REVIEW ARTICLE

# Oocyte and Embryo Cryopreservation in Porcine *In Vitro* Production Systems

Hiep Thi Nguyen[1,4], Tamas Somfai[2]*, Bui Xuan Nguyen[3], Kazuhiro Kikuchi[2], Nguyen Thi Uoc[3], Nguyen Viet Linh[1], Nguyen Thi Nhung[1], Nguyen Thi Hong[1], Nguyen Van Hanh[1]

## Abstract

Cryopreservation of oocytes and embryos, in addition to sperm, are important for gene banking and flexible use of time and space. In pigs, *in vitro* production (IVP) is not only important system for the utilization of sperm kept in gene banks, but also is one of basic technologies for the production of genetically modified, disease models or xeno-transplantation materials for human. Porcine oocytes and embryos are characterized by high lipid content that are very sensitive with cooling. Besides, *in vitro* embryos are weaker than those derived *in vivo* in term of cell number. Therefore, cryopreservation by vitrification is preferred to slow freezing. Especially, cryopreservation of porcine oocytes is possible only by vitrification and germinal vesicle (GV) stage is better than MII stage in terms of cytoskeletal organization and subsequent blastocyst formation. There are several different vitrification methods that have been applied on porcine IVP embryos based on the difference in cryoprotectants or devices to date. In this review, we discuss about recent IVP system, cryopreservation of oocytes and embryos in pigs.

**Key words:** Cryopreservation, Embryos, *In vitro*, Porcine oocytes, Vitrification.

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## Introduction

Cryopreservation is a process by which biological cells or tissues are preserved at sub-zero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods (Armitage, 1987). Cryopreservation of sperm by traditional freezing is the basic means for gene banking in farm animals. *In vitro* production (IVP) is the technology of production of embryos from gametes under laboratory conditions. Briefly, the porcine oocytes are collected from ovaries obtained from slaughterhouse. The oocytes are then subjected to *in vitro* maturation (IVM) for 44-46 h. The matured oocytes are proceeded subsequently for *in vitro* fertilization (IVF) in 3 h and *in vitro* culture (IVC) until Day 6 (the day of IVF is defined as Day 0) for the blastocyst stage. The IVP system has been shown in Figure 1.

In pigs, IVP is an important technology for the utilization of frozen sperm kept in gene banks (Kikuchi *et al*., 2016). Also, IVP is a basic technology necessary for the production of genetically modified pigs, which have great importance for human biomedical research (Prather *et al*., 2003). Furthermore, IVP embryos can be used to make animal disease models or xeno-transplantation materials for humans by genetic modification. The first successful piglet production by the transfer of *in vitro* produced blastocyst-stage embryos was reported by Marchal *et al.* (2001). The most common source of oocytes for IVP is ovaries of pigs from commercial slaughterhouses; however, harvesting immature oocytes from live pigs by ovum pick up is also possible (Yoshioka *et al*., 2020). Although fresh sperm can be utilized for IVF to generate embryos, it is more common to use frozen sperm, both after ejaculation and collection from epididymides. The efficacy of IVP is usually evaluated by the percentage of blastocyst formation from the total number of oocytes. In the last decades, the blastocyst formation rates after *in vitro* fertilization (IVF) have been improved and can be achieved up to 30% to 40% (Grupen, 2014; Yuan *et al*., 2017). However, the *in vitro* culture conditions in pigs are still considered to be imperfect (Grupen, 2014; Chen *et al*., 2021).

Cryopreservation of oocytes and embryos is also very important. The purposes of cryopreservation for oocytes and embryos are: 1) gene banking of female germplasm; 2) the temporary suspension of the IVP process; 3) to facilitate

[1]Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

[2]Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan

[3]Southest Asia Biotechnology Center, Hanoi, Vietnam

**Corresponding Author:** Tamas Somfai, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan. e-mail: somfai@affrc.go.jp

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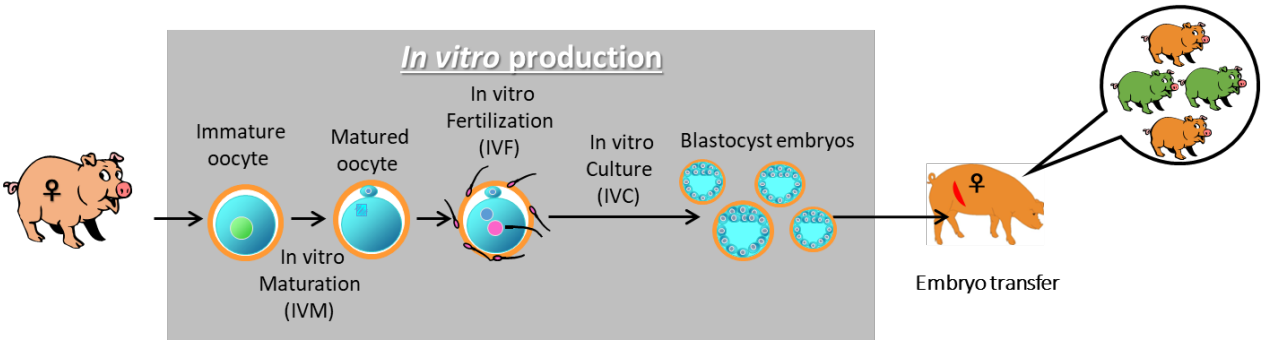


**Fig. 1:** IVP system in pigs

embryo transfer in IVP systems both by logistics (since it allows the storage and transportation of embryos) and the accumulation of embryo numbers to maximize numbers for one embryo transfer. The last point is important in pigs which are multiparous, and many embryos must be transferred into one recipient to achieve pregnancy (Kikuchi *et al.*, 2016; Nohalez *et al.*, 2018).

However, cryopreservation causes damage in cells (Mazur, 1984). Two types of damages are suggested: 1) the physical damage induced by the formation of ice crystals and 2) the chemical damage caused by changes in concentration of intracellular solute (Mazur, 1984; Mandawala *et al.*, 2016). The two strategies of cryopreservation of mammalian oocytes and embryos are slow freezing and vitrification (Moussa *et al.*, 2014). Slow (or "equilibration") freezing is a technique during which cells or tissues are kept in the presence of cryoprotective chemical agents (CPAs) and cooled slowly at a rate (0.5-1.5 °C/min) which allows extracellular ice crystal formation at a pace that dehydrates the cells sufficiently to maintain the chemical potential of their intracellular water close to that of the water in the partly frozen extracellular solution. This results in a controlled non-lethal ice crystal formation (Whittingham *et al.*, 1972).

Compared with slow freezing, vitrification applies high concentrations of CPAs followed by high cooling rate (10,000-24,000°C/min) achieved by direct plunging of the samples into liquid nitrogen. As a result, the liquid phase solidifies into a "glassy" state without ice crystal formation (Rall and Fahy, 1985). Vitrification has several advantages over slow freezing that are simple, cheap and less time consuming (Huang *et al.*, 2019). Moreover, for porcine oocytes and embryos, vitrification is preferred because they are characterized with high lipid content in the cytoplasm which makes them very sensitive to chilling during slow freezing (Nagashima *et al.*, 1994).

## Cryopreservation of Porcine IVP Embryos

In pigs, embryos are more tolerant to slow freezing than oocytes because of the smaller size of cells. Slow freezing has been efficiently used only in *in vivo* produced porcine blastocysts (Vajta *et al.*, 1997; Cuello *et al.*, 2004; Nohalez *et al.*, 2018). On the other hand, *in vitro* produced embryos are generally weaker than *in vivo* ones and are characterized with low cell numbers (Bauer *et al.*, 2010). Therefore, for the cryopreservation of porcine IVP embryos, vitrification is preferred (Esaki *et al.*, 2004).

It is well known that cryopreservation of porcine embryos at the blastocyst stage is more effective than that at cleavage stage (Nagashima *et al.*, 1992; Kuwayama *et al.*, 1997; Dobrinsky *et al.*, 2000; Cuello *et al.*, 2004). In particular, it was reported that porcine embryos at the perihatching stage are the most resistant to freezing (Nagashima *et al.*, 1992; Kuwayama *et al.*, 1997). Likewise, Cuello *et al.* (2004) demonstrated that expanded blastocysts showed the best survival and hatching rates after vitrification compared with morula and early blastocysts.

To date, several different vitrification methods have been applied on porcine IVP embryos (Vajta *et al.*, 1997; Cuello *et al.*, 2004; Bartolac *et al.*, 2015; Mito *et al.*, 2015). These methods differ in the CPAs treatment protocol and the device (carrier) used for vitrification. The most efficient method appears to be the "minimum volume cooling" using the Cryotop device (Mito *et al.*, 2015) achieving over 80% survival. It must be noted, however, that different commercially available devices seem to be equally effective for the vitrification of IVP porcine blastocysts (Bartolac *et al.*, 2015).

Vitrification of zygotes (*i.e.* fertilized oocytes at the 1-cell stage) is also very efficient (Somfai *et al.*, 2009). Vitrification of zygotes has certain advantages to blastocyst vitrification because it does not compromise post-warming cell proliferation (Nguyen *et al.*, 2018). Recently, a deviceless vitrification method called "microdrop vitrification" has been applied for porcine IVP zygotes (Somfai *et al.*, 2023). This method allows the bulk cryopreservation (*i.e.* hundreds) of IVP zygotes in microdrops of vitrification solution without reducing developmental competence to the blastocyst stage (Somfai *et al.*, 2023). Such vitrified zygotes could be used even for genome editing (Haraguchi *et al.*, 2024).

## Cryopreservation of Porcine Oocytes

Oocyte cryopreservation is the basic means for the *in vitro* gene banking of female germplasm safe from epidemic diseases. Porcine oocytes do not tolerate traditional slow

freezing methods (Didion *et al*., 1990). Porcine oocytes can be cryopreserved only by vitrification (Zhou and Li, 2009). In humans and cattle, vitrification of oocytes at mature metaphase-II (MII) stage is advantageous because they are ready to use for fertilization without the need for IVM. Hence, in these species, vitrification of oocytes at the MII stage is the gold standard for oocyte cryopreservation. On the other hand, in pigs, oocyte vitrification at the MII stage is associated with very low embryo development despite of high survival rates (Somfai, 2024). In brief, vitrification at the MII stage triggers parthenogenetic activation and damages of meiotic spindle in porcine oocytes. These lead to fertilization failure/abnormality and the failure of second polar body extrusion (Somfai, 2024). Further research is needed to address these problems.

Vitrification of porcine oocytes at the immature germinal vesicle (GV) stage has been established (Somfai *et al*., 2010). It was demonstrated that vitrification of porcine oocytes at the GV stage is clearly superior to that at the MII stage in terms of cytoskeletal organization and subsequent blastocyst formation (Egerszegi *et al*., 2013). The procedure was later modified to improve vitrification efficacy (Somfai *et al*., 2014; Appeltant *et al*., 2018; Nguyen *et al*., 2022; Nguyen *et al*., 2023) and live piglets could be produced even from such vitrified oocytes (Somfai *et al*., 2014). Therefore, vitrification of oocytes at the GV stage has been utilized for *in vitro* preservation for genetic resource of Vietnamese native pigs (Somfai *et al*., 2019). However, even at the GV stage, vitrification exerts negative effects on oocytes which are manifested in reduced blastocyst development. For instance, vitrification triggers premature meiotic resumption during IVM (Nguyen *et al*., 2022). Therefore, synchronization of nuclear and cytoplasmic maturation of vitrified oocytes during subsequent IVM has key importance for embryo production from the vitrified oocytes (Nguyen *et al*., 2022). It must be noted that intact compartment of cumulus cells around the oocytes is important during IVM for cytoplasmic maturation (Maedomari *et al*., 2007) which also regulates the meiosis resumption of oocytes (Appeltant *et al*., 2016). Therefore, maintaining the cumulus layers around the oocytes during and after vitrification is important. Denudation before vitrification reduced the survival rate of oocytes after warming (Nguyen *et al*., 2021). Moreover, the preservation of mitochondrial properties using cyclosporine A and docetaxel could improve developmental competence of vitrified oocytes (Nguyen *et al*., 2023). To our knowledge, the most efficient protocol for immature porcine oocyte vitrification was reported by Nguyen *et al*., (2022) achieving over 94% survival rate after warming and over 20% blastocyst production by subsequent IVM and IVF.

## Conclusion

In pigs, IVP is important technology for basic research in developmental biology and the utilization of frozen sperm in gene banks. Cryopreservation by vitrification of oocytes and embryos is advantageous for gene banking and logistics. However, developmental competence of vitrified oocytes/embryos is still inferior to those of fresh oocytes/embryos. Therefore, further research is needed to improve their efficacies.

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