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Factors Influencing the Efficiency of *In Vitro* Embryo Production in the Pig

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ABSTRACT

Pigs are considered an ideal source of human disease model due to their physiological similarities to humans. However, the low efficiency of *in vitro* embryo production (IVP) is still a major barrier in the production of pig offspring with gene manipulation. Despite ongoing advances in the associated technologies, the developmental capacity of IVP pig embryos is still lower than that of their *in vivo* counterparts, as well as IVP embryos of other species (e.g., cattle and mice). The efficiency of IVP can be influenced by many factors that affect various critical steps in the process. The previous relevant reviews have focused on the *in vitro* maturation system, *in vitro* culture conditions, *in vitro* fertilization medium, issues with polyspermy, the utilized technologies, etc. In this review, we concentrate on factors that have not been fully detailed in prior reviews, such as the oocyte morphology, oocyte recovery methods, denuding procedures, first polar body morphology and embryo quality.

(Key words : Oocyte recovery, *In vitro* maturation, *In vitro* fertilization, Somatic cell nuclear transfer, Pig)

INTRODUCTION

The pig is regarded as the best candidate species for xenotransplantation and transgenic animal-based creation of human-relevant proteins, largely because pigs are anatomically and physiologically similar to humans. They also have the benefits of offering a short gestation time, being inexpensive and easy to raise, etc. (Jin *et al.*, 2003; Ahn *et al.*, 2011). Numerous modifications have been used to optimize the efficiency of *in vitro* embryo production (IVP) in pigs. However, the developmental capacity of IVP porcine embryos remains extremely low compared to their *in vivo*-produced counterparts and *in vitro* produced embryos of other species (Dang-Nguyen *et al.*, 2011). This is because the existing *in vitro* culture system is suboptimal for porcine embryos, which are generally more sensitive than the embryos of other domestic animals (Dang- Nguyen *et al.*, 2011; Lee *et al.*, 2013). A variety of factors acting on multiple steps in the process can influence the efficiency of porcine IVP. This review focuses on some of these factors, particularly those that have been largely overlooked in the previous reviews. The topics addressed include the cumulus-oocyte-complex (COC) morphology, oocyte recovery method, denuding procedure, morphology of the first polar body, and relationship between embryo quality and culture duration.

FOLLICLE DIAMETER

Porcine oocytes are usually recovered from antral follicles on the ovary surface. Their diameters are often classified as < 3 mm, 3～6 mm, and > 6 mm, and the relationship between follicle diameter and developmental competence has been widely studied inpigs(Wu *et al.*, 2006; Bagg *et al.*, 2007; Kwak *et al.*, 2014). These studies have consistently demonstrated that oocytes derived from small follicles (< 3 mm) possess lower maturation and developmental competences than those derived from larger follicles (≥ 3 mm). When performing oocyte collection, therefore, researchers have typically recovered porcine oocytes from antral follicles having diameters of 3～6 mm. However, a recent study showed that oocytes recovered from follicles > 8 mm in diameter required 18 hours to complete their matu-

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ration *in vitro*, and had a higher developmental competence than those isolated from 3~7 mm follicles (Kwak *et al.*, 2014).

COC MORPHOLOGY

COC morphology is a criterion for evaluating oocyte quality. COCs are generally classified according to the number of cumulus cell layers: Class I oocytes have five or more layers of cumulus cells; Class II oocytes have three to five layers of cumulus cells; Class III oocytes have one to two layers of cumulus cells; and Class IV oocytes are either denuded or have a partial cumulus cell layer. Class I and II COCs are considered good quality, whereas Class III and IV COCs are considered poor quality. In a human oocyte study examining the relationship between COC morphology and the developmental capacity of the embryo, Sato (Sato *et al.*, 2007) reported that COC morphology did not influence the oocyte maturation rate, but the post-*in vitro* fertilization (IVF) blastocyst formation rate was significantly higher in the good morphology group compared to the poor morphology group. In pig and ovine embryo studies, oocytes with poor morphology (i.e., one or two layers of cumulus cells) showed decreased rates of meiotic resumption and cytoplasmic maturation (Alvarez *et al.*, 2009; Dadashpour Davachi *et al.*, 2012). Oocytes with poor morphology do not undergo complete maturation, perhaps because they secrete insufficient maturation factors. We have recently found that poor morphology COCs failed to undergo proper *in vitro* maturation in pigs. However, the co-culture of oocytes with both good and poor morphology oocytes greatly improved the utilization rate of the latter, which showed no reduction in maturation or subsequent developmental capacity (our unpublished data).

OOCYTE RECOVERY METHOD

The availability of sufficient oocytes is a precondition for successful IVP, because only 30% of recovered embryos typically develop into blastocysts (Davachi *et al.*, 2012). Thus, oocyte recovery is an important component of the IVP process, which can include *in vitro* maturation (IVM), IVF, somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI), etc. Although oocytes are most often recovered from antral follicles on the ovarian surface, they may also be collected from live animals using the ovum pickup (OPU) technique or multiple-ovulation protocol. In livestock, the recovery rate of oocytes from antral follicles is very poor because most of the follicles on the ovary surface are small, early follicles [follicles < 3 mm in diameter are considered early antral follicles; (Wu *et al.*, 2006)]. To obtain numerous high quality oocytes from follicles on the ovarian surface, various oocyte recovery methods have been developed, including aspiration, slicing and centrifuge methods (Arav, 2001; Wang *et al.*, 2007; Davachi *et al.*, 2012). The IVM preantral follicle strategy can provide a large number of oocytes for IVP procedures (Wu *et al.*, 2001; Hirao *et al.*, 2013; Mochida *et al.*, 2013; Tasaki *et al.*, 2013), and isolation methods and culture systems for preantral follicles have been developed in various species, including mice, cattle, pigs, horses, dogs, cats, camels, buffalos, etc. (Gupta and Nandi, 2012).

IVM OF OOCYTES

Efficient IVM, which involves both nuclear and cytoplasmic maturation, is essential for the successful production of live animals via IVP. During oocyte maturation, cumulus cells can maintain the meiotic block at the germinal vesicle (GV) stage or trigger the resumption of meiosis by secreting appropriate factors (Xia *et al.*, 2000; Tanghe *et al.*, 2002; Dang-Nguyen *et al.*, 2011). One remarkable sign of oocyte nuclear maturation is the extrusion of the first polar body (PB1); however, extrusion of PB1 does not ensure a normal chromosome number in the resultant embryo, because some aneuploid oocytes can extrude PB1 (Dang-Nguyen *et al.*, 2011). Despite oocytes with visible PB1s *in vitro*, they show a reduced development capacity compared with their *in vivo* matured counterparts (Dang- Nguyen *et al.*, 2011), suggesting poor cytoplasmic maturation is thought to be mainly responsible for the low developmental capacity of post-parthenogenetic activation (PA), post-IVF and post-SCNT]. Numerous factors can affect the cytoplasmic maturation of pig IVM oocytes. Reactive oxygen species (ROS) and intracellular levels of glutathione (GSH) critically affect oocyte maturation and the subsequent developmental capacity of the embryo following PA, IVF, and SCNT. More specifically, the cytoplasmic maturation of oocytes is improved by downregulation of ROS and upregulation of GSH (Kobayashi *et al.*, 2006; You *et al.*, 2010; Kwak *et al.*, 2012a). The distribution of cortical granules (CGs) is also considered to be a very important marker for the completion of cytoplasmic maturation in oocytes (Zhang *et al.*, 2010; Biswas and Hyun, 2011; Dey *et al.*, 2012). IVM porcine oocytes that fail to complete proper

cytoplasmic maturation often contain abnormal microtubules; this can influence the formation of the spindle, leading to aberrant chromosomal segregation after fertilization and reducing the ability of the embryos to develop to the blastocyst stage (Ueno *et al*., 2005; Miao *et al*., 2009; Zhang *et al*., 2010).

DENUDING PROCEDURES

Oocytes derived from IVM must undergo a denuding procedure prior to being manipulated for IVP. Vortexing, pipetting and pre-denuding are the main methods (Lin *et al*., 2015). Porcine embryos are generally more fragile and sensitive than those of other domestic animals, and vigorous denuding procedures can injure or even destroy porcine oocytes. Our previous study in porcine oocytes (Lin *et al*., 2015) indicated that vortexing or pipetting were the superior denuding methods for PA or IVF, while oocyte enucleation (pre-denuding) was preferable for SCNT. Furthermore, we found that the utilized denuding procedure not only had the potential to damage the oocyte, it also affected the position of the first polar body with respect to the nuclear materials, the spindle morphology, and the CG distribution.

MORPHOLOGY OF THE FIRST POLAR BODY

The relationship between the morphology of PB1 and the fertilization rate and/or quality of the generated embryo has been widely studied in the context of human assisted reproduction techniques (Navarro *et al*., 2009; Younis *et al*., 2009), but there is limited information available with respect to porcine embryo development. The quality of porcine oocytes can be evaluated based on the morphology of PB1. PB1 can be classified into five morphological categories (Lin *et al*., 2013): Grade I, round or ovoid PB1 with an intact smooth membrane; Grade 2, round or ovoid PB1 with an intact membrane; Grade 3, broken PB1 with a small PB1 fragment; Grade 4, broken PB1 with a large PB1 fragment; and Grade 5, fully fragmented PB1. Oocytes with Grade 1 or 2 PB1 are considered to be good quality. In our laboratory, porcine oocytes with differences in PB1 morphology were found to yield embryos with different levels of developmental competence. We identified simple criteria for PB1 morphology that could be used to rapidly choose good-quality oocytes, in the hopes of improving the efficiency of porcine SCNT. The formation rates and cell numbers of blastocysts were significantly higher after porcine SCNT of the good-PB1-morphology group compared to the poor-PB1-morphology group (our unpublished data).

EMBRYO CULTURE MEDIUM AND CONDITIONS

Embryo culture media can be classified as defined (protein-free), semi-defined (albumin added) and undefined (serum added). Porcine IVP systems most often use NCSU-23, PZM-3 and TCM199 media, which have large proportions of serum or albumin. Although serum can provide some beneficial factors (e.g., proteins, growth factors, vitamins, amino acids, etc.), it also contains embryotoxic factors (Camargo *et al*., 2006). Thus, many researchers use bovine serum albumin (BSA) to provide amino acid substrates for embryonic metabolism (Orsi and Leese, 2004). In efforts to improve the *in vitro* developmental potential and quality of porcine oocytes, many laboratories have added chemical supplements and/or changed the osmolarity of the IVM medium (Hwang *et al*., 2007; Naruse *et al*., 2007a; Naruse *et al*., 2007b; Biswas *et al*., 2011; Nguyen *et al*., 2011; Wu *et al*., 2011; Kwak *et al*., 2012a; Lin *et al*., 2014). It is widely accepted that the optimal incubation conditions for *in vitro* culture of porcine embryos include a temperature under 38.5 or 39℃, and air containing 5% CO_2.

RELATIONSHIP BETWEEN EMBRYO QUALITY AND CULTURE TIME

The frequency of apoptosis (Hao *et al*., 2004; Fabian *et al*., 2005), the total cell numbers in blastocysts (including the inner cell mass and trophectoderm cells) (VanSoom *et al*., 1996), and the blastocyst diameter (Hazeleger *et al*., 2000; Kidson *et al*., 2004; Hao *et al*., 2006) are usually used to judge embryo quality. Porcine embryos were reported to have more nuclei on Days 7 and 8 of culture versus Days 5 and 6 of culture, but the rates of apoptosis were significantly higher in Day 7 and 8 blastocysts versus Day 5 and 6 blastocysts (Hao *et al*., 2003; 2004). Usually, blastocysts with larger diameters have more nuclei than smaller blastocysts, whereas larger embryos tend to show more apoptosis (Kidson *et al*., 2004). In a recent study, we found that porcine PA blastocysts derived from a

group with delayed blastulation and smaller diameters also exhibited higher rates of apoptosis (our unpublished adta).

IN VITRO FERTILIZATION

In vitro fertilization, during which mature oocytes are fertilized by sperm in a lab, is a complex series of procedures used to circumvent fertility or genetic problems. However, IVF embryos frequently suffer from polyspermy due to the rapid (and often simultaneous) sperm penetration that can occur during the co-culture of oocytes with an overabundance of sperm in a small drop of IVF medium (Dang-Nguyen *et al*., 2011). The sperm concentration and duration of IVF must therefore be controlled in order to ensure acceptably high rates of fertilization and monospermy (Dang-Nguyen *et al*., 2011).

Various procedures have been used to prevent polyspermic penetration in IVF systems. Sperm penetrate between 3 and 6 h after insemination, and the incidence of polyspermic penetration increases with the co-culture duration (Funahashi *et al*., 2000). Although numerous laboratories have incubated gametes for ~6 h, reduction of the co-incubation time from 6 to 3 h was shown to decrease the polyspermy rate without changing the male pronucleus (MPN) formation rate, the penetration rate or the overall production efficiency of normal embryos by IVP (Kazuhiro Kikuchi *et al*., 2006). In the pig, successful penetration with a low incidence of polyspermy has been achieved by incubating oocytes with sperm for 20 min at 39℃ in an atmosphere containing 5% CO_2 and 95% air, and then washing the oocytes and incubating them without sperm for an additional 5 to 6 h (Kwak *et al*., 2012a; Kwak *et al*., 2012b).

The presence of cumulus cells is essential for oocyte IVM, fertilization and embryo development (Tanghe *et al*., 2002; Wongsrikeao *et al*., 2004; Li *et al*., 2006; Jeon *et al*., 2011). In cattle, cumulus-cell-free oocytes reportedly showed a reduced fertilization rate in an IVF system (Fatehi *et al*., 2002). In addition, cumulus cells are known to play important roles in fertilization and MPN formation in IVM oocytes (Kikuchi *et al*., 1993). However, Dang-Nguyen *et al*. found that the removal of cumulus cells prior to IVF did not reduce the penetration rate when using frozen sperm (Dang-Nguyen *et al*., 2011).

PARTHENOGENETIC ACTIVATION

Oocyte activation comprises a series of processes that occur in the oocyte during fertilization. In mammals, oocyte activation may be caused by the entry of sperm, which triggers the release of calcium into the oocyte, or by artificial stimulation. Artificial (parthenogenetic) activation of oocytes is a critical step in the SCNT procedure. PA embryos resemble IVF embryos during the early stages of development, are relatively easy to create, and are associated with fewer ethical problems than IVF embryos; thus, PA embryos are often used as a model system for investigating *in vitro* culture conditions (Gupta *et al*., 2008; Zhang *et al*., 2012a).

Numerous methods have been used to induce PA (Somfai *et al*., 2006; Juhi Pathak *et al*., 2013), including mechanical stimulation, electrical pulses, ultrasound, chemicals (e.g., calcium ionophores, or calcium ionophores plus cycloheximide and the protein phosphorylation inhibitor, 6-dimethylaminopurine), etc. The ionophore-mediated activation of oocytes is a simple method that does not require expensive equipment, but the activation efficiency and the developmental capacity of the activated oocytes tend to be low (Sedmikova *et al*., 2003). Most often, electrical pulses have been used to activate reconstructed embryos for porcine SCNT procedures.

Oocyte activation relies on the elevation of intracellular calcium; this alters the activity levels of maturation promoting factor (MPF) and cytostatic factor, which are responsible for the meiotic block at metaphase II (Hashimoto and Kishimoto, 1988). After electrical pulse stimulation, an influx of extracellular calcium ions increases intracellular calcium. Ionophore treatment, in contrast, induces the release of calcium from internal deposits to generate a single large spike in the intracellular calcium level. These increases in intracellular calcium trigger several calcium-dependent proteolytic pathways, leading to the destruction of cyclin B, a reduction in MPF activity, and the resumption of meiosis (Juhi Pathak *et al*., 2013). Thus, PA methods increase intracellular calcium levels in oocytes by releasing calcium from cytoplasmic stores or allowing the entry of extracellular calcium. This mimics the activating calcium spike seen when an oocyte is penetrated by a spermatozoon during the fertilization process.

SOMATIC CELL NUCLEAR TRANSFER

SCNT has been successfully applied to variety of areas, including agricultural research, biotechnology, genetic conservation and medical science. Since the first cloned animal, Dolly, was created by a SCNT-based

method, live cloned offspring have been produced from cattle, mice, goats, sheep, pigs, dogs, cats, rabbits and horses (Campbell *et al*., 2007a). SCNT was first reported in 1996 (Campbell *et al*., 1996a; b), but the first SCNT-based cloned pig was not reported until 2000 (Onishi *et al*., 2000; Polejaeva *et al*., 2000). These results indicated that, production cloned pig by SCNT method is more difficult than other animals. Although pigs have been cloned by somatic cell nuclear transfer method, the overall efficiency of SCNT is still very low due to poor *in vitro* and *in vivo* embryo development. The reason of low efficiency of SCNT is still unclear, although decades of work trying to finding this problem. Lots of factors influence the efficiency of SCNT, including animal species, the source of the recipient oocytes, the donor cell type, the protocol used to treat the donor cells prior to SCNT, the utilized PA method and the embryo culture conditions. Complications are also possible, such as donor cell losses, reprogramming failure, placental abnormalities, maternal inheritance of the mitochondrial NDA, and trophoblastic defects (Lai and Prather, 2004; Lin *et al*., 2011; Ogura *et al*., 2013).

Many researchers have attempted to improve the efficiency of SCNT by optimizing the operational procedures (e.g., the enucleation, fusion and activation methods) (Li *et al*., 2004; Campbell *et al*., 2007b; Keefer, 2008; Song *et al*., 2011). In addition, handmade cloning, which is an easy, inexpensive alternative for SCNT, has been successfully applied to several species, including pigs, cattle, and sheep (Vajta, 2007; Zhang *et al*., 2012b; Zhang *et al*., 2013). Chemical supplementation of the embryo culture medium has been extensively examined, with many studies seeking to improve cloning efficiency by using histone deacetylase (HDAC) inhibitors [e.g., trichostatin A (TSA), scriptaid, sodium butyrate and valproic acid] to induce histone acetylation (Zhao *et al*., 2009; Zhao *et al*., 2010; Diao *et al*., 2013; Song *et al*., 2014), thereby improving the epigenetic reprogramming of donor nuclei in developing reconstructed embryos. However, the reprogramming efficiency of HDAC inhibitors is often dependent on the animal species (Song *et al*., 2014).

IN SUMMARY

IVP is an important tool for the study of animal embryology and the propagation of mammalian species. The IVP-based generation of transgenic pigs is particularly interesting because such animals could potentially provide organs for human transplantation. Although the use of IVP is still limited by various barriers, future advances should enable IVP technology to better serve the needs of agriculture, biotechnology, medicine and our human community.

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