polyvinylpyrrolidone, the majority of vitrified GV stage oocytes failed to reach the MII stage whereas reasonable maturation rates were obtained in defined POM medium (Fig. 4) although both media provided over 60% maturation for non-vitrified oocytes (Yoshioka *et al.* 2008). Furthermore, the addition of 10 % (v/v) pFF to NCSU 37 medium dramatically increased the maturation ability of vitrified immature oocytes (Fig. 4). Also, preservation of mitochondrial functions during IVM by the inhibition of mitochondrial permeability transition helps vitrified immature porcine oocytes to regain their meiotic competence demonstrating that cytoplasmic recovery can be enhanced during IVM by specific reagents (Nakagawa *et al.* 2008). Understanding and enhancement of recovery mechanisms during IVM of vitrified immature oocytes may be a potential way to improve developmental rates to the blastocyst stage in the future. Furthermore, the transfer of their nuclear material from oocytes compromised by vitrification into cytoplasts of healthy oocytes offers an alternative way to preserve genetic material of female germline in pigs (Nakagawa *et al.* 2011).

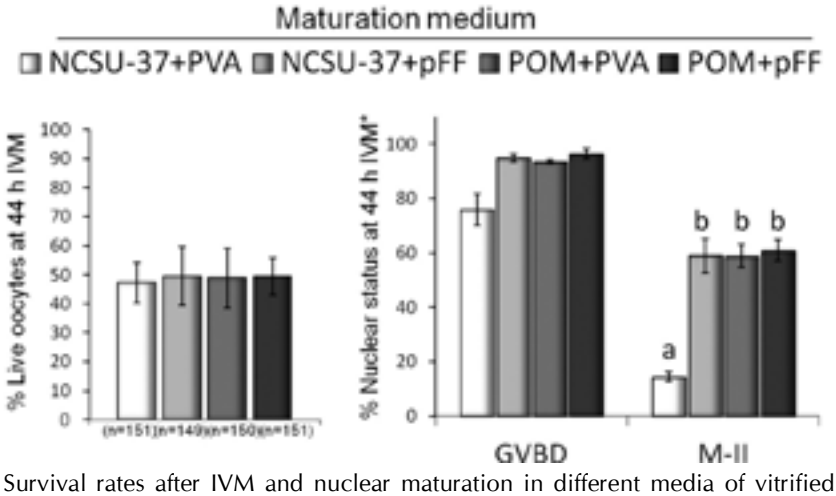

Fig. 4. Survival rates after IVM and nuclear maturation in different media of vitrified immature porcine oocytes.

Porcine oocytes were vitrified by SSV using a combination of EG and PG as permeable CPA. Four replications were performed. Data are presented as mean ± SEM. Different superscripts denote significant difference at $P < 0.05$.

GVBD = germinal vesicle breakdown

M-II = metaphase-II

Cryopreservation of ovarian tissues

Cryopreservation of ovarian tissues and whole ovaries is an emerging technology for fertility preservation in humans and gene banking in animals (Johnson & Patrizio 2011; Santos *et al.* 2010). The ability of immature oocytes to recover from vitrification-related damages during post-warming culture suggests that the earlier the developmental stage in which the female germplasm is cryopreserved the better chance it may have to regain its developmental competence if proper conditions are provided. This suggestion justifies the idea of preservation of ovarian tissue segments or whole ovaries in pigs. Although there have been some attempts in pigs to preserve ovarian tissues and whole ovaries either by slow-freezing or vitrification, these cryopreserved tissues were not utilized for practical reproduction (reviewed by Santos *et al.* 2010). Utilization of cryopreserved tissues for reproduction requires the immediate transfer after thawing or warming of cryopreserved samples into recipients or an advanced technology for tissue culture after thawing. However, in pigs, *in vitro* culture of ovarian tissues and follicles is not in an advanced stage yet and, unlike in mice or cattle, there is no report on live offspring from *in vitro* grown porcine oocytes to date (Miyano 2005; Hirao 2011). On the other hand, as an alternative to *in vitro* culture, ovarian tissue segments (both fresh and vitrified) can be transplanted into immune-deficient mice (a technique called "xenografting") which can start folliculogenesis under the proper hormonal environment (Kaneko *et al.* 2003, 2006; Moniruzzaman *et al.* 2009). In our laboratory, we have applied a vitrification protocol for the cryopreservation of ovarian tissue segments (Kikuchi *et al.* 2010). The ovarian tissue from 20 days old piglets was minced into cubes of about 1.0 to 2.0 mm. The vitrification procedure was similar to that described earlier for oocytes with modifications. The tissues were incubated in the vitrification solution employing 35% EG as permeable cryoprotectant either for 45 s or 7 min (45-s and 7-min immersion groups, respectively), then dropped with about 4

μL of vitrification solution into liquid nitrogen. After storage for several months, micro-droplets containing an ovarian tissue segment were warmed the same way as described for oocytes. Twenty to 30 pieces of tissue were grafted into the kidney capsules of ovariectomized nude mice. When antral follicles were evident in the grafts, oocytes were collected, matured and fertilized *in vitro* using our standard protocol (Kikuchi *et al.* 2002). Ovaries containing antral follicles were obtained between 2 to 4 months after grafting from 6 out of 12 mice in both the 45-s and 7-min groups. The collected oocytes were cultured and the maturation rates calculated on the basis of 1st polar body extrusion were 18% (7/39) and 33% (16/49), respectively. After IVF, rates for sperm-penetrated oocytes (83% and 88%, respectively) and those with normal male and female pronuclei (100% of penetrated oocytes in both groups) did not differ between in the two immersion groups. These results demonstrate that fully grown porcine oocytes can be collected from primordial follicles that have been cryopreserved and xenografted into nude mice. Despite of their low ability to undergo maturation, those which reach the matured stage during IVM gain the ability to be fertilized by IVF irrespective of the period of immersion. Further research is necessary to optimize vitrification/warming parameters and IVM culture systems to increase maturation rates for oocytes obtained from vitrified ovarian tissue segments.

Summary

Despite of recent advances in embryo vitrification in pigs, cryopreservation technology for female germplasm requires substantial development for future success. The application of high capacity containers with high cooling/warming rates enabling the effective and quick vitrification of oocytes at large quantities would be advantageous. Also, further works are required to find the optimum CPA treatment and warming procedures which provide the highest survival and embryo development rates. Improvement of culture systems to support post-warming recovery processes in surviving vitrified oocytes may be another potent way to increase efficacy. To do so, further research is necessary to understand the effects of cryopreservation on cellular processes and to characterize recovery mechanisms in oocytes. Emerging technologies, such as cytoplasm transfer may offer alternative ways to reset the developmental competence of oocytes compromised by cryopreservation.

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