Studies on Porcine Embryonic Development after Intracytoplasmic Sperm Injection using Freeze-dried Sperm

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Abstract

Cryopreservation has been proved as an indispensable tool in the research of animal reproductive biology and in conservation of animal genetic resources. However, cryopreservation has some disadvantages for storage and transportation. These issues emphasize the need for alternative safe and low-cost storage systems for biological specimens. Therefore, freeze-drying (FD) of sperm without liquid nitrogen has been special of interest.

Sperm DNA is the most important molecule to ensure normal fertilization and subsequent development. It is reported that defects in the structure of chromatin can severely reduce fertility and early embryo development, increase spontaneous abortions and birth defects. The maintenance of DNA integrity during FD procedures and storage, thus, is extremely important. The objectives of this study were to expand basic knowledge of FD associated sperm DNA damage and improve the efficacy of sperm preservation method by FD.

In Chapter II, we examined the effect of a combination of ethylene glycol-bis(2-aminoethylether)-N,N,N0,N0-tetraacetic acid and different concentrations of trehalose in FD medium on sperm DNA integrity and the *in vitro* development of IVM (*in vitro* maturation) porcine oocytes after intracytoplasmic sperm injection (ICSI) using freeze-dried boar sperm. Ejaculated sperm from a boar were suspended in basic FD medium supplemented with different concentrations of trehalose and freeze-dried. The results showed that the level of DNA damage, assessed by Halomax kit, in the 15 mM group was significantly lower than that in the 0 mM (control) group, and no difference was observed between the 15, 7.5, and 3.75 mM groups. Moreover, there were no significant differences in the DNA damage level among 0, 3.75 mM, and the other groups. When freeze-dried sperm were used for ICSI, the fertilization rates and blastocyst formation rates in the 7.5 and 15 mM groups were not different from those in the control group. These results suggest that FD medium supplemented with trehalose at appropriate concentrations improves sperm DNA integrity, but does not improve fertilization and preimplantation embryo development of IVM oocytes following ICSI.

Previous studies demonstrated that the oocytes have ability to repair DNA damaged sperm. The DNA damaged sperm may activate maternal DNA repair genes when injected to oocytes. Accompany with the results from Chapter II, we hypothesized that different levels of DNA damage in sperm may lead to various expression levels of DNA repair genes in

oocytes injected with those sperm. Therefore, in Chapter III, we investigated the expression profile of some DNA repair genes in porcine oocytes after ICSI. Firstly, the expression levels of *MGMT*, *UDG*, *XPC*, *MSH2*, *XRCC6*, and *RAD51* genes that concerned with the different types of DNA repair were examined in IVM oocytes injected with ejaculated sperm, or freeze-dried sperm with or without trehalose. Quantitative RT-PCR revealed that expression of six DNA repair genes in the oocytes at 4 hour after injection did not differ among the four groups. Next, we investigated the gene expression levels of these genes at different stages of maturation. The relative expression levels of *UDG* and *XPC* were significantly up-regulated in mature oocytes compared with earlier stages. Furthermore, there was an increased tendency in relative expression of *MSH2* and *RAD51*. These results suggested two possible mechanisms that mRNA(s) of DNA repair genes are either accumulated during IVM to be ready for fertilization or increased expression levels of DNA repair genes in oocytes caused by suboptimal IVM conditions.

In order to improve the efficacy of *in vitro* porcine embryonic production following ICSI using freeze-dried sperm, the effects of freeze-dried sperm treatment with Triton X-100 (TX) were examined in Chapter IV. Freeze-dried sperm with (15 mM group) or without (0 mM group) trehalose were rehydrated and centrifuged and sonicated to isolate the heads from their tails and treated with 0, 0.5% or 1.0% TX for 10 min at room temperature. The percentages of oocytes displaying two polar bodies and two pronuclei after injection and electrical stimulation were highest in the 15 mM trehalose group treated with the 0.5% TX (77.52%) but not significantly different from the remaining sperm-injected groups. In conclusion, the present study showed that freeze-dried boar sperm treated with TX at different concentrations did not improve normal fertilization of IVM oocytes after ICSI.

In Chapter V, the effects of centrifugation and electric stimulation on ICSI-oocytes were examined. Firstly, the effects of oocyte centrifugation before injection were assessed. Freeze-dried sperm after 0 mM trehalose treatment were injected into 3 different layers of centrifuged oocytes. The results showed that there were no differences in normal or abnormal fertilization among all groups compared with the control. Next, the effects of oocyte centrifugation after ICSI were investigated. Freeze-dried sperm from the 15 mM trehalose group were used for injection. One hour after injection, the oocytes were subject to centrifugation (CF+) or without centrifugation (CF–) combined with electrical activation (EA+) or without electrical activation (EA–) and cultured *in vitro* for 9 hours to examine fertilization status. Normal fertilization rates were significantly higher in the groups with electric activation than those of without electric activation. In addition, these rates were

significantly higher in the control group compared with the remaining groups. The results confirmed the importance of additional electric stimulation to activate the porcine ICSI-oocytes. In addition, centrifugation showed a negative effect on normal fertilization in case of electric activation and no effect on normal fertilization in case of no electric activation was applied.

In parallel, we tried to transfer embryos obtained after ICSI using freeze-dried sperm into recipients to generate live piglets in Chapter VI. Freeze-dried sperm from the 0 mM and 15 mM trehalose groups were used for this experiment. Sperm-injected oocytes and parthenogenetic embryos were co-transferred into the oviducts of recipients to observe full-term development. After 7 trials, we have not been succeeded in producing live piglets. Consider that blastocyst quality may responsible for failed outcome of embryo transfers, we continued to conduct the experiments in Chapter VII to compare DNA fragmentation level (apoptosis) in nuclei of blastocysts obtained from different types of sperm both in IVF and ICSI assessed by TUNEL assay. However, our results suggest that blastocyst quality (total cell number and nucleus DNA fragmentation) might not a reason for failure in embryo transfer in this research.

In conclusion, the positive effect of trehalose on DNA integrity of freeze-dried sperm provides important information for the improvement of FD methods associated FD medium; expand basic knowledge of FD associated sperm DNA damage in pigs. This research also has contributions in practice since FD will greatly simplify the establishment and management of biobanks. It also helps to minimize the environmental costs arising from the production and use of liquid nitrogen and other activities for maintenance of facilities of cryopreservation system.

Keywords: trehalose, freeze-drying, pig, gene expression, sperm treatment, oocyte treatment, embryo transfer, TUNEL assay

Abbreviations used in the dissertation

ART	assisted reproductive technique
ATP	adenosine-5'-triphosphate
BER	base excision repair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CF	Centrifugation
COC	cumulus-oocyte complex
CSC	chromosome-spindle complex
DDT	Dithiothreitol
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DSBs	double stranded breaks
DSBR	double strand break repair
eCG	equine chorionic gonadotropin
EGA	embryonic genome activation
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N0,N0-tetraacetic acid
ET	embryo transfer
FAO	Food and Agriculture Organization of the United Nations
FD	freeze-drying
FPN	female pronucleus
γH2A.X	histone H2A.X phosphorylated at serine 139
FSH	follicle-stimulating hormone
GSH	gluthathione
GV	germinal vesicle
GVBD	germinal vesicle break down
GVL	late germinal vesicle
hCG	human chorionic gonadotropin
ICSI	intracytoplasmic sperm injection
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production

LH	luteinizing hormone
LN	liquid nitrogen
M-I	metaphase-I
M-II	metaphase-II
M-III	metaphase-III
MAPK	mitogen-activated protein kinase
MMR	mismatch repair
MPF	maturation promoting factor
MPN	male pronucleus
mRNA	messenger Ribonucleic acid
NCSU	North Carolina State University
NER	nucleotide excision repair
PB	polar body
PBs	polar bodies
PCR	polymerase chain reaction
PLCζ	phospholipase C zeta
PMSG	pregnant mare serum gonadotropin
PN	Pronucleus
PNs	Pronuclei
PVP	Polyvinylpyrrolidone
RT-PCR	reverse transcription polymerase chain reaction
SOAF	sperm-borne oocyte-activating factor
SEM	standard error of the mean
SSBs	single stranded breaks
TCM	tissue culture medium
TUNEL	terminal deoxynucleotidyl transferase
TX	triton X- 100
ZGA	zygote genome activation

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Chapter I

General Introduction

1.1. Pig as a good model for bio-medical research

Pigs play an important role not only in meat supply for human beings but also in biomedical research. Pigs contribute about 37.7% of meat consumption of the world (FAO, 2009). However, according to a report of FAO in 2000, 47 pig breeds are categorized as critical, another 85 breeds are endangered and 151 breeds have become extinct (FAO, 2000). These statistics show clearly a globally serious reduction of pig biodiversity. In Vietnam, there are about 27.6 million pigs, ranked the fourth in the world in number of pigs and contributes 3.03 million ton pork per year, ranked the sixth in the world in pork production (FAO, 2009). According to latest data of FAO, the number of pigs slightly decreased to 26.3 million, supplied 3.22 million ton pork in 2013. In general, pigs always play the most important role in livestock animals in Vietnam and make a very far distance in number of heads related to cattle, buffalos and goats (FAO, 2013). Vietnam is also the country where many endemic breeds are found: I pig, Mongcai pig, Muongkhuong pig, Meo pig, Soc pig, Vanpa or mini pig, Co pig (Dang Nguyen et al. 2010). Exceptional characteristics of local breeds are well-adaptable to changes of environmental condition, good severe tolerance, specific pathogen tolerance, especially good taste of meat. However, slow converting ability from energy into muscle and protein of local breed, rapid development of hybrid and high-yield breeds and the poor management of breed conservation are main reasons leading to serious reduction in pig biodiversity in Vietnam. With above dominant characteristics and high genetic diversity, long-term conservation of Vietnamese pigs for future generation is important and urgent.

Pigs are also one of animals suitable as a valuable preclinical model for biomedical research because they own the physiological, genomic (Humphray et al. 2007), and anatomical similarities to humans (Swanson et al. 2004). Pigs, therefore, have become increasingly important as potential xenograft donors and transgenic animals to produce specific proteins (Hornak et al. 2012). Traditionally, toxicity tests often utilize rat and dog models without considering whether there is an alternative species that might be more appropriate for testing a specific compound. While no animal model can completely recapitulate the effects of every drug administered to humans, previous research have shown

that large animals are better preclinical models for drug toxicity than rodents (Olson et al. 2000). In this respect, pigs have been proved the most suitable choice.

Potential of minipig as a platform for future developments in genomics, transgenic technology, *in vitro* toxicology and related emerging technologies has been recognized since some decades ago (reviewed by Forster et al. 2010). This is due to the fact that the current knowledge bases for the most of the field of study such as genomics, reproductive biology, immunology and genetic manipulation is significantly greater for the pigs than the dogs or non-human primates.

In addition, domestic pigs are plentiful and inexpensive, because they are well-established as a food source for human beings. They are used in medical training programs with a mature body weight of approximately 100 kg (Kobayashi et al. 2012). More suitable for medical research than domestic pigs, however; miniature pigs with a mature body weight of 40 to 50 kg are more expensive because of limited annual production. Under both the economic aspect and the ethical point of view, the pattern of sharing and reuse of miniature-pig tissues and cells for research are recommended to reduce the total number of pigs needed for medical research (Kobayashi et al. 2012).

The other advantages of utilization the pigs in research are also recognized. Research with human materials (oocytes and sperm) is basically limited; the use of domestic species, therefore, will rapidly promote our understanding of the fundamental mechanism of intracytoplasmic sperm injection (ICSI) (Catt & Rhodes 1995). Pig ovaries from slaughter-house are waste materials and abundant sources. This is also a great advantage of *in vitro* research related to pig ovaries compared with other livestock. In the studies related to female reproductive tract, porcine Fallopian tubes are prominent and tortuous, making them readily accessible to surgical procedures. In pharmaceutical safety studies, non-human primates are also often used; however, pigs are ideal substituted animals because they are sexually mature at about 4–6 months of age while the age of non-human primate is much later (for example, 3–4 years for macaques). The expense for housing and feeding for pigs can be reduced significantly. Additionally, pigs have multipule litters rather than single birth of primates potentially reduced genetic variability between the litters.

In brief, with the importance mentioned above, conservation activities including the establishment of gene bank for long-term storage and the utilization of assisted reproductive technologies (ARTs) are extremely necessary. In the effort to conserve minipigs in Vietnam, ARTs have been used to produce embryos *in vitro* from Ban pigs (Nguyen et al. 2007; Dang-Nguyen et al. 2010).

1.2. Freeze-drying as an effective approach for preservation of mammalian sperm

Prokaryotic and eukaryotic organisms contain approximately 70–90% water, which is required to create the intracellular aqueous environment in which all biological processes occur (Billy & Potts 2001). However, the organisms are generally unstable when kept in aqueous solutions at room temperature because of the degradation, denaturation and growth of microbial contaminants (Arakawa et al. 2007). In other words, water is an important contributing factor to the conformational stability of a protein; on the other hand, water is a destabilizing factor in the long-term preservation of the chemical and structural integrity of a protein. Therefore, various preservation methods, such as cryopreservation, vitrification, lyophilization or freeze-drying (FD), air drying or vacuum drying, have been developed to avoid/reduce the negative effect of water. Lyophilization is the process which extracts the water from a product to the level at which the biological growth and chemical reactions are no longer supported, thus the product can retain stable and easy to store. Lyophilization uses the simple principle of physics called sublimation. Sublimation is the transition of a substance from solid (ice) state to the vapor state without passing first through a liquid (water) phase. It is an effective method for preserving a wide variety of heat-sensitive materials such as proteins, microbes, pharmaceuticals, tissues and plasma. This process consists of two major steps: 1) freezing of a protein solution, and 2) drying of the frozen solid under vacuum. The drying step is further divided into two phases: primary and secondary drying. The primary drying removes the frozen water and the secondary drying removes the non-frozen ‘bound’ water (Arakawa et al. 1993).

It is recognized that the maintenance of cells in liquid nitrogen (LN) is the golden standard for storing frozen cells (Loi et al. 2008). ARTs in general and cryopreservation in particular have been proved as an indispensable tool in the animal reproduction field, not only to maintain and improve the quality of livestock animals, but also to conserve the genetic diversity of rare species, or maintain the large number of genetically modified mouse strains (Wakayama et al. 2010). Unlikely oocyte cryopreservation, sperm cryopreservation has early succeeded in retaining fertilization of sperm and in producing viable piglets (Mattioli et al. 1989; Yoshida et al. 1993b). Sperm cryopreservation is important for the following purposes: 1) maintenance of genetic diversity in domestic and wild species populations; 2) facilitating the distribution of genetically superior domestic species lines; 3) treatment of iatrogenic infertility and 4) genetic banking of genetically modified animal models of human health and diseases. The novel discovery of Polge et al. (1949) showed that

the use of glycerol and its analog could provide protection ablility to cells at low temperatures. This is often cited as the defining moment in the establishment of modern sperm cryobiology. The discovery paved the way for deep-freezing cells in LN. Then the process of finding a solution for avoiding intracellular ice formation in freezing resulted in the evolution of the freezing paradigms, such as slow freezing and rapid freezing (vitrification). Vitrification is a powerful technique for cryopreservation of mammalian sperm, oocytes and embryos (Rall & Fahy 1985; Kasai 2002). In general, the current freezing protocols are straightforward and efficient, with a good recovery rate after thawing (Shevde & Riker 2009), but cryopreservation in general and vitrification in particular are not devoid of problems (reviewed by Loi et al. 2013) because of following disadvantages: the need for a stable replacement supply of LN for storage, the expense of long-term storage of LN, the difficulties with international transportation, and the effects of unpredicted disasters. Especially, aside from these practical inconveniences, the storage of genetic materials in LN also pose an environmental concern, since industrial production of LN and the maintenance of its storage centers have a high carbon emission (Loi et al. 2013). In addition, LN which is not readily available in some parts of the world, especially in developing countries, also is a big obstacle. These issues emphasize the need for alternative safe and low-cost storage systems for biological specimens. Therefore, cheaper and safer solutions for long-term storage of sperm have been sought and deployed, among which preservation by FD without LN has been a focus of attention. However, boar semen differs from the semen of other domestic animals in several aspects. It is produced in large volumes and is extremely vulnerable to sudden cooling immediately after collection (cold shock) (Ericksson 2000). Therefore, boar semen requires special consideration in the design of freezing protocols (Pursel & Johnson 1971). Undoubtedly, the efforts to develop FD method would open the promised new perspective to the conservation of animal genetic resources.

Various reports have claimed that freeze-dried sperm which are not motile but have an intact DNA were able to fertilize oocytes. Katayose et al. (1992) firstly demonstrated that hamster and human sperm can form a pronucleus (PN) following microinjection into hamster eggs and prior storage in the dehydrated state for 12 months at 4°C. Offsprings after microinjection of freeze-dried sperm that had been stored at room temperature were produced in mice (Wakayama & Yanagimachi 1998, Kusakabe et al. 2001), rabbits (Yushchenko 1957; Liu et al. 2004), and rats (Hiraybayashi et al. 2005). These findings demonstrated that nuclear and cellular viability are not equivalent. Despite drastic physical alternations in sperm structures the nuclei of freeze-dried sperm seems to be cytogenetically

intact, because 92% of oocytes injected with a rabbit freeze-dried sperm had normal chromosome constituents when examined before the first cleavage (Liu et al. 2004). In pigs, it has been reported that ICSI-oocytes using freeze-dried sperm have the ability to develop to the blastocyst stage (Kwon et al. 2004) and day 39 of gestation period after transferring to recipients (Nakai et al. 2007). Next target would be the generation of normal offsprings from freeze-dried sperm in remaining species and those experiments are currently ongoing.

Freeze-dried sperm losses of motility since process of FD deeply damages sperm membranes including acrosome membrane (Gianaroli et al. 2012). However, the sperm acrosome contains a variety of hydrolytic enzymes; the release of these enzymes into the ooplasm might be harmful (Tesarik & Mendoza 1999). Based on the findings that the injection of an intact hamster spermatozoon into a mouse oocyte leads to degeneration of the ooplasm, whereas the injection of a demembraned hamster spermatozoon without acrosome forms two normal pronuclei; Kimura et al. (1998) suggested that there is species-specific tolerance of the ooplasm to exotic acrosomal contents. Similarly, in golden hamsters, Yamauchi et al. (2002) reported that all oocytes injected with acrosome-intact sperm heads died within 3 h after injection, while those oocytes injected with acrosome-free sperm heads survived. It also has been demonstrated that the rupture of the sperm plasma membrane prior to injection allows sperm decondensing factors in oocytes an easy access to the sperm nucleus following ICSI in humans (Dozortsev et al. 1995) and presence of an intact acrosome in ooplasm might hamper this process. In FD preservation, the loss of sperm acrosome is not a challenge because acrosomal damage has no effect to fertilization ability of freeze-dried sperm injected into ooplasm; in addition, the concern about negative effect of hydrolytic enzymes in acrosome is also eliminated. More interestingly, it is reported that ability of dead, immotile and motile sperm to decondense and to form male PN after ICSI was not significantly different (Wei & Fukui 1999). Provided that DNA integrity of the sperm nucleus is maintained, embryos could be generated by ICSI technique even using severely membrane damaged sperm that are no longer capable of normal physiological activity (Kusakabe et al. 2001).

Advantages of sperm FD can be further emphasized in case of bulls with high economic importance because of the sensitivity of sperm of these individuals to the process of cryopreservation (Hochi et al. 2011). Likewise, in species whose sperm are difficult to be preserved by freezing, FD will be a good replacement. However, the high initial cost and expensive equipments may be the disadvantages of FD preservation. Also, freeze-dried sperm are dead in conventional sense after dehydration and rehydration; therefore, they

cannot be used in artificial insemination or *in vitro* fertilization (IVF) programs (Hochi et al. 2011), ICSI is the only way to use freeze-dried sperm.

The research in the field of sperm FD was intermittent until ICSI using nonmotile sperm was proven efficiently in practice starting with mice (Kimura &Yanagimachi 1995). The successful production of live offsprings derived from ICSI with freeze-dried sperm has been reported in mice (Wakayama & Yanagimachi 1998; Kusakabe et al. 2001; Kaneko et al. 2003; Ward et al. 2003), in rats (Hirabayashi et al. 2005; Kaneko et al. 2007; Hochi et al. 2008), in hamsters (Muneto et al. 2011) and in rabbits (Liu et al. 2004). In other domestic species, only blastocysts have been obtained in cattle, (Keskintepe et al. 2002; Martins et al. 2007; Hara et al. 2011) and in pigs, development to the blastocyst stage (Kwon et al. 2004) and 39-day fetuses (Nakai et al. 2007) have been reported until now. In monkeys, the pronuclear-stage zygotes have just been obtained (Sanchez-Partida et al. 2008) (Table 1- that was modified and updated from Hochi et al. 2011). There is still a problem in producing live offspring from freeze-dried sperm in many other animals such as cattles, pigs, and monkeys. In a recent study, Gianaroli et al. (2012) attempted to freeze-dry human sperm and reported that sperm viability and motility were totally compromised after FD but sperm chromatin structure was not altered in comparison with fresh sperm. The author demonstrated that FD procedure did not affect DNA integrity of human sperm.

Similar to cryopreservation, in lyophilization, discovery and selection of lyoprotectants (protectants used for lyophilization) play a very important role for the success of this methodology. Studies of substances supplemented to FD media to reduce the DNA damage of freeze-dried sperm have been of special interest. Wakayama and Yanagimachi (1998) two conventionally used culture media (CZB; Chatot Ziomek Bavister and DMEM; Dulbecco modified Eagle medium) to FD solution and produced successfully live pubs (Wakayama & Yanagimachi 1998). On the other hand, Kusakabe et al. (2001) recommended the use of a solution composed from 10 mM Tris-HCl, 50 mM NaCl and 50 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for FD of mouse sperm. During the process of FD or freezing without cryoprotectant, damaged plasma membrane released endonucleases which are responsible for DNA fragmentation (Kusakabe et al. 2001; 2008) and this enzymes are activated by divalent cation such as Ca^{2+} and Mg^{2+} (Sotolongo et al. 2005). However, activation of the endonuclease will be inhibited by the addition of chelating agents such as EGTA to the FD buffer. The positive effect of EGTA continued to be demonstrated in other species including pigs (Nakai et al. 2007) and cattle (Martins et al. 2007).

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside, molecular formula: $C_{12}H_{22}O_{11}.2H_2O$) is a non-reducing disaccharide formed by the linkage of two glucose molecules. It shows mild sweetness (45% sucrose), high solubility, low cariogenicity, low hygroscopicity, and high glass transition temperature, good stability during processing and storage and excellent protein protection properties. It is more widely applicable to food technology and biotechnology (Schiraldi et al. 2002). It was first described in the early 19th century as a component of the ergot of rye, later discovered in a great variety of species include the so-called "resurrection plant" (*Selaginella lepidophylla*), certain brine shrimps, nematodes and baker's yeast showing the incredible property of surviving for years to dehydration (reviewed by Schiraldi et al. 2002).

In previous studies, trehalose has also proved effective in the cryopreservation of sperm (Eroglu et al. 2009; Hu et al. 2009; Kozdrovski 2009), oocytes (Eroglu et al. 2002), and stem cells (Gordon et al. 2001). The high glass transition temperature is one of the major advantages of this sugar compared with conventional cryoprotectants inculuding dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propanol (PROH). In addition, mammalian cell membranes are practically impermeable to sugars; thus, it has been used as an extracellular additive (Eroglu et al. 2009). This property helps cells to minimize intracellular ice formation during cryopreservation which is one of the main reasons for reduced quality of frozen-thawed sperm.

Besides, trehalose was also reported to have notable ability in stabilizing protein structure and bio-membrane in the dry state. Trehalose has multiple functions, and some of them are species-specific. The anhydrobitic organisms are able to tolerate the lack of water owing to their ability to synthesize large quantities of trehalose including plants, fungi, bacteria and invertebrate animals, and trehalose plays a key role in stabilizing membranes and other macromolecular assemblies under extreme environmental conditions (Higashiyama 2002). Moreover, in anhydrobiosis, every metabolic process is switched off, and then can be restored without any irreversible damage upon rehydration, thus, such organisms can survive for decades in the dry state. Nematodes, when dehydrated slowly, convert as much as 20% of their dry weight to trehalose, helping them survive dehydration (Crowe et al. 1992). The large amount accumulation of trehalose is restricted to some desiccation tolerant plants such as some ferns and the angiosperm *Myrothamnus flabellifolia* (Muller et al. 1995).

Previously, trehalose was considered as a storage molecule, aiding the release of glucose for carrying out cellular functions but the role of trehalose has expanded up to now

(reviewed by Jain & Joy 2008). It is synthesized as a stress-responsive factor and help in retaining cellular integrity when cells are exposed to environmental stresses like heat, cold, oxidation and desiccation.

However, studies in some anhydrobiotic organisms have indicated that the mechanism of desiccation tolerance involves not only sugars but also several osmolytes, non-enzymatic antioxidants and even protein (Crow et al. 2005). Understanding these mechanisms have many applications in cell biology because the elucidation of how anhydrobiotic organisms escape irreversible damage caused by dehydration can contribute to the understanding of the importance of water in maintaining the structural and functional integrity of membranes and fully hydrated cells (Crowe et al. 1987).

Inspired by these survival schemes in nature, many studies have been conducted on the protective effect of trehalose on different biomolecules, mainly proteins and membranes. Accumulating evidence suggests that trehalose is very effective for cells in preventing drought injury. It also may act as a free radical scavenger (Benaroudj 2001). Although, the exact mechanism mediates the protective effects of trehalose is not completely defined.

Trehalose, even in small quantities, inhibits vesicles fusion completely and depresses the phase transition temperature of dry lipids, maintaining them in the liquid crystalline phase in the absence of water (Crowe et al. 1992). Because of its high hydration potential, trehalose may stabilize biological membranes and proteins in the dry state by hydrogen bonds of its hydroxyl groups to the polar groups of proteins and phosphate groups of membranes (Kawai et al. 1992). Moreover, trehalose has the tendency to form a protective glass-like structure that has a low reactivity, making it more stable than other disaccharides due to its non-reducing characteristic. In this hygroscopic glass-like structure, trehalose is extremely stable both at high temperature and when completely desiccated and may hold biomolecules in a form that allows them to return to their native structure and function following rehydration (Crowe & Crowe 2000). In fact, very labile proteins such phosphofructokinase which completely inactivates after dehydration, restores its complete (100%) activity when rehydrated following a FD process in the presence of trehalose, but only small (13% of the original) activity is restored if trehalose is replaced by glucose (Carpenter et al. 1987).

Although trehalose has been used as a cryoprotectant for oocyte cryopreservation, but it is the most effective in stabilizing membranes in the dry state (Crowe et al. 1992). Remarkably, protective effect of trehalose is strongly enhanced when combined with late embryogenesis abundant (LEA) proteins – a protein first identified in land plant, originally discovered in the late stages of embryo development in plant seeds and their expression is

associated with desiccation tolerance in seeds and anhydrobiotic plants. LEA protein is expressed in eukaryotes, suggesting that mammalian cells may be protected by the same mechanisms although the path to lyophilization of larger organisms is not so straightforward (Hand et al. 2011).

1.3. Intracytoplasmic sperm injection as a unique method for freeze-dried sperm

ICSI is an advanced technique in which one single sperm is injected directly into an oocyte. Firstly, Uehara and Yanagimachi (1976) carried out successfully the microinjection of human and golden hamster sperm into hamster oocytes. Since then, ICSI has been used to generate live offspring of rabbits (Deng & Yang 2001; Hosoi et al. 1988), cattle (Goto et al. 1990), humans (Palermo et al. 1992), mice (Kimura & Yanagimachi 1995), sheep (Catt et al. 1996), horses (Cochran et al. 1998), cats (Pope et al. 1998), monkeys (Hewitson et al. 1999), pigs (*in vivo* matured oocytes, Martin 2000; *in vitro* matured oocyte, Nakai et al. 2003) and golden hamsters (Yamauchi et al. 2002). This technique has widely accepted in assisted reproductive technologies in human to overcome subfertility or infertility mainly caused by male factors. Heterologous ICSI could be a powerful tool to study human sperm functionality using pigs as the oocyte donor (Canovas et al. 2007). Interestingly, while direct sperm injection into human oocytes (ICSI) results in high fertilization, development and pregnancy rates, equivalent to those found with embryos produced by conventional IVF (Van Steirteghem et al. 1993); the outcomes of ICSI in domestic species are still low. On the other hand, ICSI has become the potential tool for the conservation of endangered species (Iritani 1991), for the propagation of livestock species with the high genetic merit and for the research of fertilization mechanisms.

There are two areas where ICSI has already had a direct impact (Catt & Rhodes 1995). One is direct propagation and the other is to test of X and Y sperm separation or sex sorted semen technique. First piglets were produced with the desired sex by intracytoplasmic injection of flowcytometrically sorted sperm (Probst & Rath 2003). In some cases, the semen can be unsuitable for artificial insemination or even IVF, but ICSI can be successfully used to generate embryos. For the sperm separation technique, sperm subjecting to procedures of flowcytometry could have low motility because of their susceptibility and low numbers because of technical restriction, ICSI can be used to overcome this limitation. Especially, immature sperm, sub-fertile sperm, epididymal sperm, testicular sperm and even only sperm head (Hamano et al. 1999; Nakai et al. 2003) can be used for ICSI (Anees 2008). Injection of a sperm head produced a blastocyst rate similar to injection of a whole

spermatozoon (Lee et al. 2004). This suggests that components of the sperm tail and mid-piece are not essential for fertilization and embryo development in the case of ICSI since isolated sperm heads contains a complete haploid set of chromosomes in their nucleus, the nucleus could fuse with female chromatin and develop into an embryo. Actually, healthy piglets were produced from ICSI of a sperm head in pigs (Nakai et al. 2003).

ICSI is a procedure that bypasses not only the female reproductive tract but also sperm capacitation, acrosome reaction, zona pellucida penetration, and membrane fusion between gametes (Danan et al. 1999). Each of natural steps involved in fertilization may play an important role in the physiological control of reproduction. In natural fertilization process, a sperm head is penetrated into cytoplasm via zona pellucida with the absence of a mid-piece and a tail. However, ICSI allows the injection of either whole sperm or sperm head only into ooplasm with the same outcome. It is reported that the injection of isolated sperm head can still activate an oocyte and only the sperm head is critical for egg activation and subsequent embryonic development at least in mice (Kuretake et al. 1996; Kimura et al. 1998). Although the sperm mid-piece with paternal mitochondria is introduced into the ooplasm by ICSI in case of whole sperm injection, the mitochondria of offsprings are inherited exclusively maternally (Danan et al. 1999), therefore, deficient mitochondria diseases from paternal origin will not be a troublesome when this technique is used.

ICSI and xenografting of gonadal tissue into immunodeficient experimental animals combined with optimal *in vitro* embryo production (IVP) system have been expected to be useful for the conservation of gametes (oocyte, sperm) from important genetic resources (Kikuchi et al. 2008). ICSI using fresh xenogeneic sperm has generated live offsprings for the first time in rabbits (Shinohara et al. 2002) and pigs (Nakai et al. 2010). Xenogeneic transplantation into immunodeficient mice may become a crucial approach for the preservation of fertility in prepubertal male oncology patients and for studying spermatogenic failure in infertile men (Shinohara et al. 2002). However, the fact that immediate testis transplantation is not always possible, thus, testicular tissues may need to be stored until offspring production (Kaneko et al. 2013). Based on the fact, this researcher group used xenogeneic sperm obtained from immature pig testicular tissue after cryopreservation to produce successfully live piglets (Kaneko et al. 2013).

Besides, sperm-mediated gene transfer through ICSI technique has been successfully utilized in mice, monkeys, cattles and pigs by binding exogenous DNA to sperm prior to the fertilization/injection (Perry 1999; Chan et al. 2000; Shemesh et al. 2000; García-Vázquez et al. 2010).

It is reported that a high incidence of polyspermic penetration and a low incidence of male pronuclear formation have been observed repeatedly in porcine oocytes matured and fertilized *in vitro* (reviewed by Niwa et al. 1993; Coy & Romar 2002); ICSI is one alternative way for *in vitro* production of monospermic zygotes to solve polyspermy problem in IVF system. In addition, cryopreserved oocytes were also reported evidences of polyspermy by the early extrusion of cortical granules (Vincent et al. 1990) and zona pellucida hardening through premature cortical-granule release (Schalkoff et al. 1989) as a result, cryopreservation decreased fertilization ability by IVF. In these cases, ICSI would be a reasonable alternative. Last but not the least; if the sperm have lost their motility, ICSI is indispensable for fertilization to produce the next generation (Kikuchi et al. 2008). It is the unique method available to use immobile sperm such as round spermatid (Ogura et al. 1994; Hirabayashi et al. 2002) and freeze-dried sperm (Wakayama & Yanagimachi 1998).

1.4. Improvements for *in vitro* production of porcine embryos

Collection of oocytes or embryos from donor animals via surgery is time consuming and expensive, and numbers are limited. Alternatively, the efficient utilization of ovaries from slaughterhouse animals to generate mature oocytes and embryos via *in vitro* techniques is very important (Abeydeera 2002). Ovaries from slaughtered animals are the cheapest and the most abundant source of primary oocytes for large-scale production of embryos by IVP system. *In vitro* embryonic development depends on many factors: culture media, culture condition, physical manipulation, semen quality, oocyte quality. In general, the studies toward the improvements in IVP system have been designed based on these factors. In this study, I will focus on sperm factors and oocyte factors, treatments that have been attempted for sperm and oocytes in order to improve fertilization in particular and enhance the efficacy of IVP system in general.

In vitro developmental competence of porcine *in vitro* matured and fertilized oocytes to the blastocyst stage and the birth of live piglets were first confirmed and reported (Mattioli et al. 1989; Yoshida et al. 1993b; Kikuchi et al. 2002a). These achievements paved the way for further research in order to improve intensively IVP of porcine embryos, serve for basic and applied research. However, IVP system in pigs is still poor compared to *in vivo* counterpart (Kikuchi et al. 1999) as well as comparing to *in vitro* development of other species such as cattle or mice. This slows down the progress of other reproductive techniques such as embryo transfer (ET) and establishment of embryonic stem cells because these techniques depend on the blastocyst yield and quality as the material source.

Indeed, various modifications to IVP system have been attempted with significant findings, starting from *in vitro* maturation (IVM). IVM of oocyte is a critical component of IVP system. The immature oocytes collected from ovaries at slaughterhouse are subject to *in vitro* maturation from the germinal versicle (GV) stage to the metaphase-II (M-II) stage and ready for fertilization and subsequent development. The oocyte quality directly influences on the outcome of IVP. Many studies have been conducted to enhance oocyte quality after IVM as well as to improve the effectiveness of IVM. For example, in order to increase maturation rate and developmental ability, selection of oocytes based on morphological criteria, the modification of culture media, the supplementation of hormones (LH, FSH, PMSG, hCG and so on) or some substances (cysteine, cysteamine, vitamins, porcine follicular fluid (pFF), serum and so on) into IVM media has been investigated intensively in the literature until now. Nuclear maturation of oocyte along with cytoplasmic maturation is important for the completion of meiotic division for the success of fertilization. Nuclear maturation can be determined visually by the extrusion of the first polar body (PB). However, nuclear maturation is not a prerequisite determinant of oocyte developmental potential. Inadequate cytoplasmic maturation of *in vitro* matured oocytes and/or suboptimal embryo culture condition may be responsible for poor embryo quality (Abeydeera 2002). Unfortunately, indicators for cytoplasmic maturation are unable to be accessed by vision. For examples, oocyte glutathione content is considered as an indicator of cytoplasmic maturation of oocytes (Funahashi et al. 1994) but not visually inspected. The oocytes with full cytoplasmic maturity can only be determined after fertilization by the presence of a male PN, a female PN and two PBs. In the efforts to improve the cytoplasmic maturation of *in vitro* matured oocytes, various meiotic inhibitors (roscovitine-Coy et al. 2005; butyrolactone I and cycloheximide-Marques et al. 2007) have been used for porcine oocytes which maintain the oocyte at the germinal vesicle stage, trying to mimic the *in vivo* conditions of the follicles, and increase the cytoplasmic maturation period.

Recently, assessment of oocyte quality and maturity based on molecular markers has become more important. It is reported that genes involved in metabolism such as nucleotide, carbonhydrate and protein metabolism were reported to be decreased in prepubertal female derived porcine oocytes (ovaries without corpora lutea) compared with the oocytes derived from cyclic females (Paczkowski et al. 2011). The importance of metabolism in oocyte maturation and the relationship between metabolic rates and developmental competence were well-documented in previous studies (Krisher & Bavister 1999; Preis et al. 2005). Therefore, compromised developmental competence of *in vitro* vs. *in vivo* mature oocytes

and prepubertal vs. cyclic derived oocytes is attributed to the non-fulfillment of metabolic requirement for fertilization and embryo development. The next key objective toward achieving more efficient IVM will be to establish the molecular determinants/markers of oocyte quality (Lee et al. 2008).

Regarding to sperm factors, in fact, differences in sperm treatments prior to ICSI may result in different amount of damages to sperm membrane and thereby affect the calcium oscillation, oocyte activation and PN formation after ICSI (Yanagida et al. 1997; Morozumi et al. 2006). In order to improve PN formation following ICSI of porcine and bovine oocytes, various pretreatments have been attempted, including immobilizing sperm and damaging the sperm membrane by repeated freezing and thawing without cryoprotectants (Kolbe & Holtz 1999; Katayama et al. 2002b; Tian et al. 2006), treatment with Triton-X 100 (TX), for removing sperm plasma and acrosomal membrane (Lee & Yang 2004; Tian et al. 2006), using dithiothreitol (DTT) to reduce disulfide bonds (Rho et al. 1998; Suttner et al. 2000) and induce *in vitro* decondensation of the sperm nuclei (Katayose 1992; Nakai et al. 2006). Besides, pretreating sperm with calcium ionophore (Nakai et al. 2003) or with progesterone (Katayama et al. 2002b) to have acrosome-reacted sperm for the injection also have been applied to improve the efficiency of ICSI in pigs. In cattle, efficiency of ICSI can be improved by sperm pretreatment with DTT and by oocyte activation with ionomycin plus 6-dimethylaminopurine (6-DMAP), although the developmental capacity of the resulting embryos remains limited (Rho et al. 1998). However, according to results of Nakai et al. (2006), pretreatment of boar sperm with TX and DTT shifted up the timing of sperm nuclear decondensation but did not improve the development to the pronuclear and blastocyst stage *in vitro.*

After sperm penetration, the sperm undergoes chromatin decondensation, nuclear enlarging and pronuclear formation. Remodeling of sperm nuclei requires the reduction of disulfide bonds (S–S) regulated by ooplasmic glutathione (Perreault et al. 1987; 1988, Yoshida et al. 1993a) and replacement of protamines by histones, a necessary change to render the sperm nucleus transcriptionally active (Yanagimachi 1994) to form successfully a male PN. Concomitantly, the maternal genome is also modified and prepared for integration with the paternal genome.

Regarding to oocyte factors, previous studies suggested that induction of oocyte activation was one of the most important factors for male PN formation in ICSI-oocytes (Nakai et al. 2006; Lee et al. 2003). In mice (Kimura & Yanagimachi 1995, Kuretake et al. 1996), hamsters (Hoshi et al. 1992), human (Tesarik & Sousa 1995) and rabbits (Keefer

1989), ICSI alone is sufficient to activate oocytes for embryonic development. However, in pigs, artificial oocyte activation is considered essential for successful ICSI (Lee et al. 2003; Nakai et al. 2006). Numerous procedures have been developed to artificially activate oocytes, mimicking the pattern of calcium oscillation after sperm penetration in physiological fertilization process directly or indirectly. These procedures consist of mechanical (Macháty et al. 1996), chemical (Ca^{2+} ionophore, Kline & Kline 1992; Nakai et al. 2003; Ito et al. 2004, ionomycin, Cibelli et al, 1998, ethanol, Presicce & Yang 1994, strontium chloride, Wakayama et al. 1998) and electrical stimuli, in which activation by electric pulse after sperm injection has been considered as an indispensible step in IVP system in some laboratories (pig, Nakai et al. 2003; 2006; 2007, 2010; Lee et al. 2003; rabbit, Zhou et al. 2013; cattle, Hwang et al. 2000). However, there are still inconsistent data about effect of electric stimulation on efficacy of oocyte activation, fertilization and subsequent development. Kim et al. (1999) showed that electrical stimulation following sperm injection did not enhance the incidence of male PN formation or pronuclear apposition compared with sperm injection alone. In contrast, the improved fertilization rate (Lai et al. 2001; Lee et al. 2003) and blastocyst formation rate (Lai et al. 2001; Lee et al. 2003; Nakai et al. 2006) were reported *in vitro* matured porcine oocytes subject to electric stimulus after intracytoplasmic injection of frozen-thawed sperm.

Porcine oocytes are characterized by a dark, granulated ooplasm due to its high lipid content and thus, considered more difficult to manipulate than other species such as human, mice. Oocyte/embryo centrifugation facilitates the observation of sperm release from pipette into ooplasm in ICSI method (in bovine, Rho et al. 1998; in pig, Lai et al. 2001) or the visibility of nuclear elements such as GVs, metaphase spindle or PNs or other internal organelles in other micromanipulation techniques in pigs (Wall et al. 1985; Yong et al. 2005) and in bovine (Wall et al. 1988; Tatham et al. 1996). It was reported that the centrifugation of bovine, porcine and murine zygotes does not affect the subsequent development (Wall et al. 1985; Chung et al. 2001). It has been recognized to have potential applications in microinjection of DNA into zygotes in rabbits, sheep, mice and pigs (Hammer et al. 1985; Brinster et al. 1986).

1.5. Activation mechanisms of expression of DNA repair genes in oocytes

DNA integrity of a cell is threatened from three sides (Hoeijmakers 2009). Firstly, spontaneous reactions (mostly hydrolysis) intrinsic to the chemical nature of DNA in anaqueous solution create abasic sites and cause deamination (Lindahl et al. 1993). Secondly,

metabolism generates reactive oxygen and nitrogen species, lipid peroxidation products, endogenous alkylating agents, estrogen and cholesterol metabolites, and reactive carbonyl species (De Bont & van Larebeke, 2004), all of which damage DNA. Thirdly, DNA is damaged by exogenous physical and chemical agents, but this damage to some extent is avoidable. DNA damage includes single stranded breaks (SSBs) and double stranded breaks (DSBs).

DSBs arise from endogenous processes including reactive oxygen species generated during cellular metabolism, collapsed replication forks, and nucleases; and from exogenous agents including ionizing radiation and chemicals that directly or indirectly damage DNA, and can be repaired by non-homologous end-joining (NHEJ) and homologous recombination (HR), and defects in these pathways cause genome instability and promote tumorigenesis (Shrivastav et al. 2008). NHEJ and HR repair various types of double strand breaks. NHEJ simply brings two ends together, but bases may be lost or added. This inaccurate process takes place mostly before replication, in the absence of an identical copy of DNA. After replication, HR, acting through a series of complex DNA transactions, uses the identical sister chromatid to properly align the broken ends and unerringly insert missing information (Shrivastav et al. 2008).

DNA repair activities are extremely important because unrepaired DNA damage has the risks to be mutagenic, cytotoxic and carcinogenic or lead to apoptosis, necrosis or other forms of cell death. The components of the DNA repair system act in three levels: (1) arresting the cell cycle to allow time for DNA repair; (2) triggering the signal transduction events to activate the repair components; and (3) directly reversing, excising or tolerating DNA damage via constitutive and induced activities (Begley & Samson 2004).

Oocyte quality is strongly associated with advanced reproductive age. Some studies have indicated differential gene expressions between younger and older oocytes in human (Steuerwald et al. 2007; Grondahl et al. 2010) and mouse (Hamatani et al. 2004b; Pan et al. 2008). Age affects the expression of genes responsible for cell cycle regulation, cytoskeletal structure, energy pathways, transcription control, and stress responses (Steuerwald et al. 2007) in human oocytes. Compromised DNA repair has also associated with accelerated oocyte aging. The G1 phase of the first cell cycle also represents the first opportunity for the repair of DNA lesions. The early steps in preimplantation development including maturation, fertilization, and onset of first cleavage, activation of the embryonic genome, compaction, and blastocyst formation can be affected by the culture media and conditions. Microarray technologies were efficiently applied to humans and to mice to identify the differences in

developmental ability between *in vitro* versus *in vivo* matured oocytes (Jones et al. 2008; Pan et al. 2005).

DNA repair gene expression was investigated in human oocytes and blastocysts to identify the pathways involved at these stages and to detect potential differences in DNA repair mechanisms of pre- and postembryonic genome activation (EGA) (Jaroudi et al. 2009). Large numbers of repair genes were detected indicating that all DNA repair pathways are potentially functional in human oocytes and blastocysts. Expression levels of DNA repair genes at the pre- and post-EGA transcriptional level suggest differences in DNA repair mechanisms at these developmental stages (Jaroudi et al. 2009). However, the elucidation of complex mechanisms of DNA repair system remains a challenge (Begley & Samson 2004).

Problem description

A progressive loss of biodiversity is occurring at an unprecedented pace (Loi et al. 2013). Not only wildlife, even domestic animals are disappearing and being replaced by a smaller number of more productive animals. For this reason, the establishment, regulation, and management of biological resources from natural habitats for *ex situ* conservation purposes are emergent demands (Loi et al. 2013). Dry storage would greatly simplify the establishment and management of biobanks; minimize the environmental costs arising from the production and use of LN and other activities for maintenance of facilities of cryopreservation system, especially in developing countries. Stored genomes may regenerate future generations of the endangered or extinct animals, or may be used to expand animal populations through somatic cell nuclear transfer (SCNT).

It was demonstrated that sperm containing damaged DNA are able to penetrate the oocyte and fertilize it but the DNA fragmentation in sperm distinctly negatively influences the results of assisted reproduction (Henkel et al. 2004). Similarly, there was a negative correlation between the percentage of sperm with fragmented DNA and fertilization rates in IVF (Sun et al. 1997) and ICSI (Lopes et al. 1998; Sakkas et al. 1996). DNA damage leads to the failure of fertilization if sperm containing fragmented DNA are selected for injection (Lopes et al. 1998). For freeze-dried sperm, fragmentation of DNA decreased the developmental ability of the sperm-injected oocytes (Nakai et al. 2007). Defects in the structure of chromatin can severely reduce fertility and early embryo development, increase spontaneous abortions as well as birth defects (Evenson et al. 1980; Cordelli et al. 2005). The maintenance of DNA integrity during FD procedures and storage, thus, is extremely important.

Although embryo development up to the blastocyst stage is possible by *in vitro* culture, the ultimate test of embryonic viability is to establish pregnancies and live births following transfer into recipients. To date, the ability of porcine oocytes fertilized by ICSI with freeze-dried sperm to be implanted, sustain a pregnancy and develop into offsprings has not been demonstrated in the literature although the offsprings of laboratory animals such as mouse, rabbit and rat have been generated successfully quite long time ago (mouse, Wakayama & Yanagimachi 1998; rabbit, Liu et al. 2004; rat, Hirabayashi et al. 2005).

Major objectives of the study

Sperm DNA is the most important molecule to ensure normal fertilization and subsequent development. FD preservation without LN is one of the great benefits for gene-bank establishment. Minimizing sperm DNA damage during FD procedure is extremely important for any purposes of sperm usage.

This thesis focused on how to improve the DNA integrity of freeze-dried boar sperm in Chapter II; then, hypothesis testing about the relationship between DNA damage in freeze-dried sperm and the expression of DNA repair genes in oocytes injected with those sperm was conducted in experiments of Chapter III. The effect of some treatments for sperm and oocyte to improve the efficacy of *in vitro* porcine embryonic production following ICSI using freeze-dried sperm were investigated in Chapters IV and V. And finally, we have tried to produce live piglets from freeze-dried sperm by embryo transfer into recipients in Chapter VI. The general aims of this research were to expand basic knowledge of FD associated sperm DNA damage in pigs and to improve the efficacy of FD preservation.

Table 1. Achievements in freeze-drying preservation of mammalian sperm

Species	Highest achievements	References	Published year
Mouse	Live offspring	Wakayama & Yanagimachi	1998
			2001
		Kusakube et al.	2003
		Kaneko et al.	2003
		Ward et al.	
Rabbit	Live offspring	Liu et al.	2004
Rat	Live offspring	Hirabayashi et al.	2005
		Kaneko et al.	2007
		Hochi et al.	2008
	(heat-dried sperm)	Lee et al.	2013
Hamster	Live offspring	Muneto et al.	2011
Horse	Live offspring	Choi et al.	2011
Cattle	Blastocyst	Keskintepe et al.	2002
		Martins et al.	2007
		Hara et al.	2011
Pig	Blastocyst and pregnancy	Kwon et al.	2004
		Nakai et al.	2007
Monkey	Pronuclear zygote	Sanchez-Partida et al.	2008

Chapter II

Effect of trehalose on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection

Introduction

Cryopreserved boar sperm have been commercially available since 1975 (Johnson & Larsson, 1985). The ultimate goal of semen preservation is to achieve, using artificial insemination, a pregnancy rate equivalent to that after natural mating. Although cryopreservation of sperm has approached to achieve this goal, certain disadvantages with this approach still exist, for example, the need for a constant replacement supply of liquid nitrogen (LN) for storage, the expense of long-term storage using LN, difficulties with international transportation, and the effects of unexpected disasters. Therefore, cheaper and safer solutions for long-term storage of sperm have been sought (Wakayama & Yanagimachi 1998), among which preservation by FD without LN has been a focus of attention. It is believed that sometime in the future it will be possible to store freeze-dried sperm indefinitely at ambient temperature and to ship it all over the world without the need for dry ice or LN (Kusakabe et al. 2008). If the DNA integrity of the sperm nucleus could be maintained, embryos could be generated by ICSI technique using severely damaged sperm that are no longer capable of normal physiological activity (Kusakabe et al. 2001).

Interestingly, damage to the sperm plasma membrane appears to facilitate oocyte activation rather than the case for membrane-intact sperm (Morozumi et al. 2006), especially in species such as cattle and pigs, their sperm have stable membranes. However, Nakai et al. (2006) have reported in pigs that development to the pronuclear and blastocyst stages after ICSI *in vitro* was not improved even by pretreatment of sperm with 1% Triton X-100 and 5mM dithiothreitol to remove the plasma membrane and promote decondensation of sperm chromatin. More importantly, even if the chromatin organization in somatic cells is extremely vulnerable to combined osmotic/dehydration stress during the FD process (Loi et al. 2008), sperm cells in which the chromatin is extremely condensed due to cross-linked disulfide bonds of protamines may be much more resistant to potentially damaging agents. Because of this prevailing property, efforts to preserve sperm by FD may hold promise for conservation of animal genetic resources. A combination of FD and ICSI might facilitate such an approach, especially for precious and endangered breeds.

Although ICSI in human oocytes results in high rates of successful fertilization, yielding a pregnancy rate equivalent to that of conventional IVF (Van Steirteghem et al. 1993), the outcome of ICSI in domestic species is still low. Successful production of viable piglets after ICSI using *in vivo* matured (Kolbe & Holtz 2000; Martin 2000) and *in vitro* matured oocytes (Nakai et al. 2003) has been reported. However, when freeze-dried boar sperm is used, the outcome of ICSI is still poor in terms of fertilization and blastocyst formation (Kwon et al. 2004). Generation of offspring using freeze-dried sperm has been achieved in experimental animals (mouse, Wakayama & Yanagimachi 1998; rabbit, Liu et al. 2004; rat, Hirabayashi et al. 2005) but not in large domestic animals. So far, no healthy piglets, only 39 days fetuses of gestation, have been produced after transfer to recipients (Nakai et al. 2007). The reasons(s) for this poor developmental competence is unclear; however, during the FD procedure, DNA in the sperm head might become further damaged by both freezing and drying stresses in comparison with cryopreserved sperm after conventional freezing. Therefore, it is important to identify the strategies that would reduce damage to sperm DNA.

There have been several attempts to reduce DNA damage during FD procedures. Sperm endonucleases are released from plasma membrane-damaged sperm during FD or freezing without a cryoprotectant and these can be activated by divalent cations such as Ca^{2+} and Mg^{2+} (Sotolongo et al. 2005). Studies using mice (Kusakabe et al. 2001) and pigs (Nakai et al. 2007) have reported that when ethylene glycol-bis (2-aminoethylether) -N,N,N',N'-tetraacetic acid (EGTA), as a typical chelating agent, is added to FD medium, Ca^{2+} is chelated, thus protecting sperm DNA from degradation. Trehalose is a non-reducing disaccharide exhibiting a high glass transition temperature and stability during processing and storage and is capable of stabilizing and protecting membranes and proteins under extreme environmental conditions, allowing anhydrobiotic organisms to survive cycles of dehydration-rehydration (reviewed by Jain & Roy 2008). The natural process by which trehalose helps anhydrobiotic organisms to survive dehydration (Crowe et al. 1992) has attracted a lot of interest with regard to its potential role in protecting biomolecules, including sperm DNA. Application of this reagent to the FD procedure is also expected; so far, however, there have been no detailed reports about the effects of EGTA combined with trehalose in FD medium on sperm DNA integrity, IVF, and subsequent development of ICSI-oocytes in pigs.

The objective of this Chapter was to clarify whether trehalose would improve the DNA integrity of freeze-dried boar sperm in combination with EGTA and also to assess its

effect on IVP following ICSI using the freeze-dried sperm. The integrity of DNA in a male PN in zygotes was also examined by immunostaining of histone H2A.X phosphorylated at serine 139 (γH2A.X), which is a marker of DNA double-strand breaks (DSBs) responsible for serious damages affecting fertilization and subsequent development (Enciso et al. 2009).

Materials and Methods

Chemicals and media

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise stated. The FD medium contained 10 mM Tris-HCl buffer supplemented with 50 mM EGTA (346-01312; Dojindo Laboratories, Kumamoto, Japan) and is referred to as basic FD medium hereafter. This medium was then supplemented with different concentrations of trehalose (T0167; 0 as the control, 3.75, 7.5, 15, 30, 60 and 90 mM) and also NaCl (50, 47.5, 45, 40, 33.3, 16.7, and 0 mM, respectively). The pH and osmolality of the final solutions were adjusted to 8.0 to 8.5 and 265 to 270 mOsm/kg, respectively, and then they were filtered and stored at 4 °C. These media were prepared from stock solutions of 1.0 M Tris-HCl, 250 mM EGTA, 1.0 M trehalose and 1.0 M NaCl.

The maturation medium was modified North Carolina State University (NCSU)-37 solution containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 mM β-mercaptoethanol, 1 mM dibutyl cAMP, 10 IU/mL eCG (Serotropin; ASKA Pharmaceutical Co. Ltd., Tokyo, Japan) and 10 IU/mL hCG (Puberogen 500 units, Novartis Animal Health, Tokyo, Japan) (Kikuchi et al. 2002a). Two media were used for IVC of sperm-injected oocytes: the first one was modified NCSU-37 supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/mL bovine serum albumin (BSA), 50 mM β-mercaptoethanol (IVC-PyrLac), and the second one was modified NCSU-37 supplemented with 5.55 mM glucose (IVC-Glu) (Kikuchi et al. 2002a). The osmolality of these media was adjusted to 285 mOsm/kg.

Sperm collection and FD

Ejaculated semen was collected from a Landrace boar and transferred to the laboratory within 1 h. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × *g* at 30°C and the seminal plasma was removed by aspiration of the supernatant. The pellet were resuspended in FD medium containing different concentrations of trehalose prewarmed to 30°C, and the supernatant was removed after

centrifugation. The final pellet was resuspended again in FD media containing different concentrations of trehalose (0, 3.75, 7.5, 15, 30, 60, and 90 mM) to obtain a final concentration of 4×10^8 cells/mL (control, 3.75, 7.5, 15, 30, 60, and 90 mM groups, respectively). One milliliter of sperm suspension from each group was placed into an individual glass vial (5-mL glass vial, Maruemu Corporation, Tokyo, Japan). The vials were covered with aluminum foil and placed in a refrigerator at −80°C for at least 4 h. The aluminum foil was removed, and a rubber cap (Maruemu Corporation, Tokyo, Japan) was placed on the vial, enabling passage of the air through small gaps between the cap and vial containing the specimens, and then they were placed in an FD system (FTS systems DuraDry μP, SP Scientific, Warminster, PA, USA). The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at −30°C and then increased to 30°C during the last 1 h 20 min (0.75°C/min). After flushing with inactive N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps (Maruemu Corporation, Tokyo, Japan) using a crimping tool. The freeze-dried samples were transferred to a refrigerator at 4°C and stored under dark conditions until the time of experiments.

Rehydration of freeze-dried spermatozoa

For rehydration, the same volume (1 mL) of deionization distilled water was added to vials immediately after opening the rubber and aluminum caps using a decapper. The sperm suspension was centrifuged for 2 min at 600 × *g* and the supernatant was removed. The sperm pellet was resuspended and diluted in PBS (−) to a final concentration of 3×10^6 cells/mL. DNA damage was detected in all groups using a Halomax kit (Sperm-Halomax for analysis in *Sus scrofa* sperm, Halotech DNA SL, Tres Cantos, Madrid, Spain) modified from the sperm chromatin dispersion test (Fernández et al. 2005).

Halomax kit for detecting sperm DNA fragmentation

Firstly, the lysis solution was allowed to reach room temperature (22°C). Then, a plastic tube containing agarose (supplied in a kit) was placed in a water bath at 90°C to 100°C for 5 min or until the agarose had fully melted, and then equilibrated in a water bath at 37°C for 5 min. Meanwhile, 25 μL of each diluted sperm sample was added to an empty tube, and 50 μL of liquefied agarose was then added followed by gentle mixing, and the temperature of the tubes was maintained at 37°C. Then, 2 μL of the mix was placed in drop form onto marked wells and each drop was covered with an 18 × 18 mm glass coverslip. The

slides were held in a horizontal position throughout the entire process. The slides were placed on a cold surface precooled at 4°C in a refrigerator for 5 min to solidify the agarose and to produce a thin microgel. The coverslips were gently removed and the slides were fully immersed horizontally in lysis solution for 5 min to remove the sperm membrane and partially deproteinize the nuclei. After washing for 5 min in a tray containing an excess of distilled water, the slides were dehydrated in increasing concentrations of ethanol (70% and 100% for 2 min each), air-dried, and immediately observed or stored at room temperature in tightly closed dark boxes. The slides were stained using a mixture of 5 μg/mL Hoechst 33342 and DABCO antifade mounting medium (1:1) just before observation by fluorescence microscopy. Additionally, all sperm images in each group were captured on the same day, under the same conditions, using a CCD camera (CoolSNAP CF; Roper Scientific, USA) connected to RS Image as the operating software (Roper Scientific). Freshly ejaculated sperm without FD was used as the control. These images were converted to measurable format using Photoshop (version 5.0, Adobe Systems Incorporated, San Jose, CA, USA), and then ImageJ (version 1.41, National Institutes of Health, Bethesda, MD, USA) was used to measure the area of both the DNA dispersion halo and the sperm head (not including the tail), referred to as the "DNA dispersion halo" hereafter. Only a single spermatozoon was measured, and overlapped ones were avoided. Sperm located close to a dark background were excluded. The area of the DNA dispersion halo was measured in terms of pixels.

Oocyte collection and IVM

Oocyte collection and IVM were conducted as described (Kikuchi et al. 2002a). Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COCs) were collected from follicles 2-6 mm in diameter in Medium 199 (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 20 mM Hepes (Dojindo), 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 μL of maturation medium for 20-22 h in four-well dishes (Nunclon Multidishes; Kamstrupvej, Roskilde, Denmark). The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39°C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, the cumulus cells were removed from the oocytes by repeated

pipetting in Medium-199 in air supplemented with 150 IU/mL hyaluronidase. Denuded oocytes with the first polar body (PB) were harvested under a stereomicroscope and used as IVM oocytes.

ICSI and oocyte stimulation

Freeze-dried sperm were rehydrated with 1 mL deionization water and centrifugation for 2 min at 600 × *g* and then the supernatant was removed. The sperm pellet was resuspended in PBS (−) with 5 mg/mL BSA and kept at room temperature for no longer than 2 h during ICSI, which was carried out as described (Nakai et al. 2003; Nakai et al. 2006; Nakai et al. 2010) with minor modifications. Briefly, the basic medium for sperm treatment was IVC-PyrLac supplemented with 20 mM Hepes and 4% (w/v) polyvinylpyrrolidone (MW 360,000) (IVC-PyrLac-Hepes-PVP). About 20 IVM oocytes were transferred to a 20-μL drop of Medium 199 (with Hanks' salts). The solution containing the oocytes was placed on the cover of a plastic dish (Falcon 35-1005; Becton Dickinson and Company, Franklin Lakes, NJ, USA). A small volume (0.5 μL) of the sperm suspension was transferred to a 2-μL drop of IVC-PyrLac-Hepes-PVP, which was prepared close to the drops containing the oocytes. All drops were covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Inc., Kyoto, Japan). A single sperm in the suspension was aspirated from its tail into the injection pipette, and the pipette was moved to the drop containing the oocyte. The sperm was injected into the ooplasm using a piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd., Tsuchiura, Japan). This process was repeated until all the oocytes in the droplet had been injected. ICSI was completed within 2 h after rehydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. As control groups, IVM oocytes were injected with a small amount of injection solution without sperm (sham-injection group) or injected with ejaculated sperm (fresh sperm group). One hour after the injection, the oocytes in all groups were separately transferred to an activation solution consisting of 0.28 M *d*-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.1 mg/mL BSA. Once the oocytes were sunk down on the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs under the same condition using a somatic hybridizer (SSH-10; Shimadzu, Kyoto, Japan), then washed three times and cultured in IVC-PyrLac.

IVC of sperm-injected oocytes

Sperm-injected oocytes after electrical stimulation were cultured *in vitro* in IVC-PyrLac for 48 h and then transferred to IVC-Glu for subsequent culture for 144 h (6 days) at 38.5°C under 5% O_2.

DNase I treatment

Sperm treated with DNase I to induce purposely the DNA damage was used as a control group according to Villani et al. (2010) with some modification. Briefly, freeze-dried sperm was rehydrated and centrifuged to remove supernatant. Sperm pellet was resuspended for 2 min in a permeabilizing solution consisting 0.1% sodium citrate and 0.1% Triton X-100 and digested for 60 min at room temperature with 1000 IU/mL of DNase I (2000 IU/mg, Roche Diagnostics) in PBS with 5 mM $MgCl_2$. To stop the activation of DNase I, 50 mM EDTA was added to the sperm suspension and incubated for 2 min. The mixture was then centrifuged and resuspended in PBS-BSA twice to remove supernatant before injection to the matured oocytes.

Immunofluorescence staining

Freeze-dried sperm-injected oocytes were stained for γH2A.X to assess the DSBs level among groups. Freeze-dried sperm treated with DNase I was used as positive control. The immunostaining were carried out mainly according to the procedures of Somfai et al. (2011) with some modification. Briefly, pronuclear stage oocytes at 10 h after injection were fixed in 4% paraformaldehyde in PBS for at least 60 min, washed twice in PBS with 0.2% Triton X-100, and incubated in permeabilizing blocking solution PBS (−) with 5 mg/mL BSA and 0.2% Triton X-100 overnight at 4°C. They were then washed twice with the medium and incubated with the primary antibody, anti-phospho-histone H2A.X (Ser 139) raised in mice (1: 250 dilution, Clone JBW301, Millipore) at 37°C for 2 h. After the embryos were washed twice in PBS with 0.2% Triton X-100, they were incubated with Alexa-Flour-488-conjugated anti-mouse secondary antibody (1: 200 dilution, Molecule Probe) at 37°C for 1 h in dark. For negative control group, some oocytes were stained with secondary antibody without primary antibody incubation. The embryos were briefly washed twice and stained with Hoechst 33342 and DABCO (1:1) for several minutes and mounted on glass slides and observed under fluorescent microscope.

Fertilization assessment

Some oocytes were taken out from the culture medium at 10 h after injection and fixed in fixative solution with a 1:3 mixture of acetic acid: ethanol (v/v) under vaseline-supported coverslips for several days. The oocytes were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for several minutes, and then their fertilization status was examined using phase-contrast microscopy.

Assessment of embryonic development

The injected oocytes cultured for 6 days were mounted on glass slides, fixed, and stained using the same procedures as described above. The proportion of oocytes developing to blastocysts and the mean number of cells in each blastocyst were counted in all groups. In this study, only embryos with a clear blastocoel were considered to be blastocysts.

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989), then subjected to ANOVA using the general linear model procedure and analyzed by Tukey's multiple range test (version 9.3; Statistical Analysis System Institute, Cary, NC, USA). Differences were considered significant at $P<0.05$.

Results

DNA damage in freeze-dried boar sperm

After rehydration, stereomicroscopic observations revealed that there were no differences in morphological change of the sperm among all groups (Fig. 1). The sperm tail was not separated from the sperm head after FD procedure and rehydration. Use of the Halomax kit yielded clear figures reflecting the DNA fragmentation in each sperm head (Fig. 2). The bigger size of halos indicates the more accelerated level of DNA fragmentation (arrow in Fig. 2b and 2e). The measurements of halo area (pixels) reflecting freeze-dried sperm fragmentation in different concentrations of trehalose are summarized in Fig. 3. When trehalose was added to FD medium at different concentrations, the results indicated that the protective effect of trehalose against DNA damage was dependent on its concentration. The DNA damage levels in the 7.5 and 15 mM groups were significantly lower than those in the control (0 mM) group ($P<0.05$). However, the halos were increased in size after treatment with higher concentrations (30, 60, and 90 mM), and were not different from that in the control group. DNA damage level of fresh sperm group was significantly lower than those of

the freeze-dried sperm groups (P<0.05). Within the trehalose concentrations tested, the sperm DNA fragmentation level was lowest in the 15 mM group; however, the level was not different from that in the 7.5 mM group. Therefore, freeze-dried sperm in these groups and also control group were used for injection into IVM oocytes for examination of *in vitro* subsequent developmental competence in Experiment 2.

Pronuclear label of zygotes with γH2A.X

At the pronuclear stage, the normality of zygotic PNs was examined using immunostaining with marker of DSBs, γH2A.X. Embryos containing DSBs at different levels showed the various γH2A.X expressions (Fig. 4 and 4'). The signal of DSBs in a male PN was very strongly expressed in positive control group in which DNase I-treated sperm were injected to the oocytes; however, the signal from female PN was not detected (Fig. 4'c'). There was no difference in the expression level of γH2A.X in a male PN at 10 h after injection between 0 and 15 mM trehalose groups (Fig. 4a' and 4b'). Moreover, when two PNs have not entered fusion stage yet, as shown in Figure 4a' (0 mM trehalose) and 4b' (15 mM trehalose), a female PN was localized close to a PB, and we found that the signals seemed to be similar in both male and female PNs (both PNs show an equal distribution and intense immunostaining against γH2A.X).

Fertilization status of IVM-oocytes following ICSI

The oocytes with two PBs and two PNs (defined as male and female PN) after injection and IVC for 10 h were considered to have undergone normal fertilization (Fig. 5a, 5b, and 5c). As given in Table 2, the fertilization rate of fresh sperm-injected group did not differ from those of freeze-dried sperm-injected groups, except for 0 mM group. Moreover, there were no significant differences in term of fertilization rate among 0, 7.5, and 15 mM groups. A smaller percentage of oocytes with two PBs and two PNs was also observed in the sham-injection group. The rate was significantly lower than those in the groups injected with freeze-dried sperm (8.1% vs. 45.9% for 0 mM group, 52.9% for 15 mM group, and 60.8% for 7.5 mM group, P<0.05). On the other hand, the percentage of oocytes with two PBs and one PN, in other words, oocytes that had been activated without sperm participation in the sham-injection group, was higher than in the other groups (P<0.05).

Ability of ICSI oocytes to develop to the blastocyst stage

Blastocyst formation rates after 6 days of ICSI were not different in all groups injected with freeze-dried sperm and sham-injection group. However, the rate was

significantly lower in freeze-dried sperm-injected group compared with those in fresh sperm-injected group ($P<0.05$) (Table 3). On the other hand, blastocyst quality measured by cell number in blastocyst were not different among all groups (Fig. 5a', 5b', and 5c').

Discussion

In this study, we found that trehalose exerted a concentration-dependent protective effect against DNA fragmentation during FD procedures. Unexpectedly, within the dose range of trehalose investigated, optimal sperm DNA protection was achieved at 7.5 and 15 mM, whereas lower or higher concentrations were not effective. It has been reported that sperm containing damaged DNA still have the ability to fertilize oocytes after ICSI (Zini et al. 2005). However, the ICSI oocytes showed poor embryo development and high pregnancy loss rates after embryo transfer (Sakkas et al. 1998). FD medium supplemented with trehalose is able to reduce the degree of DNA damage occurring during the FD in both somatic cells (Loi et al. 2008) and sperm (Martins et al. 2007). The effect of trehalose supplementation in FD medium is immediately evident from the morphological appearance of the dried cells observed by scanning electron microscopy (Loi et al. 2008; Martins et al. 2007). It is suggested that trehalose molecules not only interact with DNA but also form a large number of hydrogen bonds among themselves, forming a glassy/viscose medium, thereby reducing structural fluctuations of DNA and preventing its denaturation (Loi et al. 2008). Previous findings have also suggested that the structure of DNA is well-protected in a dry state by trehalose supplementation. Trehalose is also thought to increase cytoplasmic viscosity, thus reducing the likelihood of intracellular ice crystal formation, which is often fatal. It also may act as a free radical scavenger, as free radicals are known to damage proteins, lipids, and nucleic acids (Cui et al. 2000). The exact mechanism by which trehalose stabilizes living systems during freeze-thawing, heat-cooling, or dehydration-rehydration cycles remains a matter of debate, and no consensus has yet emerged (Pereira et al. 2004). However, the water-replacement hypothesis has been widely accepted as a suitable explanation for the DNA-trehalose system. Trehalose is thought to replace the water shell around proteins/membranes and to preserve the three dimensional structure of biomolecules by formation of hydrogen bonds between trehalose and the phosphate groups of DNA (Jain & Roy 2008). Moreover, rapid freezing also results in release of intracellular enzymes (Pursel & Johnson 1971) or lipids (Darin-Bennett 1973), and leads to redistribution of ions (Hood et al. 1970), the latter facilitating the formation of hydrogen bonds among trehalose, DNA and water molecules, thus protecting DNA from potential damage during FD.

The results of the present study indicated that the DNA integrity of freeze-dried sperm was improved by adding a combination of EGTA and trehalose to FD medium. There are possibilities that EGTA reduces sperm DNA fragmentation induced by the activity of endonucleases through its chelating effect and also that trehalose protects sperm DNA from potential damage induced by the FD process and storage as a result of its known stabilizing properties. It has been suggested that the calcium ion-chelating capacity of EGTA would have a negative impact on the outcome of fertilization, as calcium ions play an important role in the fertilization process. This possibility was obviated in our experiments because the freeze-dried sperm was rehydrated, centrifuged, and washed to eliminate the EGTA and trehalose before injection into oocytes. However, it is not easy to explain why higher concentrations ($\geq$ 30 mM) of trehalose were not effective in protecting sperm DNA in comparison with lower concentrations such as 7.5 and 15 mM. As it is known that the pH and osmolality of FD medium have a very important impact on FD procedures, all FD media were adjusted to the same range of pH and osmolality to eliminate the influence of these factors. Furthermore, the concentration at which trehalose exerts optimal protection is dependent on species, cell type, cell state and the method of preservation (Mittal & Devireddy 2008). For example, in cryopreservation, Badr et al. (2010) have indicated that addition of trehalose (optimal at 100 mM) to the freezing extender leads to reduction of cryodamage and oxidative stress, and showed improvement of viability and *in vitro* embryonic development of cryopreserved buffalo sperm. In contrast, Kozdrovski et al. (2009) added 0, 50, and 100 mM trehalose to the freezing extender of European brown rabbit sperm and found that motility and progressive motility were lowest for semen frozen with 100 mM trehalose, as assessed using a computer-assisted semen analysis system. Thus, it can be suggested that the optimum concentration of trehalose differs among species. In addition, it is of interest that, unlike cryopreservation, FD utilizes a simple principle of physics known as sublimation, which is the transition of a substance from the solid (ice) state to the vapor state without passing through the liquid (water) phase. The fact that higher concentrations of trehalose had no protective effect on sperm DNA may be due to the fact that sublimation may result in crystallization of the trehalose, thus disturbing the hydrogen bonding (Jain & Roy 2008).

As I had expected, our results indicated that damage to sperm DNA decreased sharply when sperm was freeze-dried in the presence of trehalose at suitable concentrations (7.5 and 15 mM). More fragmentation of sperm DNA was observed if the FD medium contains only EGTA (Fig. 2a, 2b and 2e). However, the DNA damage level of freeze-dried

sperm in all groups was significantly higher than those of fresh sperm. These results are consistent with some previous reports in other species such as sheep (Iuso et al. 2013) and mice (Kusakabe et al. 2001). In sheep, Iuso et al. (2013) used a transmission electron microscopy to evaluate the ultrastructure of fresh, frozen thawed and freeze-dried lymphocytes and reported that plasma membrane and mitochondria of freeze-dried cells were degraded, whereas these anomalies were totally absent in fresh samples, and occasionally observed in frozen thawed cells. FD procedures most likely lead to a significant increase of DNA damage because of mechanical injury during desiccation. The fertilization after ICSI and development to the blastocyst stage did not differ significantly among 0, 7.5, and 15 mM groups. This finding indicated that DNA damage to sperm, at certain level, does not compromise fertilization, blastocyst yield or quality after ICSI. Our findings also support the results by Zini et al. (2005) who suggested that DNA fragmentation has no impact on the fertilization rate during ICSI. This can be explained by the fact that fertilized oocytes have the capacity to repair DNA damage in both parental genomes.

All cells, except viruses and mature sperm, possess a variety of enzymatic mechanisms for repair of damaged DNA (Ashwood-Smith & Edwards 1996). These complex DNA repair mechanisms are important for maintaining genomic integrity and limiting the introduction of mutations into the gene pool. In the present study, we assumed that DNA damage in freeze-dried sperm might be repaired in porcine oocytes through pre- and postreplication repair mechanisms, as reported by Brandiff & Pedersen (1981) and Genesca et al. (1992). This repair capacity depends mainly on the extent of sperm DNA fragmentation, and the cytoplasmic and genomic quality of the oocyte. Loi et al. (2008) also confirmed this repair capacity because 16% of reconstructed embryos developed to the blastocyst stage, whereas 60% of donor nuclei had obvious DNA damage. SSBs are quickly repaired by oocytes after fertilization, but DSBs could be responsible for chromosome aberrations and loss of genetic materials, because the repair of DSBs in oocytes is more difficult than that of SSBs (Enciso et al. 2009). The result of zygote pronuclear labeling with γH2A.X, a marker of DSBs, indicated that there was no or very little signal of DSBs in a male PN formed in the oocytes at 10 h after injection with freeze-dried sperm in 0 and 15 mM group. The difference in DNA damage level of sperm between 0 and 15 mM groups might be neutralized by the DNA repair ability of oocytes after fertilization. Because it is confirmed that damaged DNA repair in the oocytes occurs during first few hours of first cell cycle after fertilization (Ashwood-Smith & Edwards 1996). Therefore, most of damage (if any) was repaired before DNA replication takes place. However, if serious damage is

expressed in sperm (as in the positive control), it is most likely unable to be repaired by the oocytes, as a result, subsequent development of zygotes would be influenced. On the other hand, inefficient zygotic DNA repair increases the risk of inherited chromosomal aberration and the efficiency depends on the quality of oocyte. Wossidlo et al. (2010) reported in detail of a very dynamic pattern of γH2A.X appearance during zygotic pronuclear development in mice and suggested that DNA demethylation may be mediated to a large extent by DNA repair-induced mechanisms.

In a previous study, it was reported that 23.1% of oocytes developed to the blastocyst stage after injection of freeze-dried boar sperm head in medium containing 50 mM EGTA (Nakai et al. 2007). Embryos obtained after ICSI with freeze-dried sperm head developed to the early fetal stage (39 days after ICSI and putative zygote transfer), but no viable offspring had been produced yet. In the present study, the blastocyst formation rate was 12.6% using this type of FD medium (0 mM group). The lower percentage in the present study might be attributable to the injection procedures using whole sperm, leading to introduction of a larger volume of injection medium into the ooplasm. Thadani (1980) reported that injection of excessive amounts of micromanipulation medium into oocytes appeared to have an adverse effect on subsequent embryonic development. The quantity of the medium injected with a whole spermatozoon was believed to be proportional to the length of the sperm tail, suggesting that diminishing the length of the sperm tail by cutting it could help minimize the volume of injected medium. A relatively high percentage of lysed oocytes (5%–20%, data not shown) following ICSI and electric pulse also reflects the hypothesis mentioned above. The blastocyst formation rate of the oocytes injected with fresh sperm was significantly higher compared with freeze-dried sperm-injected groups (Table 3). This can be explained due to induced chromosomal damage and deterioration of the sperm-borne oocyte-activating factor during the FD process (Liu et al. 2004) or phospholipase C zeta (PLCζ), an oocyte-activating factor in sperm which was reported to be leaked or reduced in quantity after some pretreatment procedures of sperm (Nakai et al. 2011), might be also reduced after FD procedures. The lyophilization process may introduce chemical changes to the sperm plasma and nuclear membranes, rendering them more difficult to be dissolved than those of fresh, intact sperm (Liu et al. 2004). There is another possibility that the fresh sperm used for injection were sonicated several seconds to immobilize the sperm before injection to oocyte; however, sonication makes sperm head isolated from tail; therefore, only sperm heads were injected to the oocytes. And as mentioned above, this may lead to the reduction of injection

medium (containing PVP) amount into oocytes, thus, less harmful than whole sperm injection.

Blastocyst quality measured in terms of the average number of cells in blastocysts in the freeze-dried injected groups did not differ from the other groups, and was comparable to the result obtained by Nakai et al. (2007), being 33.1 cells on average. In general, imbalance of nuclear and cytoplasmic maturation (Kikuchi et al. 2008) and the use of prepubertal oocytes, which have a poor quality with respect to adult oocytes (Ptak et al. 2006) in *in vitro* systems, are still the major causes of reduced fertilization and subsequent development in comparison with *in vivo* systems. The poor outcome of ICSI in this study may also be partly attributable to this. This study indicated that the percentage of oocytes with two PNs at 10 h after injection in the sham-injection group was significantly lower than those in the groups injected with freeze-dried sperm, whereas the blastocyst formation rates did not differ among these groups. It is presumed that two PN embryos with the presence of both male and female PNs are usually diploid (Lee et al. 2004). Therefore, most of the blastocysts obtained from sham injection seem to be euploid (haploid) because of the absence of a male PN. When a direct current pulse of 1.5 kV/cm was applied for 20 µs to ICSI oocytes matured *in vitro* for 44 h, Nakai et al. (2010) found that the rates of diploid blastocysts in the ICSI group (ranged from 48.9% to 60.6%) were significantly higher than those in sham group (28.0%). When ICSI is used, increased emphasis is placed on the quality of the sperm chromatin and the ability of the oocyte to initiate decondensation and PN formation (Nakai et al. 2007). Therefore, protection of sperm DNA integrity is important for maintaining the fertility of freeze-dried sperm. Although this study did not clarify the proportion of sperm with fragmented DNA, the data were sufficient to allow us to conclude that addition of trehalose to FD medium sharply reduced the degree of sperm DNA fragmentation in a concentration-dependent manner.

Although the Halomax kit is easy and convenient to handle, its inability to differentiate between SSBs and DSBs in the same sperm cell was a limitation of the present study. The type of damage occurring in freeze-dried sperm may be correlated with the protective role of trehalose, and thus a suitable assay that can characterize the types of DNA fragmentation is needed. The previous study have also suggested that DNA damage might contribute to early postimplantation death (Singer et al. 2006) and full term development of oocytes injected with these sperm. Therefore, further research is necessary to investigate the effect of the combination of EGTA and trehalose in FD medium on the *in vivo* developmental competence of oocytes injected with the resulting freeze-dried sperm. In

conclusion, addition of trehalose to FD medium at suitable concentrations improves sperm DNA integrity after FD procedures, but does not promote fertilization and subsequent development to the blastocyst stage. To our knowledge, this is the first study to have examined the combined effect of EGTA and trehalose in FD solution on the DNA integrity of freeze-dried sperm and *in vitro* embryonic development of porcine IVM oocytes that have been injected with freeze-dried sperm.

Table 2. Female and male PN formation[a] of IVM porcine oocytes injected with fresh or freeze-dried sperm

Group	No. of oocytes examined	% of oocytes with 2PB and 2PN	% of oocytes with 2PB and 1PN
Fresh sperm	92	71.9 ± 2.0^{c}	3.4 ± 2.0^{c}
Trehalose 0 mM[b]	145	45.9 ± 3.6^{d}	$11.7^{a} \pm 2.3^{c}$
Trehalose 7.5 mM[b]	41	60.8 ± 6.3^{cd}	$13.3^{a} \pm 3.0^{c}$
Trehalose 15 mM[b]	137	52.9 ± 3.8^{cd}	$13.5^{a} \pm 3.2^{c}$
Sham injection	100	8.1 ± 3.2^{e}	$45.2^{b} \pm 6.8^{d}$

Means ± SEM are presented

Data were analyzed by ANOVA followed by Tukey's multiple range tests. At least three replications were carried out in each group.

PB: polar body; PN: pronucleus.

[a] PN formation was examined after 10 h of IVC.

[b] Freeze-dried sperm at different concentrations of trehalose.

[c-e] Values with different superscripts within each column differ significantly ($P<0.05$).

Table 3. Preimplantation development of IVM porcine oocytes injected with fresh or freeze-dried sperm

Group	No. of oocytes examined	Blastocyst %	Mean cell number
Fresh sperm	95	32.7 ± 1.5[b]	41.2 ± 3.9
Trehalose 0 mM[a]	236	12.6 ± 2.0[c]	39.4 ± 4.0
Trehalose 7.5 mM[a]	68	12.3 ± 1.5[c]	36.4 ± 6.3
Trehalose 15 mM[a]	227	12.2 ± 2.2[c]	28.9 ± 3.1
Sham injection	173	15.5 ± 3.3[c]	41.7 ± 3.3

Means ± SEM are presented

Data were analyzed by ANOVA followed by Tukey's multiple range tests. At least three replications were carried out in each group. In this study, embryos with a clear blastocoel were defined as blastocysts after 6 days of IVC.

[a] Freeze-dried sperm at different concentrations of trehalose.

[b,c] Values with different superscripts within column differ significantly ($P<0.05$).

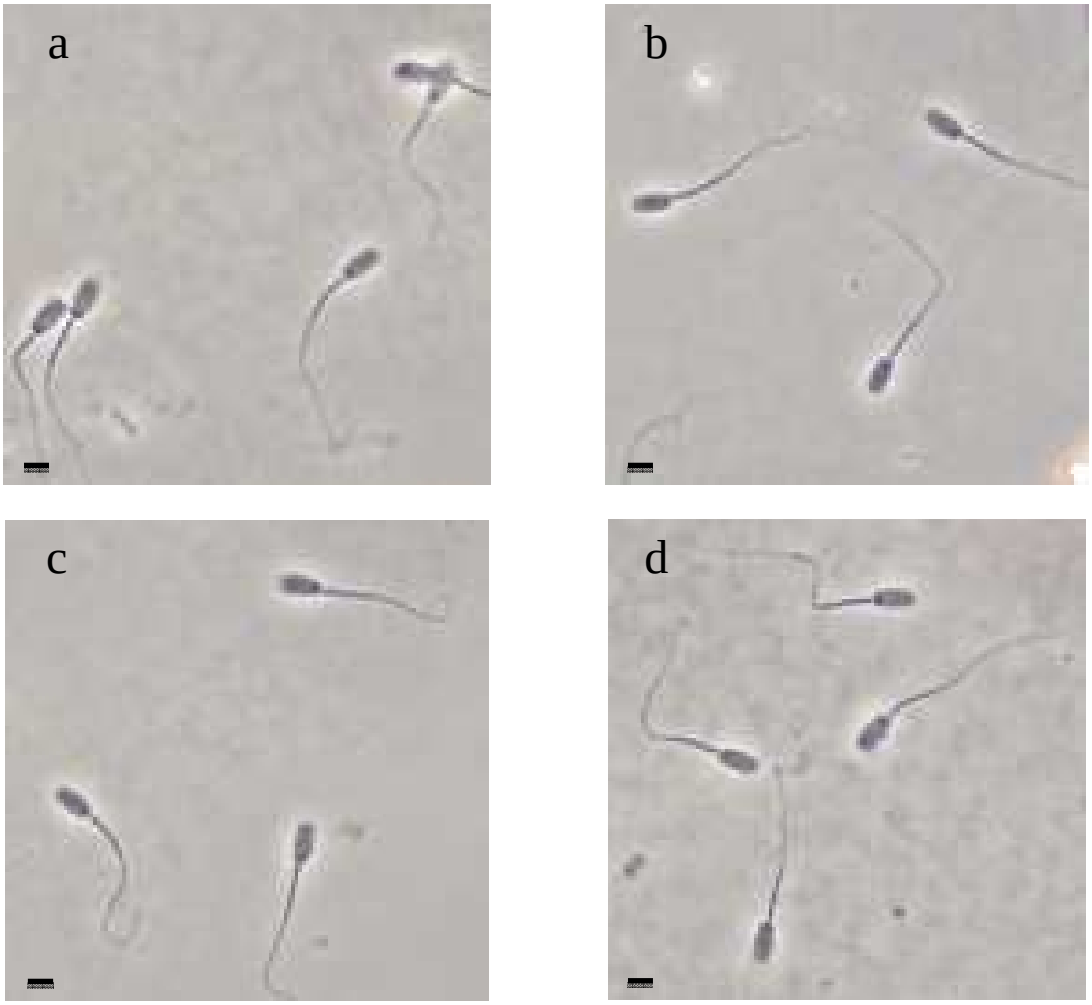

Fig. 1. Freeze-dried sperm after rehydration. Freeze-dried sperm after rehydration in all groups exhibited no morphological difference by naked eye. Trehalose (a) 0 mM, (b) 7.5 mM, (c) 15 mM, and (d) 90 mM group. Scale bar: 5 μm.

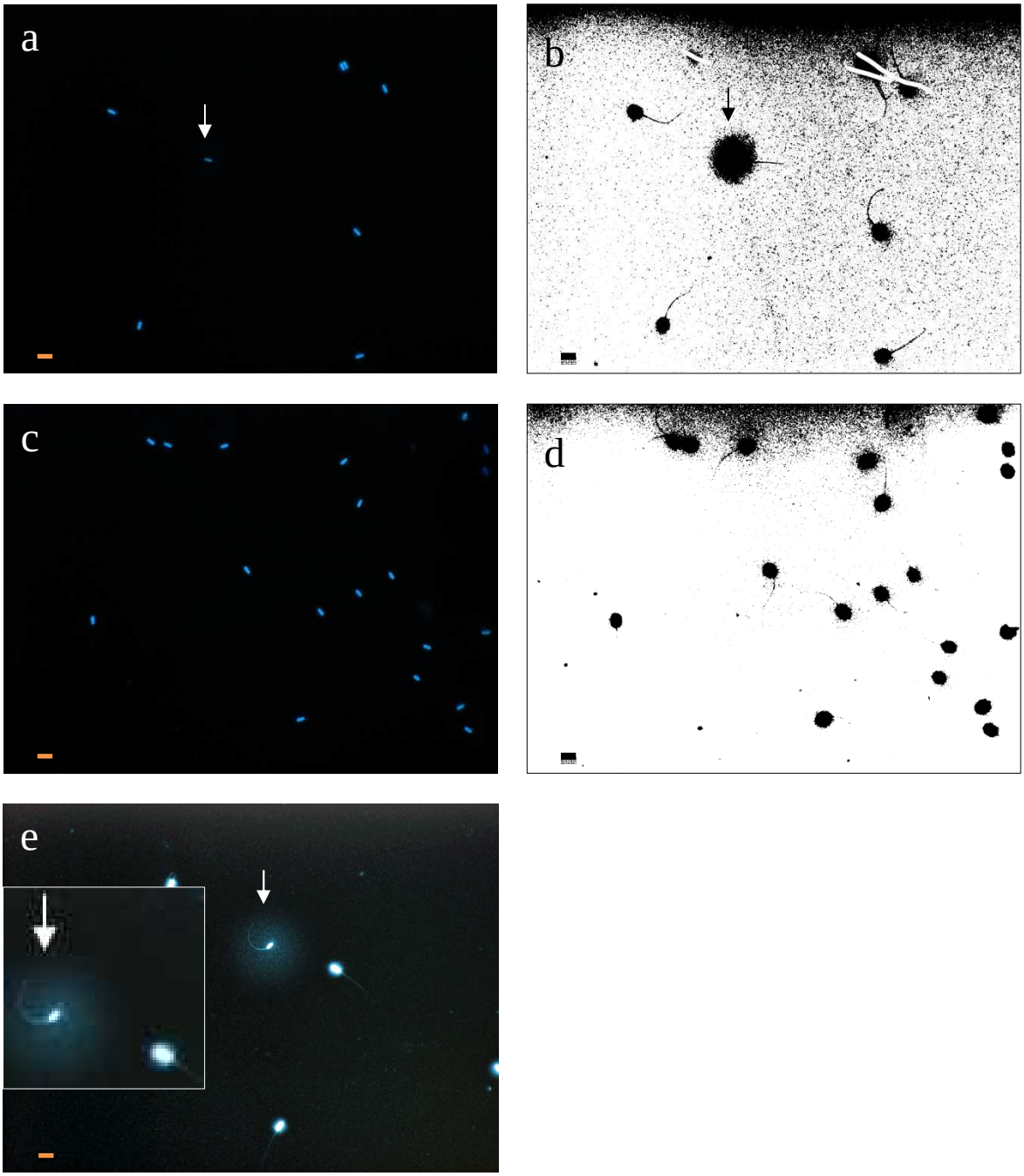

Fig. 2. DNA fragmentation of freeze-dried sperm assessed by Halomax kit. DNA fragmentation of freeze-dried sperm in the control (a, b, and e) and 15 mM trehalose (c and d) groups. (a and c) DNA chromatin was stained with Hoechst 33342. (b and d) after converting to measurable images by Photoshop 5.0. (e) A very large DNA dispersion halo is evident in 0-mM group using the filter function of Photoshop (arrow). Scale bar: 10 µm.

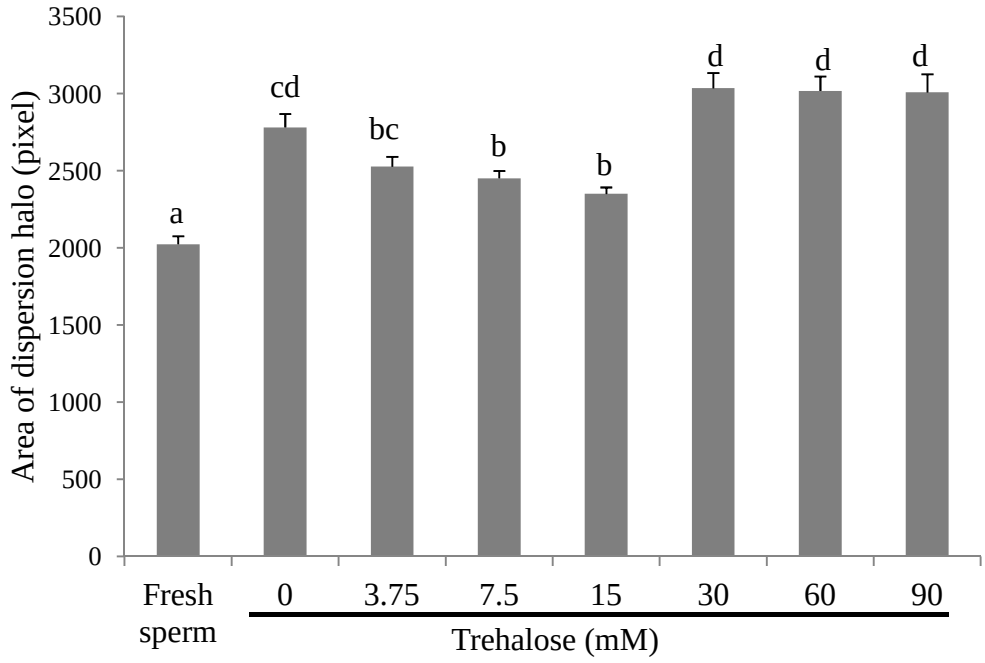

Fig. 3. DNA damage to sperm freeze-dried in the presence or absence of trehalose, as assessed using the Halomax kit. Data were analyzed by ANOVA followed by Tukey's multiple range tests. $^{a\text{-}d}$ different superscripts show a significant difference ($P<0.05$). At least three replicate trials were performed for each group.

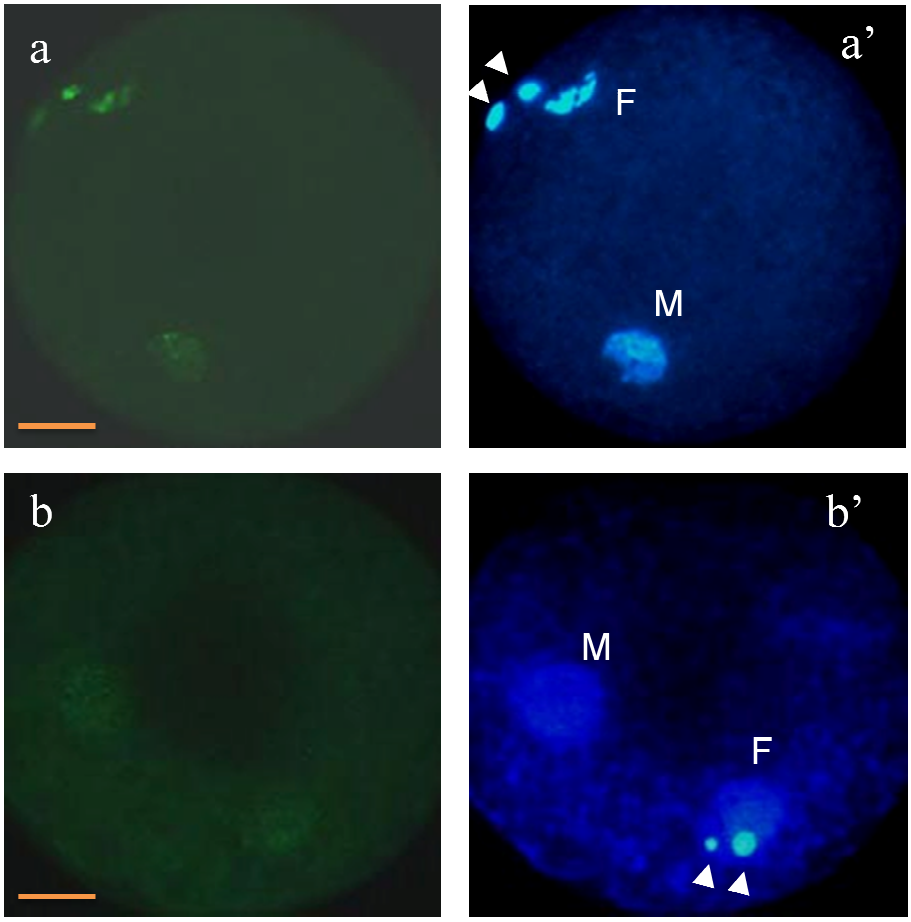

Fig. 4. DSBs in embryos produced by ICSI with freeze-dried sperm at 10 h after injection completion, stained for histone H2A.X phosphorylated at serine 139 (γH2A.X). γH2A.X signal in a PN of the oocyte injected with sperm freeze-dried in (0 mM (a) and 15 mM (b) trehalose). γH2A.X forms "foci" at DSBs site in a male PN induced by DNase I digestion. Chromatin is counterstained with Hoechst 33342 (a'-b'). M: male pronucleus; F: female pronucleus; arrow head: polar body. Scale bar: 20 μm.

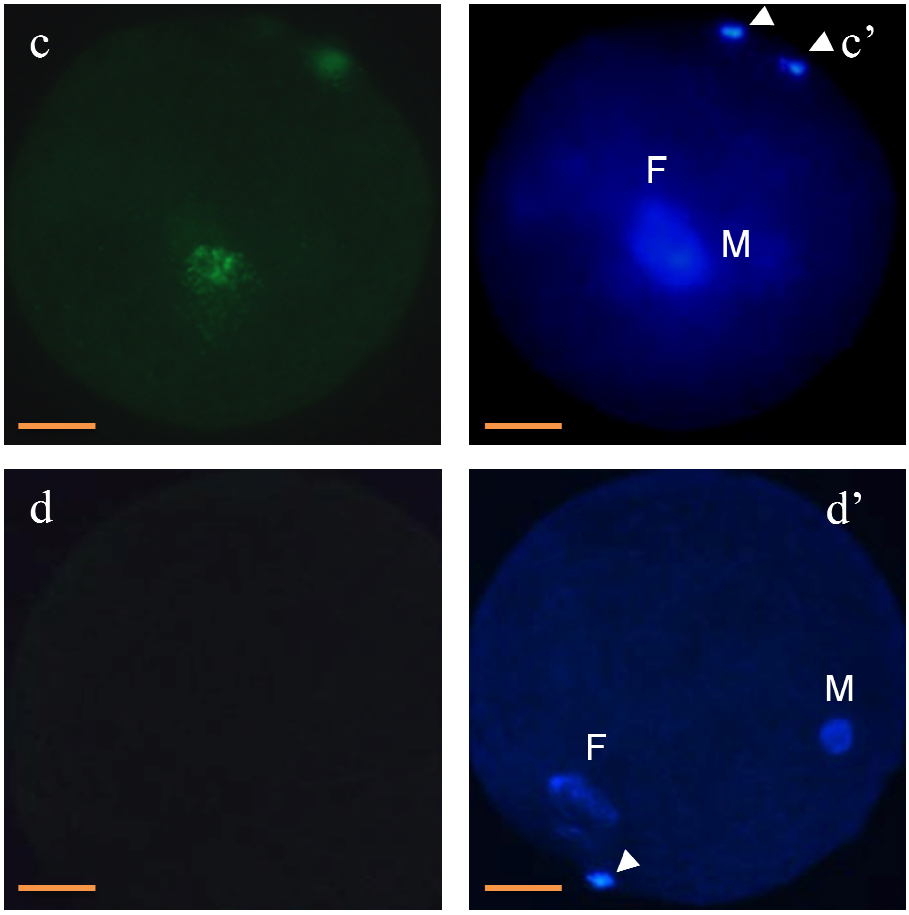

Fig. 4'. DSBs in embryos produced by ICSI with freeze-dried sperm at 10 h after injection completion, stained for histone H2A.X phosphorylated at serine 139 (γH2A.X). γH2A.X signal in a PN of the oocyte injected with sperm freeze-dried in (0 mM treated with 1000 IU/mL DNase I for 60 min at room temperature (c). Primary antibody was omitted in some embryos to prevent nonspecific binding (d). γH2A.X forms "foci" at DSBs site in a male PN induced by DNase I digestion. Chromatin is counterstained with Hoechst 33342 (c'- d'). The signal of DSBs in a male PN was very strongly expressed in positive control group in which DNase I–treated sperm were injected to the oocyte. However, the signal in female PN (overlapped to male PN) is not detected. M: male pronucleus; F: female pronucleus; arrow head: polar body. Scale bar: 20 μm.

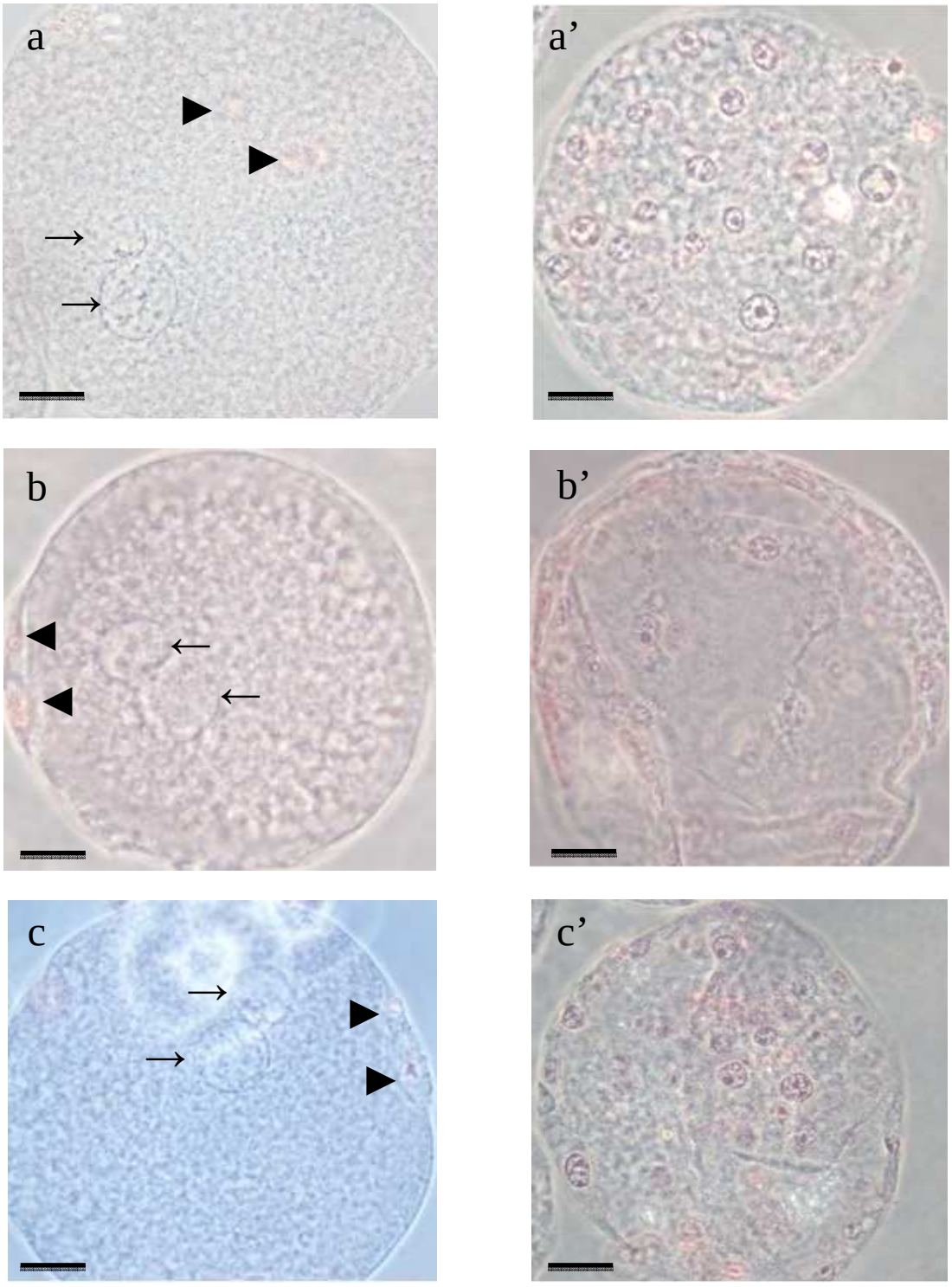

Fig. 5. Pronuclear stage (10 h after injection, a - c) and blastocyst stage (6 days after IVC, a’ - c’). Embryos obtained from the oocyte injected with freeze-dried sperm treated with 0 mM (a and a’), 7.5 mM (b and b’) and 15 mM (c and c’) trehalose. In pronuclear-stage embryos, two PBs (arrow head) and two PNs (arrow) were clearly visible. Scale bar: 20 μm

Chapter III

Expression of DNA repair genes in porcine oocytes before and after fertilization by intracytoplasmic sperm injection using freeze-dried sperm

Introduction

It is reported that newly fertilized oocytes in some species possess the ability to sense, respond to, and repair at least some types of DNA damage, particularly in the incoming sperm DNA (Zheng et al. 2005). DNA repair is an essential process for maintenance of genomic integrity in the preimplanation embryos to correct the damage existed in the gametes. The damage may be either inherent or arose during DNA replication and is also caused by genotoxic agents (Zheng et al. 2005). The DNA damage needs to be repaired before the first round of DNA replication of zygotes to minimize the mutation load of the developing embryos (Zheng et al. 2005). There are several DNA repair pathways in mammalian cells: direct reversal of damage, nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand break repair (DSBR) (Jaroudi et al. 2009). Direct reversal of damage is the simplest form of DNA repair and also the most energy efficient method; it does not require a reference template as the other single strand repair mechanisms. O-6-methylguanine-DNA methyltransferase (known as *MGMT*) a specific DNA repair enzyme can remove the alkyl group from the O^6-position of the guanine, thereby preventing its mutagenic and carcinogenic effects (Zuo et al. 2004), belonging to direct reversal pathway. There are a number of regulatory elements in the *MGMT* promoter region, and a number of stimuli may increase *MGMT* expression, such as irradiation, glucocorticoid exposure, and cAMP (Liu et al. 2012).

Expression profile of maternal DNA repair genes correlates the ability of the oocytes to recognize and repair DNA damage at certain stages (oocyte/blastocyst). For instance, Jaroudi et al. (2009) demonstrated that the mRNA level for most repair genes was higher in oocytes compared with blastocysts in human and this is to ensure sufficient availability of template until zygotic or embryonic genome activation (so-called zygotic or embryonic genome activation (ZGA or EGA, respectively) (Zheng et al. 2005), and that the DNA repair transcripts accumulated in the human oocyte play an important role in chromatin remodeling and maintain chromatin integrity during fertilization. Furthermore, when the DNA damage caused during fertilization is recognized as irrepairable, embryos are excluded by cell cycle arrests or activation of apoptotic pathways (Jaroudi et al. 2009). To our knowledge, there are

a few works about the expression profiling of DNA repair transcripts in only human oocytes and early embryos due to the rare availability of the materials and the ethical considerations (Li et al. 2006). Usage of non-human primates, laboratory or domestic animals in this research area is more feasibled and has a great importance on providing novel knowledge on this field.

It is considered that DNA repair ability of oocytes correlates with the amount of maternal repair mRNA in the cytoplasm which accumulated during the growth phase of oocytes and follicles, and required for completion of the meiotic cell cycle (Zheng et al. 2005). Moreover, *in vitro* culture of oocytes and embryos may lead to dysregulation of many genes (Zheng et al. 2005; Jones et al. 2008, Salhab et al. 2013), resulting in low cellular viability and long-term embryo viability by the impaired competence for the repair of the DNA damage. Recent studies suggested the differential expression of several repair genes between *in vivo* matured and IVM oocytes in cattle (Thelie et al. 2007), in human (Jones et al. 2008) and in non-human primates (Zheng et al. 2005). However, the expression of DNA repair-related genes after fertilization has not been examined in porcine oocytes.

In Chapter II, I have shown that sperm freeze-dried in the basic medium containing 15 mM trehalose showed less DNA damage compared with control group without trehalose treatment. However, normal fertilization and subsequent embryonic development were not different between both two groups. It is still unknown that whether higher DNA damage level of sperm in the control group compared with 15 mM trehalose group leads to the upregulation of expression of DNA repair genes in oocytes after injection of a spermatozoon from this group. The objective of this study was to estimate the expression levels of DNA repair-related genes in porcine oocytes after fertilization by ICSI before early cleavage stage. In addition, the expression profile of these genes was also detected in different stages of oocyte maturation.

Based on previous studies (Harrouk et al. 2000; Wood et al. 2001; Zheng et al. 2005; Jaroudi & SenGupta 2007), I focused on the expression of six DNA repair genes related to repair ability of different kinds of DNA damage: *MGMT* (for direct reversal), *UDG* (for NER), *XPC* (for BER), *MSH2* (for MMR), *XRCC6* (for DSBR by homologous recombination) and *RAD51* (for DSBR by non-homologous end-joining) (Tables 4 and 5).

Many reports showed that DNA repair in oocytes occurs in the first few hours after fertilization (in rats, Harrouk et al. 2000; in mice, Derijck et al. 2006), or prior to S-phase (pronuclear stage) (in humans, Aitken & Koppers 2011). Therefore, in this Chapter, mRNAs were extracted from oocytes at 4 h after sperm injection (Experiment 1) and at four time

points of oocyte maturation (Experiment 2) and were subjected to quantitative RT-PCR to examine the expression of these genes.

Materials and methods

Chemicals and media

All chemicals were obtained from Sigma-Aldrich, unless otherwise stated. The FD medium contained 10 mM Tris-HCl buffer supplemented with 50 mM EGTA and is referred to as basic FD medium. This medium was then supplemented with different concentrations of trehalose (0 (referred as the control) and 15 mM) and also NaCl (50 and 40 mM, respectively). The osmolality and pH of the final solutions were adjusted to 265 to 270 mOsm/kg and 8.0 to 8.5, respectively, and then they were filtered and stored at 4^{o}C. The IVM medium oocytes was NCSU-37 solution containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 mM β-mercaptoethanol, 1 mM dibutyl cAMP, 10 IU/mL eCG, and 10 IU/mL hCG (Kikuchi et al. 2002a). IVC medium for sperm-injected oocytes was modified NCSU-37 supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/mL BSA and 50 mM β-mercaptoethanol (IVC-PyrLac) (Kikuchi et al. 2002a).

Sperm collection and FD

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Tsukuba, Japan. Sperm collection and FD were conducted as described previously (Men et al. 2013). In brief, ejaculated semen was collected from a Landrace boar, which is used for reproductive program at National institute of Livestock and Grassland Science, Tsukuba Japan, and transferred to the laboratory within 30 min. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × *g* at 30^{o}C and the seminal plasma was removed. The pellet was re-suspended in FD medium containing trehalose prewarmed at 30^{o}C, and the supernatant was removed after centrifugation. The final pellet was then re-suspended in FD media containing 0 or 15 mM trehalose at a final concentration of 4×10^{8} cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial, then the vials were covered with aluminum foil and placed in a refrigerator at –80^{o}C for at least 4 h. The aluminum foil was replaced by a rubber cap with small gaps between the cap and the vial, and then they were placed in a FD system. The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at –30^{o}C and then increased to 30^{o}C during the last 1 h 20 min (0.75^{o}C/min).

After filling with N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps. The freeze-dried samples were transferred to a refrigerator and stored at 4°C under dark condition until usage.

Oocyte collection and* in vitro *maturation (IVM)

Oocyte collection and IVM were conducted as described (Kikuchi et al. 2002a). In brief, ovaries were obtained from prepubertal crossbred gilts (Landrace-Large White-Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COCs) were collected from follicles 2 - 6 mm in diameter in Medium 199 (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 µL of maturation medium for 20 - 22 h in four-well dishes. The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39°C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, the cumulus cells were removed from the oocytes by repeated pipetting in Medium 199 supplemented with 150 IU/ mL hyaluronidase. Denuded oocytes with the first PB were harvested under a stereomicroscope and used as IVM oocytes.

ICSI and oocyte stimulation

Freeze-dried sperm were re-hydrated with deionization distilled water. The sperm suspension was centrifuged for 2 min at 600 × *g* and the sperm were washed with PBS (–) containing 5 mg/mL BSA, then re-suspended in the same buffer. The sperm suspension was sonicated for about 5 to 10 sec to isolate a sperm head and kept at room temperature prior to ICSI, which was carried out as previously described (Nakai et al. 2006) with some modifications (Men et al. 2013). In brief, sperm were kept in IVC-PyrLac- Hepes-PVP. About 30 IVM oocytes were transferred to a 20-µL drop of Medium 199. A small volume (0.5 µL) of the sonicated sperm suspension was transferred to a 2-µL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil. A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator. ICSI was completed within 2 h after re-hydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. The end of injection was considered as 0 h postinjection. As a control group, mature oocytes were injected with ejaculated sperm (fresh sperm group). One hour postinjection, the oocytes were transferred to an activation solution consisting of 0.28 M

d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk down the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 µs under the same condition for each group using a somatic hybridizer, then washed three times and cultured in IVC PyrLac for 3 h before RNA extraction.

RNA extraction, purification, cDNA systhesis and quantitative RT- PCR (qRT-PCR)

Total RNAs of the pooled oocytes or putative zygotes were purified using an RNeasy Micro Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Reverse Transcription was performed to synthesize cDNA using a Primescript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) according to manufacturer's instructions. qRT-PCR was performed using a LightCycler® 480 SYBR Green I (Roche, Indianapolis, IN, USA) according to standard protocols. For each gene, the quantities of transcript were normalized to the reference transcript and tubulin α1 was used to standardize the data.

Decondensation status of sperm head

For assessment of fertilization status, putative zygotes were fixed at 4 h postinjection (3 h after electric activation) in fixative solution with a 1:3 mixture of acetic acid: ethanol (v/v) under vaseline-supported coverslips for several days. They were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for several minutes, and then their fertilization status was examined using a phase-contrast microscopy

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989) then subjected to one way-ANOVA using R packages 3.0.1 (R Core Team 2013). As the difference is found in groups by ANOVA, further analysis is conducted by Tukey's posthoc test using the R packages. Differences at $P<0.05$ were considered to be significant.

Results

Expression levels of DNA repair genes in IVM- oocytes injected with sperm

Damaged DNA in sperm should be repaired by oocytes before pronuclear formation. According to Nakai et al. (2006), the rate of pronuclear formation sharply increased at 4 h after electric stimulation, therefore, we analyzed the expression of DNA repair genes at the time point of 3 h after stimulation. There were no differences in expression level of 6 investigated genes in oocytes at 4 h postinjection (Fig. 6).

Decondensation status of sperm head at 4 h postinjection (3 h after electric activation)

DNA damage is believed to be detected during decondensation of the sperm head and induces activation of essential DNA repair pathways. Therefore, the decondensation status of freeze-dried sperm head and the pronuclear formation rate were also examined at 4 h postinjection (Fig. 7A and 7B). As shown in Table 6, relatively high percentages of decondensed sperm head were observed in all three groups (62 to 71%) and there were no differences among the groups. Similarly, the rate of zygotes with two PBs and two PNs was also not different and ranged from 8 to 20% among groups. Newly formed PNs at 4 h postinjection were smaller, separately located and the female or male pronucleus was easily distinguishable (Fig. 7C).

Results of nuclear status of oocytes at different maturational stages

The data obtained from fixation and staining of oocytes (Table 7) showed that all COCs were at the GV stage just after collection and at the GVL (later of germinal vesicle) stage 20 h after IVM. And then 5.3% of oocytes had the first PN at 33 h of IVM and 61.8% of oocytes show the first PB at 44 h of IVM under light microscopic observation. At 33 h of IVM, oocytes had the first PB were removed and the remaining oocytes were fixed and stained. The data reveal that 95.7% of these remaining oocytes were at the metaphase-I (M-I) stage and 4.3% were at the GVL stage (Table 7).

Expression levels of DNA repair genes in oocytes at different maturational stages

The expression level of DNA repair genes in oocytes at different maturational stages were summarized in Fig. 8. Of the six genes investigated, expression levels of *UDG* and *XPC* were significantly up-regulated in M-II oocytes compared with earlier stages but did not differ between GV and GVL stage. There was an increased tendency in relative expression of *MSH2* and *RAD51* over time during IVM process although no differences in *MGMT* and *XRCC6*.

Discussion

It is generally accepted that there are mechanisms in a cell to maintain genome integrity including DNA damage detection, repair, cell cycle arrest and apoptosis. Such mechanisms coordinate together to protect the fetus from potential DNA damage originating either in parental gametes or in the embryo's somatic cells (reviewed by Jaroudi & SenGupta 2007). Moreover, the cell cycle of embryonic cells is much shorter than that of adult cells. The integrity of its genome is thus easy to be influenced. In other word, DNA repair at the

early stages is of great significance for the later development. During the early embryonic development, three main transitions occur in preimplantation development, and each transition is reflected by changes in gene expression patterns (Zheng et al. 2005). The first transition is the maternal to zygotic transition. The second transition occurs during compaction at the 8-cell stage (Fleming et al. 2001) and the last one occurs during blastocyst formation at the 32- to 64-cell stage (Zheng et al. 2005). In the first transition, mRNAs and proteins that are maternal factors and stored in the cytoplasm of the oocyte during oogenesis, and necessary for oocyte maturation, homeostasis and transision to the first stages of embryogenesis but become unnecessary or potential deleterious as the embryo develops are destroyed and replaced by novel transcripts which are specific to the zygotes or embryos (ZGA or EGA, respectively) (Zheng et al. 2005). It is well-documented that genome activation is an essential event in order to synthesize new protein preparing for the first cell division and subsequent events as well. The phenomenon of *in vitro* cultured mouse 1-cell embryos which were arrested at the 2-cell stage (2-cell block) was found to be related to the delay of ZGA (Qiu et al. 2003). The initiation of EGA varies between species. In mice, event of genome activation begins during the 1-cell stage and becomes evident by the 2-cell stage with a transcriptional and translational burst (Schultz 2002). In porcine embryos, EGA occurs during the 4-cell stage, promoting a dramatic reprogramming of gene expression accompanied by the generation of novel transcripts that are not expressed in the oocytes (Jarrell et al. 1991; Hyttel et al. 2000). A recent study uncovered a series of successive waves of embryonic transcriptional initiation that occur as early as the 2-cell stage in human preimplantation embryos (Vassena et al. 2011); in contrast to the previously accepted time point of embryonic genome activation at the 4- to 8-cell stage (Braude et al. 1988). EGA occurs in bovine embryos by the 8- to 16- cell stage (Memili & First 2000). In the present study, we attempted to investigate the expression pattern of DNA repair genes in porcine zygotes before genome activation. Previously, we showed that sperm freeze-dried in the presence of trehalose showed less DNA fragmentation than that in the absence of trehalose. However, the rates of oocytes with two PBs and two PNs (referred as normal fertilization), and blastocyst formation were not different between the two groups (Men et al. 2013). Therefore, we speculated that DNA damage in freeze-dried sperm might be repaired through pre- and postreplication repair mechanisms in oocytes (Genesca et al. 1992). This repair capacity depends mainly on the extent of sperm DNA fragmentation and the cytoplasmic quality of the oocyte. SSBs could be quickly repaired by oocytes after fertilization, but DSBs

could be responsible for chromosome aberrations and loss of genetic materials, thus the repair of DSBs in oocytes is more difficult than that of SSBs (Enciso et al. 2009).

The objective of this study initially was to compare the induction of DNA repair genes in newly fertilized oocytes by ICSI of sperm freeze-dried in the presence or absence of trehalose because of observed different DNA integrity of these two groups after FD. Six candidate genes participating in the repair of various types of DNA damage were selected and their expressions were analyzed at a given time after fertilization. Three genes (*MGMT*, *UDG* and *XPC*) are candidate genes for repair of SSBs; one gene for mismatch repair (*MSH2*) and two remaining genes (*XRCC6* and *RAD51*) are for repair of DSBs. As a result, there were no differences in relative gene expression level of six genes in the sperm injected oocytes at 4 h postinjection and mature oocytes. This means the expression level of these genes might have already been abundant in the oocytes matured at the M-II stage. Also probably, at 4 h postinjection, these genes have not been induced significantly or the DNA fragmentation of fresh sperm and two types of freeze-dried sperm was not sufficient to induce the differential expression. Harrouk et al. (2000) indicated that fertilization with sperm exposed to a DNA damaging agent alters the expression of DNA repair genes as early as the 1-cell stage in the rat preimplantation embryo. However, the expression of DNA repair genes in the 1-cell embryos is limited since 1-cell embryos are completely dependent on maternal proteins for DNA repair. The zygotes may be able to regulate its repair efficiency only after the first cell division (Harrouk et al. 2000).

In the genes investigated in this study, *MGMT* works as DNA methyltransferase with the function of direct reversal alkylation at the O^6 position of guanine, and it has an important role to avoid the lethal cross-linking resulting in enhanced resistance to alkylating agents (Pegg et al. 1995). The expression level of *MGMT* gene was not different in all groups. *UDG* gene was expressed abundantly in oocytes and zygotes and revealed a significantly higher level in the M-II oocytes compared with those at the earlier stages. Similarly, expression level of *XPC* gene in the M-II oocytes was significantly higher than those in oocytes at the earlier stages. For *MSH2* gene, the expression of this gene had an increased tendency in the M-II stage oocytes relative to oocytes at the GV, GVL and M-I stages. Zheng et al. (2005) showed that the *MSH2* gene was expressed throughout development from oocytes at the GV stage to the hatched blastocyst stage, with a transient increase in expression in embryos at the 8-cell and morula stages in a non-human primate, and our data were in agreement with this report. *RAD51* is involved in the homologous recombination pathway of DSBR, and is essential for embryo viability (Zheng et al. 2005; Jaroudi &

SenGupta 2007). In the rhesus monkey, this gene was expressed in abundance in oocytes, but its expression decreased during oocyte maturation and then increased again at the 8-cell stage (Zheng et al. 2005). Unlike observed patterns in the rhesus monkey, our data in pigs revealed that the expression of *RAD51* tends to up-regulate during IVM. According to results of Jaroudi et al. (2009), *RAD51* and *MSH2* were expressed at high levels in both human oocytes and blastocysts, on the other hand, *XRCC6* had medium to high expression levels in the M-II oocytes and blastocysts.

In the present study, it can not be denied that relative expression value of *UDG* gene in the M-II oocytes in Experiment 1 was almost two-fold lower than those in Experiment 2. The M-II oocytes used in Experiment 2 were denuded at 44 h of IVM and immediately subjected to RNA extraction. The M-II oocytes used in Experiment 1 were also denuded at 44 h of IVM but subjected to RNA extraction at about 2 h later when ICSI was completed. As mentioned before, this gene showed highly expression in the M-II oocytes in Experiment 2 relative to other genes at 44 h of IVM. It may be possible that lower expression value of the M-II oocytes in Experiment 1 was attributed to age-related degeneration of some DNA repair mRNAs in oocytes or the expression of this gene reached peak at 44 h then decreased. This explanation is clearly supported by the fact that the relative expression of this gene in the other groups of Experiment 1 also did not achieve the similar level of Experiment 2 irrelevant to treatments.

There are two possible explanations for the maturational stage-dependent changes of most of DNA repair genes in IVM oocytes observed in Experiment 2. One possibility is that during IVM, oocytes have accumulated mRNAs of DNA repair genes being ready for fertilization. The other possibility is that suboptimal IVM system stresses oocytes and it might induce up-regulation of these DNA repair genes. The later appears to be a more satisfactory explanation. Indeed, Jones et al. (2008) reported that several genes involved in many signaling pathways, such as response to stress, cell cycle, cell proliferation, cell division and cell death and so on, were up-regulated in IVM oocytes compared with *in vivo* matured oocytes, and this up-regulation may attribute to dysregulation occurring during IVM. Cumulus cells play an essential role in whole process of oocyte growth and maturation; therefore, their gene expression profiles according to IVM condition have been also investigated in detail (Tesfaye et al. 2009; Ouandaogo et al. 2012). In bovine cumulus cells, isolated from the COCs after IVM culture, genes involved in response to stress were up-regulated and genes related to cumulus expansion and oocyte maturation were down-regulated compared with the cumulus cells isolated from the COCs produced *in vivo*

(Tesfaye et al. 2009). Similarly, expression of genes involved in DNA replication, recombination and repair in human cumulus cells isolated from *in vivo* and *in vitro* at different nuclear maturation stages were up-regulated in cumulus cells after IVM (Ouandaogo et al. 2012).

It should be noted that there are so many DNA repair genes and enzymes in the oocytes and zygotes in addition to those examined in the present study. Expression level of these genes may depend on unknown cellular signals related to normal development or delayed development in the oocytes injected with sperm that have various types or levels of DNA damage. The expression of those genes depends on the embryo developmental stage and/or influenced by many other unknown factors.

In conclusion, the present study revealed that expression of DNA repair genes in fertilized oocytes at 4 h after ICSI using fresh sperm, freeze-dried sperm in the presence or absence of trehalose was not different. Likewise, no difference was observed in the expression of DNA repair genes between the sperm injected groups and the M-II oocyte group without sperm injection. On the other hand, during IVM, the expression of *XPC* was significantly increased in the M-II oocytes compared with earlier stages. The expression of *UDG* was significantly increased from the GV through GVL, M-I and M-II stages. Further experiments are needed to confirm whether increased expression levels of DNA repair genes in the oocytes are caused by suboptimal IVM conditions or their accumulation. If increased level of DNA repair genes is confirmed to be caused by IVM conditions, the efficient improvement of IVM system can be achieved based on expression profile of DNA repair genes.

Table 4. Repairable DNA damage type, proper functions, accession number and primer sequence of six investigated genes.

Genes symbol	Full name	DNA damage type	Proper function	References
MGMT	O-6-methylguanine-DNA methyltransferase	Direct reversal: alkylated guanine	Removes alkylating lesions at the O6 of guanine residues	Wood et al. 2001; Jaroudi et al. 2009
UDG	Uracil -DNA glycosylase	Single strand break (Base)	DNA glycostylase, recognize and remove uracil opposite A	Harrouk et al. 2000; Wood et al. 2001; Zheng et al. 2005; Jaroudi et al. 2009
XPC	Xeroderma pigmentosum, complementation group C	Single strand break (Nucleotide)	DNA binding	Harrouk et al. 2000; Wood et al. 2001; Zheng et al. 2005;
MSH2	mutS homolog 2, colon cancer	Mispairing	Mismatch and loop recognition	Harrouk et al. 2000; Wood et al. 2001; Zheng et al. 2005; Jaroudi et al. 2009
XRCC6	X-ray repair cross-complementing 6	Double strand break	Non-homologous end joining	Jaroudi et al. 2009
RAD51	Sus scrofa *RAD51* homolog (S.cerevisiae)	Double strand break	Homologous recombination	Wood et al. 2001; Zheng et al. 2005; Jaroudi et al. 2009
α TUB	Alpha tubulin	–	Reference gene	

Table 5. Repairable DNA damage type, proper functions, accession number and primer sequence of six investigated genes

Gene symbol	Genebank accession no.	Primers	Product Size (bp)
MGMT Direct Reversal	XM_003483574	F/acttgcaggtccagaggaga R/tgcagcagcttccataacac	168
UDG Base excision repair	XM_003132925	F/cagctccgtcaagaagatcc R/gctgaggtgcttcttccaac	175
XPC Nucleotide excision repair	AF041032	F/atccgacgaagattctgagc R/tcttcttgcctcctttacgc	179
MSH2 Mismatch repair	NM_001195357	F/tggtcccaatatgggaggta R/catttcagccatgaatgtgg	184
XRCC6 Non-homologous end-joining	NM_001190185 XR_045703	F/aacggaaggtgccctttact R/cttttagccattgcctcagc	223
RAD51 Homologous recombination	NM_001123181	F/attctgaccgaggcagctaa R/atgggaagctggcatgttac	224
TUBAl* Cell cycle	NM_001044544	F/tggaccacaagtttgacctgatg R/gtcctcacgggcctcagaaa	101

*indicate endogenous reference gene

Table 6. Decondensation status of sperm head at 4 h postinjection (3 h after electric activation)

Treatment	No of examined oocytes	Oocyte with decondensed sperm head (%)	Oocyte with 2PB+2PN (%)
Fresh	108	62.49 ± 9.97	12.80 ± 5.29
Tre 0 mM	123	71.53 ± 10.39	8.86 ± 5.21
Tre 15 mM	130	67.23 ± 6.61	20.44 ± 6.10

Data were presented as mean ± SEM of 5 replicates.

PB; polar body, PN; pronucleus, Fresh; ejaculated fresh sperm, Tre 0 mM; sperm freeze-dried in basic freeze-drying medium without trehalose, Tre 15 mM; sperm freeze-dried in basic freeze-drying medium supplemented with 15 mM trehalose

Table 7. Nuclear status of oocytes assessed by staining at different maturational stages

Time of IVM (h)	No of oocyte examined	Maturational stages			
		No (%) GV	No (%) GVL	No (%) M I	No (%) M-II
0	120	120 (100 ± 0.0)	–	–	–
20	122	–	122 (100 ± 0.0)	–	–
33	116	–	5 (4.3± 0.3)	111 (95.7 ± 0.3)	#
44	309	–	–	118 (38.2 ± 0.9)	191 (61.8 ± 0.9)

GV; germinal vesicle, GVL; late germinal vesicle, M-I; metaphase-I and M-II; metaphase-II stages. #: at 33 h of IVM, M-II oocytes were removed by observation (5.3%) before staining.

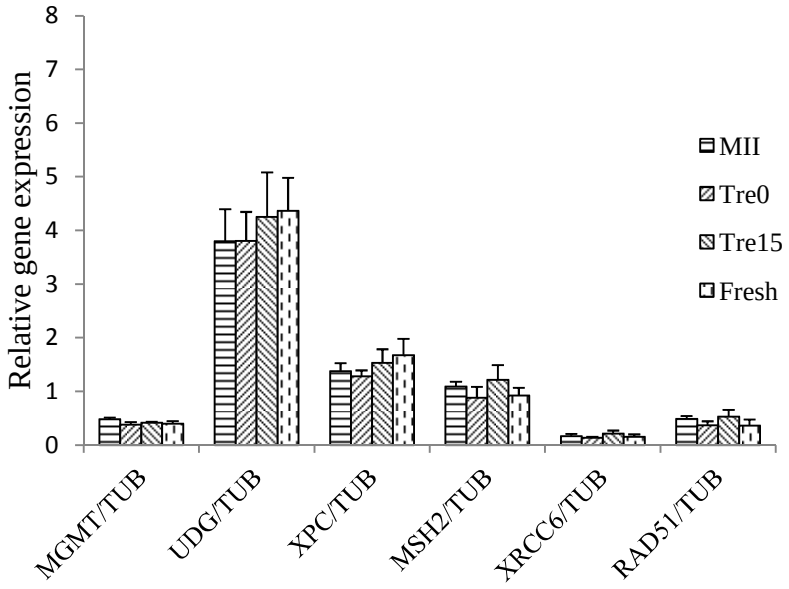

Fig. 6. Relative gene expression of 6 DNA repair genes in oocytes at 4 h after ICSI. Data were obtained from 3 biological samples with duplicate.

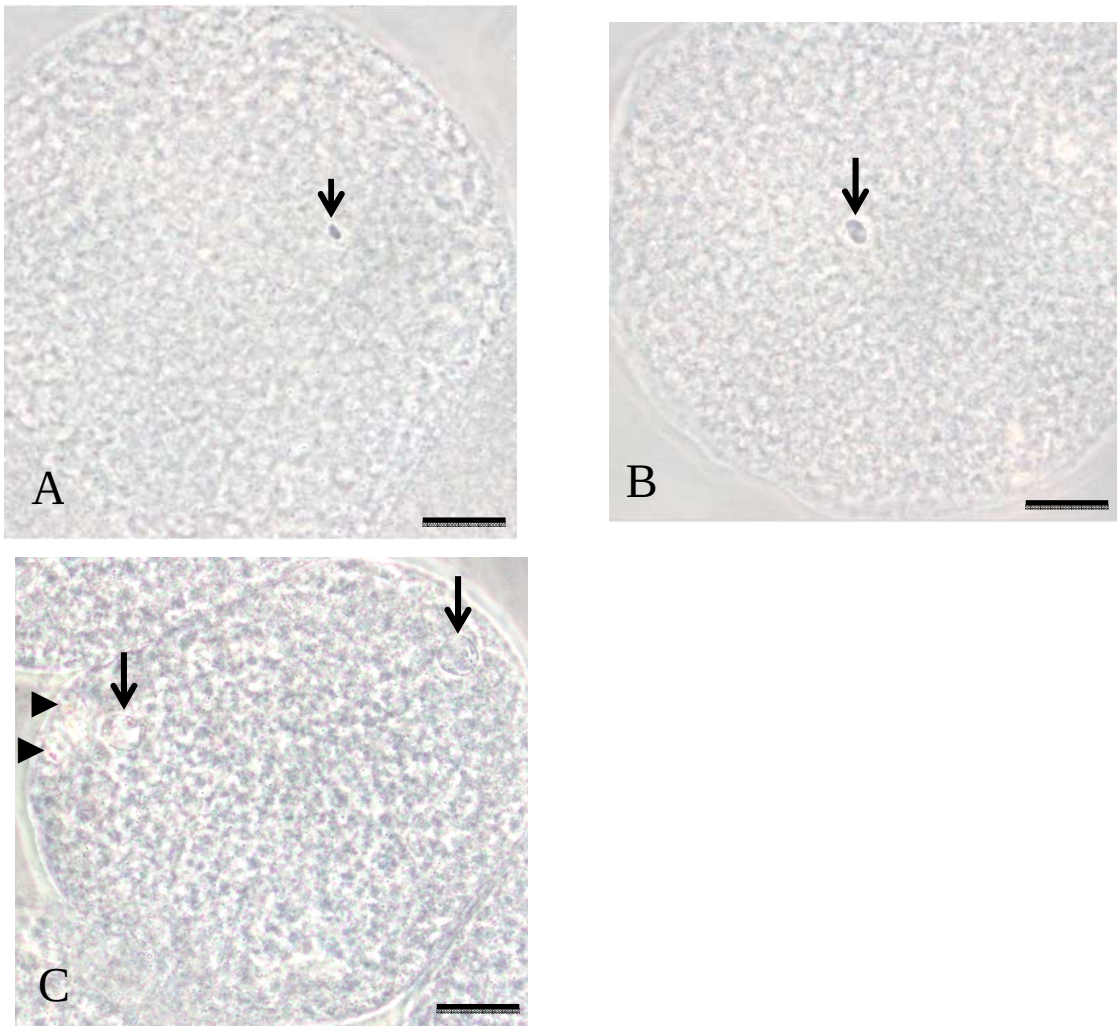

Fig. 7. Decondensation status and PN formation of sperm at 4 h after ICSI. Intact sperm head and decondensed sperm head (arrows in A and B, respectively). Newly formed female (left) and male (right) pronuclei (arrows in C); the first (upper) and second (lower) polar bodies (arrowheads in C). Scale bar = 20 μm.

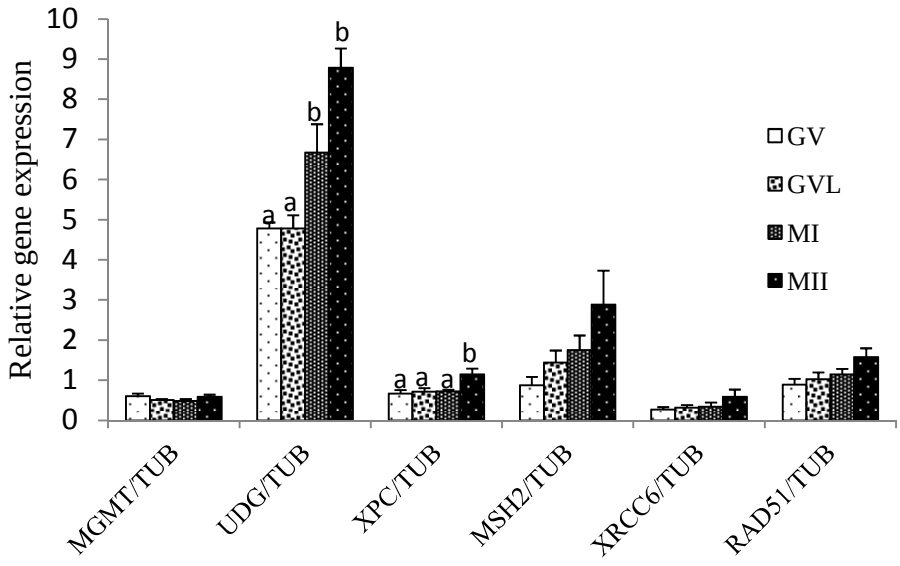

Fig. 8. Relative gene expression of DNA repair genes in oocytes at different stages of *in vitro* maturation. Data were obtained from 3 biological samples with duplicate. [a, b] Data with different superscripts in each gene show statistically significant differences, $P<0.05$.

Chapter IV

Effect of sperm treatment with Triton X-100 to remove plasma membrane on fertilization of IVM porcine oocytes

Introduction

The successful decondensation of sperm nuclear after being injected into the oocytes is a prerequisite factor for the following events of ZGA. The pretreatment of frozen-thawed sperm with Triton X-100 (TX) for disrupting sperm membrane has been applied to improve the fertiation ablility of ICSI-oocytes with the certain findings. In mice, removal of both sperm membranes and acrosome before the injection procedure improved the success of ICSI (Morozumi & Yanagimachi 2005). Lee and Yang (2004) also found that an intact membrane and tail structure of boar sperm are not essential for embryo development by ICSI, even dead porcine sperm, at the early stage of necrosis caused by plasma membrane damage, support better embryo development compared with live non-damaged sperm. This result suggested that prior removal or damage of sperm membrane would increase the success rate of ICSI in species whose sperm have stable membranes. Membrane-damaged boar sperm are, thus, beneficial to sperm decondensation during ICSI.

Sperm pretreatment might help to enhance sperm head decondensation, PN formation and subsequent development of sperm-injected oocytes. The chemicals such as calcium ionophore (Wei & Fukui 1999; Nakai et al. 2003), Triton X-100 (Nakai et al. 2006; Watanabe et al. 2010) and dithiothreitol (DDT) (Rho et al. 1998; Wei & Fukui 1999; Nakai et al. 2006; Watanabe & Fukui 2006; Watanabe et al. 2010) can be used to increase the plasma membrane permeability and sperm head decondensation. DTT is a disulfide reducing agent that induces the reduction of the protamine disulfide bonds in sperm nuclei and leads to the decondensation of the sperm heads. The targets of each treatment in each study were different; sperm plasma membrane for TX, and the disulfide bond in sperm nuclei for DTT. Therefore, it was inferred that DTT was the most severe treatment compared with the others (Watanabe et al. 2010). There is supporting evidence that prolonged DTT treatment (50–60 min; Yong et al. 2005, Watanabe & Fukui 2006) degraded the fertilization ability and developmental competence of porcine oocytes. Similarly, a combination of TX and DTT treatments (Nakai et al. 2006) did not increase the efficacy of embryo production in pigs. In addition, when examined at DNA and chromosomal levels, prolonged DDT

treatment also induced chromosome aberration with 83.0% in the group treated for 60 min (Watanabe et al. 2010).

According to Kasai et al. (1999), TX treatment promoted oocyte activation and sperm decondensation of human sperm when they were injected into mouse oocytes. In contrast to this study, some other studies showed that sperm treatmens with TX (Szczygiel & Ward 2002; Tian et al. 2006) and with TX combined DDT (in mice, Szczygiel & Ward 2002; in pigs, Nakai et al. 2006) were not beneficial to the development of ICSI-derived embryos. In addition, Szczygiel & Ward (2002) indicated that simultaneous treatment of sperm with TX and DTT induces extensive chromosomal breakage and then suggested that such a treatment should not be attempted in human ICSI. Therefore, only TX for sperm treatment without DDT was used in the present experiment.

Although effect of TX treatment on decondensation, male PN formation and subsequent development of frozen-thawed sperm were reported in different species by many research groups. Taking into consideration that response of freeze-dried sperm (with motionless status) to TX treatment may be different from frozen-thawed sperm (with motion status). Moreover; there has been no report about effect of TX treatment for freeze-dried sperm on fertilization of IVM porcine oocytes after ICSI. The objective of this Chapter was to assess effect of TX treatment on fertilization of IVM oocytes injected with freeze-dried sperm pretreated with different concentrations of TX (0, 0.5% and 1%).

Materials and Methods

In vitro *matured (IVM) oocyte preparation*

Oocyte collection and IVM were conducted as described (Kikuchi et al. 2002a). Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace - Large White - Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35^{o}C. Cumulus-oocyte complexes (COCs) were collected from follicles 2-6 mm in diameter in Medium 199 in air (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum, 20 mM Hepes, 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 µL of maturation medium for 20-22 h in four-well dishes. The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39^{o}C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, cumulus cells were removed from oocytes by repeated pipetting in Medium-199 in air supplemented with 150

IU/ mL hyaluronidase. Denuded oocytes with the first PB were harvested under a stereomicroscope and used as IVM oocytes.

Sperm collection, FD and rehydration

Sperm collection and FD were conducted as described previously (Men et al. 2013). Briefly, ejaculated semen was collected from a Landrace boar and transferred to the laboratory within 1 h. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × g at 30^{o}C and the seminal plasma was removed. The pellet was resuspended in FD medium containing trehalose prewarmed at 30^{o}C, and the supernatant was removed after centrifugation. The final pellet was then resuspended in FD media containing 0 or 15 mM trehalose at a final concentration of 4x10^{8} cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial, then the vials were covered with aluminum foil and placed in a refrigerator at –80^{o}C for at least 4 h. The aluminum foil was replaced by a rubber cap with small gaps between the cap and the vial, and then they were placed in a FD system. The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at –30^{o}C and then increased to 30^{o}C during the last 1 h 20 min (0.75^{o}C/ min). After flushing with inactive N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps. The freeze-dried samples were transferred to a refrigerator and stored at 4^{o}C under dark condition until usage. For rehydration, the same volume (1 mL) of deionization distilled water was added to vials immediately after opening the rubber and aluminum caps using a decapper. The sperm suspension was centrifuged for 2 min at 600 × g and the sperm were washed with PBS (–) containing 5 mg/mL BSA, then resuspended in the same buffer. The sperm suspension was sonicated for several seconds to isolate sperm heads and kept at room temperature prior to ICSI.

Pretreatment of freeze-dried sperm with Triton X-100

Sperm freeze-dried in FD medium containing 0 and 15 mM trehalose were used in this experiment. After rehydration and centrifugation to remove FD medium, sperm were resuspended in PBS (–) containing 0, 0.5 and 1 % TX for 10 min at room temperature. After exposure to TX, sperm were washed twice in PBS-BSA, sonicated several seconds to isolate the sperm head and maintained at room temperature prior to injection to IVM oocytes.

ICSI and oocyte stimulation

ICSI was carried out as previously described (Nakai et al. 2006) with some modifications (Men et al. 2013). Briefly, sperm were kept in IVC-PyrLac -Hepes-PVP. About 30 IVM oocytes were transferred to a 20-μL drop of Medium 199 in air. A small volume (0.5 μL) of the sonicated sperm suspension was transferred to a 2-μL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil. A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator. ICSI was completed within 2 h after rehydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. The end of injection was considered as 0 h postinjection. As a control group, mature oocytes were handled the same procedure without sperm (sham group). One hour postinjection, the oocytes were transferred to an activation solution consisting of 0.28 M d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk down the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs under the same condition for each group using a somatic hybridizer, then washed three times and cultured in IVC PyrLac.

Assessment of fertilization status

For assessment of fertilization status, the oocytes injected with freeze-dried sperm from trehalose 0 and 15 mM groups that had pretreated with different concentrations of TX were fixed at 10 h postinjection (i.e. 9 h after electric activation) in fixative solution with a 1:3 mixture of acetic acid:ethanol (v/v) under vaseline-supported coverslips for several days. They were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for several minutes, and then their fertilization status was examined using phase-contrast microscopy.

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989) then subjected to one way-ANOVA using R packages 3.0.1 (R Core Team 2013). As the difference is found in groups by ANOVA, further analysis is conducted by Tukey's posthoc test using the R packages. Differences at $P<0.05$ were considered to be significant.

Results

Fertilization status of oocytes injected with freeze-dried sperm after Triton X-100 treatment

The oocytes with 2 PB and 2 PN were considered as fertilized normally. Oocytes with more than 2 PB and/or 2 PN or without PN were considered as abnormally fertilized oocytes. Results are shown in Table 8. Normal fertilization rate in the 15 mM trehalose group treated with 0.5% TX was highest among groups but no significant difference was found. There were no significant differences in the rates of normal fertilization and abnormal fertilization of porcine oocytes at 10 h after ICSI between the 0 mM and 15 mM trehalose groups. Moreover, when freeze-dried sperm were incubated in different concentrations of TX for 10 min at room temperature, these parameters also did not differ among groups treated with 0, 0.5 and 1 % TX. The rate of oocytes with 2 PB and 2 PN in the sham group was significantly lower than those of other sperm injected groups ($P<0.001$). The rate of the oocytes with 2 PB and one female PN (considered as activated oocytes) in sham group was 67.5% (data not shown in the tables or figures).

Discussion

The result of this experiment showed that freeze-dried boar sperm treated with TX at different concentrations did not improve normal fertilization. It is reported that the delay in oocyte activation and decondensation of sperm chromatin after sperm injection is caused by the existence of the sperm plasma membrane (Katayama et al. 2002b). Removal of plasma membrane by treatments with different surfactants have been attempted to promote sperm chromatin decondensation and oocyte activation. Previous studies suggest that removal of sperm plasma membrane by treatment with TX most likely facilitates the exposure of soluble sperm factors in the cytoplasm of the sperm to the ooplasm which is considered to be beneficial for nuclear decondensation and subsequent zygote activation. Kuretake et al. (1996) demonstrated that in mouse ICSI, sperm treated with TX could fertilize oocytes with the same efficiency as intact sperm heads, resulting in normal preimplantation and postimplantation development (i.e. live offsprings) and the result was comparable to that of the control group in their experiments (Kuretake et al. 1996; Kimura et al. 1998). Moreover, treating human sperm with TX before ICSI resulted in the fastest and most efficient oocyte activation and sperm head decondensation, suggesting that TX is considered to be beneficial rather than detrimental (Kasai et al. 1999).

However, the result obtained in this experiment suggested that the disruption of plasma membrane by FD process seems to have a similar effect as exposure with TX of membrane intact sperm. Therefore, further treatments to the membrane of freeze-dried sperm did not improve male PN formation as well as normal fertilization. Damages to sperm membrane resulting from dehydration can be attributed to the changes in the physical state of lipid membrane. However, different findings about effect of treatments for sperm were reported in different laboratories and seem to depend on species and sperm types. Because the physical and chemical properties of the sperm plasma membrane are varied from species to species (Kasai et al. 1999). It is well-documented that active sperm-borne oocyte-activating factors (SOAFs), important components of the male gamete, appear during transformation of the round spermatid into the spermatozoon (Kasai et al. 1999). SOAFs are partly localized in the perinuclear theca in the postacrosomal region (Kimura et al. 1998) and under the plasma membrane over the equatorial segment of the acrosome (Sutovsky et al. 2003). Interestingly, the action of SOAFs is not highly species-specific since mouse oocytes are activated by injection of sperm from foreign species, such as the hamster, rabbit, pig, human and even fish (Kimura et al. 1998). Then, Kim et al. (1999) also reported a same conclusion about no strictly species-specificity of SOAFs by the series of experiments of injection of sperm from mice, cattle or human into porcine oocytes and indicated that sperm of these species can activate porcine oocytes. In this study, male PN formation and pronuclear apposition were observed in porcine oocytes following injection of porcine, bovine, mouse or human sperm although none of the porcine oocytes form any mitotic metaphase nor developed to the two-cell stage. Among SOAFs, PLCζ is thought to be the strongest candidate as a sperm factor that trigers oocyte activation and following early embryonic development (Yoneda et al. 2006). The location or distribution of PLCζ differs among species, for examples, between rodents (almost present in sperm head; Kuretake et al. 1996; Yamauchi et al. 2002) and domestic animals (not only present in sperm head, but also in tail; pigs: Nakai et al. 2011 and horses: Bedford-Guaus et al. 2011). For sperm types, fresh sperm, frozen-thawed sperm and freeze-dried sperm are supposed to contain different amount of SOAFs. This leads to different outcomes of fertilization and subsequent development when these sperm were used for injection.

Nakai et al. (2011) demonstrated that treatments for frozen-thawed boar sperm leads to a reduced ability to induce oocyte activation and that this should be taken into consideration when preparing samples for ICSI. However, whether similar findings obtained

when using freeze-dried sperm have not been clarified. The findings in this experiment showed that TX treatment for freeze-dried boar sperm had no positive effect on normal fertilization and abnormal fertilization.

Table 8. Fertilization rate of oocytes injected with freeze-dried sperm head at 10 h after ICSI

Treatment	Total	No (%) normal Fertilization	No (%) abnormal fertilization
Tre 0mM + 1%TX	114	77 (67.54±2.3)	23 (20.18±3.0)
Tre 0mM + 0.5%TX	122	77 (63.11±4.4)	26 (21.31±4.2)
Tre 0mM + 0%TX	94	64 (68.09±5.0)	20 (21.28±5.6)
Tre 15mM + 1%TX	93	66 (70.97±7.7)	17 (18.28±6.7)
Tre 15mM + 0.5%TX	129	100 (77.52±4.6)	19 (14.73±2.7)
Tre 15mM + 0 %TX	68	51 (75.00±9.7)	10 (14.71±4.1)
Sham injection	80	0 (0.00*±0.0)	8 (10.00±7.0)

Normal fertilization: oocytes with 2 PB and 2 PN visible;

Abnormal fertilization: oocytes with more than 2 PB and/or 2 PN or without PN.

*shows significant difference within the column (ANOVA-Tukey posthoc test, $P<0.001$)

At least 4 replicates were done in each group

Tre 15 mM: 15 mM of trehalose included in sperm freeze-drying medium

0, 0.5, 1% were concentrations of Triton X-100 (TX) used in experiments

Sham: same handling for injection conducted without sperm

Chapter V

Effect of oocyte treatment by centrifugation and electric activation on fertilization of IVM porcine oocytes

Introduction

Centrifugation to stratify cytoplasm facilitates the observation of PN in oocyte or sperm penetrated in ooplasm, especially useful for species such as pigs and cattle whose oocytes has dark ooplasm with rich lipid dorplets (Tatham et al. 1995; 1996; Rho et al. 1998). The centrifugation for polarization of lipid droplets in the cytoplasm before cryopreservation was reported to increase significantly the cleavage rate of oocytes that survived after freezing-thawing and also the development rates to blastocysts (Otoi et al. 1997).

For some species such as mice (Kimura & Yanagimachi 1995; Kuretake et al. 1996), hamsters (Hoshi et al. 1992), human (Tesarik & Sousa 1995) and rabbits (Keefer 1989), ICSI alone is considered to be sufficient to activate oocytes for embryonic development. Even external activation stimulus(li) was not required for the development to the blastocyst stage after injection of freeze-dried sperm in rats (Kaneko et al. 2009) and mice (Wakayama & Yanagimachi 1998, Kawase et al. 2007). It seems that manipulations associated with ICSI may have provided sufficient stimulus for oocyte activation in these species.

However, it is reported that external activation stimulus(li) (by sperm extract) is required for blastocyst development after ICSI with freeze-dried sperm in horses (Choi et al. 2011). In rabbits, chemical activation treatments such as calcium ionophore with cycloheximide and 6-DMAP, were essential for blastocyst production (Liu et al. 2004). In cattle, necessity for additional oocyte activation before or after the ICSI procedure was also confirmed (Suttner et al. 2000). In pigs, artificial oocyte activation after ICSI was reported to result in better fertilization and embryonic development (male PN formation and blastocyst development) compared with injection alone (Lee et al. 2003; Nakai et al. 2003). Catt and Rhodes (1995) also showed that boar sperm injected into oocytes after ICSI had arrested in early decondensation state without any stimulation. Protocols employing Ca^{2+} ionophore, Ca^{2+} ion containing solution and electrical stimulation have been developed for artificial induction of activation in porcine oocytes (Probst & Rath 2003). The result showed that, when sperm injected oocytes were treated with Ca^{2+} ion containing solution, activation and fertilization rates were significantly higher as compared with those without treatment.

However, a similar rate of activation and a decreased rate of fertilization were recorded in the Ca^{2+} ionophore treated group in relation to non-treated group. For electrical activation, both activation and fertilization rates were significantly increased. These findings explained the importance of artificial activation on preimplantation development of porcine ICSI oocytes. Among the methods of artificial activation, electrical stimulation is the most common especially for producing somatic cell nuclear transfer embryos (Lee & Yang 2004) and is very effective for embryo production after ICSI (Lee et al. 2003; Nakai et al. 2003; Kurebayashi et al. 2000; Lee & Yang 2004). Several factors are reported to influence an oocyte's response to electrical activation, including oocyte age, applied voltage/field strength, and the pulse number and its duration. In addition, there might be interactions between these factors (Zhu et al. 2002).

Electrical stimulation to oocytes is consiered to be resulted in the formation of pores in the plasma membrane, which allows the uptake of extracellular calcium (Onodera & Tsunoda 1989) leading to an increase in intracellular calcium concentration and finally oocyte activation. The Ca^{2+} oscillation in the ooplasm following sperm-oocyte interaction at fertilization is reported to be the essential regulator of oocyte activation and is responsible for postfertilization events including meiotic resumption and cortical granule exocytosis (Hoodbhoy & Talbot 1994).

Failure of formation of a male PN was the major cause for fertilization failure in ICSI porcine embryos (Lee et al. 2003). Sperm nuclear decondensation is reported to be delayed in ICSI compared with IVF (Katayama et al. 2002a); electric stimulation is considered to promote the intracellular calcium influx and sperm decondensation process. In a study of effect of oocyte activation by electric stimulus on embryo development of ICSI-derived porcine oocytes, blastocyst formation rate of sperm injected oocytes was much higher in the group with electric stimulus in relation to those of without electric stimulus (27.1% vs. 4.1%, respectively, Nakai et al. 2006). However, the rate of normal fertilization, defined by formation of two (first and second) PBs and two (male and female) PNs was not examined in this study. It is important to assess sperm head decondensation and pronuclear formation in oocytes before focusing on the development to the blastocyst stage. Because, electric stimulation was applied for oocytes in all my experiments, it cannot be ruled out that blastocyst obtained after culture for 6 days might be parthenogenetic origin. The rate of oocytes with 2 PBs, a male PN and a female PN (referred to normal fertilization) has been used as a good parameter to evaluate the fertilizability of sperm and oocytes after ICSI (Heuwieser et al. 1992; Catt & Rhods 1995).

Concerning to strategies to improve *in vitro* embryonic development using freeze-dried sperm, the objective of this Chapter was to examine the effect of oocyte centrifugation and electric stimulation on fertilization by ICSI of IVM porcine oocytes.

Materials and Methods

Preparation of in vitro *matured (IVM) oocytes*

Oocyte collection and IVM were conducted as previously described (Kikuchi et al. 2002a). Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace - Large White - Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35^{o}C. Cumulus-oocyte complexes (COCs) were collected from follicles 2–6 mm in diameter in Medium 199 in air (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum, 20 mM Hepes, 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 µL of maturation medium for 20–22 h in four-well dishes. The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39^{o}C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, cumulus cells were removed from oocytes by repeated pipetting in Medium 199 in air supplemented with 150 IU/ mL hyaluronidase. Denuded oocytes with the first PB were harvested under a stereomicroscope and used as IVM oocytes.

Sperm collection, FD and rehydration

Sperm collection and FD were conducted as described previously (Men et al. 2013). Briefly, ejaculated semen was collected from a Landrace boar and transferred to the laboratory within 1 h. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × g at 30^{o}C and the seminal plasma was removed. The pellet was resuspended in FD medium containing trehalose prewarmed at 30^{o}C, and the supernatant was removed after centrifugation. The final pellet was then resuspended in FD media containing 0 or 15 mM trehalose at a final concentration of 4×10^8 cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial, then the vials were covered with aluminum foil and placed in a refrigerator at –80^{o}C for at least 4 h. The aluminum foil was replaced by a rubber cap with small gaps between the cap and the vial, and then they were placed in a FD system. The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at –30^{o}C and then increased to 30 ^{o}C

during the last 1 h 20 min (0.75 ^{o}C/ min). After flushing with inactive N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps. The freeze-dried samples were transferred to a refrigerator and stored at 4 ^{o}C under dark condition until usage. For rehydration, the same volume (1 mL) of deionization distilled water was added to vials immediately after opening the rubber and aluminum caps using a decapper. The sperm suspension was centrifuged for 2 min at 600 × g and the sperm were washed with PBS (–) containing 5 mg/mL BSA, then resuspended in the same buffer. The sperm suspension was sonicated for several seconds to isolate sperm head and kept at room temperature prior to ICSI.

Oocyte centrifugation to stratify cytoplasm before and after ICSI

IVM oocytes with the first PB were subjected to centrifugation according to Fahrudin et al. (2007); Viet Linh et al. (2011) with some modifications. In brief, IVM oocytes were transferred to a 1.5-ml micro centrifugation tube (20–30 oocytes in one tube) and then centrifuged at 10,000 × g for 20 min at 37^{o}C in Medium 199 to stratify the cytoplasm (Experiment 1 and 2). For Experiment 3, sperm injected oocytes were centrifuged at 10,000 × g for 5 min at 37^{o}C in Medium 199, followed by electrical activation and *in vitro* culture. In this experiment, cytochalasin was not used during centrifugation.

ICSI and oocyte stimulation

ICSI was carried out as previously described (Nakai et al. 2006) with some modifications (Men et al. 2013). Briefly, sperm were kept in IVC-PyrLac- Hepes-PVP. About 30 IVM oocytes were transferred to a 20-µL drop of Medium 199 in air. A small volume (0.5 µL) of the sonicated sperm suspension was transferred to a 2-µL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil. A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator. ICSI was completed within 2 h after rehydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. The end of injection was considered as 0 h postinjection. One hour postinjection, the oocytes were transferred to an activation solution consisting of 0.28 M d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk down the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 µs under the same condition for each group using a somatic hybridizer then washed three times and cultured in IVC PyrLac.

Assessment of fertilization status

For assessment of fertilization status, the oocytes were fixed at 10 h postinjection in fixative solution with a 1:3 mixture of acetic acid: ethanol (v/v) under vaseline-supported coverslips for several days. They were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for several minutes, and then their fertilization status was examined using phase-contrast microscopy.

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989) then subjected to one way-ANOVA using R packages 3.0.1 (R Core Team 2013). As the difference is found in groups by ANOVA, further analysis is conducted by Tukey's posthoc test using the R packages. Differences at $P<0.05$ were considered to be significant.

Results

Relative distance of chromosome spindle complexes relative to PB before and after centrifugation

Three types of location were classified (type A: chromosome spindle complexes (CSC) are closest to PB, type B: CSC are located at the same side with PB compared with equatorial line, type C: CSC are located at the other side with PB compared with equatorial line). The ratio of each type was shown in Fig. 9. Before centrifugation (Fig. 10A), most of oocytes show type A (76.3%; Fig. 10C) and the remaining oocytes show type B (23.7%; Fig. 10D). No oocyte with type C was observed. However, after centrifugation, the ratio of oocytes with type B increased twice in relation to before centrifugation and a small portion of type C appears (1.8%). Centrifugation made the distance between CSC and PB become much farther. On the other hand, after centrifugation, three distinctive layers of cytoplasm were observed including 'transparent', mitochondria and lipid layer, as presented in Fig.10B).

In this experiment, we also investigated the distribution of CSC in cytoplasm after centrifugation and staining with aceto-orcein. The result showed that the oocytes with CSC located in the 'transparent' layer occupied about 73.5% (Fig.11B) and the oocytes with CSC located in the mitochondria layer occupied about 18.2% (Fig.11A) and the remaining oocytes with CSC were located in the lipid layer (9.4%) (data not shown in tables or figures).

Effect of sperm injection into 3 different layers of centrifuged oocytes on fertilization of IVM porcine oocytes (effect of centrifugation before injection)

As shown in Fig. 12, normal fertilization and abnormal fertilization rates were not significantly different among the control (non-centrifugation) and experimental groups. In term of normal fertilization, the lowest rate (61.6%) was recorded in the group that sperm injected into mitochondrial layer and the highest rate (68.9%) was recorded in the group that sperm injected into lipid layer. An example of an abnormally fertilized oocyte was shown in Fig. 13D.

When sperm were injected into 3 layers of oocytes after centrifugation, sperm heads could be visible clearly in live cytoplasm when they were injected into the mitochondrial layer (Fig. 13A) and the transparent layer (Fig. 13C). However, in case of sperm injection into lipid layer, the sperm head is not visible (Fig. 13B).

Effect of oocyte centrifugation after ICSI and of electric stimulation on fertilization of IVM porcine oocytes (effect of centrifugation after injection)

The rates of normal fertilization, abnormal fertilization and metaphase-III (M-III) arrest were shown in Fig. 14. The rates of normal fertilization in control group was significantly higher ($P<0.05$) than those of remaining groups. The normal fertilization rate was significantly higher ($P<0.05$) in the groups with electric activation applied compared with no electric activation, irrespective of centrifugation (63.0% vs. 29.2% for centrifuged groups and 71.9% vs. 31.8% for non-centrifuged groups, respectively). However, the abnormal fertilization rate in the groups subjected to both centrifugation and electric activation was significantly higher ($P<0.05$) than those of the groups without electric activation, irrespective of centrifugation and was not different from the control group (non-centrifugation, with electric activation).

The oocytes with the presence of metaphase-like chromosome plates (Fig. 15) at 10 h after injection are referred to as M-III arrested oocytes. The rates of the M-III arrested oocyets were significantly higher ($P<0.001$) in the groups without electric activation compared with those in the groups subjected to electric activation, irrespective of centrifugation (40.5% vs. 6.9% for centrifuged groups and 35.8% vs. 4.5% for un-centrifuged groups, respectively).

Discussion

Centrifugation facilitates the visible of sperm release in ooplasm and therefore may reduce the amount of medium injected to oocytes. However, the findings in this experiment showed that there were no benefits of oocyte centrifugation before ICSI on normal fertilization (Fig. 12). In addition, centrifugation makes the distance between CSC and PB further, thus, may induce the higher risk of disruption of CSC by the injection procedure. And redistribution of cytoplasm after centrifugation did not promote decondensation and PN formation; in contrast, it delayed the oocyte activation and probably interrupted the normal decondensation process necessary for PN formation.

Oocyte centrifugation after ICSI also was not beneficial on normal fertilization (Fig. 14). Even when the oocytes were centrifuged, the abnormal fertilization rate was significantly higher in the group applied for electric activation than those of the groups received no electric activation. In the category of abnormal fertilization examined in this experiment, most of oocytes presented 3 PN with only one PB. Because only one sperm head was injected to one oocyte, it is suspected that the oocytes did not complete meiosis; as a consequence, no second PB was extruded. Such oocytes would have 2 female PNs, and one male PN. There are two possibilities to explain for the higher rate of the oocytes with 3 PN in the electric activated group. Normally, electric pulse induces intracellular cacium oscillation and promotes the sperm chromatin decondensation and female PN and also male PN formation if pserm has been in ooplasm. However, when electric stimulus was applied for oocyte activation after centrifugation, it is most likely that the normal resumption of meiosis, chromatin remodeling and PN formation are disrupted or inhibited. Centrifugation coupled with electric stimulation would disturb the extrusion of the second PB. Oocyte centrifugation more likely triggers the disruption of CSC into 2 or more pieces, resulting in the embryos with more than two PNs.

The result in present study was in agreement with the findings of Yong et al. (2005) who reported that transformation from sperm head decondensation to male PN was delayed in centrifuged oocytes compared with non-centrifuged oocytes. The delay may be caused by slow or irreversible redistribution of internal organelles including lipid droplets of ooplasm after centrifugation. Because the homogeneous distribution of ooplasmic lipids plays an important role in the transformation of sperm head into male PN (Kikuchi et al. 2002b). In addition, centrifuged oocytes might also lose the opportunity of syngamy with sperm nuclei at appropriate time point (Yong et al. 2005). Interestingly, although a significantly lower rate

of male PN was observed in centrifuged oocytes, development to the blastocyst stage was not affected by centrifugation in that work. This is attributed to the parthenogenetic development in these oocytes. Therefore, we suggest that, before discovery of an appropriate method for distinguishing, the blastocysts derived from parthenogenetically activated oocytes or from sperm-injected oocytes, probably, examination of the presence of a male PN in oocytes after fertilization would be more significant.

In cattle, Chung et al. (2001) suggested that centrifugation of oocytes matured *in vitro* has no detrimental effect on fertilization and subsequent early embryonic development. In addition, Wei & Fukui (1999) also reported that the proportions of bovine oocytes that were successfully injected, survived or showed two PNs were higher when centrifuged oocytes were used in conventional ICSI. Our results revealed that there were no differences in term of normal fertilization rate in porcine oocytes injected with freeze-dried sperm between centrifuged and non-centrifuged oocytes without electric stimulation.

Normal fertilization rate was significantly improved in the groups with electric activation applied compared with no electric activation group, confirming the importance of artificial electric stimulation to porcine ICSI-oocytes. In contrast to the report of Yong et al. (2005) who revealed that there was no effect of electric activation on normal fertilization of porcine ICSI-oocytes, even detrimental effect on oocyte survival, the present result showed that electric stimulation increased significantly normal fertilization rate, irrespective of centrifugation. This difference can be explained by the fact that this research group performed modified ICSI – with the injection of head membrane damaged sperm but not conventional ICSI.

After centrifugation, the clear zone (referred to as transparent layer in our pevious study) could be the site of ICSI of mature oocytes in order to visualize the injected sperm (Fig. 10). This is impossible in non-centrifuged oocytes, which have vesicular elements and lipid droplets that make the ooplasm opaque, unlike in human and mouse oocytes (Tatham et al. 1996). Ooplasm stratification after centrifugation would make it possible to remove these lipid droplets (delipidization) prior to the freezing or vitrification of oocytes or embryos by either further centrifugation or micromanipulation (Nagashima et al. 1994; Pangestu et al, 1995). Since the existence of lipid droplets in the oocytes or embryos subjecting to cryopreservation reduces their survival potential. Tatham et al. (1996) indicated that maturation spindles and cortical cytoskeleton were mostly intact and were firmly anchored to the oocyte cortex beneath the oolemma, even after centrifugation.

The percentage of oocytes enter to the M-III stage, characterized by metaphase-like chromosomes with an elongated metaphase spindle, was significantly higher in the groups without electric activation, suggesting that mechanical injection procedure is not sufficient to induce oocyte activation to proceed to the PN stage. It is reported that activity of maturation promoting factor (MPF) in non-activated oocytes gradually decreases during arrest at the M-II stage, but it is high enough to maintain the M-phase (Kikuchi et al. 1995). This high MPF activity makes the injected sperm nuclei either recondensed into a chromatin mass or changed into metaphase-like chromosomes in mice (Clarke & Masui 1987), pigs (Kikuchi et al. 1999a) and cattle (Abeydeera & Niwa 1992). In cattle, according to Liu et al. (1998), when oocytes were full activated (defined as pronuclear formation), MPF inactivation followed by MAPK (mitogen-activated protein kinase) inactivation occurred quickly; whereas the oocytes undergoing partial activation (defined as exit from the M-II arrest but no PN formation, so called the enter to the M-III stage), MPF was inactivated but MAPK activity remained high. They suggest that a decrease in MPF activity coincided with M-II exit and a decrease in MAPK activity coincided with PN formation (i.e. the inactivation of two these kinases is independent).

However, a small portion of sperm injected oocytes also showed the entering to the M-III stage even after the oocytes had been electrically activated. This might be caused by inconsistent changes in activity of MPF and MAPK in some oocytes after sperm injection and oocyte stimulation. In some cases, electrical stimulation alone triggers a temporary decrease in MAPK activity, but the resumption of its activity was detected after stimulation (Collas et al. 1993; Ito et al. 2004; Nanassy et al. 2007). On the other hand, some porcine IVM oocytes may have reduced levels of sperm decondensing factor(s) because of precocious activation of the oocytes prior to and during ICSI, due to prolonged handling of oocytes and extended exposure to room temperature as demonstrated by Lee et al. (2003). Probably, amount of oocyte-activating factor(s) released by the injected sperm was only enough to partially initiate the physiological cascade of normal fertilization, (i.e. to release the oocyte from M-II arrest).

In conclusion, oocyte centrifugation before ICSI did not improve normal fertilization rate. Oocyte centrifugation after ICSI showed a negative effect on normal fertilization in case of electric activation and no effect on normal fertilization in case of no electric activation applied. Moreover, the significantly higher percentage of the oocytes at the M-III stage in the groups without EA confirmed the importance of additional electric stimulation to activate the porcine ICSI oocytes.

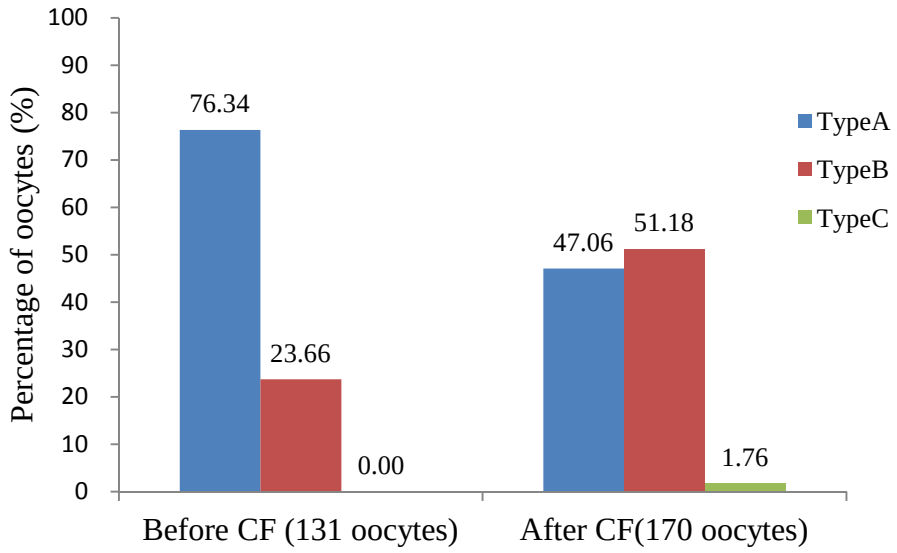

Fig. 9. Location of CSC relative to PB in oocyte before and after CF at 10,000 × g for 20 min. Data are collected from 6 replicates with 131 oocytes for before CF group and 7 replicates with 170 oocytes for after CF group.

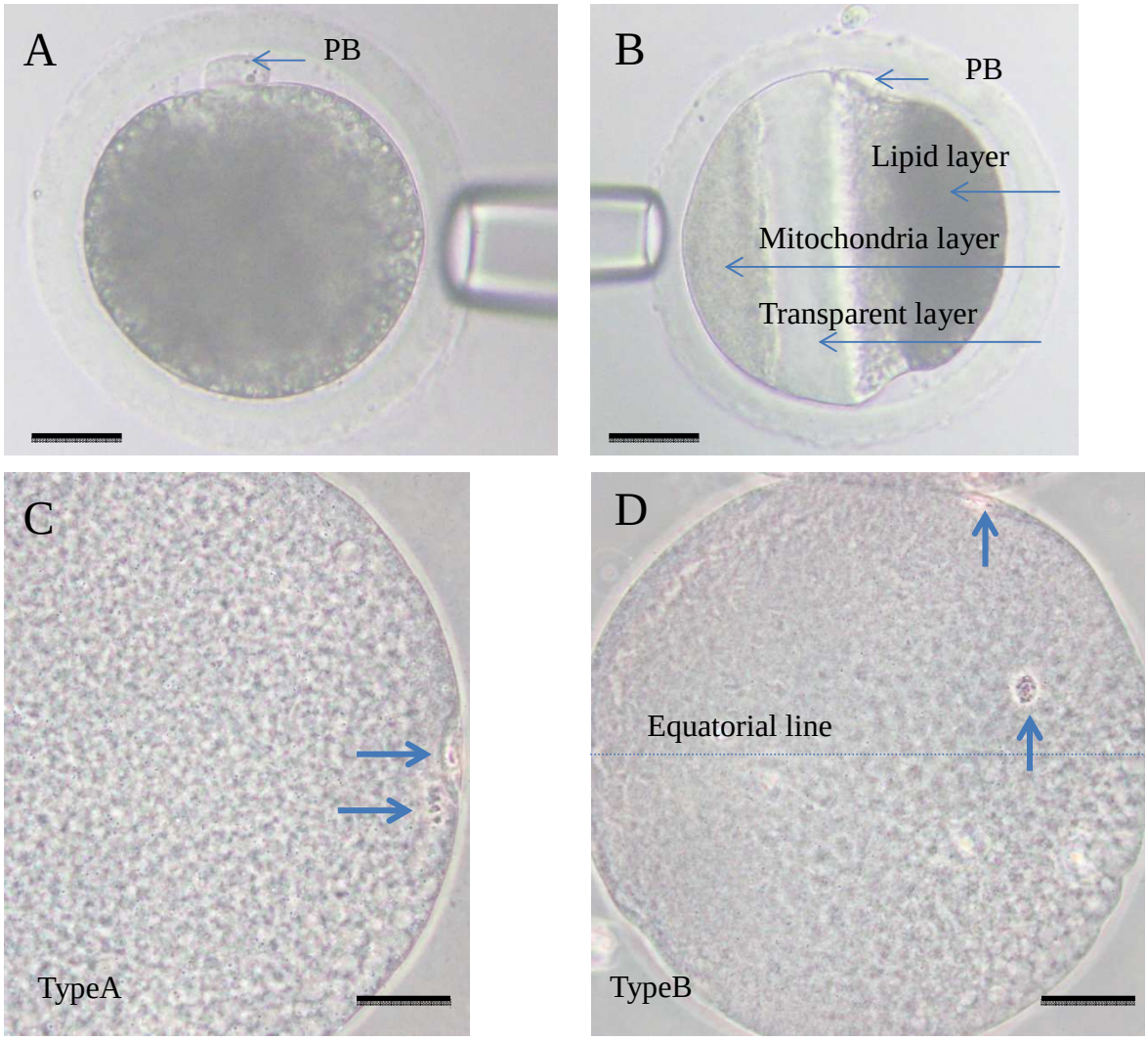

Fig. 10. *In vitro* matured oocytes before (A) and after (B) centrifugation were observed by stereo-microscope (A and B) and stained with aceto-orcein (C and D). After centrifugation at 10,000 × g, for 20 min, at 37^{o}C, three distinctive layers could be observed including lipid layer, mitochondria layer and transparent layer. Before centrifugation, most of oocytes showed with type A (CSC adjacent with PB; C) but after centrifugation, the rate of oocytes with type B increased (CSC migrated farther the way to PB; D). Scale bar: 20 μm.

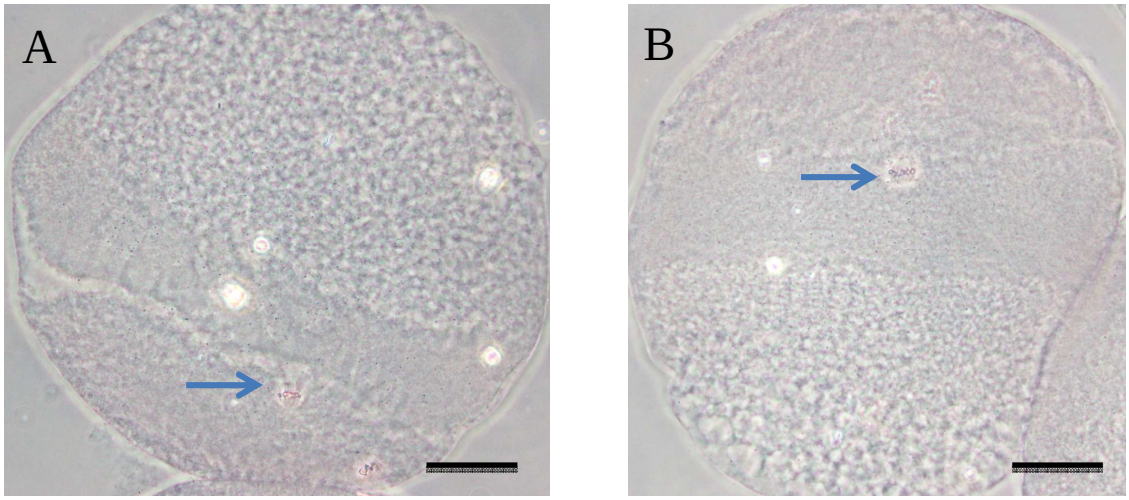

Fig. 11. *In vitro* matured oocytes after centrifugation and stain with aceto-orcein. CSC was located in the mitochondria layer (A) and the transparent layer (B). Scale bar: 20 μm.

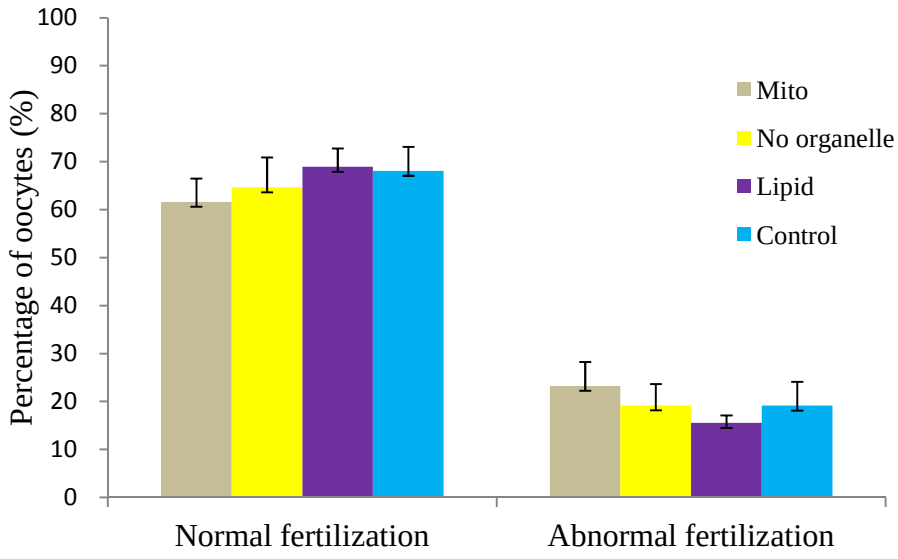

Fig.12. Effect of sperm injection into 3 different layers of centrifuged oocytes on fertilization of IVM porcine oocyte. Data were given as mean ± SEM of 5 replicates in each group.

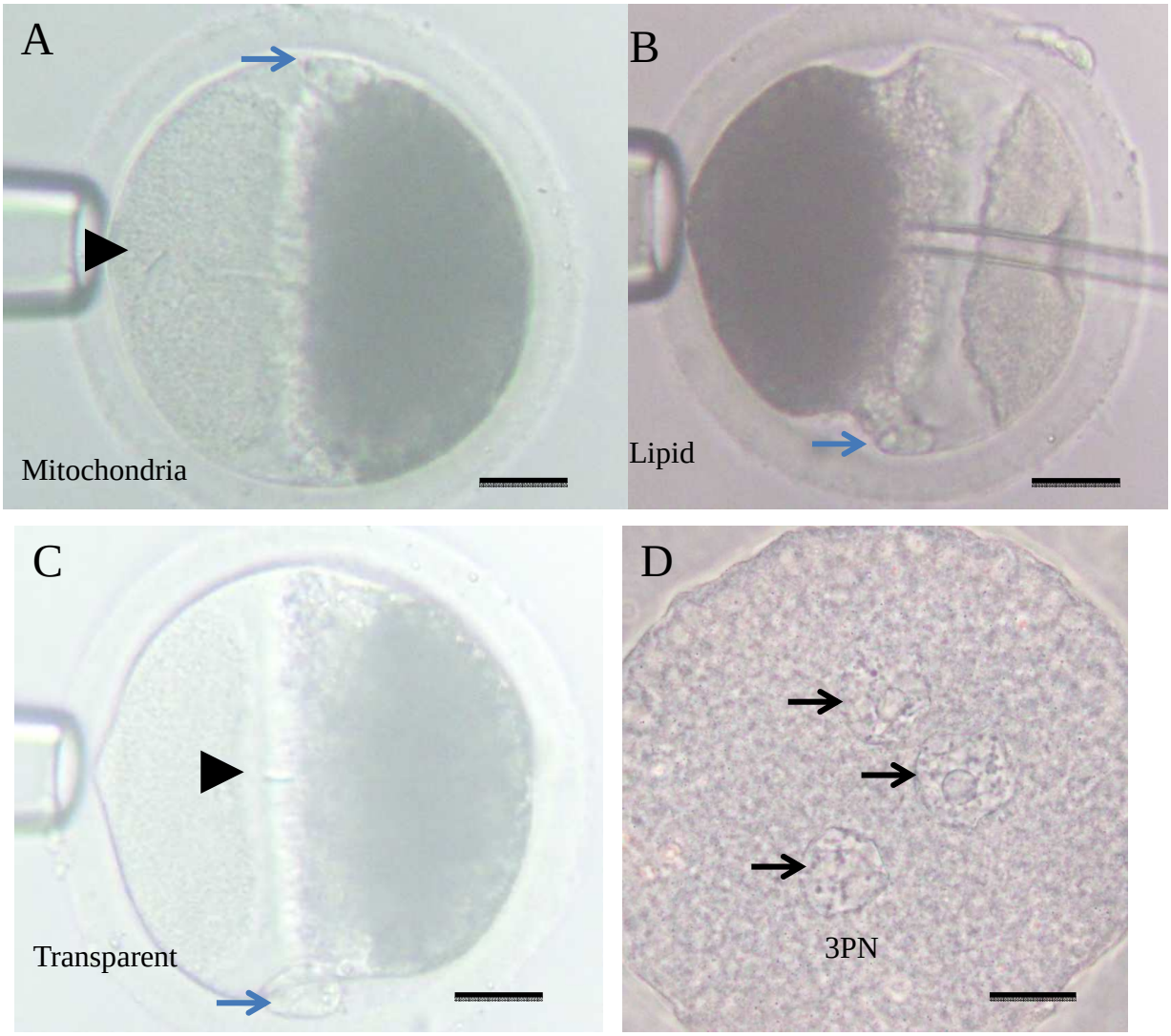

Fig. 13. Sperm injection into 3 layers of oocytes after centrifugation. Sperm heads (arrowhead) could be visible clearly in cytoplasm when they were injected into the mitochondrial layer (A) and the transparent layer (C). In case of sperm injection into lipid layer, the sperm head is not visible (B). An abnormally fertilized embryo with two PB (invisible in this focus) and three PN (D). Scale bar: 20 μm.

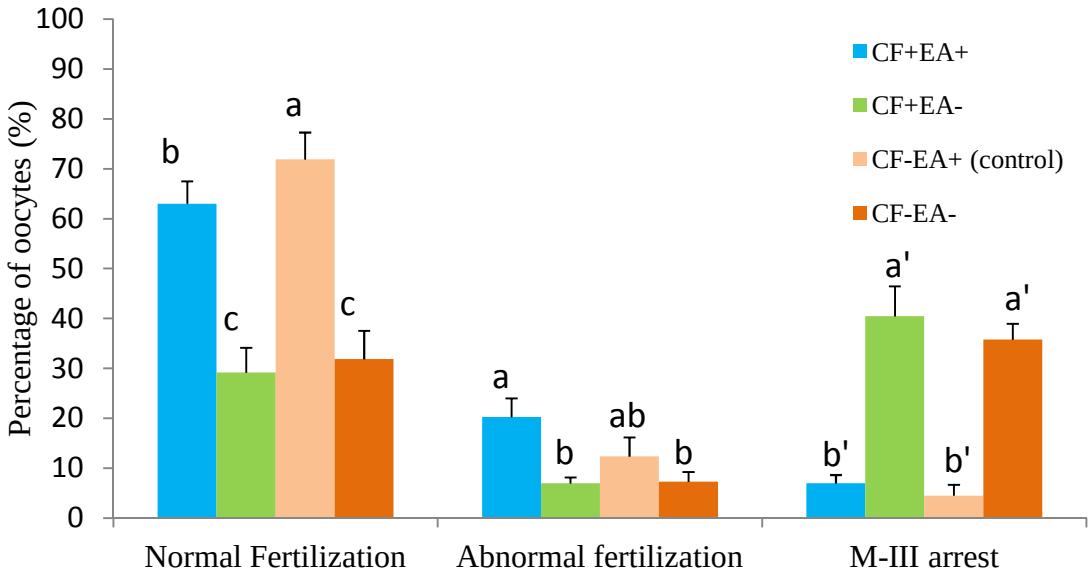

Fig.14. Effect of centrifugation and electric stimulation on fertilization of IVM porcine oocytes. CF: centrifugation, EA: electric activation, +: with CF/EA, -: without CF/EA,

Data were given as mean ± SEM of at least 7 replicates in each group

[a,b,c] statistically significant differences exists among groups (P<0.05)

[a', b'] statistically significant differences exists among groups (P<0.001).

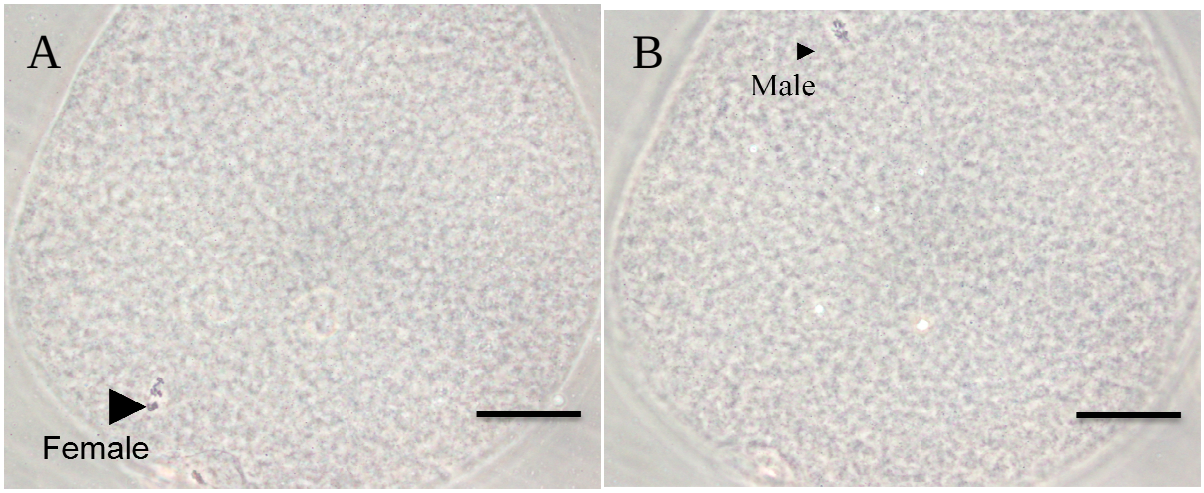

Fig. 15. An oocyte arrested at M-III stage with 2 PB and 2 metaphase-like structures. Oocyte obtained in the group without electric activation. Two metaphase-like structures include one from female origin that is close to PB (A) and the other from male origin (B). Two focuses of one oocyte. Scale bar: 20 μm.

Chapter VI

In vivo developmental ability of freeze-dried sperm injected oocytes after embryo transfer to recipients

Introduction

Because of the high fecundity of pigs, the needs for extra offspring per breeding female are less than in cattle breeding. Moreover, the procedures for embryo collection and transfer mainly require surgical operation, leading to embryo transfer technique in pigs being developed only to a limited extend (Brüssow et al. 2000). However, the increased needs for transfer of porcine genetic material around the world with minimal health risks and low costs, lead to a stronger motivation to use new reproduction technologies in breeding and production in pigs. In embryo transfer technology in pigs, three main techniques have been performed and developed with more improved tendency. Each technique has both advantages and drawbacks as described below.

Surgical procedures for commercial applications have only been used to a limited extent. Gilts are often used as recipients because they are easy to handle and tolerate anesthesia and surgery better than sows. The embryos are transfered in the oviducts or tip of the uterine horns, depending on the developmental stage of the embryos. On average, the pregnancy rate is about 60%, and the litter size is 6.5 piglets, with a range from 17% with 2.4 piglets to 100% with 10.8 piglets (reviewed by Hazeleger & Kemp 2001).

Endoscopic procedures for embryo transfer have been developed also as done by surgical procedures. Nevertheless, the endoscopic procedures require anesthesia and other surgical precautions similar to those of the conventional surgical approaches, and are therefore less applicable for use on individual farms.

Nonsurgical embryo transfer was firstly reported by Polge & Day (1968). However, the poor transcervical accessibility of the uterus of non-estrous sows was mentioned as a major problem for a long time.

In vivo matured oocytes and uterus-flushing *in vivo* derived embryos is the best option for research in which the oocytes and embryos are used as materials. However, *in vivo* embryos collection offers the limited number of embryos and its cost for these procedures is very expensive. Therefore, the usage of them has no high applicability and practicality. Efforts to improve the developmental ability of IVM oocytes have been continued indefatigably. To have large numbers of embryos available, embryos may be

produced *in vitro* by IVM-IVF and IVM-ICSI of oocytes collected from ovaries of slaughtered pigs. Successful production of live piglets by those techniques has been reported (Mattioli et al. 1989; Nakai et al. 2003, respectively); however, overall success rates are still low.

The classical methods to select healthy embryos under IVF and ICSI conditions are based on morphological criteria such as early embryonic cleavage, the number and size of blastomeres, fragmentation degree, and the presence of multi-nucleation at the 4- or 8-cell stages (Fenwick et al. 2002). However, most studies suggest that embryos with proper morphological appearance alone are not sufficient to predict a successful implantation. The application of transcriptomic, proteomic and metabolomic approaches have greatly broadened our understanding of early human embryo development. These hi-technologies may ultimately lead to non-invasive evaluations for oocyte or embryo quality revealing previously hidden information concerning developmental competences of both oocytes and embryos (Assou et al. 2011) in the procedures of embryo selection for the embryo transfer program.

Previous studies suggest that co-transfer of parthenogenetically activated embryos (parthenotes) and embryos derived from *in vitro* fertilization to recipients helps to increase the chance of pregnancy and supports for full-term development (King et al. 2002). Parthenogenesis is the development of an embryo without paternal contribution (Kaufman & Sachs 1975). When they are transferred into the uterus of a surrogate mother, mammalian parthenogenetic embryo will develop to different stages depending on the species but never to term (Kono 2006). Parthenogenetic mouse blastocysts could develop only 11 days *in vivo* after the embryo transfer (Kaufman et al. 1977). The development of parthenogenetic embryos also ends at the 11 day of gestation in rabbits (Onodera & Tsunoda 1989) and at 25 or 26 days of gestation in sheep (Loi et al. 1998). Pig parthenogenetic oocytes derived by various activation methods can develop to the morula or blastocyst stage. Kurebayashi et al. (2000) confirmed that parthenogenetic porcine diploid embryos have the ability to develop up to day 29 after transfer. Besides, parthenogenetic activation is used as a functional assay of cytoplasmic maturation of oocytes and also of early embryonic development. An increased incidence of parthenogenetic activation by aged oocytes was attributed in part to the gradual decrease of MPF activity in porcine oocytes during prolonged culture (Kikuchi et al. 2000). The aged oocytes cause the failure of male PN formation and decreased embryo viability after ICSI.

The objective of this Chapter was to evaluate the *in vivo* developmental ability of oocytes following ICSI with freeze-dried sperm heads. Embryo transfer has been carried out in the effort to generate at least one live piglet following ICSI using freeze-dried sperm and IVM oocytes.

Materials and methods

Preparation of in vitro *matured (IVM) oocytes*

Oocyte collection and IVM were conducted as previously described (Kikuchi et al. 2002a). Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace - Large White - Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35^{o}C. Cumulus-oocyte complexes (COCs) were collected from follicles 2 - 6 mm in diameter in Medium 199 in air (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum 20 mM Hepes, 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 µL of maturation medium for 20 - 22 h in four-well dishes. The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39^{o}C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, cumulus cells were removed from oocytes by repeated pipetting in Medium-199 in air supplemented with 150 IU/ mL hyaluronidase. Denuded oocytes with the first polar body (PB) were harvested under a stereomicroscope and used as IVM oocytes.

Sperm collection, FD and rehydration

Sperm collection and FD were conducted as described previously (Men et al. 2013). Briefly, ejaculated semen was collected from a Landrace boar and transferred to the laboratory within 1 h. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × g at 30^{o}C and the seminal plasma was removed. The pellet was resuspended in FD medium containing trehalose prewarmed at 30^{o}C, and the supernatant was removed after centrifugation. The final pellet was then resuspended in FD media containing 0 or 15mM trehalose at a final concentration of 4x10^{8} cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial, then the vials were covered with aluminum foil and placed in a refrigerator at – 80^{o}C for at least 4 h. The aluminum foil was replaced by a rubber cap with small gaps between the cap and the vial, and then they were placed in a FD system. The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at - 30^{o}C and then increased to 30^{o}C

during the last 1 h 20 min (0.75^{o}C/ min). After flushing with inactive N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps. The freeze-dried samples were transferred to a refrigerator and stored at 4^{o}C under dark condition until usage. For rehydration, the same volume (1 mL) of deionization distilled water was added to vials immediately after opening the rubber and aluminum caps using a decapper. The sperm suspension was centrifuged for 2 min at 600 × g and the sperm were washed with PBS (-) containing 5 mg/mL BSA, then resuspended in the same buffer. The sperm suspension was sonicated for several seconds to isolate sperm head and kept at room temperature prior to ICSI.

ICSI and oocyte stimulation

ICSI was carried out as previously described (Nakai et al. 2006) with some modifications (Men et al. 2013). Briefly, sperm were kept in IVC-PyrLac- Hepes-PVP. About 30 IVM oocytes were transferred to a 20-μL drop of Medium 199 in air. A small volume (0.5 μL) of the sonicated sperm suspension was transferred to a 2-μL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil. A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator. ICSI was completed within 2 hs after rehydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. The end of injection was considered as 0 h postinjection. One hour postinjection, the oocytes were transferred to an activation solution consisting of 0.28 M d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk down the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs under the same condition for each group using a somatic hybridizer, then washed three times and cultured in IVC PyrLac until the pooling the putative zygotes for embryo transfer.

Transfer of sperm injected oocytes to recipients

Estrus synchronization of the recipient gilts was carried out basically as reported previously (Kikuchi et al. 1999b; Kashiwazaki et al. 2001; Nakai et al. 2003). In brief, an intramuscular injection of 1000 IU of eCG (Nihon Zenyaku Kogyo) and, 72 h later, an injection of 500 IU of hCG (Sankyo) were given to nonpregnant gilts (5–6 months old, 100–110 kg). Ovulation was expected at 40–45 h after the hCG injection. The embryos after IVC were transferred to freshly prepared transfer medium, NCSU-37 solution supplemented with 4 mg/ml BSA and 20 mM Hepes, adjusted to 285 osm/kg. Sperm injected and

stimulated oocytes were transported to the farm at 37°C in IVC PyrLac-Hepes. At 3 h after stimulation, the oocytes were transferred to both oviducts of estrous-synchronized recipient gilts, in which ovulation was confirmed. We also co-transferred parthenogenetic oocytes with the sperm-injected oocytes to all recipients to increase the chance of pregnancy (King et al. 2002). Parthenogenetic embryos were generated by electro-stimulation with a direct current pulse of 2.2 kV/cm for 30 μsec and incubated in IVC-PyrLac-Hepes supplemented with 10 μg/ml cytochalasin B at 37°C for 3 h.

Results

The data of trials of embryo transfer were shown in Table 9. Some recipients showed delayed estrus but returned their estrus on the second cycle (about day 56–60 after embryo transfer). After 7 trials with the number of oocytes transferred from 96 to 120 per recipient, the ability of ICSI-oocytes using freeze-dried sperm to generate a live piglet has not been demonstrated in this experiment.

Discussion

The ability of ICSI-oocytes using freeze-dried sperm to generate a live piglet has not been achieved after embryo transfer in this study. IVM-IVF oocytes were cultured for 36–48 h (Mattioli et al. 1989; Yoshida et al. 1993b; Funahashi et al. 1996; 1997) or 96 h (Day et al. 1998) and then transferred to recipients (2- to 4-cell stage embryos or 8-cell to morula stage embryos, respectively) in previous studies. However, the rate of embryo development to piglets was very low. Later, viable piglets were also generated after transfer of IVP embryos at the blastocyst stage (Marchal et al. 2001; Kikuchi et al. 2002a). However, prolonged exposure of embryos in *in vitro* culture medium is reported to decrease the subsequent development of embryo, in other words; viability of porcine IVP embryos is decreased with increasing period of IVC (Kikuchi et al. 1999b). In their study, the developmental rate to fetuses for oocytes inseminated by IVF and transferred without any culture was significantly higher than for those transferred after culture for 24 and 48 h (Kikuchi et al. 1999b). This result is attributed to the sub-optimal *in vitro* culture system. Considering this aspect, we performed the embryo transfer soon after the completion of ICSI and oocyte activation.

Probst & Rath (2003) succeeded in producing live piglets for the first time following ICSI using flowcytometrically sorted sperm with only *in vivo* matured oocytes but not IVM oocytes. The authors demonstrated that low developmental ability of these embryos might be related to insufficient IVM and IVC condition and not necessarily to ICSI itself.

Besides, oocyte quality, culture medium, embryo transportation process, number of the transferred embryos, response of ovaries with hormones, ovulation status of recipients, etc; all these factors influence the outcome of embryo transfer experiments. For instance, recipients with high plasma progesterone concentrations or with numerous follicular cysts were shown to have a reduced likelihood of remaining pregnant (Blum-Reckow & Holtz 1991). For embryo transfer medium, the parameters such as pH, osmolality, temperature, sterility and toxicity of transfer medium are important; the tolerance range of these properties of embryos is often narrow. Besides, oscillation of pH can be detrimental on embryo and fetal development and the use of buffers for embryo transfer in procedures performed outside the laboratory incubator is obligation. Taken together, optimal improvements of not only IVP system but also embryo transfer procedures have a great importance on offspring successful production. Although desired outcome has not been gained, we will continue some additional attempts in the future.

After several failed trials of embryo transfer, we considered that embryo quality may be one of factors affecting the survival of embryos after transfer; the experiments in Chapter VII were conducted to compare the blastocyst quality in term of cell numbers and DNA fragmentation index among groups using different types of sperm.

Table 9. Trials of transfer porcine oocytes after injection with freeze-dried sperm

Trials	Trehalose (mM)	No of oocytes transferred [a]	CB treated-Parthenotes	Offspring
1	15	106 (77)	29	–
2	0	103(78)	25	–
3	15	105(80)	25	–
4	15	100(84)	16	–
5	15	96(72)	24	–
6	0	108(80)	28	–
7	0	120(96)	24	–

[a]Total number of oocytes transferred to one recipient. Number in parentheses is number of ICSI- oocytes. Basic freeze-drying medium contains EGTA 50 mM in all groups. Tre 0 mM, Tre 15 mM: basic freeze-drying medium supplemented 0 or 15 mM trehalose. Day of ET is day of ICSI.

Chapter VII

Detection of DNA fragmentation in blastocyst obtained from IVM porcine oocytes injected with freeze-dried sperm

Introduction

Apoptosis occurs as a normal phenomenon during development and aging. It acts as a homeostatic mechanism to maintain cell populations in tissues and also act as a defense mechanism when cells are damaged by disease or toxic agents (Norbury & Hickson 2001). Using conventional histology, it is not always easy to distinguish apoptosis from necrosis, since they occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of ATP depletion and the availability of caspases (Zeiss 2003). Necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells. Within the scope of this study, we only discuss some points about apoptosis that had been reviewed intensively in literature until now.

In mice, DNA fragmentation in nuclei of blastocysts has been shown to be related to the method used for sperm lyophilization and the method of storage, and to be correlated with a lower ability of lyophilized sperm to yield blastocysts after ICSI (Kawase et al. 2009). Apoptosis even observed in rhesus blastocysts freshly flushed from the reproductive tracts (Ender et al. 1981). Therefore, physiological apoptosis is very important during various developmental processes. In cattle, the occurrence of apoptosis is dependent on the developmental stage of embryos. It was observed in the 8–16 cell, morula and blastocyst stage embryos but not in the stages from the zygote (two PN) before the 8 cell (Matwee et al. 2000). It is believed that apoptosis has to be tightly regulated since apoptosis may lead to pathology or developmental defects (Elmore 2007).

There are a large variety of assays available, but each assay has advantages and disadvantages which may make it acceptable to use for one application but inappropriate for another application (Watanabe et al. 2002). Assessment of embryonic cells with fragmented DNA using the terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) is a common method (Kressel & Groscurth 1994). It allows *in situ* assessment of DNA breaks in the nuclei (Farber 1994).

Interestingly, levels of cell death were similar for *in vivo* and *in vitro* cultured mouse blastocysts (Devreker & Hardy 1997). However, other studies in cattle and other mammalian species determined higher apoptotic indices in blastocysts produced *in vitro* compared to those developed *in vivo* (Fabian et al. 2005; Pomar et al. 2005), concluding that insufficient *in vitro* embryo production systems might increase apoptosis.

It is known that total cell number of blastocyst after culture is related to the viability of the fetuses (Kikuchi et al. 1999b). Additionally, Hardy et al. (2003) reported a high apoptotic index in morphologically excellent human blastocysts produced *in vitro* and proposed that programmed cell death might play an important role in normal development. Cell numbers and apoptosis levels are proposed as useful indicators of developmental potential of embryo (Neuber et al. 2002; Elmore 2007).

The objective of this Chapter was to compare the quality of the blastocysts in terms of cell number and DNA fragmentation (apoptosis) obtained by ICSI using different sources of sperm: fresh sperm, frozen-thawed sperm, freeze-dried sperm and in blastocyst obtained by conventional *in vitro* fertilization.

Materials and methods

Preparation of in vitro *matured (IVM) oocytes*

Oocyte collection and IVM were conducted as previously described (Kikuchi et al. 2002a). Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace - Large White - Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35^{o}C. Cumulus-oocyte complexes (COCs) were collected from follicles 2-6 mm in diameter in Medium 199 in air (with Hanks' salts) supplemented with 10% (v/v) FBS, 20 mM Hepes, 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs were cultured in 500 µL of maturation medium for 20–22 h in four-well dishes. The COCs were subsequently cultured for 24 h in maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39^{o}C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively. After IVM, cumulus cells were removed from oocytes by repeated pipetting in Medium 199 in air supplemented with 150 IU/ mL hyaluronidase. Denuded oocytes with the first polar body (PB) were harvested under a stereomicroscope and used as IVM oocytes.

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Sperm collection and FD were conducted as described previously (Men et al. 2013). Briefly, ejaculated semen was collected from a Landrace boar and transferred to the laboratory within 1 h. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × g at 30^{o}C and the seminal plasma was removed. The pellet was resuspended in FD medium containing trehalose prewarmed at 30^{o}C, and the supernatant was removed after centrifugation. The final pellet was then resuspended in FD media containing 0 or 15mM trehalose at a final concentration of 4×10^{8} cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial, then the vials were covered with aluminum foil and placed in a refrigerator at –80^{o}C for at least 4 h. The aluminum foil was replaced by a rubber cap with small gaps between the cap and the vial, and then they were placed in a FD system. The FD program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at –30^{o}C and then increased to 30^{o}C during the last 1 h 20 min (0.75^{o}C/ min). After flushing with inactive N_2 gas, the vials were sealed with rubber caps and further fastened with aluminum caps. The freeze-dried samples were transferred to a refrigerator and stored at 4^{o}C under dark condition until usage. For rehydration, the same volume (1 mL) of deionization distilled water was added to vials immediately after opening the rubber and aluminum caps using a decapper. The sperm suspension was centrifuged for 2 min at 600 × g and the sperm were washed with PBS (–) containing 5 mg/mL BSA, then resuspended in the same buffer. The sperm suspension was sonicated for several seconds to isolate sperm head and kept at room temperature prior to ICSI.

ICSI and oocyte stimulation

ICSI was carried out as previously described (Nakai et al. 2006) with some modifications (Men et al. 2013). Briefly, sperm were kept in IVC-PyrLac- Hepes-PVP. About 30 IVM oocytes were transferred to a 20-μL drop of Medium 199 in air. A small volume (0.5 μL) of the sonicated sperm suspension was transferred to a 2-μL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil. A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator. Ejaculated fresh sperm, frozen-thawed sperm and freeze-dried sperm were used for injection. ICSI was completed within 2 h after rehydration of freeze-dried sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical

activation. The end of injection was considered as 0 h postinjection. One hour postinjection, the oocytes were transferred to an activation solution consisting of 0.28 M d-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk down the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs under the same condition for each group using a somatic hybridizer, then washed three times and cultured in IVC PyrLac.

In vitro *fertilization (IVF) and IVC of porcine oocytes*

IVF was conducted according to Kikuchi et al. (2002a). Epididymides from a boar of the Landrace breed were collected, and epididymal sperm were collected and frozen (Kikuchi et al. 1998). Sperm were thawed and preincubated for 1 h at 37^{o}C in Medium 199 adjusted to pH 7.8. The medium for IVF was a modified Pig- supplemented with 2 mM sodium pyruvate, 2 mM caffeine, and 5 mg/ml BSA. A portion (10 μL) of the preincubated sperm was introduced into 90 μL of fertilization medium containing about 20 COCs surrounded by expanded cumulus cells. The final sperm concentration was adjusted to $1x10^5$/mL. Co-incubation was carried out at 39^{o}C under 5% O_2. After co-incubation of the gametes for 3 h, the oocytes were freed from the cumulus cells and attached sperm and were transferred into IVC medium. The day of insemination was defined as Day 0. The basic IVC medium was NCSU-37 medium containing 4 mg/mL BSA and 50 mM βmercaptoethanol. IVC was performed in IVC-PyrLac for the first two days and in IVC-Glu until day 6 at 38.5^{o}C under 5% O_2.

TUNEL assay for detection of DNA fragmentation in porcine blastocyst

DNA fragmentation of blastocyst was detected by using a combined technique for simultaneous nuclear staining and TUNEL assay according to the procedures as descried previously (Brison & Schultz 1997; Kajia et al. 2004). The embryos were washed four times in PBS containing 3 mg/mL polyvinylalcohol (PBS-PVA), and fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, they were washed four times in PBS-PVA, permeabilized in PBS containing 0.1% (v/v) TritonX-100 for 60 min, and incubated in a blocking solution (PBS containing 10 mg/mL BSA) overnight at 4°C. They were washed four times in PBS-PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo) for 1 h at 38.5°C and 5% CO_2 in air. As a positive control, some embryos per TUNEL analysis were incubated in 1000 IU/mL DNase I (deoxyribonuclease I; Roche Diagnostics) for 20 min at 38.5°C and 5% CO_2, and washed

twice in PBS-PVA before TUNEL staining. As a negative control, some embryos were incubated in fluorescein-dUTP in the absence of TdT. After that, the embryos were washed three times in PBS-PVA and counterstained with 50 μg/mL propidium iodide to label all nuclei. They were then washed in the blocking solution, treated with an anti-bleaching solution (DABCO), and mounted on a glass slide and covered with coverslip. Labeled nuclei were examined under fluorescent microscope. Two standard filter sets were used for detection of fluorescein isothiocyanate (FITC) alone (emission wavelength: 525 nm), and for detection of propidium iodide alone (emission wavelength: 560 nm). The total number of cells was determined by counting the red-stained cell by propidium iodide. The numbers of apoptotic bodies were counted for each blastocyst. DNA fragmentation index of blastocyst was calculated by dividing the number of total apoptotic bodies by the total number of cells.

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989) then subjected to one way ANOVA using R packages 3.0.1 (R Core Team 2013). As the difference is found in groups by ANOVA, further analysis is conducted by Tukey's posthoc test using the R packages. Differences at $P<0.05$ were considered to be significant.

Results

The number of cells and the amount of apoptosis in the blastocyst are important parameters of preimplantation embryonic development and health. The data about these parameters were shown in Table 10. Total cell number of blastocyst obtained from the IVF group was significantly higher ($P<0.05$) than those from the ICSI groups using freeze-dried sperm or frozen-thawed sperm but did not differ from ICSI group using ejaculated fresh sperm. However, DNA fragmentation index of blastocyst in ICSI group using freeze-dried sperm was significantly lower ($P<0.01$) compared with remaining groups. Two morphologically distinct types of apoptosis were found during observation: the apoptotic bodies (i.e. green-stained dots) and apoptotic cells (i.e. green-stained cells) (Fig. 16 A'). Results of the control staining of ICSI-blastocyst were showed in Fig. 16. For positive control, all the cells expressed strongly TUNEL stained-signals while no signal of TUNEL staining was observed in negative control group.

Discussion

DNA fragmentation in the blastocysts produced by ICSI or IVF using different types of sperm was examined by TUNEL staining to assess embryo quality. Our finding indicated that DNA fragmentation index in blastocyst was not different between ICSI and IVF using the same lot of frozen-thawed sperm. Nonetheless, when ICSI was used, the index in blastocysts in the group injected with freeze-dried sperm was significantly lower compared with the groups injected with fresh and frozen-thawed sperm. This is an unpredicted result and not easy to explain. Our previous study (Men et al. 2013) suggests that, although DNA fragmentation level in freeze-dried sperm was significantly higher than those in ejaculated fresh sperm, however; the result of fertilization was not different. For the subsequent development to the blastocyst stage, higher blastocyst formation rate was observed in fresh group. In order to explain for this result, we suggest that the quality of embryos, although considered as fertilized normally, might be different between these two groups. Probably, a higher proportion of normally fertilized oocytes in the fresh sperm group would be able to continue to development to the blastocyst stage while the oocytes in freeze-dried sperm group would mainly arrest, leading to significant difference in blastocyst formation rate. However, once blastocysts were successfully formed, DNA fragmentation originated from sperm would not be a factor affecting the blastocyst quality. This may be the most reasonable explanation for interesting finding that apoptosis index of freeze-dried sperm group was even lower than that of the fresh sperm group and other groups as well. Because programmed cell death, as evidenced by DNA fragmentation occurs spontaneously and frequently in *in vitro* produced system. Another evidence that cell number per blastocyst was not different between the fresh sperm and freeze-dried sperm groups following ICSI in this experiment, also supporting for this argument.

One limitation of the TUNEL assay is that it only provides a static picture of the blastocysts at a specific time of development, meaning that how long dead cells persist or how fast they are cleared by phagocytosis is not known (Nerber et al. 2002). Apoptotic level was reported higher in the inner cell mass than in the trophectoderm, probably due to the regulation of the inner cell mass is more sensitive than regulation of the trophectoderm, since the inner cell mass will form the fetus (Neuber et al. 2002). In that article, the majority (93%) of IVP blastocysts contained one or more apoptotic cells.

Total cell number of blastocyst obtained from IVF group was significantly higher than those from the ICSI groups using freeze-dried sperm or frozen-thawed sperm but did

not differ from ICSI group using ejaculated fresh sperm. When frozen-thawed sperm were used to fertilize IVM porcine oocytes, mean cell numbers per blastocyst obtained from IVF was significantly higher than those of ICSI using the same lot of sperm and same IVC medium. One possible reason for decreased blastocyst quality in ICSI group is that ICSI procedures used injection medium containing PVP, seemed to be more detrimental to embryo development compared with IVF group that did not use PVP. Another possibility is due to various sperm quality used for two these methods. In IVF group, the strongest sperm will have more opportunity to fertilize oocytes when co-incubated with oocytes. However, in ICSI group, the selection of one spermatozoon to inject into one oocyte does not ensure that the selected sperm are the best. We suppose this might also responsible for the different blasocyt quality between two groups.

In conclusion, the results suggest that blastocyst quality in term of total cell number and nucleus DNA fragmentation of blastocyst might be not a reason for failure in embryo transfer in this research. Further investigations are nessessary to clarify the reason of failed embryo transfer and to increase the opportunity of live piglet production by FD preservation method.

Table 10. Total cell number and DNA fragmentation index of *in vitro* produced porcine blastocyst

Group	No of blastocyst examined	Apoptotic bodies (TUNEL-positive cell)	Total cell number (PI-positive cells)	DF index (%)
ICSI-Fresh sperm	26	7.31	$39.7^{ab} \pm 3.3$	$18.4^{B} \pm 4.1$
IVF-FT sperm	63	6.89	$44.4^{b} \pm 2.5$	$15.5^{B} \pm 2.3$
ICSI-FT sperm	43	8.21	$35.9^{a} \pm 1.7$	$22.9^{B} \pm 3.0$
ICSI-FD sperm	57	3.47	$37.6^{a} \pm 2.1$	$9.2^{A} \pm 1.6$

FT: frozen-thawed sperm, the same lot of sperm for IVF and ICSI was used

FD: freeze-dried sperm, from 15 mM trehalose group used

PI: propidium iodide

DF index: DNA fragmentation index: the number of apoptotic bodies (TUNEL positive-cells or dots) was divided for the total cell number

Two (fresh group) to five replicates were conducted in this experiment

Mean ± S.E.M. are presented

[a,b] Values with different superscript letters within the column are significantly different ($P<0.05$)

[A,B] Values with different superscript letters within the column are significantly different ($P< 0.01$)

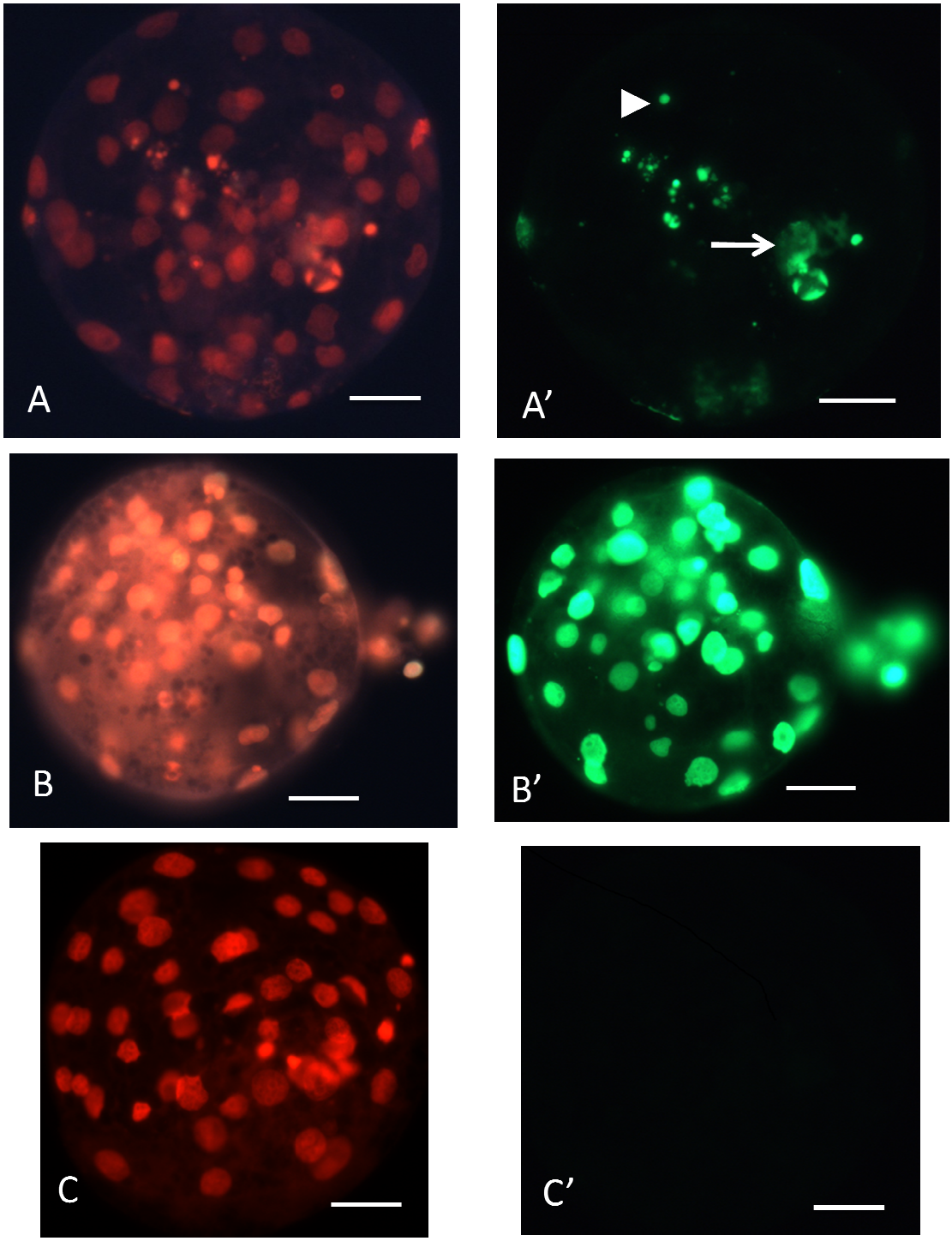

Fig. 16. DNA fragmentation in nuclei of the blastocyst derived from freeze-dried sperm. One bastocyst obtained from IVM oocytes injected with freeze-dried sperm showed many apoptotic cells (arrow) and dots (arrowhead) (A and A'). Positive (B and B') and negative control (C and C') staining of ICSI-blastocyst obtained from frozen-thawed boar sperm. A. B and C: red staining by PI; A', B' and C': green staining by TUNEL. Scale bar: 20 μm

Chapter VIII

General Discussion

Mammalian sperm preservation by FD has been considered as a safe and inexpensive approach relative to cryopreservation in recent years. It has been developed with a major concern because if sperm could be stored in a freeze-dried state without the loss of fertilization ability, the cost for maintenance and shipping could be enormously reduced.

The FD preservation ignored the motility of sperm. The resultant sperm from this technique are in the dry state, and able to store at room temperature or in ordinary refrigerator; however, ICSI must be used to introduce these sperm into the oocytes to start the fertilization process because they are motionless, membrane-damaged or "dead" in the conventional sense (Kusakabe et al. 2008).

EGTA chelates calcium, thus prevents DNA damage induced by endonucleases. It has been used as an effective lyoprotectant (Kusakabe et al. 2001; Nakai et al. 2007). Besides, trehalose, a non-reducing disaccharide, is found in a large amount in anhydrobiotic organisms. Inspired by these survival schemes in nature, many studies have been conducted on the protective effect of trehalose on different biomolecules, mainly proteins and membranes (Crowe & Crowe 2000; Crow et al. 2005). Therefore, we attempted to combine both EGTA and trehalose in FD medium to examine their synergistic effect on DNA of freeze-dried sperm.

In this thesis, the experiments in Chapter II were carried out to investigate the effect of supplement of trehalose into basic FD medium on DNA integrity of freeze-dried boar sperm, fertilization and subsequent development of IVM porcine oocytes. Firstly, various concentrations of trehalose were included (from 0 to 90 mM trehalose) in order to find the optimal concentration for freeze-dried sperm DNA protection. The results showed that the groups supplemented with trehalose 7.5 and 15 mM significantly decreased DNA fragmentation in freeze-dried sperm compared with 0 mM trehalose group. Then, we used the concentrations for further investigations about fertilization at 10 h after sperm injection and the developmental competence to the blastocyst stage after 6 day *in vitro* culture. However, there were no significantly differences in term of normal fertilization and blastocyst formation among 0, 7.5 and 15 mM trehalose groups. As a result, supplement of trehalose at appropriate concentrations improved DNA integrity but not improve fertilization and preimplantation development. Our results were in aggrement with the result of one previous study, showing that sperm with damaged DNA did not affect fertilization by

ICSI (Zini et al. 2005). The difference in DNA damage level of freeze-dried sperm between 0 mM and 15 mM trehalose group might be neutralized by DNA repair capacity of oocytes after fertilization as described in many previous studies (Brandriff & Pedersen 1981; Genesca et al. 1992; Ashwood-Smith & Edwards 1996; Iuso et al. 2013). Because all cells except viruses and mature sperm, possess a variety of enzymatic mechanisms for repair of damaged DNA. These complex DNA repair mechanisms are important for maintaining genomic integrity and limiting the introduction of mutations into the gene pool. The findings in Chapter II raised us the question that whether the different levels of DNA damage in freeze-dried sperm induce the different expression levels of DNA repair genes in oocytes injected with those sperm after fertilization. Previously, Harrouk et al. (2000) indicated that fertilization with sperm exposed to a DNA damaging agent alters the expression of DNA repair genes as early as the 1 cell stage in the rat preimplantation embryo. Our hypothesis was that higher level of DNA damage in sperm would lead to higher expression level of DNA repair genes in oocytes. Then, hypothesis testing about the relationship between DNA damage in freeze-dried sperm and the expression of DNA repair genes in oocytes was conducted in experiments in Chapter III. Four groups were designated including matured oocytes, matured oocytes injected with fresh sperm, and freeze-dried sperm from 0 mM trehalose and 15 mM treahalose group. Unexpectedly, the relative expression level of DNA repair genes in oocyted at 4 h postinjection did not differ among all groups.

A study by Zheng et al. (2005) demonstrated that DNA repair ability of oocytes correlates to the amount of maternal repair mRNA in the cytoplasm which accumulated during the growth phase of oocytes and follicles, and required for completion of the meiotic cell cycle. Therefore, amount of repair mRNA in oocytes might be also various depending on stages of maturation. This argument leads to the next experiment about the effect of maturational stages on expression of DNA repair genes. Two (*UDG* and *XPC*) of six investigated genes showed a significantly higher level of DNA repair genes in the M-II stage in relation to the earlier stage (M-I, GVL and GV stage). Two genes (*MSH2* and *XRCC6*) with the increased tendency in expression of these genes depending on maturational stages were detected. These findings in this Chapter have an oriented significance for future research. Because, within the scope of this work, the experiments to confirm whether increased expression levels of DNA repair genes in oocytes caused by suboptimal IVM conditions or their accumulation have not been conducted. If increased level of DNA repair genes is confirmed to be caused by IVM conditions, the efficient improvement of IVM system can be achieved based on the expression profile of DNA repair genes. Because one

previous study indicated that dysregulation of many genes occurs during IVM of human oocyte (Jones et al. 2008). One of ideas for future direction, to test the hypothesis, I think about the establishment an IVC system in which oocytes are subject to different stressful factors (O_2 tension, CO_2 tension, incubation temperature, detrimental agents, beneficial agents), then the expression of DNA repair genes would be detected to find any differences between the conditions. This idea also serves for the general objective; it is, to improve IVP system since the use of *in vivo* matured oocytes is expensive and often impractical.

In parallel, a series of experiments in Chapters IV and V were performed to investigate the effect of some treatments for sperm and oocytes, also in order to improve the efficacy of embryo production following ICSI using freeze-dried sperm. Some improvements in embryonic development would be very important in increasing the opportunity of producing live piglets when freeze-dried sperm and IVM oocyte are used. In mice, removal of both sperm membranes and acrosome before the injection procedure improved the success of ICSI (Morozumi & Yanagimachi 2005). However, in pigs, our results suggest that freeze-dried sperm treated with TX to remove acrosomal and plasma membrane before ICSI did not increase the incidence of normal fertilization. This result was similar to the finding in a recent study, reporting that disrupting or removing the sperm membrane by TX pretreatment did not result in a significant improvement in male PN formation (Garcia-Mengual et al. 2015).

For the oocyte treatment, although oocyte centrifugation before ICSI facilitated the visibility of sperm release in cytoplasm during ICSI procedures, it did not improve normal fertilization compared with no centrifugation group. In the experiments for evaluating the effect of combination of centrifugation and electric stimulation on fertilization by ICSI of IVM porcine oocytes, sperm injected oocytes that were subject to both centrifugation and electric activation showed a significantly lower rate of normal fertilization than the group without centrifugation. We concluded that oocyte centrifugation after ICSI have a negative effect on fertilization of IVM oocytes. This result was in agreement with the finding of Yong et al. (2005) who reported that transformation from a sperm head decondensation to a male PN was delayed in centrifuged porcine oocytes compared with non-centrifuged oocytes. In contrast, in cattle, Chung et al. (2001) reported that centrifugation of bovine IVM oocytes has no detrimental effect on fertilization and subsequent early embryonic development. In addition, Wei & Fukui (1999) also reported that the proportions of bovine oocytes with two PNs were higher when centrifuged oocytes were used in conventional ICSI. On the other hand, we also observed a significantly higher rate of normal fertilization and a significantly

lower rate of the oocytes entered to M-III stage in the groups with electric activation applied, irrespective of centrifugation. The result of this experiment confirmed that electric activation after ICSI in pigs is important for promoting the oocyte activation and male PN formation as demonstrated in the studies of Nakai et al (2003; 2006; 2007).

IVM oocytes injected with freeze-dried sperm heads were reported capable of growing to the day 39 after oocyte transfer (Nakai et al. 2007). This is the highest achievement obtaining about *in vivo* developmental competence of IVM porcine oocytes injected with freeze-dried sperm until now. Accompany with above experiments, we have tried to produce live piglets from freeze-dried sperm by embryo transfer into recipients (Chapter VI). However, no full-term development of embryos was observed from the utilization of freeze-dried sperm and IVM oocytes. Beside the sperm factor, the imbalance between nuclear and cytoplasmic maturation of IVM oocytes is most likely the main cause for poor embryo quality and impaired postimplantation development compared with *in vivo* counterparts. Indeed, bovine IVP embryos showed reduced pregnancy rates upon transfer and increased incidence of abnormal offspring, as evidenced by high mortality rate and large calves (Garry et al. 1996). Therefore, the improvement about not only in the number of embryos reaching to the blastocyst stage but also in their quality in order to better support for fetal development upon transfer to recipients was recommended (Niemann & Rath 2001). Current research is oriented towards minimizing the negative effect of IVC conditions on quality of the resultant embryos. Suspecting that blastocyst quality may be one of factors affecting the survival of embryo after transfer; the next experiments was conducted to compare the blastocyst quality in term of cell numbers and DNA fragmentation index among groups using different types of sperm (Chapter VII). Apoptosis or programmed cell death is regulated tightly since it may lead to pathology or developmental defects (Elmore 2007). Cell numbers and apoptosis levels are proposed as useful indicators of developmental potential of embryos. Our result demonstrated that cell number of blastocyst obtained from the IVF group was significantly higher than those from the ICSI groups using freeze-dried sperm or frozen-thawed sperm but did not differ from ICSI group using ejaculated fresh sperm. Even DNA fragmentation index of the blastocysts in the ICSI group using freeze-dried sperm was significantly lower compared with remaining groups, suggesting that blastocyst quality is not much different between ICSI and IVF, between freeze-dried sperm and frozen-thawed sperm. These data support for our argument that culture condition rather than types of sperm or fertilization mode would affect apoptotic index. It is reported that programmed cell death, as evidenced by DNA fragmentation occurs spontaneously and

frequently in IVP system, resulting decreased viability of porcine embryo in culture (Long et al. 1998). Probably, other factors are responsible for the failed outcome of ET. These undefined factors will be the topics for continuing research in future.

In summary, in this work, studies to improve DNA integrity of freeze-dried sperm and early embryonic development of IVM porcine oocytes were conducted. Supplement into FD medium with 50 mM EGTA and 15 mM trehalose results an optimal protection for sperm DNA after FD procedures. However, even sperm DNA integrity was maintained better, normal fertilization and subsequent development of resultant embryos following ICSI were not different between 0 and 15 mM trehalose groups. When the relationship between DNA damage in sperm and expression of DNA repair genes in oocytes is investigated, there was no significant difference in expression of DNA repair genes among groups injected with different types of sperm that showed the different levels of DNA damage (fresh or freeze-dried sperm). In general, treatments for sperm and oocyte in this study had no promoting effects on normal fertilization of IVM oocytes following ICSI. Freeze-dried boar sperm treated with TX at different concentrations did not improve normal fertilization of IVM oocytes. Any treatments to remove the sperm plasma membrane seem to be not necessary in case of freeze-dried sperm. Normal fertilization rates were significantly higher in the groups with electric activation than those of without electric activation, confirming the importance of artificial electric stimulation to porcine ICSI-oocytes. In addition, normal fertilization was significantly higher in the control group (sperm injected oocytes were electrically stimulated without centrifugation) compared with the remaining groups and centrifugation showed a negative effect on normal fertilization in case of electric activation and no effect on normal fertilization in case of no electric activation applied. Besides, there is still a problem in obtaining viable offsprings from freeze-dried boar sperm and IVM oocytes. In conclusion, the positive effect of trehalose on DNA integrity of freeze-dried sperm provide important information for the improvement of FD methods associated FD medium; expand basic knowledge of FD associated sperm DNA damage in pigs. This research also has contributions in practice since FD would greatly simplify the establishment and management of biobanking. It also helps to minimize the environmental costs arising from the production and use of LN and other activities for maintenance of facilities of cryopreservation system, especially in developing countries.

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References

Abeydeera LR, Niwa K. 1992. Ability of *in vitro* maturing bovine oocyte to transform sperm nuclei to metaphase chromosome. Journal of Reproduction and Fertility 96:565-572.

Abeydeera LR. 2002. *In vitro* production of embryos in swine. Theriogenology 57:256-273.

Aitken RJ, Koppers AJ. 2011. Apoptosis and DNA damage in human sperm. Asian Journal of Andrology 13:36-42.

Anees C, Harshan HM. 2008. Intracytoplasmic sperm injection: method and application in veterinary science. Veterinary World 1:25-27.

Arakawa T, Ejima D, Tsumoto K, Ishibashi M, Tokunaga M. 2007. Improved performance of column chromatography by arginine: dye-affinity chromatography. Protein Expression and Purification 52:410-414.

Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. 1993. Factors affecting short-term and long-term stabilities of proteins. Advanced Drug Delivery Reviews 10:1-28.

Ashwood-Smith MJ, Edwards RG. 1996. DNA repair by oocytes. Molecular Human Reproduction 2:46-51.

Assou S, Boumela I, Haouzi D, Anahory T, Dechaud H, De Vos J, Hamamah S. 2011. Dynamic changes in gene expression during human early embryo development: from fundamental aspects to clinical applications. Human Reproduction Update 17:272-290.

Badr MR, Mary G, Abd EM, Hassan MH. 2010. Effect of trehalose on cryopreservation, oxidative stress and DNA integrity of buffalo sperm. Journal of Reproduction and Infertility 1:50-57.

Bedford-Guaus SJ, McPartlin LA, Westmiller SL, Buffone MG, Roberson MS. 2011 Molecular cloning and characterization of phospholipase C zeta in equine sperm and testis reveals species-specific differences in expression of catalycally active protein. Biology of Reproduction 85:78-88.

Begley TJ, Samson LD. 2004. Network responses to DNA damaging agents. DNA Repair 3:1123-1132.

Benaroudj N, Lee DH, Goldberg AL. 2001. Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. The Journal of Biological Chemistry 276:24261-24267.

Billy D, Potts M. 2001. Life and death of dry prokaryotes. Research Microbiology 153:7-12.

Blum-Reckow B, Holtz W. 1991. Transfer of porcine embryos after 3 days of *in vitro* culture. Journal of Animal Science 69:3335-3342.

Brandiff B, Pedersen RA. 1981. Repair of the ultraviolet-irradiated male genome in fertilized mouse oocytes. Science 211:1431-1433.

Braude P, Bolton V, Moore S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 332: 459-461.

Brinster RL, Chen HY, Trumbauer ME, Yagle AK, Palmiter R D. 1986. Factors affect on the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proceedings of the National Academy of Sciences of United States of America 82:4438.

Brison DR, Schultz RM. 1997. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biology of Reproduction 56:1088-1096.

Brüssow KP, Torner H, Kanitz W, Rátky J. 2000. *In vitro* technologies related to pig embryo transfer. Reproduction Nutrition Development 40:469-480.

Canovas S, Coy P, Gomez E. 2007. First steps for the development of a functional assay for human sperm using pig oocytes. Journal of Andrology 28:273-281.

Carpenter JF, Crowe LM, Crowe JH. 1987. Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. Biochimica et Biophysica Acta 923:109-115.

Catt SL, Catt JW, Gomez MC, Maxwell WMC, Evans G. 1996. The birth of a male lamb derived from an *in vitro* matured oocyte fertilized by intra-cytoplasmic injection of a single presumptive male sperm. Veterinary Record 139:494-495.

Catt JW, Rhods SL. 1995. Comparative intracytoplasmic sperm injection (ICSI) in human and domestic species. Reproduction, Fertility and Development 7:161-167.

Chan AW, Luetjens CM, Dominko T, Ramalho-Santos J, Simerly CR, Hewitson L, Schatten G. 2000. Foreign DNA transmission by ICSI: injection of sperm bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. Molecular Human Reproduction 6:26-33.

Choi YH, Varner DD, Love CC, Hartman DL, Hinrichs K. 2011. Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. Reproduction 142:529-538.

Chung JT, Downey BR, Casper RF, Chian RC. 2001. Effect of centrifugation on early embryonic development and parthenogenetic activation of bovine oocytes matured *in vitro*. Reproduction, Fertility and Development 13:383-388.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce De Leon FAP, Robl JM. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblast. Science 280:1256-1258.

Clarke HJ, Masui Y. 1987. Dose-dependent relationship between oocyte cytoplasmic volume and transformation of sperm nuclei to metaphase chromosomes. Journal of Cell Biology 104:831-840.

Cochran R, Meintjes M, Reggio B, Hylan D, Carter J, Pinto C, Paccamonti D, Godke RA. 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. Journal of Equine Veterinary Science 18:736-740.

Collas P, Sullivan EJ, Barnes FL. 1993. Histone H1 kinase activity in bovine oocytes following calcium stimulation. Molecular Reproduction and Development 34: 224-231.

Cordelli E, Eleuteri P, Leter G, Rescia M, Spanò M. 2005. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. Contraception 72:273-279.

Coy P, Romar R, 2002. *In vitro* production of pig embryos: a point of view. Reproduction Fertility and Development 14:275-286.

Coy P, Romar R, Ruiz S, Canovas S, Gadea J, Garcia Vazquez F, Matas C. 2005. Birth of piglets after transferring of *in vitro*-produced embryos pre-matured with R-roscovitine. Reproduction 129:747-755.

Crowe JH, Hoekstra FA, Crowe LM. 1992. Anhydrobiosis. Annual Review of Physiology 54:579-599.

Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W, Tablin F. 2005. The trehalose Myth revisited: Introduction to a symposium on stabilization of cells in dry state. Cryobiology 43:89-105.

Crowe JH, Crowe LM, Carpenter JF, Aurell Wistrom C. 1987. Stabilization of dry phospholipid bilayer and protein by sugars. Biochemical Journal 242:1-10.

Crowe JH, Crowe LM. 2000. Preservation of mammalian cells-learning nature's tricks. Nature Biotechnology 18:145-146.

Cui J, Holmes EH, Greene TG, Liu PK. 2000. Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. FASEB Journal 14:955-967.

Danan Claude, Damien Sternberg, André Van Steirteghem, Cécile Cazeneuve, Philippe Duquesnoy, Claude Besmond, Michel Goossens, Willy Lissens, Serge Amselem. 1999. Evaluation of Parental Mitochondrial Inheritance in Neonates Born after Intracytoplasmic Sperm Injection. American Journal of Human Genetics 65:463-473.

Dang-Nguyen TQ, Tich NK, Nguyen BX, Ozawa M, Kikuchi K, Manabe N, Ratky J, Kanai Y, Nagai T. 2010. Introduction of various Vietnamese indigenous pig breeds and their conservation by using assisted reproductive techniques. Journal of Reproductive Development 56:31-35.

Darin-Bennett A, Poulos A, White IG. 1973. The effect of cold shock and freeze–thawing on release of phospholipids by ram, bull and boar sperm. Australian Journal of Biological Sciences 26:1409-20.

Day BN, Abeydeera LR, Johnson LA, Welch GR, Wang WH, Cantley TC, Rieke A. 1998. Birth of piglets preselected for gender following *in vitro* fertilization of *in vitro* matured pig oocytes by X and Y bearing sperm stored by high speed flow cytometry. Theriogenology 49:360.

De Bont R, van Larebeke N. 2004. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19: 169-185.

Deng M, Yang XJ. 2001. Full term development of rabbit oocytes fertilized by intracytoplasmic sperm injection. Molecular Reproduction 59:38-43.

Derijck AA, van der Heijden GW, Giele M, Philippens ME, van Bavel CC, de Boer P. 2006. Gamma H2AX signaling during sperm chromatin remodelling in the mouse zygote. DNA Repair 5:959-971.

Devreker F, Hardy K. 1997. Effects of glutamine and taurine on preimplantation development and cleavage of mouse embryos *in vitro*. Biology of Reproduction 57:921-928.

Dozortsev D, Rybouchkin A, De Sutter P, Dhont M. 1995. Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. Human Reproduction 10:2960-2964.

Elmore S. 2007. Apoptosis: A Review of Programmed Cell Death. Toxicologic Pathology 35:495-516.

Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. 2009. A two-tailed Comet assay for assessing DNA damage in sperm. Reproductive Biomedicine Online 18:609-616.

Enders AC, Schlafke. 1981. Differentiation of the blastocyst of the rhesus monkey. American Journal of Anatomy 162:1-21.

Eriksson B. 2000. Cryopreservation of Boar Semen: Studies on sperm viability *in vitro* and fertility. Doctoral Thesis, Swedish University of Agricultural Sciences Uppsala.

Eroglu A, Toner M, Toth T. 2002. Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. Fertility and Sterility 77:152-158.

Eroglu A, Bailey SE, Toner M, Toth TL.2009. Successful cryopreservation of mouse oocytes by using low concentrations of trehalose and dimethylsulfoxide. Biology of Reproduction 80:70-78.

Evenson DP, Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210:1131-1133.

Fabian D, Koppel J, Maddox-Hyttel P. 2005. Apoptotic processes during mammalian preimplantation development. Theriogenology 64:221-231.

Fahrudin M, Kikuchi K, Karja NWK, Ozawa M, Maedomari N, Somfai T, Ohnuma K, Noguchi J, Kaneko H, Nagai T. 2007. Development to the blastocyst stage of porcine somatic cell nuclear transfer embryos reconstructed by the fusion of cumulus cells and cytoplasts prepared by gradient centrifugation. Cloning and Stem Cells 9:216-228.

FAOSTAT. 2009. Food and Agriculture Organization of the United Nations. Rome, Italy; [assessed 6 Oct 2014] Available from URL http://faostat3.fao.org/download/Q/*/E.

FAOSTAT. 2013. Food and Agriculture Organization of the United Nations. Rome, Italy; [assessed 6 Oct 2014] Available from URL http://faostat3.fao.org/download/Q/*/E.

Farber E. 1994. Programmed cell death: necrosis versus apoptosis. Modern Pathology 7:605-609.

Fenwick J, Platteau P, Murdoch AP, Herbert M. 2002. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. Human Reproduction 17:407-412.

Fernández JL, Muriel L, Goyanes VJ, Segrelles E, Gosálvez J, Enciso M, Lafromboise M, Jonge DC. 2005. Simple determination of sperm DNA fragmentation with an improved sperm chromatin dispersion (SCD) test. Fertility and Sterility 84:833-842.

Fleming TP, Sheth V, Fesenko I. 2001. Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. Frontiers in Bioscience 6**:**1000-1007.

Forster R, Ancian P, Fredholm M, Simianer H, Whitelaw B. 2010. The minipig as a platform for new technologies in toxicology. J Pharmacol Toxicol Methods 62:227-235.

Funahashi H, Cantley TC, Day BN. 1997. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization. Biology of Reproduction 57:49-53.

Funahashi H, Kim NH, Stumpf TT, Cantley TC, Day BN. 1996. Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of porcine oocytes. Biology of Reproduction 54:1412-1419.

Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN, Cantley T, Rieke A. 1994. Use of low-salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after *in vitro* fertilization. Biology of Reproduction 51:633-639.

Garcia-Mengual E, Silvestre MA, Salvador I, Cebrian-Serrano A, Garcia-Rosello E. 2015. Male pronucleus formation after ICSI: effect of oocyte cysteine or sperm Triton X-100 treatments. Czech Journal of Animal Sciences 60:241-249.

García-Vázquez FA, Ruiz S, Matás C, Izquierdo-Rico MJ, Grullón LA, De Ondiz A, Vieira L, Avilés-López K, Gutiérrez-Adán A, Gadea J. 2010. Production of transgenic piglets using ICSI-sperm-mediated gene transfer in combination with recombinase RecA. Reproduction 140:259-272.

Garry FB, Adams R, McCann JP, Odde KG. 1996. Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 45:141-152.

Genesca A, Caballin MR, Miro R, Benet J, Germa JR; Egozcue J. 1992. Repair of human sperm chromosome aberrations in the hamster egg. Human Genetics 82:181-186.

Gianaroli L, Magli MC, Stanghellini I, Crippa A, Crivello AM, Pescatori ES, Ferraretti AP. 2012. DNA integrity is maintained after freeze-drying of human sperm. Fertility and Sterility 97:1067-1073.

Goto K, Kinoshita A, Takuma Y, Ogawa K. 1990. Fertilization of bovine oocytes by the injection of immobilized, killed sperm. Veterinary Record 24*:*517-520.

Gordon S, Oppenheimer S, Mackay A, Brunnabend J, Puhlev I, Levine F. 2001. Recovery of human mesenchymal stem cells following dehydration and rehydration. Cryobiology 43:182-187.

Grondahl ML , Yding Andersen C , Bogstad J , Nielsen FC , Meinertz H , Borup R . 2010. Gene expression profiles of single human mature oocytes in relation to age. Human Reproduction 25:957-968.

Hamano, K., X. Li, X. Q. Qian, K. Funauchi, M. Furudate, and Y. Minato. 1999. Gender preselection in cattle with intracytoplasmically injected, flow cytometrically sorted sperm heads. Biology of Reproduction 60:1194-1197.

Hamatani T, Carter MG, Sharov AA, Ko MS. 2004a. Dynamics of global gene expression changes during mouse preimplantation development. Developmental Cell 6:117-131.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS. 2004b. Age-associated alteration of gene expression patterns in mouse oocytes. Human Molecular Genetics 13:2263-2278.

Hammer RE, Pursel VG, Rexroad CE, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680-683.

Hand SC, Menze MA, Toner M, Boswell L, Moore D. 2011. LEA proteins during water stress: not just for plants anymore. Annual Review of Physiology 73:115-134.

Hara H, Abdalla H, Morita H, Kuwayama M, Hirabayashi M, Hochi S. 2011. Procedure for bovine ICSI, not sperm freeze-drying, impairs the function of the microtubule-organizing center. Journal of Reproductive Development 57:428-432.

Hardy K, Stark J, Winston RM. 2003. Maintenance of the inner cell mass in human blastocysts from fragmented embryos. Biology of Reproduction 68:1165-1169.

Harrouk W, Codrington A, Vinson R, Robaire B, Hales BF. 2000. Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. Mutation Research 461:229-241.

Hazeleger W, Kemp B. 2001. Recent developments in pig embryo transfer. Theriogenology 56:1321-1331.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. 2004. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertility and Sterility 81:965-972.

Heuwieser W, Yang X, Jiang S, Foote RH. 1992. A comparison between *in vitro* fertilization and microinjection of immobilized sperm from bulls producing sperm with defects. Molecular Reproduction and Developement 33:489-491.

Hewitson L, Dominko T, Takahashi D, Martinovich C, Ramalho Santos J, Sutovsky P, Fanton J, Jacob D, Monteith D, Neuringer M, Battaglia D, Simerly C, Schatten G. 1999. Unique checkpoints during the first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkeys. Nature Medicine 4:431-433.

Higashiyama T. 2002. Novel functions and applications of trehalose. Pure and Applied Chemistry 74:1263-1269.

Hirabayashi M, Kato M, Aoto T, Ueda M, Hochi S. 2002. Rescue of infertile transgenic rat lines by intracytoplasmic injection of cryopreserved round spermatids. Molecular Reproduction and Development 62:295-299.

Hirabayashi M, Kato M, Ito J, Hochi S. 2005. Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. Zygote 13:79-85.

Hochi S, Watanabe K, Kato M, Hirabayashi M. 2008. Live rats resulting from injection of oocytes with sperm freeze-dried and stored for one year. Molecular Reproduction and Developement 75:890-894.

Hochi S, Abdalla H, Hara H, Hirabayashi M. 2011. Challenging endeavour for preservation of freeze-dried mammalian sperm. Journal of Reproduction and Development 57:557-63.

Hoeijmakers JH. 2009. DNA damage, aging, and cancer. New England Journal of Medicine 361:1475-85.

Hood RD, Foley CW, Martin TG. 1970. Effects of cold shock, dilution, glycerol and dimethylsulfoxide on cation concentrations in pig sperm. Journal of Animal Science 30:91-94.

Hoodbhoy T, P Talbot. 1994. Mammalian cortical granules, contents, fate and function. Molecular Reproduction and Development 39:439-448.

Hornak M, Oracova E, Hulinska P, Urbankova L, Rubes J. 2012. Aneuploidy Detection in Pigs Using Comparative Genomic Hybridization: From the Oocytes to Blastocysts. PLoS ONE 7:e30335.

Hosoi Y, Miyake M, Utsumi K, Iritani A. 1988. Development of rabbit oocytes after microinjection of spermatozoon. Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination 3:331-333.

Hoshi K, Yanagida K, Sato A. 1992. Pretreatment of hamster oocytes with Ca^{2+} ionophore to facilitate fertilization by ooplasmic micro-injection. Human Reproduction 7:871-875.

Hu JH, Li QW, Jiang ZL, Yang H, Zhang SS, Zhao HW. 2009. The cryoprotective effect of trehalose supplementation on boar sperm quality. Reproduction in Domestic Animals 44:571-575.

Humphray SJ, Scott CE, Clark R, Marron B, Bender C, Camm N, Davis J, Jenks A, Noon A, Patel M, Sehra H, Yang F, Rogatcheva MB, Milan D, Chardon P, Rohrer G, Nooneman D, de Jong P, Meyers SN, Archibald A, Beever JE, Schook LB, Rogers J. 2007. A high utility integrated map of the pig genome. Genome Biology 8:139.

Hwang Seongsoo, Eunyoung Lee, Jongtaek Yoon, Byung-Koo Yoon, Je-Ho Lee, Dooseok Choi. 2000. Effects of electric stimulation on bovine oocyte activation and embryo development in intracytoplasmic sperm injection procedure. Journal of Assisted Reproduction and Genetics 17:310-314.

Hyttel P, Laurincik J, Rosenkranz C, Rath D, Niemann H, Ochs RL, Schellander K. 2000. Nucleolar proteins and ultrastructure in preimplantation porcine embryos developed *in*

vivo. Biology Reproduction 63:1848-1856.

Ito J, Shimada M, Terada T. 2004. Mitogen-activated protein kinase inhibitor suppresses cyclin B1 synthesis and reactivation of p34cdc2 kinase, which improves pronuclear formation rate in matured porcine oocytes activated by Ca^{2+} ionophore. Biology of Reproduction 70:797-804.

Iuso D, Czernik M, Di Egidio F, Sampino S, Zacchini F, Bochenek M, Smorag Z, Modlinski JA, Ptak G, Loi P. 2013. Genomic stability of lyophilized sheep somatic sells before and after nuclear transfer. PLoS ONE 8:e51317.

Iritani A. 1991. Micromanipulation of gametes for *in vitro* assisted fertilization. Molecular Reproduction and Development 28:199-207.

Jain NK, Roy I. 2008. Effect of trehalose on protein structure. Protein Science18:24−36.

Jaroudi S, SenGupta S. 2007. DNA repair in mammalian embryos. Mutatation Research 635:53-77.

Jaroudi Souraya, Georgia Kakourou, Suzanne Cawood, Alpesh Doshi, Domenico M. Ranieri, Paul Serhal, Joyce C. Harper, and Sioban B. SenGupta. 2009. Expression profiling of DNA repair genes in human oocytes and blastocysts using microarrays. Human Reproduction 24:2649-2655.

Jarrell VL, Day BN, Prather RS. 1991. The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, Sus scrofa: quantitative and qualitative aspects of protein synthesis. Biology of Reproduction 44:62-68.

Johnson LA, Larsson K, editors. 1985. Proceedings of the First International Conference of Deep Freezing of Boar Semen. Uppsala, Sweden: Swedish University of Agriculture Sciences Press p310.

Jones Gayle M, David S Cram, Bi Song, M Cristina Magli, Luca Gianaroli, Orly Lacham-Kaplan, Jock K. Findlay, Graham Jenkin, Alan O Trounson. 2008. Gene expression profiling of human oocytes following *in vivo* or *in vitro* maturation. Human Reproduction 23:1138-1144.

Kaneko H, Kikuchi K, Nakai M, Somfai T, Noguchi J, Tanihara F, Ito J, Kashiwazaki N. 2013. Generation of Live Piglets for the First Time Using Sperm Retrieved from Immature Testicular Tissue Cryopreserved and Grafted into Nude Mice. PLoS ONE 8:e70989.

Kaneko T, Whittingham DG, Yanagimachi R. 2003. Effect of pH value of freeze-drying solution on the chromosome integrity and developmental ability of mouse sperm. Biology of Reproduction 68:136-139.

Kaneko T, Kimura S, Nakagata N. 2007. Offspring derived from oocytes injected with rat sperm, frozen or freeze-dried without cryoprotection. Theriogenology 68:1017-1021.

Karja NWK, Wongsrikeao P, Murakami M, Agung B, Fahrudin M, Nagai T, Otoi T. 2004. Effects of oxygen tension on the development and quality of porcine *in vitro* fertilized embryos. Theriogenology 62:1585-1595.

Kasai Magosaburo. 2002. Advances in the cryopreservation of mammalian oocytes and embryos: Development of ultrarapid vitrification. Reproductive Medicine and Biology 1:1-9.

Kasai T, Hoshi K, Yanagimachi R. 1999. Effect of sperm immobilization and demembranation on the oocyte activation rate in the mouse. Zygote 7:187-193.

Katayama M, Miyano T, Miyake M, Kato S, 2002b. Progesterone treatment of boar sperm improves male pronuclear formation after intracytoplasmic sperm injection into porcine oocytes. Zygote 10:95-104.

Katayama Mika, Mitsunobu Koshida, Masashi Miyake. 2002a. Fate of the acrosome in ooplasm in pigs after IVF and ICSI. Human Reproduction 17:2657-2664.

Katayose H, Matsuda J, Yanagimachi R. 1992. The ability of dehydrated Hamster and human sperm nuclei to develop into pronuclei. Biology of Reproduction 47:277-284.

Kaufman MH, Sachs L. 1975. The early development of haploid and aneuploid parthenogenetic embryos. Journal of embryology and experimental morphology 34:645-655.

Kaufman MH, Bames SC, Surani MAH. 1977. Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. Nature 265:53-55.

Kawai H, Sakurai M, Inoue Y, Chujo R, Kobayashi S. 1992. Hydration of oliosaccharides: anomalous hydration ability of trehalose. Cryobiology 29:599-606.

Kawase Y, Hani T, Kamada N, Jishage K, Suzuki H. 2007. Effect of pressure at primary drying of freeze-drying mouse sperm reproduction ability and preservation potential. Reproduction 133:841-846.

Kawase Y, Wada NA, Jishage K. 2009. Evaluation of DNA fragmentation of freeze-dried mouse sperm using a modified sperm chromatin structure assay. Theriogenology 72:1047-1053.

Keefer CL. 1989. Fertilization by sperm injection in the rabbit. Gamete Research 22:59-69.

Keskintepe L, Pacholczyk G, Machnicka A, Norris K, Curuk MA, Khan I, Brackett BG. 2002. Bovine blastocyst development from oocytes injected with freeze-dried sperm. Biology of Reproduction 67:409-515.

Kikuchi K, Ekwall H, Tienthai P, Kawai Y, Noguchi J, Kaneko H, Rodriguez-Martinez H. 2002b. Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst *in vivo* and *in vitro*. Zygote 10:355-66.

Kikuchi K, Izaike Y, Noguchi J, Furukawa T, Daen FP, Naito K, Toyoda Y. 1995. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged *in vitro*. Journal of Reproduction and Fertility 105:325-330.

Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H. 1999b. Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured *in vitro*. Biology of Reproduction 60:336-340.

Kikuchi K, Kashiwazaki N, Nagai T, Nakai M, Somfai T, Noguchi J, Kaneko H. 2008. Selected Aspects of Advanced Porcine Reproductive Technology. Reproduction in Domestic Animals 43:401-406.

Kikuchi K, Nagai T, Ding J, Yamauchi N, Noguchi J, Izaike Y. 1999a. Cytoplasmic maturation for activation of pig follicular oocytes cultured and arrested at metaphase-I. Journal of Reproduction and Fertility 116:143-156.

Kikuchi K, Nagai T, Kashiwazaki N, Ikeda H, Noguchi J, Shimada A, Soloy E, Kaneko H. 1998. Cryopreservation and ensuing *in vitro* fertilization ability of boar sperm from epididymides stored at 4^{o}C. Theriogenology 50:615-623.

Kikuchi K, Naito K, Noguchi J, Shimada A, Kaneko H, Yamashita M, Aoki F, Tojo H, Toyoda Y. 2000. Maturation/M-phase promoting factor: a regulator of aging in porcine oocytes. Biology of Reproduction 63:715-722.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. 2002a. Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. Biology of Reproduction 66:1033-1041.

Kim NH, Jun SH, Do JT, Uhm SJ, Lee HT, Chung KS. 1999. Intracytoplasmic injection of porcine, bovine, mouse, or human spermatozoon into porcine oocytes. Molecular Reproduction and Development 53:84-91.

Kimura Y, Yanagimachi R, Kuretake S, Bortkiewicz H, Perry AC, Yanagimachi H. 1998. Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. Biology of Reproduction 58:1407-1415.

Kimura Y, Yanagimachi R. 1995. Intracytoplasmic sperm injection in the mouse. Biology of Reproduction 52:709-720.

King TJ, Dobrinsky JR, Zhu J, Finlayson HA, Bosma W, Harkness L, Ritchie WA, Travers A, McCorqquodale C, Day BN, Dinnyes A., De Sousa PA, Wilmut I. 2002. Embryo development and establishment of pregnancy after embryo transfer in pigs: coping with limitations in the availability of viable embryos. Reproduction 123:507–515.

Kline D, Kline JT. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Developmental Biology 149:80-89.

Kobayashi E, Hishikawa S, Teratani T, Lefor AT. 2012. The pig as a model for translational research: overview of porcine animal models at Jichi Medical University. Transplantation Research 1:8.

Kolbe T, Holtz W. 2000: Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI). Animal Reproduction Science 64:97–101.

Kolbe T, Holtz W. 1999. Intracytoplasmic injection of *in vivo* or *in vitro* matured oocytes with fresh ejaculated or frozen-thawed epididymal sperm and additional calcium-ionophore activation in the pig. Theriogenology 52: 671-682.

Kono T. 2006. Genomic imprinting is a barrier to parthenogenesis in mammals. Cytogenetic and Genome Research 113:31-35.

Kozdrowski R. 2009. The effect of trehalose on post-thaw viability and fertility of European brown hare (Lepus europaeus Pallas, 1778) sperm. Animal Reproduction Science 116:326-334.

Kressel M, Groscurth P. 1994. Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. Cell Tissue Research 278:549-556.

Krisher RL, BD Bavister. 1999. Enhanced glycolysis after maturation of bovine oocytes *in vitro* is associated with increased developmental competence. Molecular Reproduction and Development 53:19-26.

Kurebayashi S, Miyake M, Okabe K, Kato S. 2000. Successful implantation of *in vitro* matured, electro activated oocytes in the pig. Theriogenology 53:1105-1119.

Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. 1996. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biology of Reproduction 55:789-795.

Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. 2001. Maintenance of genetic integrity in frozen and freeze-dried mouse sperm. Proceedings of the National Academy of Sciences of United States of America 98:13501-13506.

Kusakabe H, Yanagimachi R, Kamiguchi Y. 2008. Mouse and human sperm can be freeze-dried without damaging their chromosomes. Human Reproduction 23:233-239.

Kwon IK, Park KE, Niwa K. 2004. Activation, pronuclear formation, and development *in vitro* of pig oocytes following intracytoplasmic injection of freeze dried sperm. Biology of Reproduction 71:1430-1436.

Lai L, Sun Q, Wu G, Murphy CN, Kuhholzer B, Park KW, Bonk AJ, Day BN, Prather RS. 2001. Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes. Zygote 9:339-346.

Lee JW, Tian XC, Yang X. 2004. Optimization of parthenogenetic activation protocol in pig. Molecular Reproduction and Development 68:51-57.

Lee JW, Tian XC, Yang X. 2003. Failure of male pronucleus formation is the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. Biology of Reproduction 68:1341-1347.

Lee JW, Yang X. 2004. Factors affecting fertilization of porcine oocytes following intracytoplasmic injection of sperm. Molecular Reproduction and Developement 68:96-102.

Lee YS, Latham KE, Vandevoort CA. 2008. Effects of *in vitro* maturation on gene expression in rhesus monkey oocytes. Physiology Genomics 35:145-158.

Li Steven Shoei-Lung, Yung-Hsien Liu, Chao-Neng Tseng, Sher Singh. 2006. Analysis of gene expression in single human oocytes and preimplantation embryos. Biochemical and Biophysical Research Communications 340:48-53.

Lindahl T. 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.

Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, Pfeffer R, Bormann CL, Tian XC, Yanagimachi R, Yang X. 2004. Freeze-dried sperm fertilization leads to full-term development in rabbits. Biology of Reproduction 70:1776-1781.

Liu Lili, Yuan Lin, Stanton L. Gerson. 2012. Chapter 2 - *MGMT*: A critical DNA repair gene target for chemotherapy resistance and for stem cell protection. DNA repair in cancer therapy. MR Kelley. San Diego, Academic Press:17-28.

Loi P, Ledda S, Fulka JJ, Moor RM. 1998. Development of parthenogenetic and cloned ovine embryos: effect of activated mammalian oocytes. Biology of Reproduction 58:1177-1187.

Loi P, Matsukawa K, Ptak G, Clinton M, Fulka JJ. 2008. Freeze-dried somatic cells direct embryonic development after nuclear transfer. PLoS ONE 3:2978.

Loi P, Iuso D, Czernik M, Zacchini F, Ptak G. 2013. Towards storage of cells and gametes in dry form. Trends in Biotechnology.31:688-695.

Long CR, Dobrinsky JR, Garrett WM, Johnson LA. 1998. Dual labeling of the cytoskeleton and DNA strand breaks in porcine embryos produced *in vivo* and *in vitro*. Molecular Reproduction and Development 51:59-65.

Lopes S, Andrea J, Jian-Guo S, Robert FC. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human sperm. Human Reproduction 13:896-900.

Macháty Z, Funahashi H, Mayes MA, Day B, Prather RS. 1996. Effects of injecting calcium chloride into *in vitro*-matured porcine oocytes. Biology of Reproduction 54:316-322.

Maedomari N, Kikuchi K, Ozawa M, Noguchi J, Kaneko H, Ohnuma K, Nakai M, Shino M, Nagai T, Kashiwazaki N. 2007. Cytoplasmic glutathione regulated by cumulus cells during porcine oocyte maturation affects fertilization and embryonic development *in vitro*. Theriogenology 67:983-993.

Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Miemillod P. 2001. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 56:17–29.

Marques MG, Nicacio AC, de Oliveira VP, Nascimento AB, Caetano HV, Mendes CM, Mello MR, Milazzotto MP, Assumpção ME, Visintin JA. 2007. *In vitro* maturation of pig oocytes with different media, hormone and meiosis inhibitors. Animal Reproduction Science 97:375-381.

Martin MJ. 2000. Development of *in vivo*-matured porcine oocytes following intracytoplasmic sperm injection. Biology of Reproduction 63:109–112.

Martins CF, Bao SN, Dode MN, Correa GA, Rumpt R. 2007. Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. Theriogenology 67:1307-1315.

Mattioli M, Bacci ML, Galeati G, Seren E. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31:1201-1207.

Matwee C, Betts DH, King WA. 2000. Apoptosis in the early bovine embryo. Zygote 8:57-68.

Memili E, First NL. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. Zygote 8:87-96.

Men Nguyen Thi, Kazuhiro Kikuchi, Michiko Nakai, Atsushi Fukuda, Fuminori Tanihara, Junko Noguchi, Hiroyuki Kaneko, Nguyen Viet Linh, Bui Xuan Nguyen, Takashi

Nagai, Atsushi Tajima. 2013. Effect of trehalose on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection. Theriogenology 80:1033-1044.

Mittal S, Devireddy RV. 2008. Desiccation tolerance of adult stem cells in the presence of trehalose and glycerol. Open Biotechnology Journal 2:211-218.

Morozumi K, Shikano T, Miyazaki S, Yanagimachi R. 2006. Simultaneous removal of sperm plasma membrane and acrosome before intracytoplasmic sperm injection improves oocyte activation/embryonic development. Proceedings of the National Academy of Sciences of United States of America 103:17661-17666.

Morozumi K, Yanagimachi R. 2005. Incorporation of the acrosome into the oocyte during intracytoplasmic sperm injection could be potentially hazardous to embryo development. Proceedings of the National Academy of Sciences 102:14209-14214.

Muller J, Boller T, Wiemken A. 1995. Trehalose and trehalase in plants: recent developments. Plant Science 112:1-9.

Muneto T, Horiuchi T. 2011. Full-term Development of Hamster Embryos Produced by Injecting Freeze-dried Sperm into Oocytes. Journal of Mammalian Ova Research 28:32-39.

Nagashima H, Kashiwazaki N, Ashman R, Grupen C, Seamark RF, Nottle M. 1994. Recent advances in cryopreservation of porcine embryos. Theriogenology 41:113-118.

Nakai M, Ito J, Sato K, Noguchi J, Kaneko H, Kashiwazaki N, K Kikuchi K. 2011. Pre-treatment of sperm reduces success of ICSI in the pig. Reproduction 142:285-293.

Nakai M, Kaneko H, Somfai T, Maedomari N, Ozawa M, Noguchi J, Ito J, Kashiwazaki N, Kikuchi K. 2010. Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts. Reproduction 139:331-335.

Nakai M, Kashiwazaki N, Takizawa A, Maedomari N, Ozawa M, Noguchi J, Kaneko H, Shino M, Kikuchi K. 2006. Morphological changes in boar sperm nuclei with reduced disulfide bonds in electrostimulated pig oocytes. Reproduction 131:603-611.

Nakai M, Kashiwazaki N, Takizawa A, Maedomari N, Ozawa O, Noguchi J, Kaneko H, Shino M, Kikuchi K. 2007. Effects of chelating agents during freeze-drying of boar sperm on DNA fragmentation and on developmental ability *in vitro* and *in vivo* after intracytoplasmic sperm head injection. Zygote 15:15-24.

Nakai M, Kashiwazaski N, Takizawa A, Hayashi Y, Nakatsukasa E, Fuchimoto D, Noguchi J, Kaneko H, Shino M, Kikuchi K. 2003. Viable piglets generated from pig oocytes matured

in vitro and fertilized by intracytoplasmic sperm head injection. Biology of Reproduction 68:1003-1008.

Nanassy L, Lee K, Javor A, Machaty Z. 2007. Changes in MPF and MAPK activities in porcine oocytes activated by different methods. Theriogenology 68:146-152.

Neuber E, Luetjens CM, Chan AW, Schatten GP. 2002. Analysis of DNA fragmentation of *in vitro* cultured bovine blastocysts using TUNEL. Theriogenology 57:2193-2202.

Nguyen BX, Uoc NT, Nagai T, Kikuchi K, Viet-Linh N, Dang-Nguyen QT, Duc NH, Hanh NV, Thanh NT, Bui LC, Huu QX, Minh PN. 2007. Effect of gonadotropin treatment on the oocyte collection and *in vitro* fertilization in Ban pig. Reproduction Fertility and Development 19:269-270.

Niemann H, Rath D. 2001. Progress in reproductive biotechnology in swine. Theriogenology 56:1291-1304.

Niwa K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in the pig. Journal of reproduction and fertility. Supplement 48:49-59.

Norbury CJ, Hickson ID. 2001. Cellular responses to DNA damage. Annual Review of Pharmacology and Toxicology 41:367-401.

Ogura A, Matsuda J, Yanagimachi R. 1994. Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proceedings of the National Academy of Sciences of United States of America 91:7460-7462.

Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B, Heller A. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regulatory Toxicology and Pharmacology 32:56-67.

Onodera M, Tsunoda Y. 1989. Parthenogenetic activation of mouse and rabbit eggs by electric stimulation *in vitro*. Gamete Research 22:277-283.

Ouandaogo ZG, Frydman N, Hesters L, Assou S, Haouzi D, Dechaud H, Frydman R, Hamamah S. 2012. Differences in transcriptomic profiles of human cumulus cells isolated from oocytes at GV, MI and MII stages after *in vivo* and *in vitro* oocyte maturation. Human Reproduction 27: 2438-2447.

Otoi T, Yamamoto K, Koyama N, Tachikawa S, Murakami M, Kikkawa Y, Suzuki T. 1997. Cryopreservation of mature bovine oocytes following centrifugation treatment. Cryobiology 34:36-41.

Paczkowski M, Y. Yuan, J. Fleming-Waddell, C. A. Bidwell, D. Spurlock and R. L. Krisher. 2011. Alterations in the transcriptome of porcine oocytes derived from prepubertal and

cyclic females is associated with developmental potential. Animal Science 89:3561-3571.

Palermo GD, Joris H, Devroey P, Van Steirteghem AC. 1992. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. Lancet 340:17-18.

Pan H, O'brien MJ, Wigglesworth K, Eppig JJ, Schultz RM. 2005. Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development *in vitro*. Developmental Biology 286:493-506.

Pan H, Ma P, Zhu W, Schultz RM. 2008. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. Developmental Biology 16:397-407.

Pangestu M, Lewis I, Shaw J. 1995. Delipidated pronuclear bovine embryos develop to blastocysts which survive cryopreservation. Proceedings of Fertility Society of Australia 14:116.

Pegg AE, Dolan ME, Moschel RC. 1995. Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Progress in Nucleic Acid Research and Molecular Biology 51:167-223.

Pereira CS, Lins RD, Chandrasekhar I, Freitas LC, Hünenberger PH. 2004. Interaction of the disaccharide trehalose with a phospholipid bilayer: a molecular dynamics study. Biophysical Journal 86:2273-2285.

Perreault SD, Barbee RR & Slott VL. 1988. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Developmental Biology 125: 181-186.

Perreault SD, Naish SJ, Zirkin BR. 1987. The timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content. Biology of Reproduction 36:239-244.

Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R. 1999. Mammalian transgenesis by intracytoplasmic sperm injection. Science 14:180-183.

Polge C, Day BN. 1968. Pregnancy following nonsurgical egg transfer in pigs. Veterinary Record 82:712.

Polge C, Smith AV, Parkes AS. 1949. Revival of sperm after vitrification and dehydration at low temperatures. Nature 164:666.

Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, Roelen BA. 2005. Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. Theriogenology 63:2254-2268.

Pope CE, Johnson CA, McRae MA, Keller GL, Dresser BL. 1998. Development of embryos procedure by intracytoplasmic sperm injection of cat oocytes. Animal Reproductive Science 53:221-236.

Preis KA, Seidel GJ, Gardner DK. 2005. Metabolic markers of developmental competence for *in vitro*-matured mouse oocytes. Reproduction 130:475-483.

Presicce G, Yang X. 1994. Parthenogenetic development of bovine oocytes matured *in vitro* for 24 h and activated by ethanol and cycloheximide. Molecular Reproduction and Development 38:380-385.

Probst S, Rath D. 2003. Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes. Theriogenology 59:961-73.

Ptak G, Matsukawa K, Palmieri C, Della Salda L, Scapolo PA, Loi P. 2006. Developmental and functional evidence of nuclear immaturity in prepubertal oocytes. Human Reproduction 21:2228-2237.

Pursel VG, Johnson LA. 1971. Procedure for the preservation of boar sperm by freezing. United states Department of Agriculture ARS Bull 44:1-5.

Qiu JJ, Zhang WW, Wu ZL, Wang YH, Qian M, Li YP. 2003. Delay of ZGA initiation occurred in 2-cell blocked mouse embryos. Cell Research 13:179-185.

R Core Team. R. 2013. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; [accessed 9 Sept 2013] Available from URL http://www.R-project.org/.

Rall WF, Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. Nature 313:573-575.

Rho GJ, Kawarsky S, Johnson WH, Kochhar K, Betteridge KJ. 1998. Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. Biology of Reproduction 59: 918-924.

Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Human Reproduction 11:837-843.

Sakkas D, Urner F, Bizzaro D. 1998. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Human Reproduction 13:11-18.

Salhab M, Dhorne-Pollet S, Auclair S, Guyader-Joly C, Brisard D, Dalbies-Tran R, Dupont J, Ponsart C, Mermillod P, Uzbekova S. 2013. *In vitro* maturation of oocytes alters gene expression and signaling pathways in bovine cumulus cells. Molecular Reproduction and Development 80:166-182.

Sánchez-Partida LG1, Simerly CR, Ramalho-Santos J. 2008. Freeze-dried primate sperm retains early reproductive potential after intracytoplasmic sperm injection. Fertility and Sterility 89:742-745.

Schalkoff ME, Oskowitz SP, Powers RD. 1989. Ultrastructural observation of human and mouse oocytes treated with cryopreservatives. Biology of Reproduction 40:379-93.

Schiraldi C, Isabella DL, Mario DR. 2002. Trehalose production: exploiting novel approaches. Trends in Biotechnology 20:420-425.

Schultz RM. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Human Reproduction Update 8:323-331.

Shemesh M, Gurevich M, Harel-Markowitz E, Benvenisti L, Shore LS, Stram Y. 2000. Gene integration into bovine sperm genome and its expression in transgenic offspring. Molecular Reproduction and Development 56:306-308.

Shevde LA, Riker AI. 2009. Current concepts in biobanking: development and implementation of a tissue repository. Frontiers in Bioscience (Scholar edition) 1:188-193.

Shinohara T, Inoue K, Ogonuki N, Kanatsu-ShinoharaM, Miki H. Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A. 2002. Birth of offspring following transplantation of cryopreserved immature testicular pieces and *in vitro* microinsemination. Human Reproduction 17:3039-3045.

Shrivastav M, De Haro LP, Nickoloff JA. 2008. Regulation of DNA double-strand break repair pathway choice. Cell Research 18:134-47.

Singer TM, Lambert IB, Williams A, Douglas GR, Yauk CL. Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. Mutation Research 2006;598:164-93.

Snedecor GW, Cochran WG. 1989. Statistical methods. The Iowa State University Press 8:273-296.

Somfai T, Kikuchi K , Kaneda M, Akagi S, Watanabe S, Mizutani E, Haraguchi S, Dang-Nguyen TQ, Inaba Y, Geshi M, Nagai T. 2011. Cytoskeletal abnormalities in

relation with meiotic competence and ageing in porcine and bovine oocytes during *in vitro* maturation. Anatomia, Histologia, Embryoligia 40:335-344.

Sotolongo B, Huange TTF, Isenberger E, Ward WS. 2005. An endogenous nuclease in hamster, mouse, and human sperm cleaves DNA into loop-sized fragments. Journal of Andrology 26:272-280.

Steuerwald NM, Bermudez MG , Wells D, Munne S, Cohen J. 2007. Maternal age-related differential global expression profiles observed in human oocytes. Reproductive Biomedicine Online 14:700 -708.

Sun J, Jurisicova A, Casper R. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. Biology of Reproduction 56:602-607.

Sutovsky P, Manandhar G, Wu A, Oko R. 2003. Interactions of sperm perinuclear theca with the oocyte: implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. Microscopy Research and Technique 61:362-378.

Suttner R, Zakhartchenko V, Stojkovic P, Muller S, Alberio R, Medjugorac I. 2000. Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pre-treatment and injection technique. Theriogenology 54:935-948.

Swanson KS, Mazur MJ, Vashisht K, Rund LA, Beever JE, Counter CM, Schook LB. 2004. Genomics and clinical medicine: rationale for creating and effectively evaluating animal models. Experimental Biology and Medicine 229:866-875.

Szczygiel MA, Ward WS. 2002. Combination of dithiothreitol and detergent treatment of sperm causes paternal chromosomal damage. Biology of Reproduction 67:1532-1537.

Tatham BG, Sathananthan AH, Hannawardena VD, Munesinghe DY, Lewis I, Trounson. AO. 1996. Centrifugation of bovine oocytes for nuclear micromanipulation and sperm microinjection. Human Reproduction 11:1499-1503.

Tatham BG, Dowsing AT, Trounson AO. 1995. Enucleation by centrifugation of *in vitro*-matured bovine oocytes for use in nuclear transfer. Biology of Reproduction 53:1088-1094.

Tesarik J, Mendoza C. 1999. *In vitro* fertilization by intracytoplasmic sperm injection. BioEssays 21:791-801.

Tesarik J, Sousa M. 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. Fertility and Sterility 63:343-349.

Tesfaye D, Ghanem N, Carter F, Fair T, Sirard MA, Hoelker M, Schellander K, Lonergan P. 2009. Gene expression profile of cumulus cells derived from cumulus–oocyte

complexes matured either *in vivo* or *in vitro*. Reproduction, Fertility and Development 21:451-461.

Thadani VM. 1980. A study of hetero-specific sperm-egg interactions in the rat, mouse and deer mouse using *in vitro* fertilization and sperm injection. Journal of Experimental Zoology 212:435-53.

Thelie A, Papillier P, Pennetier S, Perreau C, Traverso JM, Uzbekova S, Mermillod P, Joly C, Humblot P, Dalbies-Tran R. 2007. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. BMC Developmental Biology 7:125.

Tian JH, Wu ZH, Liu L, Cai Y, Zeng SM, Zhu SE, Liu GS, Li Y, Wu CX. 2006. Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from *in vitro*-matured oocytes and intracytoplasmic sperm injection. Theriogenology 66:439-448.

Uehara T, Yanagimachi R. 1976: Microsurgical injection of sperm into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Biology of Reproduction 15:467-470.

Van Steirteghem AC, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, Derde MP, Van Assche E, Devroey P. 1993. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. Human Reproduction 8:1055-1060.

Vassena R, Boué S, González-Roca E, Aran B, Auer H, Veiga A, Izpisua Belmonte JC. 2011. Waves of early transcriptional activation and pluripotency program initiation during human preimplantation development. Development 138:3699-3709.

Viet Linh N, Kikuchi K, Nakai M, Noguchi J, Kaneko H, Dang-Nguyen TQ, Maedomari N, Nguyen BX, Nagai T, Manabe N. 2011. Improvement of porcine oocytes with low developmental ability after fusion of cytoplasmic fragments prepared by serial centrifugation. Journal of Reproductive Development 57:620-626.

Villani P, Eleuteri P, Grollino MG, Rescia M, Altavista P, Spanò M, Pacchierotti F, Cordelli E. 2010. Sperm DNA fragmentation induced by DNAse I and hydrogen peroxide: an *in vitro* comparative study among different mammalian species. Reproduction 140:445-452.

Vincent C, Pickering SJ, Johnson MH. 1990. The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a

reduction in the number of cortical granules present. Journal of Reproduction and Fertility 89:253-259.

Wakayama T, Yanagimachi R. 1998. Development of normal mice from oocytes injected with freeze-dried sperm. Nature Biotechnology 16:639-641.

Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369-374.

Wall RJ, Pursel VG, Hammer RE, Brinster RL. 1985. Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biology of Reproduction 32, 645-651.

Wall RJ, Hawk HW. 1988. Development of centrifuged cow zygotes cultured in rabbit oviducts J Reprod. Fertil, 82, 673-680.

Ward MA1, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG, Yanagimachi R. 2003. Long-term preservation of mouse sperm after freeze-drying and freezing without cryoprotection. Biology of Reproduction 69:2100-2108.

Watanabe H, Suzuki H, Fukui Y. 2010. Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated sperm in mice. Reproduction 139:513-521.

Watanabe M, Hitomi M, van der Wee K, Rothenberg F, Fisher SA, Zucker R, Svoboda KK, Goldsmith EC, Heiskanen KM, Nieminen AL. 2002. The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. Microsc Microanalysis 8:375-391.

Wei H, Fukui Y. 1999. Effects of bull, sperm type and sperm pretreatment on male pronuclear formation after intracytoplasmic sperm injection in cattle. Reproduction, Fertility and Development 11:59-65.

Wood RD, Mitchell M, Sgouros J, Lindahl T. 2001. Human DNA repair genes. Science 291:1284-1289.

Wossidlo M, Arand J, Sebastiano V, Lepikhov K, Boiani M, Reinhardt R, Schöler H, Walter J. 2010. Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes. Embo Journal 29:1877-1888.

Yamauchi Y, Yanagimachi R, Horiuchi T. 2002. Full-term development of golden hamster oocytes following intracytoplasmic sperm head injection. Biology of Reproduction 67:534-539.

Yanagida K, Kimura Y, Katayose H, Yazawa H, Konnai K, Sato A. 1997. Fertilization using male germ line-cells. Human cell 10:255-262.

Yanagimachi R. 1994. Mammalian fertilization. In The Physiology of Reproduction, 2nd edn. Eds E Knobil, JD Neill. New York: Raven Press:189-317.

Yoneda A, Kashima M, Yoshida S, Terada K, Nakagawa S, Sakamoto A, Hayakawa K, Suzuki K, Ueda J & Watanabe T. 2006. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Czeta. Reproduction 132:393-401.

Yong HY, Hong JY, Pak SI, Kang SK, Lee BC, Lee ES, Hwang WS. 2005. Effect of centrifugation and electrical activation on male pronucleus formation and embryonic development of porcine oocytes reconstructed with intracytoplasmic sperm injection. Reproduction, Fertility and Development 17:557-563.

Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel GV. 1993a. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biology of Reproduction 49:89-94.

Yoshida M, Mizoguchi Y, Ishigaki K, Kojima T, Nagai T. 1993b. Birth of piglets derived from *in vitro* fertilization of pig oocytes matured *in vitro*. Theriogenology 39:1303-1311.

Yushchenko NP. 1957. Proof of the possibility of preserving mammalian sperm in a dried state. Proceeding of Lenin Academy of Agricultural Sciences 22:37-40.

Zeiss CJ. 2003. The apoptosis-necrosis continuum: insights from genetically altered mice. Veterinary Pathology 40:481-495.

Zheng P, RD Schramm, KE Latham. 2005. Developmental regulation and *in vitro* culture effects on expression of DNA repair and cell cycle checkpoint control genes in rhesus monkey oocytes and embryos. Biology of Reproduction 72:1359-1369.

Zhou Xiaomei, Mingru Yin, Weihua Jiang, Manxi Jiang, Shangang Li, Heping Li, and Xuejin Chen. 2013. Electrical activation of rabbit oocytes increases fertilization and embryo development by intracytoplasmic sperm injection using sperm from deceased male. Journal of Assisted Reproduction and Genetics 30:1605-1610.

Zhu Jie, Evelyn E. Telfer, Judy Fletcher, Anthea Springbett, John R. Dobrinsky, Paul A. De Sousa, Ian Wilmut. 2002. Improvement of an electrical activation protocol for porcine oocytes. Biology of Reproduction 66:635-641.

Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. 2005. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Human Reproduction 20:3476-3480.

Zuo Chunlai, Lingbao Ai, Pam Ratliff, James Y. Suen, Ehab Hanna, Thomas P. Brent, Chun-Yang Fan. 2004. O6-methylguanine-DNA methyltransferase gene: Epigenetic silencing and prognostic value in head and neck squamous cell carcinoma. Cancer Epidemiology Biomarkers and Prevention 13:967-975.