

## CRYOPRESERVATION OF *IN VITRO* PRODUCED EMBRYOS AND IMMATURE OOCYTES IN PIGS

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### ABSTRACT

*Gamete cryopreservation combined with in vitro embryo production (IVP) and cryopreservation of resultant embryos are important technologies for gene banking in domestic animals. Especially, cryopreservation of embryos and oocytes offers the possibility for the preservation of female genetic lines. Although several vitrification protocols have been developed for the cryopreservation of embryos and oocytes in pigs, their adaptation in IVP systems have resulted in only limited success. In case of IVP embryos, high frequencies of polyspermy and the sensitivity of porcine embryos to culture stresses reduce embryo competence and thus limit the efficacy of their cryopreservation. Several approaches have been introduced to improve efficacy of cryopreservation for IVP embryos such as removal of intracellular lipid, adjustments of the culture systems to improve embryo competence and artificial induction of stress tolerance. Our researches have demonstrated that IVP embryos can be both selected for monospermy and vitrified effectively at the zygote stage and their direct transfer into recipients is an effective approach to overcome the problems of polyspermic development and culture stress. Regarding oocyte cryopreservation, our researches have revealed that using current protocols, vitrification of porcine oocytes at the immature stage is more advantageous compared with vitrification at the matured stage since oocytes preserved at the immature stage show better developmental competence in the IVP system. Optimization of cryoprotectant treatment during vitrification and warming temperature after vitrification specifically for immature oocytes allowed us the production of live piglets from cryopreserved oocytes for the first time. Nevertheless, limited efficacies require further improvement of these technologies.*

Keywords: Pig, Gene Banking, Cryopreservation, *In Vitro* Embryo Production, Vitrification, Embryo, Oocyte

### INTRODUCTION

Cryopreservation of embryos and oocytes offers the possibility for the preservation and gene banking of female genetic lines and allows the flexible use of embryos and oocytes in time and space. The development of reliable protocols for the cryopreservation of porcine embryos and oocytes has been far behind those of other domestic species because the high sensitivity of porcine embryos and oocytes to low temperatures (Wilmot 1972; Polge *et al.* 1974; Didion *et al.* 1990). Nearly two decades ago, in a series of studies Dr Hiroshi Nagashima and his colleagues had demonstrated that the relatively high lipid content of porcine oocytes and embryos is the primary reason for their enormous sensitivity to chilling (Nagashima *et al.* 1994; Nagashima *et al.* 1995; Nagashima *et al.* 1999). Mechanical removal of intracellular lipid (“delipidation” or “delipidation”) has contributed the first success in cryopreservation of porcine embryos using traditional slow freezing (Nagashima *et al.* 1995). Application of vitrification technology using high cooling and warming rates in the presence of high concentrations of cryoprotectants (CPA) enabled researchers to preserve mice embryos without the formation of intracellular ice (Rall and Fahy 1985). Because of its high cooling and warming rates, vitrification was expected to overcome the problems of membrane injuries caused by chilling and has become the method of choice for the cryopreservation of porcine embryos (Dobrynsky 2001). The first piglets from vitrified embryos were obtained from *in vivo* derived blastocysts

(Dobrinsky *et al.* 2000; Berthelot *et al.* 2000; Cameron *et al.* 2000). Nevertheless, in pigs, *in vitro* production (IVP) is still the most effective way for the mass production of embryos using frozen sperm and therefore it is an essential technology for gene banking. Cryopreservation of pig IVP embryos was a step behind that of their *in vivo* derived counterparts because of the relatively late development of reliable protocols for *in vitro* fertilization (IVF) and embryo culture (IVC) and because the compromised developmental competence of its derivative embryos (Gruppen 2014). Cryopreservation of IVP embryos still represents a challenge in this species. Furthermore, despite of great efforts for the establishment of efficient protocols for the vitrification of unfertilized oocytes in the last decade (reviewed by Zhou and Li 2009), production of live piglets from vitrified oocytes has not been reported until recently (Somfai *et al.* 2014). In the chapters below, we will discuss the problems arising with the cryopreservation of *in vitro* produced embryos and oocytes in pigs, and possible strategies to overcome them.

## CRYOPRESERVATION OF IVP EMBRYOS

### Vitrification methods and the optimum embryo stage

Since the early 1990's a wide range of vitrification techniques have been applied for porcine embryos (Table 1). These methods differ from each other in their vitrification carrier and/or cryoprotectant (CPA). 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 vitrification carrier has a crucial role since it determines the cooling and warming speed which greatly affect survival. Among carriers, Cryotop and Cryoloop have been found to provide the highest cooling/warming rates in solutions with minimum CPA volume and therefore are considered to be highly effective (Sansinena *et al.* 2011). On the other hand, vitrification without carriers by dropping embryos in a CPA solution directly into liquid nitrogen (LN<sub>2</sub>) or onto a metal surface cooled by LN<sub>2</sub> is also possible (Misumi *et al.* 2003, Dinnyes *et al.* 2003). The recently reported hollow fiber vitrification (HFV) method applying a medium-permeable microtube as the carrier has been proven to be effective by allowing the quick and controlled handling of embryos in groups during CPA treatment, vitrification and warming (Maehara *et al.* 2012). Recently, increasing effort has been put in the development of vitrification systems without direct contact with LN (sanitary systems) with success both with *in vivo* (Beebe *et al.* 2005; Misumi *et al.* 2013) and *in vitro* produced embryos (Men *et al.* 2011). Regarding the optimum embryo stage, IVP embryos at the blastocyst and morula stages have been found to be equally adequate for vitrification, whereas embryos at the 2-4 cell stages show low survival rates (Sanchez-Osorio *et al.* 2008).

Table 1. Application history of main vitrification methods for the cryopreservation of porcine embryos.

Year <sup>a</sup>	Method <sup>b</sup>	CPA <sup>c</sup>	Embryo stage	Survival%	Reference
1993	Plastic straw	EG	Blastocyst	21	Yoshino <i>et al.</i> 1993
1997	Open pulled straw	EG+DMSO	Morula, blastocyst	100	Vajta <i>et al.</i> 1997
	(OPS)				
2003	Microdroplets	EG	Morula, blastocyst	91	Misumi <i>et al.</i> 2003
2003	Solid surface	EG	Blastocyst	24*	Dinnyes <i>et al.</i> 2003
2004	Cryotop	EG+DMSO	Blastocyst	83* <sup>#</sup>	Esaki <i>et al.</i> 2004
2004	Superfine OPS	EG+DMSO	Morula, blastocyst	56	Cuello <i>et al.</i> 2004
2008	Cryoloop	EG+DMSO	Blastocyst	83.3* <sup>#</sup>	Kawakami <i>et al.</i> 2008
2008	Metal mesh	EG+DMSO	Morula, blastocyst	84.3	Fujino <i>et al.</i> 2008
2010	Pullulan film	EG	Blastocyst	79.0	Sakagami <i>et al.</i> 2010
2012	Hollow fiber	EG+DMSO	Morula	77.6*	Maehara <i>et al.</i> 2012
2013	Micro volume	EG	Blastocyst	88.9	Misumi <i>et al.</i> 2013
	air cooling				

<sup>a</sup> The year of first application in porcine embryos

<sup>b</sup> Carrier or cooling device

<sup>c</sup> Cryoprotectant agent (permeable). Abbreviations: EG= ethylene glycol, DMSO= dimethyl sulfoxide

\* *In vitro* produced embryos

<sup>#</sup> Delipated before vitrification

### **The curses of IVP: polyspermy and culture stresses**

Porcine embryos produced by IVF and IVC are considered to have lower developmental competence than *in vivo* produced embryos because the high incidences of polyspermy and the stresses during culture which reduce competence of IVP embryos (Nagai *et al.* 2006). Current IVF systems in pigs still struggle with high rates of polyspermic fertilization with rates ranging between 40-70% of total fertilized oocytes. Polyspermy not only reduces the efficacy of blastocyst production but also potentially compromises the efficacy of piglet production after the transfer of blastocysts since a remarkable proportion of polyspermic oocytes can develop to blastocysts with low cell numbers and high frequencies of abnormal chromosome numbers (Han *et al.* 1999, Somfai *et al.* 2008a). Although intra-cytoplasmic sperm injection (ICSI) of a single spermatozoon is considered to be an elegant option to avoid polyspermy, the relatively slow process of micromanipulation does not allow the production of embryos in great numbers and the efficacy of this method is further limited by abnormal male pronuclear formation (Nakai *et al.* 2010). Another problem of porcine IVP embryos is their relatively high sensitivity to oxidative stress. Under suboptimal culture conditions intracellular concentrations of reactive oxygen species (ROS) increase in porcine embryos which reduces their developmental competence (Karja *et al.* 2006). In accordance, IVP embryos transferred at the 1-cell (zygote) stage show higher pregnancy rates compared with those transferred later after 2 days of culture, at the 2-4 cell stage (Kikuchi *et al.* 1999). Furthermore, cryopreservation makes porcine embryos and oocytes even more susceptible for the harmful accumulation of ROS (Somfai *et al.* 2007; Gupta *et al.* 2010).

### **Reducing lipid contents in embryos: the first success**

Delipation of porcine embryos at early stages has been reported to improve survival rates and litter sizes after the vitrification and transfer of resultant blastocysts (Ushijima *et al.* 2004; Esaki *et al.* 2004; Beebe *et al.* 2005). The first piglets obtained from cryopreserved porcine IVP embryos were also achieved by delipation at the early stages of IVP embryos followed by vitrification at the blastocysts stage (Nagashima *et al.* 2007). Alas, 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 delipation protocol without micromanipulation applying a centrifugation treatment in the presence of cytochalasin B after a treatment with trypsin which allows the intracellular lipid (polarized by centrifugation) to secede from the embryo within the intact zona pellucida. Another approach was reported by Men *et al.* (2006) who improved cryotolerance of IVP blastocysts by chemical enhancement of lipolysis using forskolin. Another drug, phenazine ethosulfate has also been reported to reduce lipid content in porcine IVP embryos; however, with only a limited effect on blastocyst survival after vitrification (Gajda *et al.* 2011).

### **Avoiding the curses: vitrification and transfer of preselected monospermic zygotes**

The above mentioned suggests that the successful vitrification and transfer of IVP embryos at the very early stages may be beneficial by avoiding stresses caused by the culture system. Based on this hypothesis, we have vitrified porcine IVP embryos at the zygote stage either at 10 h or 22 h after IVF. The vitrification technique was a modified SSV which allows the treatment and processing of zygotes in large groups (up to 100 embryo/group) in approximately 20 minutes (Figure 1). Before vitrification, IVF oocytes were centrifuged to make the cytoplasm transparent and zygotes were selected for vitrification according to the presence and number of pronuclei omitting highly polyspermic oocytes. This centrifugation treatment does not affect the developmental ability and cryo-tolerance of zygotes (Somfai *et al.* 2008 a and b). Irrespective of timing of vitrification (i.e. at 10 h or 22 h after IVF) high survival rates (over 93.4%) were achieved, and over 14 % of vitrified zygotes could develop to blastocysts with total cell numbers not different from those of non-vitrified zygotes. Demonstrating the normality of embryos developing from vitrified zygotes, we achieved 60% pregnancy after direct transfer of vitrified IVP zygotes into the oviducts of recipient females and produced a total of 17 live piglets. To our knowledge this was the first success for the cryopreservation of porcine IVP embryos produced by IVF (Somfai *et al.* 2009). This approach have several advantages as 1) culture stresses can be avoided 2) omitting of polyspermic oocytes is possible, 3) it enables the quick preservation of porcine zygotes in large quantities and 4) without the need of lipid removal.

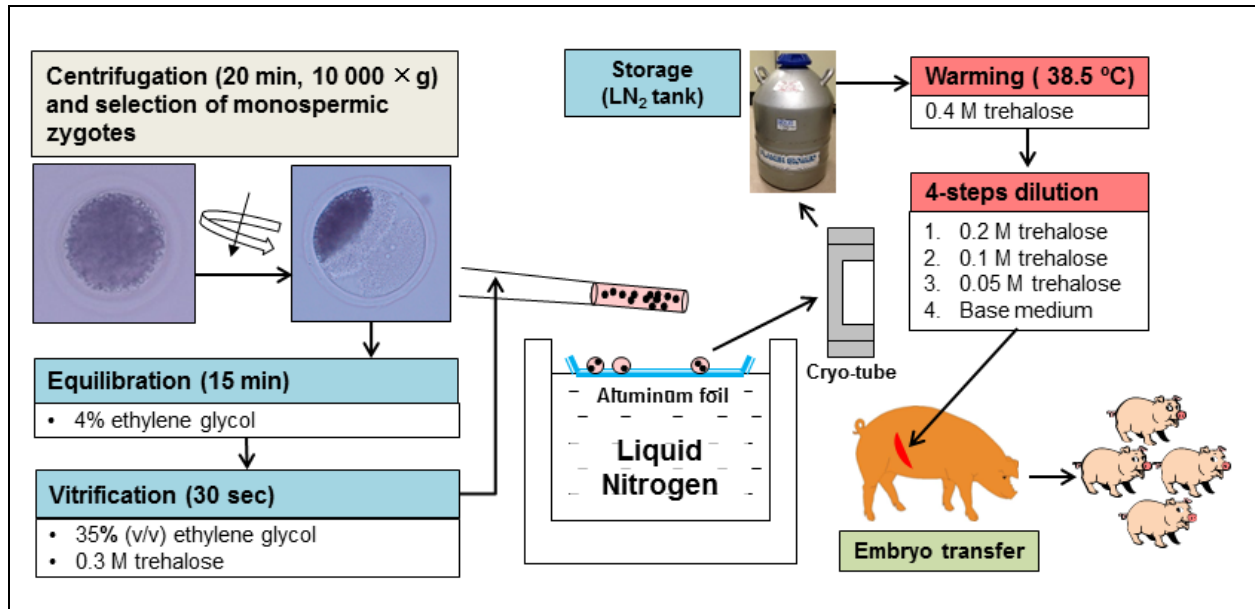


Fig. 1. The schematic overview of a solid surface vitrification protocol modified for the cryopreservation of IVF-derived porcine zygotes in large groups (Somfai *et al.* 2009).

### Vitrification of blastocysts: the impact of the culture system

The culture system used for embryo production greatly affects not only embryo development but also the ability of resultant embryos to survive vitrification. Besides the fact that the actual embryo stage is affected by the culture system, other factors such as the presence of serum (Men *et al.* 2005) and glucose (Castillo-Martín *et al.* 2013) in the medium during embryo culture have also been reported to be important for the success of vitrification of IVP blastocysts. On the other hand, current trends in the development of porcine IVP systems aim the development of chemically defined media by omitting of undefined components such as sera. Further controversies may also arise with the supplementation of embryo culture media with glucose which – especially at early stages – can exert negative effects on development reducing blastocyst yields (Kikuchi *et al.* 2002, Karja *et al.* 2006). Accumulation of intracellular ROS during culture and further exposure of resultant blastocysts to CPA may trigger apoptosis (indicated by DNA fragmentation) in cells (Guerin *et al.* 2001; Rajaei *et al.* 2005). Accordingly, the rate of apoptotic cells reportedly increase in vitrified porcine blastocysts (Fabian *et al.* 2005; Cuello *et al.* 2007). Supplementation of the embryo culture medium with the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone has been reported to increase the ability of porcine IVP blastocysts to recover from vitrification (Men *et al.* 2006). In a recent study, supplementation of culture media with MEM vitamins also improved survival rates of IVP blastocysts (Cuello *et al.* 2013). Further modification of the culture systems to achieve increased tolerance of IVP blastocysts to vitrification and early pre-selection of the monospermic ones for embryo culture may be possible ways to improve efficacy of their cryopreservation.

### Induced stress tolerance

A fundamentally novel innovation to improve cryo-tolerance of porcine embryos was introduced by Pribenszky *et al.* (2010) who induced stress resistance of mammalian cells by applying a non-lethal stresses to them, such as high hydrostatic pressure, osmotic, heat and oxidative stress. Such treatment is believed to enhance the cellular production of stress-proteins which, in turn, increase their tolerance to cryopreservation. This approach has been applied in several cell types including embryos, embryonic stem cells, spermatozoa, and oocytes in several species including mouse, cattle and pig. High hydrostatic pressure treatment of porcine oocytes improved the cryo-tolerance of embryos produced from them by nuclear transfer (Du *et al.* 2008). Although the results are promising, to our knowledge this method has not been tested on porcine IVM/IVF embryos and cellular and subcellular mechanisms underlying this approach require further clarification.

## CRYOPRESERVATION OF IMMATURE OOCYTES

### Why immature oocytes?

There are two reasons which justify the importance of immature oocyte cryopreservation. First, cryopreservation of oocytes at the immature stage is inevitable when only immature oocytes are ready at hand and the technological background is not given for IVM at the site of sampling. Second, recent results indicate, that porcine oocytes vitrified at the immature germinal vesicle (GV) stage possess higher competence for fertilization and embryo development than those vitrified by the same method at the mature metaphase-II (MII) stage (Somfai *et al.* 2012; Egerszegi *et al.* 2013). Despite of the higher survival rates, the cooling/warming process during vitrification of MII stage oocytes causes a combination of different types of alterations in the cytoplasm (including ROS accumulation, spindle malformations and parthenogenetic activation) which greatly compromise the ability of surviving oocytes for normal fertilization and subsequent embryo development (Somfai *et al.* 2012). On the other hand, our results have confirmed that vitrification at the GV stage followed by IVM of vitrified/warmed oocytes is an efficient approach to prevent these vitrification-induced cytoplasmic alterations and to produce high quality blastocyst stage embryos *in vitro* (Somfai *et al.* 2012; Egerszegi *et al.* 2013).

### Difficulties and key points

Contraversially to the above mentioned, porcine oocytes (similarly those of other mammalian species) show higher tolerance to vitrification at the MII stage than at the GV stage in terms of their survival as measured by membrane integrity. We applied our SSV protocol (previously used for zygote preservation) without centrifugation treatment but with a pre-treatment with cytochalasin B for the preservation of immature cumulus-enclosed oocytes. When EG was used as the permeable CPA, a remarkably higher oocyte survival (70%) was achieved at the MII stage whereas only 25% of GV stage oocytes survived vitrification (Somfai *et al.* 2012). The plausible reason for this phenomenon is believed to be the difference in the permeability of the oocyte membrane to water and cryoprotectant between the GV and MII stages that has been described in some mammalian species (Le Gal *et al.* 1994; Agca *et al.* 1998). The membrane of GV oocytes is considered to be less permeable to CPAs but more permeable to water compared with that of the MII oocytes. Accordingly, the replacement of EG with the more permeable propylene glycol (PG) dramatically improved the survival rates; however, embryo development was very low because the high toxicity of PG (Somfai *et al.* 2013a). On the other hand, vitrification in a combination of EG and PG resulted in improved (over 40 %) survival compared with the use of EG only, without any toxic effect on embryo development. In general, the combination of different permeable CPA is known to be more effective for vitrification than individual CPA and the optimum CPA combination is yet to be defined. Another important point for the success of vitrification is a high warming rate, which has been identified as a key factor that determines the success of oocyte vitrification in mice (Seki and Mazur 2009). Increasing the temperature from 38 C to 42 C of the hotplate used to keep warming dishes during warming of vitrified oocytes dramatically increased the survival rates of vitrified porcine GV-stage oocytes up to 86.4 % (Somfai *et al.* 2014). The early timing of vitrification is crucial to maintain competence of GV-stage oocytes as 20 h pre-maturation of oocytes before vitrification greatly compromises their meiotic competence after warming (Somfai *et al.* 2012). Regarding the optimum IVM culture for vitrified oocytes, our results have demonstrated that supplementation of the IVM medium with porcine follicular fluid (pFF) is important when simple culture media, such as North Carolina State University -37 (NCSU37; Petters and Wells 1993) is used (Somfai *et al.* 2013b). Recently, we used porcine oocyte medium (POM; Yoshioka *et al.* 2008) a more complex medium for the IVM of vitrified/warmed porcine GV-stage oocytes. This medium allows normal nuclear maturation and subsequent fertilization by IVF of vitrified oocytes even without supplementation with pFF. Using this system, blastocysts produced *in vitro* from oocytes vitrified at the GV stage into 5 recipients resulted in 5 pregnancies and a total of 21 live piglets (Table2, Figure 2). To our knowledge these are the first - and to date the only piglets produced from cryopreserved oocytes.

Table 2. Birth of piglets after transfer of blastocyst stage embryos produced *in vitro* from oocytes vitrified at the immature stage.

Recipient	Embryos transferred	Embryo stage	Live piglets born	Birth weight (kg) <sup>a</sup>
#1	16	Blastocyst	4	1.50±0.04
#2	27	Blastocyst	6	1.53±0.06
#3	17*	Blastocyst	5	1.34±0.2
#4	13*	Blastocyst	3	1.37±0.04
#5	23*	Blastocyst, morula	2	1.40±0.0

A part of these results (Recipient #1–#4) are cited from Somfai *et al.* 2014

<sup>a</sup> mean ± SEM

\*Embryos produced in a defined IVP system without the use of pig follicular fluid.



Fig. 2. The first piglets produced from cryopreserved oocytes 5 days after delivery. The oocytes were vitrified at the immature (GV) stage and stored in liquid nitrogen for several weeks. After retrieval, they were warmed at 42 °C and used for *in vitro* maturation followed by *in vitro* fertilization and embryo culture. Cultured embryos at the blastocyst stage were transferred into recipient females.

## CONCLUSION

In the last decade, vitrification technology has been successfully integrated in IVP technology for the cryopreservation of oocytes and embryos. Nevertheless, since IVP embryos and immature oocytes still have limited viability and stress tolerance to vitrification, further improvements of these methods are required to maximize survival rates. Base research will be essential to understand vitrification-related alterations at cellular and molecular levels in oocytes and embryos. Based on these results, culture systems (such as media) may be modified in the future to address these alterations. Also, vitrification protocols may be customized according to the needs/characteristics of oocytes/embryos specific for developmental stages. Novel approaches such as induced stress resistance will provide

possibilities for further improvements in the future.

Furthermore, it is important to point out that in pigs, the applicability of IVP and cryopreservation methods on the practical level is still limited by the difficulty of embryo transfer by non-surgical methods. Especially, warming and transfer of embryos preserved by current vitrification procedures are considered to be relatively difficult for technicians as it usually requires embryo handling under microscope and the use of other laboratory equipment. In this respect, further efforts are needed towards the development of simplified vitrification methods applicable even under farm and field conditions.

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