Studies on preservation of porcine zygotes for embryo production by interspecies somatic cell nuclear transfer

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CONTENTS

LIST OF ABBREVIATION

ANOVA	analysis of variance
BSA	bovine serum albumin
CB	cytochalasin B
CGA	chlorogenic acid
CO_2	carbon dioxide
COCs	cumulus oocyte complexes
^{O}C	celsius
DMEM	dulbecco's modified eagle's medium
DMD	duchenne muscular dystrophy
DMSO	dimethyl sulfoxide
DPBS	dulbecco's phosphate buffered saline
ET	embryo transfer
FBS	foetal bovine serum
FCS	foetal calf serum
Fw	forward primer
g	gram
GGTA1	glycoprotein galactosyltransferase alpha 1, 3
GHR	Growth hormone receptor

GLM	general linear model
h	hour
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
LN2	liquid nitrogen
M	molar
MI	metaphase of the first meiotic division
MII	metaphase of the second meiotic division
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
n-3	omega-3
OPS	open pulled straw
PCR	polymerase chain reaction
PBM	porcine blastocyst medium
PFF	porcine follicular fluid
PFM	porcine fertilization medium

PZM-5	porcine zygote medium
Rv	reverse primer
SAS	statistical analysis system
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
TCM-199	tissue culture medium 199
TUNEL	terminal transferase dUTP nick end labelling
µg	microgram
µL	microliter
µM	micromolar
µsec	microsecond
µg/mL	microgram/milliliter
UV	ultraviolet
V	volt
v/v	volume/volume
W/v	weight/volume
WOW	well-of-the-well
ZP	Zona pellucida

CHAPTER 1. INTRODUCTION

1.1. Introduction

Mammals are remarkable organisms that have different morphologies with important characteristics allowing scientists to create animal model systems. The establishment of these animal systems is especially important for further investigations because it is exceedingly difficult to establish an appropriate model for research in complex animal species, including humans. In recent years, the major challenge for the field of reproductive biotechnology has been exploring the molecular and cellular mechanisms that are involved in controlling the quality of oocytes. The mammalian oocyte is a specific structure consisting of cytoplasmic organelles that communicate among themselves and are spatially associated. The porcine species represents an excellent experimental model for primary and biomedical research because of their physiological and immunological resemblances with humans [1, 2]. Pigs are already popular as models for cardiovascular disease, cutaneous pharmacology and toxicology, lipoprotein metabolism, and pathobiology of intestinal transport, injury, and repair [3].The porcine embryo production *in vitro* is particularly important in reproductive biotechnology for cloning, transgenesis, since these animals are

used as donors of organs for xenotransplantation, embryonic stem cell lines, and also as a model of human diseases.

1.2. Objectives of the Thesis

Hypothermic preservation is preferable for short-term shipment or storage of cells. Hypothermic preservation comes with many advantages compared with cryopreservation. It is an inexpensive technique, easy to perform on cells or tissue, does not require cytoprotective agents, and has high cell viability after storage. Thus, several storage solutions are used worldwide, including Euro-Collins [4], Viaspan [5], Celsior [6]. However, it is known that many current problems confronting hypothermic storage must be addressed, such as a less toxic cytoprotective agent, inhibiting the several-day decline in cell viability often noted under extended preservation regimes once cells are returned to normothermic temperatures, and resolving the critical limitation of the cryopreservation cap. Therefore, it is necessary to research whether to store embryos at room temperature to store them in a chemical solution. Chapter 3).

During storage, many factors may affect the resulting quality of embryos, and in particular, the zona pellucida. The zona pellucida (ZP) (in oocytes of mammals) is the outer extracellular layer that protects the inner ovule. Structurally, the zona pellucida membrane is a complex of three classes of

glycoproteins ZP1, ZP2, ZP3, which are synthesized, secreted, and added in stages of the oocyte's development during oocyte production. The ZP supports communication between oocytes and follicle cells during oogenesis, protects oocytes, eggs, and embryos during development, and regulates interactions between ovulated eggs and free-swimming sperm during subsequent fertilization [7]. Additionally, there were reports of ZP malfunction and anti-zonal antibodies that caused infertility and failure of *in vitro* fertilization (IVF) [7, 8]. Escaping from the ZP membrane is a necessary condition for an embryo to implant into the lining of the uterus. Embryo cryopreservation is an essential technique for assisted reproduction. The assisted reproductive technique has helped to produce many embryos in the cycle with stimulation of the ovary, and excess embryos can be cryopreserved and transferred to patients in other cycles, increasing the chance of pregnancy by IVF. However, the embryo cryopreservation process can lead to a stiffening of the ZP. Therefore, it is suspected that removal of the ZP is an efficient method for overcoming infertility caused by ZP abnormality in humans and other mammals [8].

Oocyte cryopreservation is favorable for the utilization of valuable genetic resources. Pigs are one of the laboratory animals that have become widely recognized for this purpose. Furthermore, the pig genome is a useful resource for the establishment of human disease models and research on human

regenerative medicine. In recent years, scientists have developed the vitrification cryopreservation method, which is regarded as the most effective for porcine embryos [8]. Published data also indicate that porcine embryos could be successfully vitrified as zygotes [9]. These observations suggest that the complete removal of the ZP is a realistic possibility to eliminate the shortcomings caused by the *in vitro* culture environment [10]. The disadvantage of a culture without the ZP is that the culture must be done in small droplets [11]. However, reports about the effectiveness of porcine embryo culture vitrification at the zygote stage are limited. One of the aims of this thesis is to elucidate the functions of the ZP during vitrification of zygotes and to determine the appropriate volume of medium for individually cultured ZP-intact and ZP-free zygotes (Chapter 4).

Somatic cell nuclear transfer (SCNT) is not only a valuable tool for producing animals with the same genetic traits, but it is also an alternative technique to help preserve endangered animals. On the other hand, this study also proposed to identify the ability of porcine ooplasm to support the *in vitro* development of embryos, to the blastocyst stage, using interspecific SCNT (iSCNT) of somatic cells from various unrelated wild animal species (Chapter 5).

CHAPTER 2. LITERATURE REVIEW

2.1. Cryobiology

Cryobiology is a branch of biology that studies the effect of low temperatures on living things. In practice, cryobiology is the study of biological material or systems at temperatures below physiological conditions. Materials or systems studied include proteins, cells, tissue, organs, or whole organisms. There are six major areas of cryobiology: 1) study of cold-adaptation of microorganisms, plants, and animals, 2) cryopreservation of cells, tissues, gametes, and embryos of animal and human origin, 3) storage of organs under hypothermic conditions for transplantation, 4) lyophilization (freeze-drying), 5) cryosurgery and 6) physics of super cooling, ice nucleation, ice growth and mechanical engineering aspects of heat transfer during cooling and warming. Cryopreservation is an application of cryobiology where cells or whole tissues are preserved by cooling to low sub-zero temperatures, for example, –196 °C (the boiling point of liquid nitrogen, LN_2). At these low temperatures, any biological activities, including the biochemical reactions that would lead to cell death, are effectively stopped. Until the mid-twentieth century, it was believed that extremely low temperatures would only harm cells and tissue. Achievements and advances in cryobiology and the possibilities that the field has created were considered unimaginable.

Birth of the first live offspring from cryopreserved embryos opened the door to potentially applying this technology to animal breeding and clinical assisted reproduction [12, 13]. Since the birth of the first mice from cryopreserved embryos live births have been reported in over 10 mammalian species (Table 1). In 2004 about 550 thousand bovine embryos were transferred into recipients [14], and about half of those were cryopreserved [15]. Successful cryopreservation of embryos have been reported in several species including cattle [15, 16], sheep [17, 18], pigs [19], horses [20], mice [21], and humans [22].

Table 1. Mammalian species yielding healthy offspring following the transfer of cryopreserved embryos to foster mother

Species	**Year**	**Reference**
Rabbit	1974	Bank et al. (1974) [23]
Sheep	1976	Willadsen et al. (1976) [24]
Goat	1976	Bilton and Moor (1976) [25]
Human	1984	Zeilmaker et al. (1984) [26]
Monkey	1986	Balmaceda et al. (1986) [27]
Cat	1988	Dresser el al. (1988) [28]
Pig	1989	Hayashi el al. (1989) [29]
Mouse	1995	Bos-Mikich et al. (1995) [30]
Hamster	1999	Lane et al. (1999) [31]
Mongolian gerbil	1999	Mochida et al. (1999) [32]
Bovine	2005	Abe et al. (2005) [33]
Buffalo	2007	Boonkusol et al. (2007) [34]
Equine	2009	Campos-Chillon et al. (2009) [35]

The principles of cryopreservation are believed to be similar for all living cells. The most crucial consideration is removing most of the water from the

cells before they freeze intracellularly. Most cryopreservation strategies are based on the following two key factors: cryoprotectant and cooling-warming rates. Currently, there are three strategies for oocyte and embryo storage slow-freezing, conventional vitrification (vitrification in-straw), and ultra-rapid vitrification (Fig.1).

Slow-freezing was the first system used for embryo cryopreservation. In this system, controlled cooling rates allow extra-cellular and intra-cellular fluid exchange without serious osmotic effects and changes in cell shape; this system's alternate name-equilibrium freezing-reflects this effect [11]. An important constraint in the use of conventional slow-freezing is the critical period of oocyte exposure to the cryoprotectant and the necessity to perform the process only in a laboratory equipped with an automated freezing system so that the cooling rate can be tightly controlled [36].

In 1985, Rail and Fahy devised an innovative method called vitrification (ice-free rapid solidification), in which oocytes or embryos suspended in a highly concentrated solution, are loaded in a straw and directly plunged into LN_2 starting with a temperature above 0 °C [37]. Significantly higher levels of oocyte and embryo viability can be maintained through this technique. The biggest obstacle to this approach is the toxicity of the high concentrations of cryoprotectant (5 - 8 mol/L) used to prevent ice formation [36].

In some species, the low survival rate is due to the sensitivity of oocytes and embryos to chilling. These species include porcine embryos, bovine oocytes, and bovine embryos at early cleavage stages [38]. Another reason for the low survival rate is lower permeability of the cell membrane, which could lead to the formation of intracellular ice and osmotic swelling. A third reason for the low survival rate is the toxicity of the cryoprotectant during exposure of the cells to the concentrated vitrification solution [36]. A new strategy aims to overcome these injuries by markedly increasing both cooling and warming rates. Using this approach, critical temperatures at which the cells are injured could be passed quickly, so the formation of intracellular ice might be avoided even in less concentrated cells, and the use of lower concentrations of cryoprotectant could be possible [36].

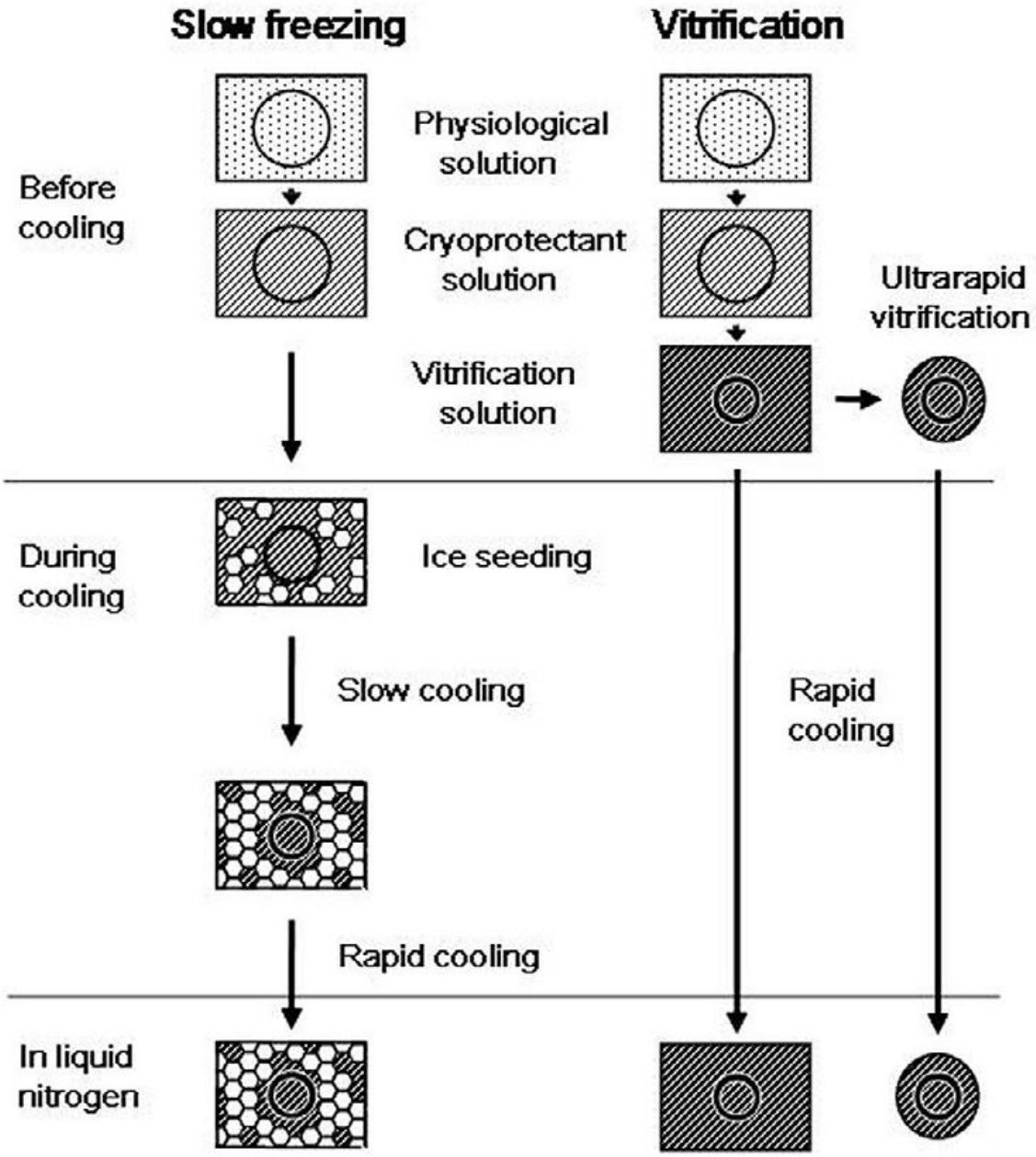

Figure 1. Schematic presentation of an embryo (circle) before cooling, during cooling, and in liquid nitrogen using the processes of slow-freezing, conventional straw vitrification, and ultra-rapid vitrification. White hexagons represent ice crystals [39].

2.2 Somatic cell nuclear transfer (SCNT)

SCNT, one of the assisted reproductive technologies, is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm (Fig. 2). SCNT in mammals was not achieved until more than four decades after the initial reports from Briggs and King of the production of adult frog clones using embryonic nuclei [40], which sparked the long-held passion of researchers working with mammalian eggs. This technique has succeeded in an ever-growing list of species. In each case, an enucleated oocyte has effectively reset the nucleus of a somatic cell such that the embryonic program could progress to the production of a live offspring [41]. The first live cloned offspring produced from the differentiated cell populations were two lambs born in 1995 using cultured embryonic cells as nuclear donors and nucleated unfertilized eggs (metaphase II oocytes, MII) as recipient cytoplasts [42]. The following year, offspring were produced using the cultured cell populations derived from fetal and adult lamb tissue [43].

Since that time, SCNT has been successfully applied to many species including cattle [44], a mouse [45], a goat [46], a pig [10], a cat [47], a rabbit [48], a horse [49], a rat [50], a dog [51], and a ferret [52] using a range of cell types. Even though the frequency of new developments has not dramatically

increased, the modification and improvements of techniques are still ongoing, including (1) simplifying the methodology, (2) reducing costs, and (3) improving survival following birth. The production of cloned embryos involves many steps, and each of these may influence the successful outcome [53].

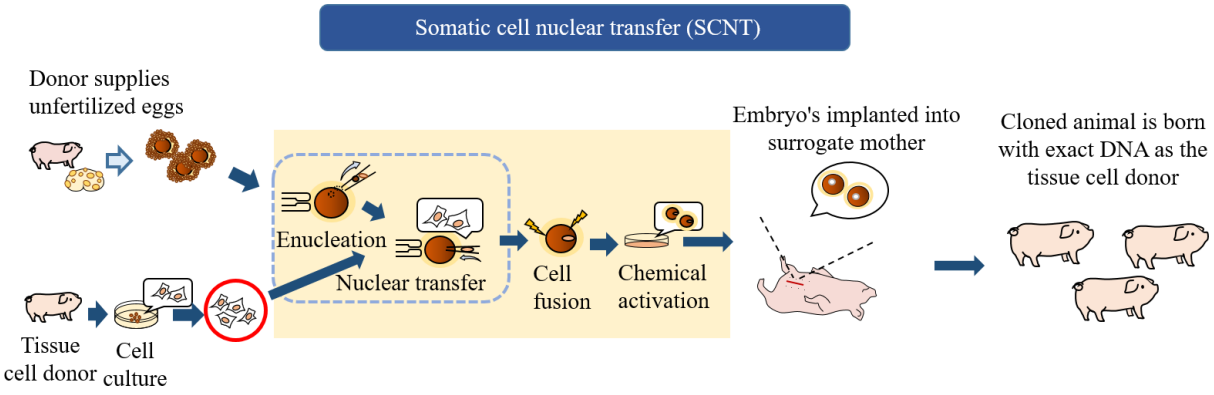

Figure 2. The somatic cell nuclear transfer (SCNT) animal is produced by transferring the nucleus from diploid to an unfertilized oocyte from which the chromosomes have been removed. The chromosomes are remodeled and developed to a particular stage before being transferred to a surrogate mother. The cloned offspring are born with the exact DNA of the donor cell.

2.3 Interspecies SCNT (iSCNT)

iSCNT has been continually developed for endangered animal conservation as well as for analyzing the interactions between the donor nucleus and the recipient cytoplasm [54]. This technology was established for many reasons, including the production of embryos from species whose oocytes are challenging to obtain or where their collection is under restricted control [55].

Although no pregnancies were reported, Domiko et al. (1999) [56] were the first to attempt iSCNT in which somatic cells of monkey, sheep, and pig were transferred into the enucleated bovine oocyte resulting in viable embryos which showed varying degrees of early embryo development *in vitro.* Subsequent reports successfully produced iSCNT embryos using different species of a donor cell and oocytes such as chicken-rabbit [57], panda-rabbit [58], human-rabbit [59], rhesus monkey-bovine [60], human-bovine [61], human-goat [62]. Tajia Dominko et al. (1999) [63] reported that in cattle enucleated hos oocytes have been fused to fibroblast cells from sheep, pig, monkey, and rat to assess the viability of cross-species zygote reconstruction. Panda or cat iSCNT into rabbit oocytes can develop to blastocyst *in vitro* [64]. Zhao et al. (2006) [65] showed that the rabbit oocyte cytoplasm is capable of dedifferentiating somatic cell nuclei from camel and Tibetan antelope. Reconstructed gaur, banteng, and yak embryos were obtained after transfer of cells into enucleated domestic cow oocytes [66]. However, complete nuclear reprogramming, low blastocyst rate, and abnormal epigenetic reprogramming remain significant problems for this technique [67]. Live offspring from a few endangered mammalian species have been produced by iSCNT (Fig. 3).

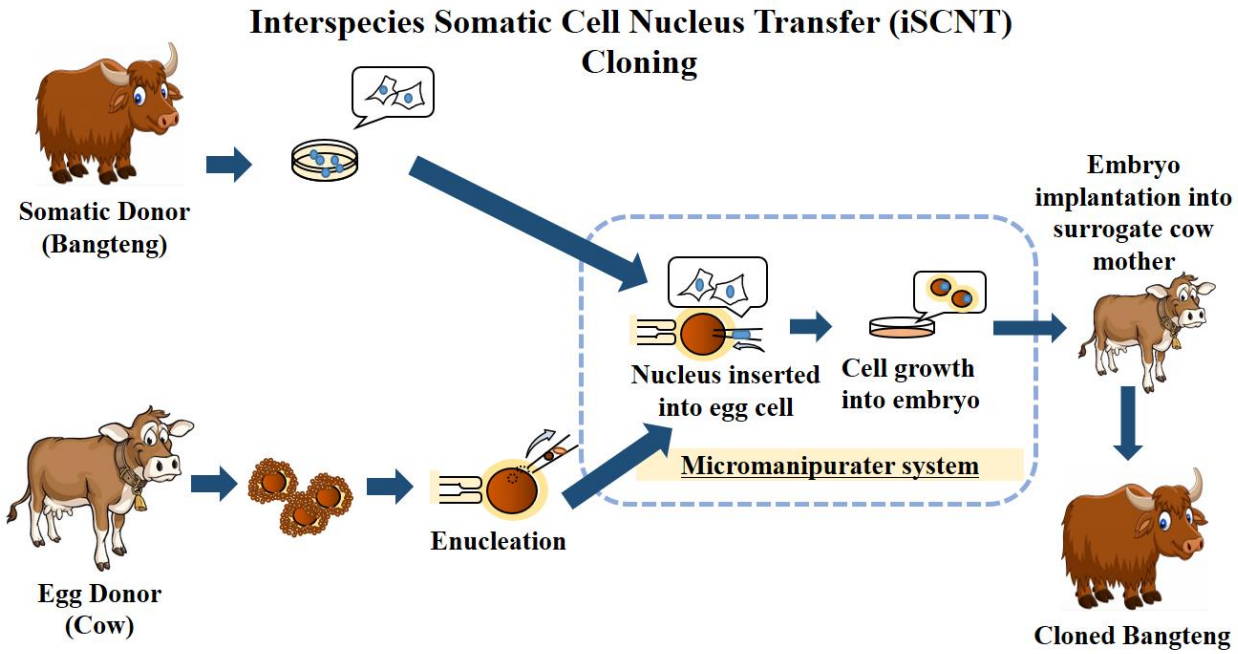

Figure 3. The process of interspecies somatic cell nuclear transfer (iSCNT)

CHAPTER 3. HYPOTHERMIC STORAGE OF PORCINE ZYGOTES IN SERUM SUPPLEMENTED WITH CHLOROGENIC ACID

3.1 Abstract

The current study was conducted to investigate the effects of 100% foetal bovine serum (FBS) and 100% porcine follicular fluid (pFF) as a storage medium on the developmental competence of porcine zygotes stored at 25°C for 24 h. Moreover, we evaluated the additive effects of chlorogenic acid (CGA) in the storage medium. When in vitro-produced zygotes were stored at 25°C for 24 h in tubes containing either tissue culture medium (TCM) 199 supplemented with 1 mg/mL bovine serum albumin (BSA), 100% of FBS or 100% of pFF, the rate of blastocyst formation was significantly higher in 100% of FBS than in BSA-containing TCM 199. When the effects of CGA supplementation in 100% of FBS on the development of zygotes stored at 25°C for 24 h was evaluated, more zygotes stored with 50 μM CGA developed to blastocysts compared with the other concentrations of CGA. When the formation date and quality of blastocysts derived from zygotes stored in 100% of FBS supplemented with 50 μM CGA were investigated, the highest ratio of blastocysts formation in the storage group appeared 1 day later than in the non-stored control group.

However, a higher proportion of blastocysts with apoptotic nuclei was observed in the stored group as compared to the non-stored group.

3.2 Introduction

Production of pig embryos by *in vitro* fertilization (IVF) has been studied from these late 20th centuries and conducted as an important tool for reproduction and, up to now, *in vitro* production (IVP) system for porcine embryos has been studied with the desire to produce large numbers of pigs and better quality pigs for basic research as well as biomedical research [19, 68]. The eggs of some mammals also contain quite large amounts of lipid. Especially, porcine oocytes have rich in lipids and lipid droplets, which are very sensitive to low temperatures [38, 88].

Porcine long-term preservation techniques such as cryopreservation and vitrification in liquid nitrogen (LN2) provide promising results [68]. Nevertheless, there is interest in porcine embryo preservation with maintenance of embryo development for 24 h. The method for storing and temporarily arresting embryo development enables the manipulation to be performed at a convenient time or place that is different from the site of embryo production. Moreover, the preservation of embryos without LN2 is simpler, less expensive and does not require the use of special equipment. However, research concerning short-term storage of porcine embryos has been limited. Recently, pre-implantation embryos at the zygote stage have been frequently used for

production of transgenic pigs by using a genome editing system [69, 70]. Therefore, it becomes increasingly necessary to develop a hypothermic storage medium that can maintain the post-stored viability of in vitro produced zygotes for the manipulation at a convenient time. Serum additives or bovine serum albumin (BSA) based preparations have been used as supplements for the hypothermic preservation of mammalian embryos [71, 72]. Ideta et al. (2013) [61] reported that the addition of serum with a high concentration in the storage medium enhances viability of bovine embryos after hypothermic preservation compared with BSA addition. They suggested that bovine embryos could be stored in 100% bovine serum at hypothermic temperatures. Serum provides energy substrates and amino acids for metabolic and anabolic processes as well as chelation of heavy metal ions or other toxins [73]. On the other hand, it has been demonstrated that porcine oocytes could be matured in 100% of porcine follicular fluid (pFF) [74]. Follicular fluids also contain growth factors, electrolytes, hormones, amino acids and unknown factors [75, 76]. If embryos could be preserved in 100% serum or pFF for a short period, the procedure of hypothermic preservation becomes simpler. However, storage medium for in vitro-produced embryos have not been well studied, and, specifically, little is known about the hypothermic storage of porcine zygotes. To our knowledge, little information is available concerning the usage of 100% serum or pFF as a storage medium. During hypothermic storage, the advantages of decreasing metabolic rates for preservation becomes a disadvantage, leading to increased

cellular perturbations, which are associated with an increase of available cellular labile iron pool, initiating the formation of reactive oxygen species (ROS). The increase in ROS levels is usually followed by massive lipid peroxidation and alteration of mitochondrial function, which can lead to cell necrosis in hypothermia or apoptosis upon rewarming [77]. Phenolic compounds in coffee beans such as chlorogenic acid (CGA), which is the main class responsible for antioxidant activity, are ROS scavengers [78]. Chlorogenic acid has in vitro free radical scavenging properties and prevents the propagation of oxidative processes [79]. Chlorogenic acid has been shown to limit apoptosis related to oxidative stress by reduced ROS production and by an increase of intracellular glutathione levels in cells [80]. In the current study, we therefore investigated the effects of 100% foetal bovine serum (FBS) and pFF as a storage medium on the developmental competence of porcine zygotes stored at 25°C for 24 h. Moreover, we evaluated the effects of various concentrations of CGA on the post stored development of the zygotes.

3.3 Materials and methods

There were no live animals used in this study, so no ethical approval was required.

3.3.1 In vitro maturation

Porcine ovaries were obtained from approximately 6-month-old gilts at a local slaughterhouse and were transported within 3 h to the laboratory in physiologic saline at 30°C. Ovaries were washed three times with modified phosphate buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/mL streptomycin sulphate (Meiji). The cumulus-oocyte complexes (COCs) were collected from ovaries. The follicles of the ovarian surface were sliced using a surgical blade on the sterilised dish. Only COCs with a uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected under a stereomicroscope. Approximately 50 COCs were then cultured in 500 μL of maturation medium, consisting of 25 mM HEPES tissue culture medium 199 with Earle salts (tissue culture medium [TCM] 199; Invitrogen Co., Carlsbad, CA, USA), supplemented with 10% (v/v) porcine follicular fluid; 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA); 50 μM sodium pyruvate (Sigma-Aldrich); 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd.); 1 μg/mL 17β-oestradiol (Sigma-Aldrich); 10 IU/mL equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan); 10 IU/mL human chorionic gonadotropin (Kyoritu Seiyaku); and 50 μg/mL gentamicin (Sigma-Aldrich), for 22 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred

into maturation medium without hormone supplementation and cultured for an additional 22 h. Cumulus-oocyte complexes were incubated at 39°C in a humidified incubator containing 5% CO_2 in air.

3.3.2 In vitro fertilization

In vitro fertilization was performed according to methods described by Nguyen et al. (2018). Frozen-thawed spermatozoa were transferred into 5 mL of fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) in a 15-mL test tube and were then washed by centrifugation at 500 *g* for 5 min. The pellets of spermatozoa were resuspended in fertilization medium to obtain a final sperm concentration of 1×10^7 cells/mL. Some of the spermatozoa (50 μL) were added to 50 μL of fertilization medium containing 10–20 matured oocytes. The final sperm concentration was adjusted to 5×10^6 cells/mL. The oocytes were co-incubated with spermatozoa at 39°C in a humidified incubator containing 5% CO_2, 5% O_2 and 90% N_2. Following co-incubation with spermatozoa for 5 h, the presumed zygotes were denuded from the cumulus cells and attached spermatozoa by mechanical pipetting.

3.3.3 IVC and assessment of blastocyst quality

The zygotes were subsequently transferred to 100-μL droplets of PZM-5 (Research Institute for the Functional Peptides Co.). Each droplet contained approximately 10 presumed zygotes. The zygotes were cultured continuously in vitro at 39°C in a humidified incubator containing 5% CO_2, 5% O_2 and 90% N_2. All the cleaved embryos were transferred into 100-μL droplets of PBM (Research Institute for the Functional Peptides Co.) at 72 h after the start of culture and cultured for an additional 4 days to evaluate their ability to develop to the blastocyst stage. To evaluate the total cell number and DNA fragmentation in the blastocysts, all embryos at the blastocyst and expanded blastocyst stages were fixed at the end of culture and were analysed using a combined technique for simultaneous nuclear staining with DAPI (Invitrogen) and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) according to methods described by Do et al. (2015) [81]. The apoptotic index was calculated by dividing the number of cells containing apoptotic nucleus (labelled by TUNEL) by the total number of cells (Fig. 4).

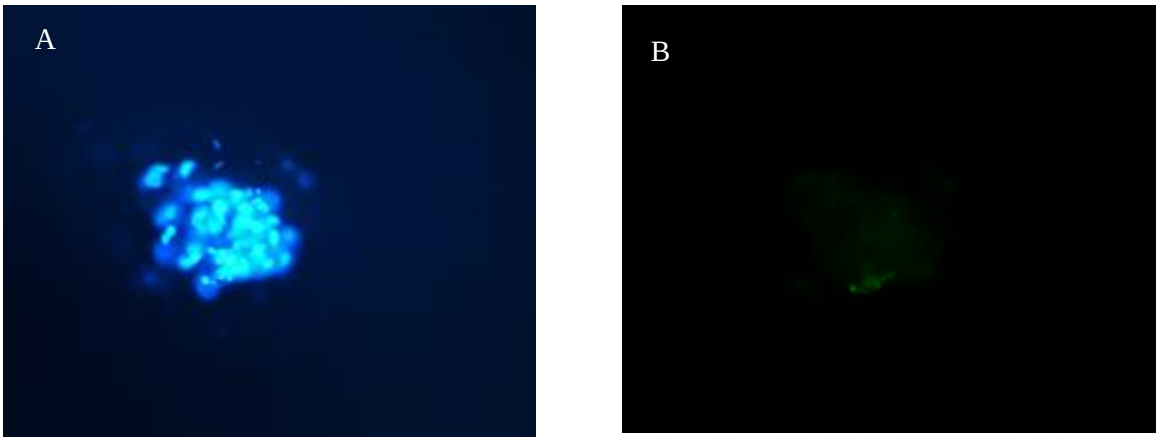

Figure 4. Representative images of porcine blastocysts stained by DAPI (A) and TUNEL (B). Blue (A) and green (B) colors represent regular nuclei and apoptotic nuclei, respectively.

3.3.4 Experimental design

In the first experiment, we examined the effects of storage medium on the development of zygotes stored at 25°C for 24 h. After removal of cumulus cells from zygotes inseminated for 5 h, the zygotes were cultured in PZM-5 for an additional 5 h. Then, the zygotes were randomly allocated to four groups and transferred into 0.6 mL tubes (Fukase Kasei, Hyogo, Japan) containing either TCM 199 supplemented with 1 mg/mL BSA (Sigma-Aldrich), 100% FBS (HyClone, Logan, Utah, USA) or 100% pFF. The tubes were placed in an incubator (Cool-Incubator A1201, Ikuta Sangyo, Nagano, Japan) at 25°C for 24 h. After 24 h of storage, the zygotes were transferred to 100 μL droplets of PZM-5 and then cultured for 7 days to evaluate their developmental ability as

described previously. As a control group, the zygotes were cultured for 7 days without the storage for 24 h. In the second experiment, we evaluated the supplementation of CGA in the storage medium on the development of zygotes stored at 25°C for 24 h. The zygotes were stored in 100% FBS because we found that more embryos could develop to the blastocyst stage after storage at 25°C in the first experiment. The zygotes were randomly allocated to five groups and stored in either FBS (control), FBS supplemented with 1 μl/mL dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc. Kyoto. Japan), FBS supplemented with 10 μM CGA diluted in DMSO, FBS supplemented with 50 μM CGA in DMSO or FBS supplemented with 100 μM CGA in DMSO. After storage in each medium at 25°C for 24 h, the zygotes were cultured for 7 days. In the third experiment, we investigated the formation date and quality of blastocysts derived from zygotes with and without storage treatment. The zygotes were stored in 100% FBS supplemented with 50 μM CGA because we found that more embryos could develop to the blastocyst stage after storage at 25°C in the second experiment. The date of blastocyst appearance was recorded, and the apoptotic nuclei in blastocysts were analysed as described previously.

3.3.5 Statistical analysis

Each experiment was repeated six to seven times. Statistical significance was inferred from analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Percentage data were subjected to arcsine transformation before statistical analysis. Data from apoptotic indices were evaluated using the *t* test. Differences with a value of $p \leq 0.05$ were regarded as significant.

3.4 Results

3.4.1 Experiment 1

As shown in Table 2, when porcine zygotes were stored for 24 h, the rate of blastocyst formation in the FBS group was significantly higher ($p < 0.05$) than that in the TCM 199 group. However, the rates of blastocysts formation in the storage groups were significantly lower than those of the control group without the storage treatment ($p < 0.01$), irrespective of the storage medium. The rates of cleavage and mean total cell numbers in blastocysts did not differ among the groups.

Table 2. Effect of storage medium on the development of zygotes stored at 25°C for 24 h*

Storage medium	No. of oocytes examined	No. (%) of embryos		Total cell number of Blastocysts
		Cleaved	Developed to blastocysts	
Control**	502	341 (68.8 ± 3.8)	63 (12.7 ± 0.5)[a]	38.6 ± 1.6
TCM 199	497	325 (62.5 ± 5.4)	7 (1.3 ± 0.7)[b]	30.1 ± 2.6
pFF	508	346 (66.3 ± 4.9)	13 (2.4 ± 1.0)[bc]	34.2 ± 2.6
FBS	485	315 (65.1 ± 4.4)	23 (5.1 ± 1.7)[c]	36.1 ± 1.8

Note. FBS: foetal bovine serum; pFF: porcine follicular fluid; TCM: tissue culture medium.

*Six replicate trials were carried out. Data are expressed as mean ± *SEM*.

**As control, the zygotes were cultured for 7 days without the storage treatment.

[a-c]Values with different superscripts in the same column are significantly different ($P < 0.05$).

3.4.2 Experiment 2

As shown in Table 3, when porcine zygotes were stored in 100% FBS supplemented with or without various concentrations of CGA for 24 h, the rate

of blastocysts formation from zygotes stored with 50 μM CGA was significantly higher ($p < 0.01$) than that from zygotes stored with the other concentrations of CGA. However, the rates of cleavage and mean total cell numbers in blastocysts did not differ among the groups.

Table 3. Effect of chlorogenic acid supplementation in storage medium on the development of zygotes stored at 25°C for 24 h *

Concentration of CGA (μM)	**No. of oocytes examined**	**No. (%) of embryos**		**Total cell number of blastocysts**
		Cleaved	**Developed to blastocysts**	
Control	249	195 (78.2 ± 1.5)	14 (5.7 ± 0.2)[a]	38.0 ± 1.7
0	256	199 (77.5 ± 1.7)	19 (7.4 ± 0.4)[b]	38.3 ± 2.0
10	248	196 (79.0 ± 1.9)	14 (5.7 ± 0.1)[a]	35.9 ± 2.2
50	247	195 (79.1 ± 1.8)	25 (10.1 ± 0.6)[c]	37.8 ± 1.6
100	257	198 (76.8 ± 1.5)	14 (5.4 ± 0.6)[a]	37.4 ± 2.6

Note. As control, the zygotes were stored in foetal bovine serum (FBS) without dimethyl sulfoxide (DMSO) and CGA for 24 h and then cultured for 7 days.

[a–c]Values with different superscripts in the same column are significantly different ($p < 0.01$).

*Seven replicate trials were carried out. Data are expressed as mean ± *SEM*.

3.4.3 Experiment 3

When the formation date and quality of blastocysts derived from zygotes with or without storage treatment were examined, the highest ratio of blastocysts formation was observed on day 5 (day 0 = insemination) in the fresh control group (No. of whole blastocysts = 116, 83.6% ± 4.6%), whereas the highest ratio in the storage group (No. of whole blastocysts = 59, 71.2% ± 2.3%) appeared 1 day later (Day 6; Figure 5). When apoptotic nuclei were examined in all blastocysts, the proportion of apoptotic nuclei in blastocysts was significantly higher ($p < 0.05$) in the storage group (11.2% ± 1.0%) than in the control group (8.3% ± 0.6%).

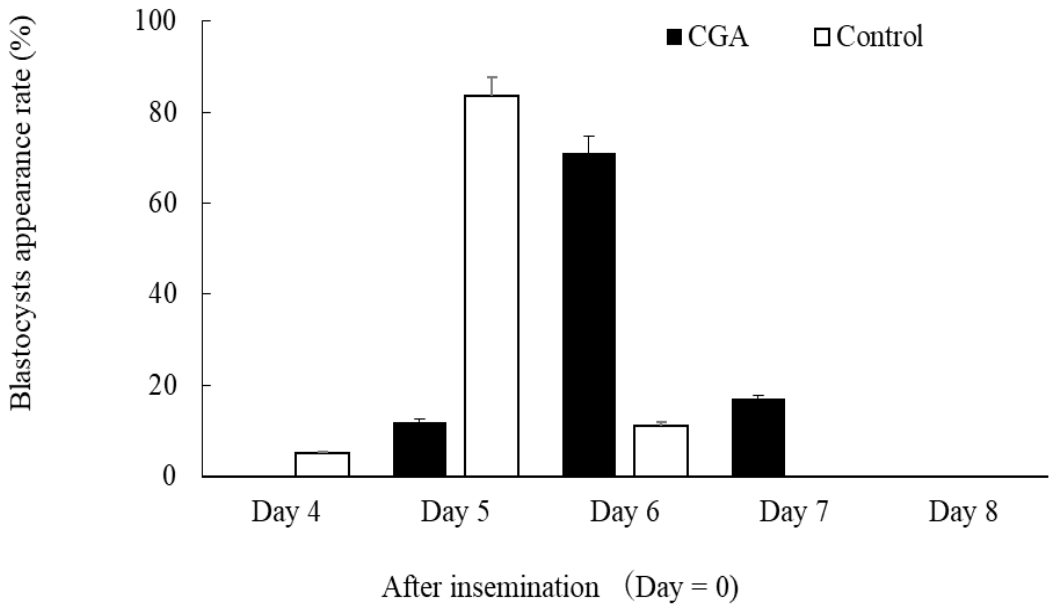

Figure 5. Kinetics of blastocyst formation of zygotes with (black bar) or without (white bar) storage treatment at 25°C for 24 h. Zygotes (*n* = 679) were stored in foetal bovine serum supplemented with 50 μM chlorogenic acid (CGA) and then cultured for 7 days. As control, the zygotes (*n* = 499) were cultured for 7 days without the storage treatment. Six replicate trials were carried out. Each bar represents the ratio (mean ± *SEM*) in whole blastocyst formation.

3.5 Discussion

Several types of serum-containing or BSA-containing media and several temperatures have been used for short-term storage of mammalian embryos [82, 83, 84]. It is well-known that porcine oocyte/embryos are more sensitive to low temperatures than those of other mammalian species, and especially, porcine

embryos are sensitive to cooling to <15°C [19]. Pomar et al. (2004) [85] reported that short storage at 25°C is more suitable for maintaining the quality and development of porcine in vivo-produced embryos. Moreover, storage medium has been suggested to play an important role for short storage of porcine embryos at hypothermic temperatures [72, 85]. In the current study, we found that when the porcine zygotes were stored at 25°C for 24 h, more zygotes stored in 100% FBS developed to the blastocyst stage compared with BSA-containing TCM. An elevation of pH in hypothermic medium during preservation has been suggested to impair the viability of embryos after the storage [61]. It has been shown that serum contains a wide variety of substances, including amino acids that play important roles as osmolytes and pH buffers [86]. Therefore, the serum as a storage medium may be effective for short-term storage of porcine embryos. However, the serum causes alterations in mitochondrial structure of embryos during in vitro culture, resulting in reduced ability of bovine embryos to metabolise lipids [87]. Moreover, serum concentration changes the viscosity of the medium, which may impair the developmental ability of stored embryos [83]. Ideta et al. (2013) [61] suggested that the concentration of FBS in hypothermic medium affected the survival rate of bovine embryos after storage at 4°C. They reported that bovine embryos could be stored in 100% of FBS at hypothermic temperatures, but the optimal concentration was 50% of FBS. In the current study, the development of zygotes

stored in only 100% FBS was examined because the preparation of storage medium becomes simpler. Therefore, further experiment is required to determine which concentration of FBS has a beneficial effect on the development of porcine embryos after short storage at 25°C.

A previous study reported that short-term storage of porcine in vivo-produced embryos at the morula and blastocyst stage at 25°C for 24 h had no detrimental effects on the viability and quality of embryos [85]. In contrast, our previous study demonstrated that the short-term storage at 25°C for 24 h decreased the development of porcine in vitro-produced embryos at the morula stage [84]. In the current study, we found that the rates of blastocysts formation of zygotes stored at 25°C for 24 h decreased as compared with those of fresh zygotes, irrespective of the storage medium. The morphology and number of cytoplasmic lipid droplets in porcine embryos have been shown to differ between in vivo- and in vitro-produced embryos [88]. Therefore, the differences in the development of porcine embryos after short-term storage may be due to the difference in the lipid composition, which is related to sensitivity to hypothermic conditions.

In our previous study, we reported that CGA is an effective antioxidant that improves the developmental competence of porcine oocytes and protects oocytes from DNA fragmentation caused by H_2O_2 exposure [89]. In the current study, similarly, we found that supplementation of the storage medium with 50

μM CGA significantly improved the rates of blastocyst formation of zygotes after storage at 25°C for 24 h. Considerable evidence in animal studies indicates that supplementation of antioxidants, vitamins C and E, amino acids or ROS scavengers can be alternative treatment strategies that help to reduce oxidative stress and can be beneficial to embryonic survival and blastocyst formation rates [79]. The beneficial effects of antioxidant CGA on post-storage development of zygotes in this study could be explained through the action of tripeptide glutathione (GSH), which is an important modulator of DNA repair activity [90]. Phenol derivatives, including CGA, in coffee beans, have exhibited enhanced GSH levels and offer protection against DNA damage [91, 92]. Therefore, it is highly possible that CGA at a suitable concentration has an important role to prevent DNA fragmentation by enhancing GSH levels and combating oxidative stress during short storage. In the current study, our results demonstrated that the highest ratio of blastocyst formation appeared 1 day later. When the development of zygotes stored in FBS supplemented with or without 50 μM CGA was evaluated at 12 h, 24 h and 48 h (24-h culture after storage) after the start of storage (153-155 zygotes examined per each hour), all of the stored and fresh zygotes did not cleave at 12 h (Data not shown). There were no differences in the cleavage rates (60%-80%) at 24 h and 48 h after the start of storage between the stored and fresh zygotes. However, the development to more than 8-cell stage of stored zygotes at 48 h after the start of storage was significantly

lower than that of fresh zygotes (9.8%-12.0% vs. 59.3%, $p < 0.01$, Chi-square analysis), irrespective of the CGA treatment. Therefore, our results indicate that the storage at hypothermic temperatures could induce temporary developmental arrest in porcine zygotes. On the other hand, the quality of embryos from zygotes stored for 24 h decreased as compared with that from fresh zygotes, even when the zygotes were stored with an adequate concentration of CGA. Previous studies indicate that suboptimal conditions for porcine short-term embryo storage affect embryo viability and quality [93]. Therefore, it is important to improve the conditions that allow zygotes to retain their full viability during storage in vitro.

CHAPTER 4. IN VITRO DEVELOPMENT OF ZONA PELLUCIDA-FREE PORCINE ZYGOTES INDIVIDUALLY CULTURED AFTER VITRIFICATION.

4.1 Abstract

The present study was designed to investigate whether or not the removal of zona pellucida (ZP) affects the development of porcine zygotes after vitrifying and warming. In the first experiment, we examined the adequate volume of culture medium for individual culture of ZP-intact and -free zygotes. After in vitro maturation, the cumulus-enclosed oocytes were denuded and ZP of some oocytes was removed. The ZP-intact and -free oocytes were fertilized with spermatozoa, and then cultured individually in microdrops with different volumes (5 µL, 10 µL, 15 µL, and 20 µL per one embryo). Results showed that the volume of culture medium influenced the development of ZP-intact zygotes, in which a volume of 15 µL was most suitable for their development and quality. However, the volume of culture medium had no effects on the development of the ZP-free zygotes. In the second experiment, we evaluated the mechanical protection effect of ZP during cryopreservation on the development of vitrified-warmed zygotes. The ZP-intact and -free zygotes collected 10 h after insemination were vitrified and warmed by the Cryotop method. There was no

difference in the blastocyst formation rates of vitrified-warmed embryos between the ZP-free and ZP-intact zygotes. Moreover, the blastocyst formation rates of vitrified-warmed embryos were similar to those of fresh embryos in each type of zygotes. Our results suggest that the removal of ZP had no detrimental effects on the develpment of zygotes vitrified by the Cryotop method, when the embryos are individually cultured by adequate volume of culture medium.

4.2 Introduction

Recently, there is an increase in demand for reliable cryopreservation protocols for porcine embryos at the early stage because transgenic or cloned embryos are generally transferred to recipients at the zygote or early cleavage stages. The vitrification method is now common for the cryopreservation of oocytes and embryos, instead of conventional freezing methods. To date, many special techniques, such as open pulled straw (OPS), solid-surface vitrification, and Cryotop, have been developed as vitrification method [94]. It has been reported that porcine embryos could be successfully vitrified at the zygote stage by the Cryotop method, resulting in acceptable survival rates [95].

The zona pellucida (ZP) in the oocyte of mammals is the outer extracellular layer that plays important roles in mechanical protection, sperm

binding, acrosome reaction, prevention of polyspermic fertilization, maintenance of a normal cleavage pattern, and prevention of embryo fusion during in vitro development. Removal of the ZP may lose these functions and change the physiological pathways of early embryo development. However, ZP-free oocytes can provide more options for improvement in somatic cell nuclear transfer (SCNT), such as handmade cloning [8]. The handmade cloning method is zona-free cloning without the use of a micromanipulator, in which the ZP-free oocytes are halved by a microblade and two enucleated halves are fused together with the donor cell. To avoid aggregation during the culture, the zona-free embryos must be cultured individually, either one embryo per microdrop or the wells of the well (WOW) system [11]. The development of zona-free embryos produced by somatic cell nuclear transfer (SCNT) has been reported to be similar to that of zona-intact embryos [31].

The questions of whether or not the removal of ZP affects the development of vitrified-warmed embryos at the one-cell stage remain because the deficit of ZP changes the mechanical protection during vitrification. This study is expected to improve our understanding of the ZP functions during vitrification of embryos. Before the vitrification examination, moreover, we determined the adequate volume of culture medium for individual culture of ZP-intact and -free zygotes in our culture system.

4.3 Materials and methods

4.3.1 Oocyte collection and in vitro maturation

Collection and in vitro maturation (IVM) of oocytes were performed following the procedure described by Nishio et al. [96]. Briefly, pig ovaries were obtained from pre-pubertal cross-bred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse. Cumulus-oocyte complexes (COCs) with uniform ooplasm and compact cumulus cell mass were cultured in maturation medium at 39°C, in a humidified incubator containing 5% CO_2. The maturation medium consisted of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA), supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd.), 10 IU/mL equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/mL gentamicin (Sigma-Aldrich). After their maturation for 20-22 h, COCs were cultured for an additional 24 h in the maturation medium without hormones under the same conditions.

4.3.2 Preparation of zona pellucida-free oocytes

The matured oocytes in vitro were partially denuded by brief treatment with 0.1% (w/v) hyaluronidase, then gently pipetted using a fine glass pipette, and exposed to 0.5% (w/v) actinase-E (Kaken-Seiyaku Corp.) in Dulbecco's PBS (Nissui Pharmaceutical, Tokyo, Japan) for 20-30 sec. The oocytes with expanded and deformed ZP were then transferred to TCM-199 without actinase-E and freed completely from their ZP by gentle pipetting. The ZP-free oocytes were incubated for 1 h in the maturation medium at 39°C, in a humidified incubator containing 5% CO_2 and 5% O_2. ZP-intact oocytes, consisting of mature oocytes that did not undergo ZP removal, were designated as controls.

4.3.3 In vitro fertilization and in vitro culture

For in vitro fertilization (IVF), frozen-thawed spermatozoa obtained from a boar were transferred into 6 mL of porcine fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × g for 5 min. The pelleted spermatozoa were re-suspended in PFM and adjusted to 5×10^6 cells/mL. ZP-intact and -free oocytes were then transferred to the sperm-containing PFM and co-incubated for 5 h at 39°C, in a humidified incubator containing 5% CO_2, 5%

O_2, and 90% N_2. After IVF, the zygotes were washed with pig zygote medium (PZM-5; Research Institute for the Functional Peptides Co.) and individually cultured at 39°C, in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. All cleaved embryos were transferred into droplets of porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) on day 3 of culture, with fertilization considered to occur on day 0. Embryos were subsequently cultured for an additional 4 days to evaluate their ability to develop to the blastocyst stage. On day 7, all blastocysts with clear blastocoel were stained with Hoechst 33342, according to procedures described by Murakami et al. [97], and their total cell number was counted.

4.3.4 Vitrification and warming

Cryopreservation of zygotes was carried out using the Cryotop method [98, 99] using the Porcine Embryo Vitrification Solution Kit (PEV-SK; Research Institute for Functional Peptides Co.), according to manufacturer's instructions. Briefly, approximately ten zygotes were equilibrated with equilibration solution-1 (PES-1; 1.8-M ethylene glycol) and equilibration solution-2 (PES-2; 1.8 M ethylene glycol, 0.3 M trehalose, and 1% [w/v]) polyethylene glycol) for 5 min each at room temperature. The equilibrated

zygotes were exposed to a room-temperature vitrification solution containing 6 M ethylene glycol, 0.6 M trehalose, and 2% (w/v) polyethylene glycol and were loaded onto the tip of the Cryotop device (Kitazato BioPharma, Shizuoka, Japan) with a minimum volume of solution. The Cryotop device containing the zygotes was plunged into liquid nitrogen within 1 min of exposure to the vitrification solution. The Cryotop device was then protected with a plastic cap and stored in liquid nitrogen for at least 2 days. Zygotes were thawed by immersing the tip of the Cryotop device directly into a warming solution (PWDS; 1.8 M ethylene glycol and 0.3 M trehalose) at 39°C for 3 min. They were then cultured in PZM-5 medium for 7 days as described above.

4.3.5 Experimental design

In the first experiment, we determined the appropriate volume of the corresponding medium for the individual culture of ZP-intact and -free embryos. After fertilization, ZP-intact and -free zygotes were randomly allocated to four groups and individually cultured in micro-drops with different volumes of PZM-5 (5, 10, 15, and 20 μL per embryo). Only cleaved embryos were transferred into droplets with the same volumes of PBM on day 3 of culture after fertilization. Embryos were subsequently cultured for an additional 4 days as described above.

In the second experiment, we evaluated the protection effect of ZP during cryopreservation on the resulting development of the vitrified-warmed zygotes, using the 15-μL volume of culture medium that we found to be most suitable for the development of embryos in the first experiment. ZP-intact and -free zygotes were collected 10 h after insemination and then vitrified and warmed as described above. After warming, embryos were individually cultured in micro-drops with 15 μL of culture medium for 7 days (Fig. 5).

4.3.6 Statistical analysis

The experiments were repeated six times for individual culture of embryos and five times for vitrification. Data on embryonic development and total cell number of blastocysts were evaluated using analysis of variance with a general linear model (GLM) procedure in SAS software (SAS for Windows, version 9.1, SAS Institute Japan, Tokyo, Japan). The statistical model considered two main factors, including the volume of medium and ZP presence in the first experiment and vitrification and ZP presence in the second experiment. It also considered two-way interactions. Non-significant interactions were excluded from the model. Differences with a value of $P < 0.05$ were regarded as significant.

4.4 Results

4.4.1 Experiment 1

No significant interaction was observed between the volume of culture medium and the presence or absence of ZP, as indicated by the rates of cleavage and blastocyst formation and the total cell number of blastocysts. There were no differences between ZP-free and -intact zygotes in the rates of blastocyst formation, irrespective of medium volume (Table 4). In ZP-intact zygotes, blastocyst formation rates of embryos cultured in 15 and 20 μL culture medium increased as compared with embryos cultured in 5 μL medium. The total cell number of blastocysts from embryos cultured in 15 μL of culture medium was the highest among the ZP-intact zygote groups. In the ZP-free zygotes, however, the volume of culture medium had no apparent effects on the blastocyst formation rates or the total cell number of blastocysts.

Table 4. Effects of volume of culture medium on the development of zona-free and -intact porcine embryos at the one-cell stage*

Volume of medium (μL)	Zona pellucida	No. of oocytes examined	No. (%) of embryos		Total cell number of blastocysts
			Cleaved	Developed to blastocyst	
5	Intact	153	140 (91.6 ± 2.2)^{a,c}	10 (6.5 ± 1.9)^{a}	34.3 ± 2.8^{a,b}
	Free	152	147 (96.7 ± 1.6)^{b}	6 (3.8 ± 0.9)^{a}	31.0 ± 3.7^{a}
10	Intact	147	131 (88.7 ± 2.5)^{c}	15 (10.2 ± 2.0)^{a,b}	34.6 ± 2.4^{a}
	Free	155	149 (96.2 ± 0.9)^{a,b}	9 (5.7 ± 1.5)^{a}	33.8 ± 2.8^{a,b}
15	Intact	145	137 (93.2 ± 1.3)^{a,c}	23 (15.9 ± 3.3)^{b}	41.4 ± 3.3^{b}
	Free	147	140 (95.2 ± 1.7)^{a,b}	14 (9.6 ± 1.8)^{a,b}	31.1 ± 1.8^{a}
20	Intact	145	131 (89.4 ± 1.8)^{c}	23 (16.3 ± 4.4)^{b}	33.6 ± 1.6^{a}
	Free	155	151 (97.6 ± 1.2)^{b}	17 (10.7 ± 2.5)^{a,b}	31.2 ± 1.7^{a}

*Six replicate trials were carried out. Data are expressed as the means ± SEM. No significant volume of medium x zona pellucida interaction was observed to affect the rates of cleavage and blastocyst formation and total cell number of blastocysts ($P > 0.05$). $^{a-c}$Values with different superscripts in the same column are significantly different ($P < 0.05$).

4.4.2 Experiment 2

Rates of cleavage, blastocyst formation, and total cell number of blastocysts indicated no appreciable interaction between vitrification and retention or removal of the ZP (Table 5). There was no difference in blastocyst

formation rates of vitrified-warmed embryos between the ZP-free and ZP-intact zygotes. Moreover, blastocyst formation rates of vitrified-warmed zygotes were similar to those of fresh zygotes in each type of embryo. The vitrification treatment and the existence of ZP had no apparent effects on the total cell number of the resulting blastocysts

Table 5. Development of zona-free and -intact porcine embryos at the one-cell stage after vitrification with Cryotop*

Zona pellucida	Vitrification	No. of embryos vitrified	No. (%) of warmed embryos		Total cell number of blastocysts
			Cleaved	Developed to blastocyst	
Intact	Fresh	226	198 (88.6± 3.2)[a,b]	43 (19.0 ± 3.2)[a]	36.3 ± 1.7
	Vitrification	150	119 (78.6 ± 3.5)[a]	21 (13.6 ± 2.1)[a,b]	38.5 ± 2.8
Free	Fresh	235	216 (91.8 ± 3.9)[b]	28 (12.2 ± 1.7)[b]	37.8 ± 3.1
	Vitrification	145	119 (81.5 ± 2.5)[a]	13 (8.9 ± 1.0)[b]	34.6 ± 3.9

*Five replicate trials were carried out. Data are expressed as the means ± SEM. No significant vitrification x zona pellucida interaction was observed to affect the rates of cleavage and blastocyst formation and total cell number of blastocysts ($P > 0.05$). [a-b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

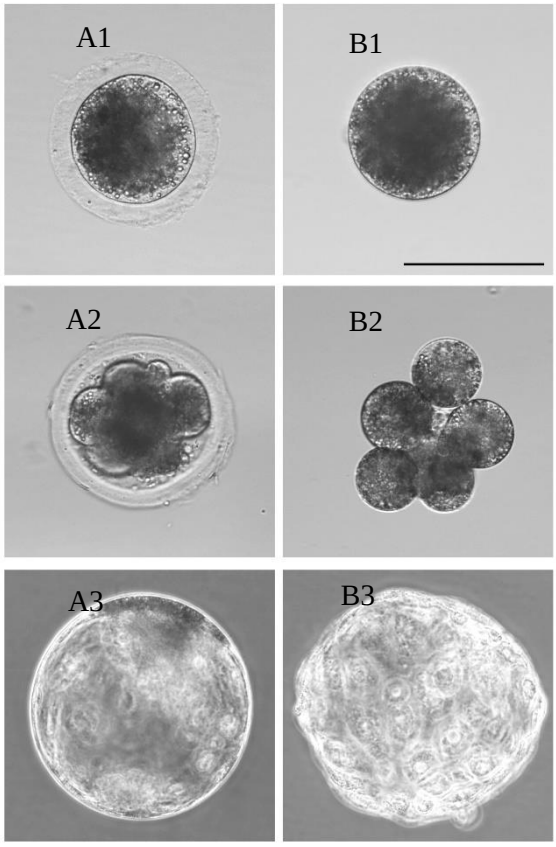

Figure 6. The development of zona pellucida (ZP) -free and -intact porcine zygotes after vitrification using the Cryotop method. ZP-intact zygotes (A1) and -free zygotes (B1) were vitrified. After warming, ZP-intact and -free zygotes cleaved (A2 and B2, respectively) and developed to the blastocyst stage (A3 and B3). Scale bar indicates 100 μm.

4.5 Discussion

The mammalian ZP is an extracellular glycoprotein structure which is formed during oocyte development. In most species, the ZP encloses oocyte and embryo until the beginning of implantation and protects them from mechanical damage during ovulation and transport along the female reproductive tract

[100]. However, it has been demonstrated that ZP-free oocytes can be fertilized under suitable conditions after ZP removal [101], and the presence of the zona pellucida is not necessary for embryo development in vitro [102, 11]. Moreover, ZP-free oocytes can develop normally to full-term after in-vitro fertilization and culture [103]. In the present study, we also found that the development of ZP-free zygotes was similar with that of ZP-intact zygotes, irrespective of medium volume, when the embryos were individually cultured after in-vitro fertilization. In the conventional group culture system, paracrine factors released by embryos promote growth of neighbouring embryos [104, 105] , whereas individual culture system could support the embryonic development by the accumulation of autocrine factors secreted from embryos themselves [11]. In the individual culture system, therefore, the volume of culture medium or density of embryos is considered a particularly critical factor that affects the embryo development and quality of resulting blastocysts [106]. In the present study, we observed that the volume of culture medium influenced the embryonic development in the ZP-intact embryo group, in which a volume of 15 μL was most suitable for the development and quality of embryos. However, the volume of culture medium had no effects on the embryonic development in the ZP-free embryo group. The reason for the difference on the volume effects between ZP-intact and -free embryos is unclear. It has been suggested that the metabolism of ZP-free

embryos are accelated and exchanged after ZP removal, wheares ZP-intact embryos save energy for the process of ZP expansion during early embryo development [52]. Therefore, one possible explanation could be due to the difference of embryonic metabolism.

The ZP surrounding embryos acts as a natural barrier, hindering the free movement of water and cryoprotectants between the intra- and extracellular compartments. Removal of ZP seems to result in the loss of mechanical protection during cryopreservation and thus decrease the survival rates of embryos after freezing and thawing. To date, it has been demonstrated that, in mouse and rabbit, relatively high survival rates after vitrification have been achieved with ZP-free blastocysts [102, 107]. Post-thaw survival of bovine ZP-free embryos produced by handmade cloning method has been also reported to be similar to that of ZP-intact embryos produced by IVF [57]. To our knowledge, no information is available concerning cryopreservation of ZP-free zygotes/embryos in pigs. Moreover, porcine embryos are very sensitive to damage caused by low temperature and osmotic stress [38]. In the presrent study, we observed that the blastocyst formation rates of vitrified-warmed zygotes with or without the ZP were < 14%, but the rates of vitrified-warmed zygotes were similar to those of fresh zygotes. Moreover, there was no difference in the blastocyst formation rates of vitrified-warmed embryos and the

total cell number of resulting blastocysts between the ZP-free and ZP-intact zygotes. These results may be supported by previous reports which demonstrated that a high blastocyst development of cropreserved porcine zygotes could be obtained by vitrification method [108, 109] . The use of a small volume of vitrification solution in the container is a key to achieving vitrification at a high success rate. Thus, many devices and methods for the vitrification have been developed to decrease the total volume of vitrification solution [11]. Cryotop method used in the present study has been suggested to be one of the most powerful and superior devices for vitrification for pig embryos at the pronuclear stage [110]. The Cryotop method based on minimum volume procedure produces both high cooling and warming rates [98], which may contribute positively to the survival of ZP-free zygotes. Taken together, our results indicate that when the zygotes were vitrified by the Cryotop method, the removal of ZP before vitrification had no detrimental effects on the embryonic develpment and the quality of resulting blastocysts.

In conclusion, the present study demonstrated that the removal of ZP does not affect the development of vitrified-warmed zygotes, indicating that ZP has no mechanical protection effects during cryopreservation. Moreover, the acceptable development of vitrified-warmed embryos without ZP may be

obtained using Cryotop method when the embryos are individually cultured by adequate volume of culture medium.

CHAPTER 5. PRODUCTION OF CLONED ELEPHANT EMBRYOS BY INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER USING ENUCLEATED PIG OOCYTES

5.1 Abstract

In this study, we examined the feasibility of using domestic elephant fibroblast cell injection electrofusion for iSCNT to produce elephant embryos. More than 69 % of domestic elephant fibroblast cells successfully fused with the porcine oocytes following electrofusion, and 0.6 % of the embryos were able to reach the blastocyst stage. The development of elephant-porcine iSCNT embryos up to the blastocyst stage proved that elephant reconstructed embryos using porcine cytoplast is feasible. This study is the first demonstration that SCNT elephant somatic cell nuclei can be reprogrammed and develop to the blastocyst stage.

5.2 Introduction

SCNT is an efficient technique for assessing the development potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm [111]. SCNT provides not only a valuable tool for producing animals with the same genetic traits but also an opportunity to

develop iSCNT, which involves the transfer of donor cell nuclei from one species to enucleated oocytes of another species [112]. Furthermore, iSCNT provides an opportunity for molecular tracking of nucleocytoplasmic interaction and the transmission of two cytoplasmic populations. This methodology influences embryonic development and survival post-implantation and provides an enabling technology to explore fundamental aspects of developmental biology in mammals. In the future, iSCNT seems to be a more valuable tool for many reasons including the production of embryos from species with limited availability of oocytes either because their oocytes are challenging to obtain or because their collection is under restricted control [55, 112]. Regarding reproductive goals, iSCNT has been applied to the conservation of wildlife.

The transfer of somatic cells to enucleated oocytes requires a process termed nuclear reprogramming to transform the differentiated cell nuclei to the ooplasm before converting to a totipotent state and inducing somatic gene expression [61]. Incomplete donor nuclei reprogramming and abnormal epigenetic reprogramming are thought to be related to the low efficiencies in SCNT and iSCNT cloned embryos [113].

Elephants are exceptionally charismatic megavertibrates; the Asian (*Elephas maximus*) and African bush (also called savanna, *Loxodonta africana*) species are listed respectively as endangered and vulnerable by the International

Union for Conservation of Nature. Many researchers are trying to study semen collection, evaluation, cryopreservation, and artificial insemination to conserve these species [114]. However, in female elephants, it is possible to study their hormonal profile to detect the estrous cycle and pregnancy prognosis [115]. Other assisted reproduction biotechnologies, such as *in vitro* embryo production, embryo transfer and cloning techniques, are not easily applied to the rescue of the elephant species. The porcine oocytes have been considered "universal recipient cytoplasm" in cloning research because they represent an abundant resource. Abul Hashem et al. (2007) [116] demonstrated the ability of porcine oocytes to reprogram the nucleus of another species. Thus, it may be feasible to use porcine oocytes to create an elephant-porcine iSCNT embryo, thereby offering a model system to address fundamental questions about differentiation and its reversibility in both cellular and molecular aspects of iSCNT.

5.3 Materials and methods

5.3.1 Oocyte collection and in vitro maturation

Collection and *in vitro* maturation (IVM) of oocytes were performed following the procedure described by Nishio et al. (2018) [96]. Pig ovaries were obtained from pre-pubertal cross-bred gilts (Landrace × Large White × Duroc

breeds) at a local slaughterhouse. Cumulus-oocyte complexes (COC) with uniform ooplasm and compact cumulus cell mass were cultured in maturation medium at 39 °C, in a humidified incubator containing 5 % CO_2 (Fig. 7A). The maturation medium consisted of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA), supplemented with 10 % (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 µM sodium pyruvate (Sigma-Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 50 µM β-mercaptoethanol (Wako Pure Chemical Industries Ltd.), 10 IU/mL equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Kyoritu Seiyaku), and 50 µg/mL gentamicin (Sigma-Aldrich). After maturation for 20 - 22 h, COCs were cultured for an additional 24 h in the maturation medium without hormones under the same conditions.

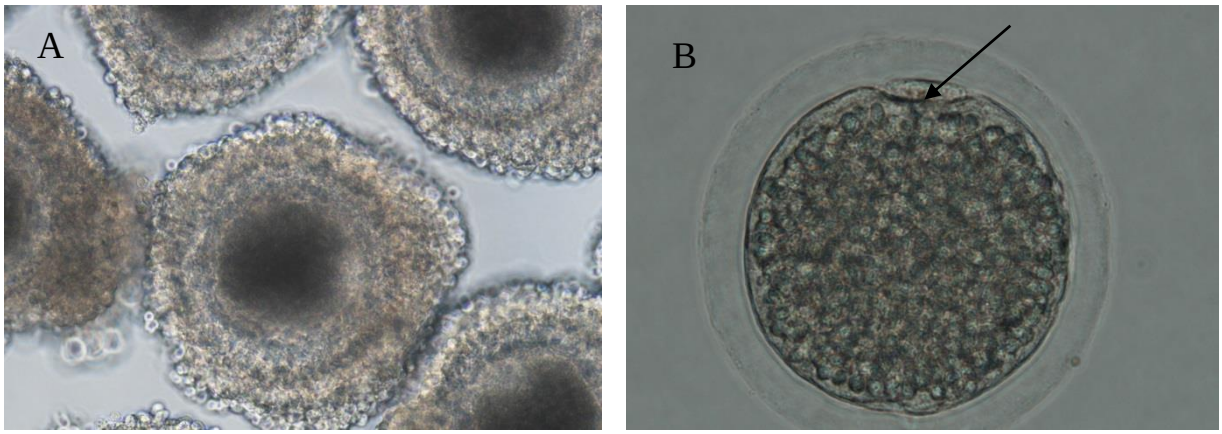

Figure 7. Matured porcine oocytes; porcine oocytes with compact cumulus cells of more than two layers, and homogenous dark ooplasm were selected and

cultured for 20 - 22 h and 24 h, respectively (x200) (A). Mature oocytes presenting the first polar body (arrow) were selected for NT (x400) (B).

5.3.2 Enucleation of recipient oocytes

After maturation culture, porcine COCs were mechanically denuded in a TCM199 medium supplemented with 0.1% (w/v) hyaluronidase (Sigma-Aldrich). Oocytes with the first polar body extrusion were collected (Fig. 7B) and then incubated for 10 min in 3 μg/mL of Hoechst 33342 diluted in manipulation medium (PBS supplemented with 5% FBS and 5 μg/mL of cytochalasin B [Sigma-Aldrich]). Oocytes were secured with a holding pipette and rotated as needed into a position suitable for enucleation (Fig. 8A). The zona pellucida near with the first polar body was cut by a transfer pipette and a small volume (approximately 5% - 10%) of cytoplasm nearby the first polar body was took out of first polar body and MII chromosome and checking enucleation by low-light filtered fluorescence (Fig. 8B).

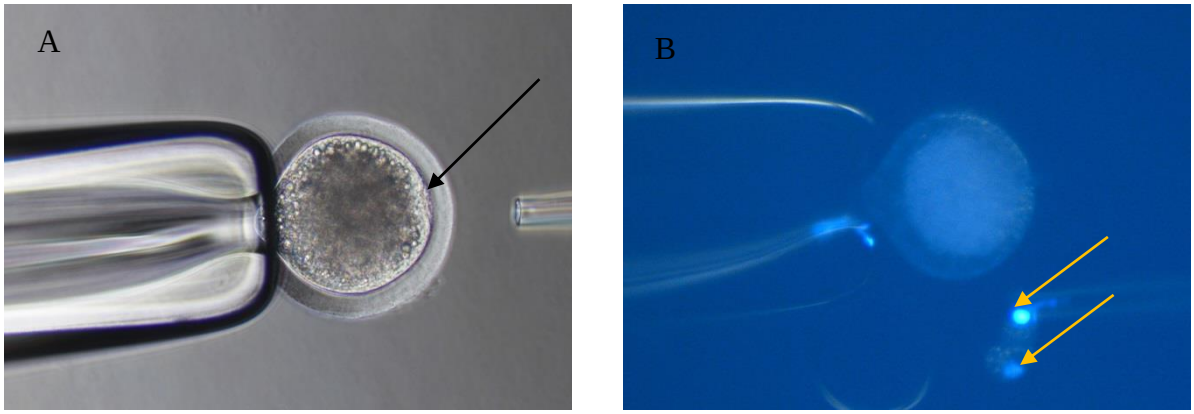

Figure 8. Enucleation of porcine oocytes. The oocyte was secured with a holding pipette and rotated as needed into a position suitable for enucleation (A); a transfer pipette cut the zona pellucida near with the first polar body (black arrow), and a small volume of cytoplasm nearby the first polar body was removed. The first polar body and MII chromosome (yellow arrows) (×200) (B).

5.3.3 Donor cell preparation

5.3.3.1 Preparation of domestic elephant somatic cells

Domesticated elephant fibroblast cells were collected from fibroblast-like cells derived from African bush (savanna) elephants. The domesticated elephant fibroblast cells were cultured in plastic 35 mm Petri dishes (Falcon 3530001) containing Dulbecco`s Modified Eagle Media (DMEM) supplemented with 10 % (v/v) FBS and 42 µg/mL L-serine, 30 µg/mL Glycine, and 110 µg/mL sodium pyruvate at 37 °C in a humidified atmosphere containing 5 % CO_2 for

three days. The monolayer fibroblast cells were washed twice with DMEM and then incubated in 0.25 % trypsin-EDTA for 3 minutes at 37 °C. After trypsinization, washing medium (DMEM supplemented with 10 % FBS, 42 µg/mL L-serine, 30 µg/mL Glycine, and 110 µg/mL sodium pyruvate) was used. The cells were pelleted by centrifugation at 600×g for five minutes, re-suspended in the washing medium, and then maintained in this medium until SCNT manipulation.

5.3.3.2 Preparation of porcine fetal fibroblast cells (PFF)

Porcine fetal fibroblast (PFF) cells were cultured in DMEM supplemented with 10 % (v/v) FBS at 37 °C, in a humidified atmosphere containing 5 % CO_2. PFF derived cells were collected by the 0.05 % trypsin-EDTA treatment for 3 minutes at 37 °C. After washing with culture medium, the PFF cells were pelleted by centrifugation at 600 g for five minutes, re-suspended in culture medium (DMEM supplemented with 10 % (v/v) FBS), and then maintained in a culture medium until SCNT manipulation.

5.3.4 Nuclear transfer, fusion, activation and embryos culture

The elephant cells were transferred into the spaces of enucleated porcine oocytes, and a single elephant donor cell was placed inside the plasma of the

enucleated porcine oocytes (Fig. 9A and Fig. 9B). Nuclear transfer couplets were washed three times in mannitol fusion medium (0.28 M D-mannitol, 0.05 mM $CaCl_2•2H_2O$, 0.1 mM $MgSO_4•7H_2O$, 0.1 mg/mL BSA) and then placed in the fusion chamber. The couplets were fused simultaneously with a single direct current pulse of 1.5 kV/cm for 50 μsec by using an electro cell fusion generator (LF101; Nepagene, Chiba, Japan). After that, the reconstructed embryos were washed three times before being cultured in PZM-5 medium supplemented with 5 μg/mL of Cytochalasin B for two hours to chemical activation. After two hours of activation, the embryos were washed three times and cultured in PZM-5 for three days. After three days of culturing, the embryos were washed and cultured in PBM medium for another four days. Embryo development was monitored in culture for seven days (Fig. 11A and Fig. 11B). The numbers of cleaved embryos and blastocysts were recorded.

PFF cells were processed using the same method as described above. PFF cells derived embryos served as embryo developmental controls.

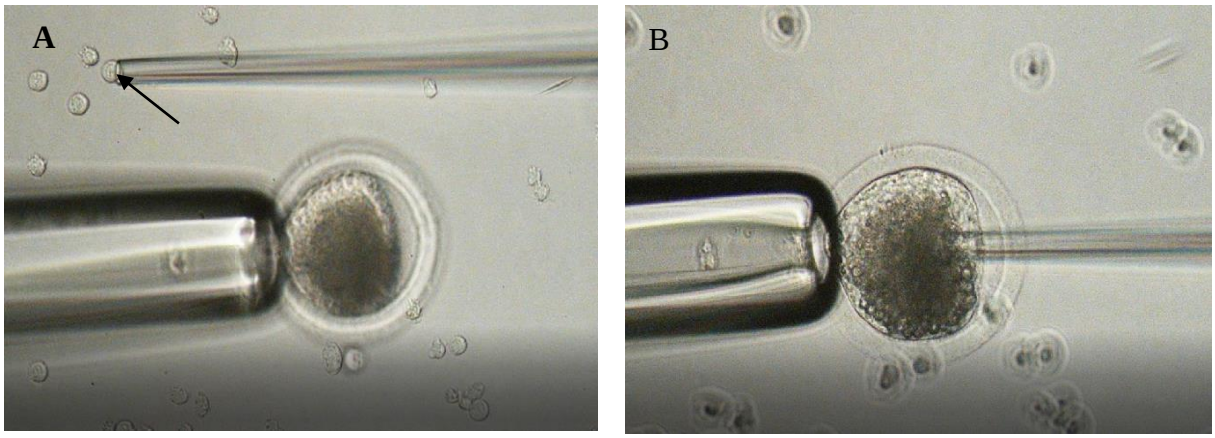

Figure 9. Nuclear transfer method; a single round donor cell (arrows) was selected (A) and a single elephant donor cell was placed inside the plasma of enucleated porcine oocytes (B) (x200).

5.3.5 Transgene detection

Detection of the transgene in the cloned elephant was carried out by polymerase chain reaction (PCR). Genomic DNA of blastocyst was extracted using heat treatment in 50 mM NaOH after neutralization. The DNA sample was subjected to PCR using Quick Taq HS DyeMix (Toyobo, Osaka, Japan) with T100 Thermal Cycler (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. Primers used for amplification were listed in Table 6. The PCR condition was 94 ℃ for 2 minutes initially, then 40 cycles of 98 ℃ 10 seconds, 65 ℃ for 30 seconds and 68 ℃ for 2 minutes.

Table 6. Primers used for PCR

Animal	Gene	Fw/Rv	Primer sequence ('5' to 3')	Product length (bp)
Porcine	GHR	Fw	TGCCCCTATCTTTTTGATGG	534
		Rv	ATCGCACCTACCTTTGCTGT	
	DMD	Fw	TGCCTCATACTTCCCAGGAC	624
		Rv	ACTGTACCACCTGCCCTGTC	
	GGTA1	Fw	ATCCGGACCCTGTTTTAAGG	626
		Rv	AAAAGGGGAGCACTGAACCT	
Elephant	GHR	Fw	ATCTGTGTTGAGGGCAGTCC	665
		Rv	GGTCAGGGGAGACATGCTAA	
	DMD	Fw	TGGTCAAGGAACACCACTGA	490
		Rv	CCCCTCAACCAACTCAGAAA	
	GGTA1	Fw	CGGGAGCACCTTTACTTCTG	544
		Rv	ACCCTTCCAAATCAGCACAG	

Note: GHR: growth hormone receptor; GGTA1: glycoprotein galactosyltransferase alpha 1, 3; DMD: duchenne muscular dystrophy. Fw: forward primer; Rv: reverse primer.

5.3.6 Experimental design and statistical analysis

Six replicates were carried out to investigate the development of iSCNT embryos produced by transferring domestic elephant fibroblast cells into enucleated porcine oocytes. The development of the iSCNT embryos was monitored and compared with PFF cells embryos. For DNA analysis, the blastocyst stage was used. The fusion and developmental rates of the iSCNT and parthenogenic embryos were compared by chi-square analysis. The quantity of elephant and porcine DNA in the iSCNT embryos was expressed as the mean ± SD. Significance was established at $P<0.05$.

5.4 Results

5.4.1 Embryo development

As shown in Table 5, a total of 243 elephant-porcine iSCNT couplets were examined, and 69.8 % of them were cleaved. There was no difference in the cleavage formation rate of elephant-porcine iSCNT embryos and parthenogenic embryos. There was a difference in the blastocyst formation rate of parthenogenic embryos, which was significantly higher than elephant-porcine iSCNT embryos ($P<0.05$).

Table 7. Development of elephant-porcine iSCNT and parthenogenic embryos*

Donor cell	No. of couplets examined	No. of fused couplets	No. (%) of embryos**	
			Cleaved	Developed to blastocyst
Elephant	243	243	168 (69.8 ± 4.7)	1 (0.6 ± 0.6)[a]
PFF	310	310	242 (75.2 ± 5.9)	21 (6.9 ± 1.4)[b]

* Data are expressed as means ± SEM. Six replicated trials were carried out

** Development rate of embryos; number of embryos/no. of couplets examined

[a-b] values with different superscipts in the same column are significantly different ($P < 0.05$)

5.4.2 Analysis of DNA

PCR confirmed the genomic DNA of the blastocyst. Amplification with specific primers was designed for each species (elephant and porcine). The products were analyzed by gel electrophoresis. Three species-specific primer pairs were each used in single. The resulting DNA only of the elephant blastocyst was amplified (Fig. 10). The result demonstrated that elephant somatic cell nuclei could be reprogrammed to develop to the blastocyst stage.

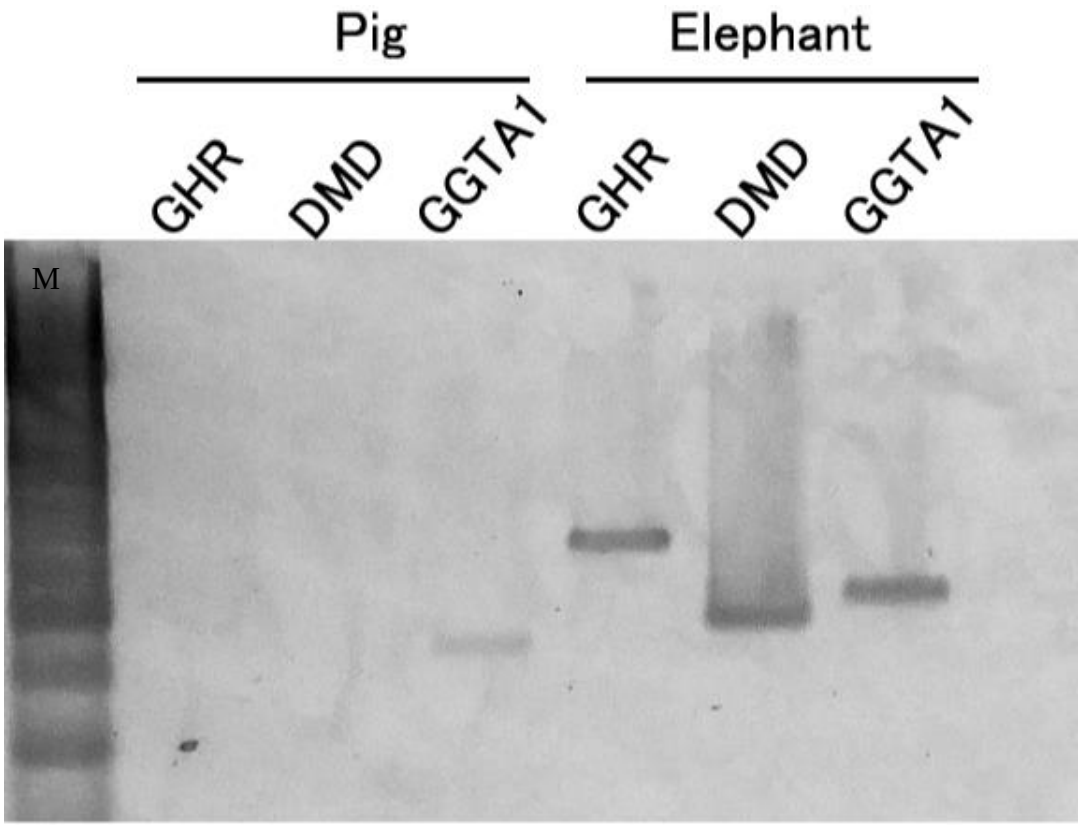

Figure 10. Polymerase chain reaction (PCR) of the transgene integrated into the genome of a cloned elephant. The products were analyzed by gel electrophoresis. Molecular size markers M (100-basepair [bp] ladder). Negative control (A) the DNA was DNA of a porcine blastocyst. The experimental group (B) the DNA was DNA of elephant blastocyst.

5.5 Discussion

This study examined the feasibility of using domestic elephant fibroblast cell injection electrofusion for iSCNT to produce elephant embryos. iSCNT is an invaluable tool for studying nucleus-cytoplasm interactions and may provide an alternative for cloning endangered animals whose oocytes are challenging to obtain. The development ability of iSCNT embryos decreases with increases in

taxonomic distance between the donor cell and recipient species. In this study, more than 69 % of the domestic elephant fibroblast cells successfully fused with the porcine oocytes following electrofusion, and 0.6 % of the embryos were able to reach the blastocyst stage. This study is the first demonstration, using SCNT, that elephant somatic cell nuclei can be reprogrammed to develop to the blastocyst stage (Fig. 10). However, the low development to the blastocyst stage of iSCNT when compared with parthenogenic porcine embryos may be a result of inefficient nuclear reprogramming, mitochondrial heteroplasmy, and incompatibilities between the donor nucleus and recipient cytoplast. African bush (savanna) elephant fibroblast cells could be reprogrammed in porcine cytoplasts.

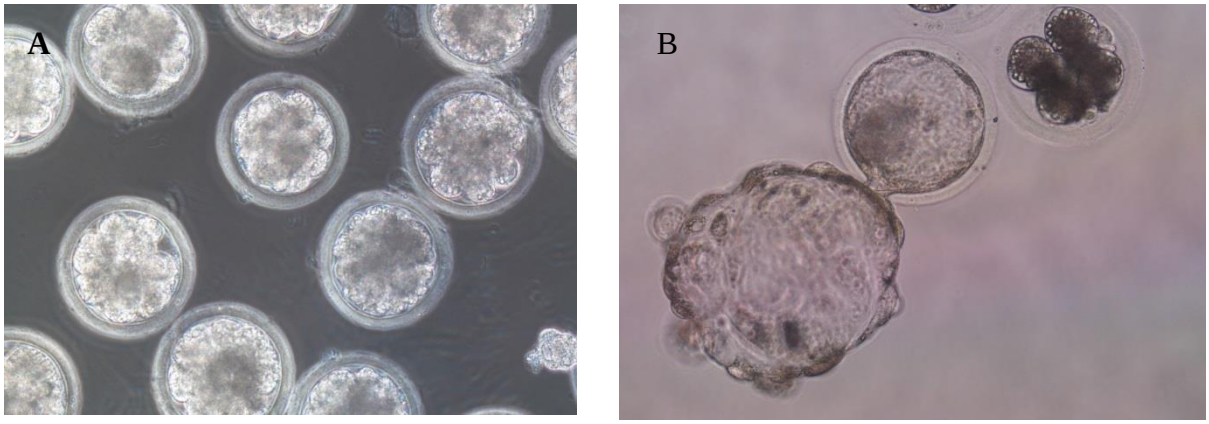

Figure 11. Various stages of elephant-porcine interspecies Somatic cell nuclear transfer (iSCNT) embryos obtained after three days (A) and blastocyst stage of elephant-porcine iSCNT embryos obtained after seven days (B**)** (×200).

Earlier studies reported that PHA-P was used to improve the efficiency of electrofusing elephant fibroblast cells into distinct species [117]. PHA has been widely used in nuclear transfer with humans [118] and in bovine oocytes [119, 120] to increase the fusion rate and developmental competency of nuclear transfer embryos.

The cell cycle of the donor cells has a considerable influence on the reprogramming process and subsequent blastocyst development. Further research is required to establish iSCNT and further study of molecular (DNA methylation, histone acetylation), and cellular mechanisms are needed to determine if interspecies embryos express proper reprogramming factors for other somatic nuclei after iSCNT.

Elephant embryos can be produced by iSCNT of African bush (savanna) elephant fibroblast cells into porcine cytoplasts. These achievements open a new way to more widely use elephant iSCNT embryos to derive elephant stem cells.

CHAPTER 6. SUMMARY

Reproduction is a characteristic of all living things and is a mechanism to maintain the species. In animal husbandry, reproduction plays an extremely significant role in many ways. For example, without reproduction, there is no proliferation of a herd, and thus no commercial cattle industry. Reproduction is also a mechanism to improve genetics and enhance livestock breeds. Therefore, the topic of reproduction has attracted significant attention from scientific researchers who are studying many technologies in this field.

Mammals are impressive organisms that have different morphologies with important characteristics allowing scientists to create animal model systems. The establishment of these animal systems is especially important for further investigations because it is exceedingly difficult to establish an appropriate model for research in complex animal species, including humans. In recent years, the major challenge for the field of reproductive biotechnology has been to explore the molecular and cellular mechanisms that are involved in controlling the quality of oocytes. The mammalian oocyte is a specific structure consisting of cytoplasmic organelles that communicate among themselves and are spatially associated. Thus, this thesis proposes the development of technologies for generating genetically modified pigs, specifically, and animals in general.

Chapter 3 reported the effects of 100 % fetal bovine serum (FBS) and 100 % porcine follicular fluid (pFF) as a storage medium for the development of porcine zygotes stored at 25 °C for 24 hours. Moreover, the study evaluated the additive effects of chlorogenic acid (CGA) in the storage medium. Results showed that 100 % of FBS was superior to BSA-containing TCM 199 as a storage medium for the storage of porcine zygotes at 25 °C for 24 hours. Moreover, the supplementation of 50 μM CGA to FBS has favorable outcomes on the post-storage development of zygotes, but the quality of embryos developed from stored zygotes decreased.

Chapter 4 investigated whether the removal of the ZP affects the development of porcine zygotes after their vitrification and warming and determined the appropriate volume of the corresponding medium for the individual culture of ZP-intact and ZP-free embryos and evaluated the protective effect of ZP during cryopreservation on the resulting development of the vitrified-warmed zygotes. Results show that the volume of culture medium influenced the development of ZP-intact zygotes, and a volume of 15 μL was most suitable for their development. However, the volume of the culture medium did not modify the development of ZP-free zygotes. The removal of the ZP before vitrification did not adversely affect embryonic development or quality of the resulting blastocysts.

Further, chapter 5 purposed to examine the feasibility of using domestic elephant fibroblast cell injection electrofusion for interspecies somatic cell nuclear transfer (iSCNT) to produce elephant embryos. Interspecies somatic cell nuclear transfer (iSCNT) is an invaluable tool for studying nucleus-cytoplasm interactions and may provide an alternative for cloning endangered animals whose oocytes are challenging to obtain. The development ability of iSCNT embryos decreases with increases in taxonomic distance between the donor cell and recipient species. In this study, more than 69 % of the domestic elephant fibroblast cells successfully fused with the porcine oocytes following electrofusion, and 0.6 % of embryos were able to reach the blastocyst stage. This is the first reported demonstration of using SCNT to reprogram elephant somatic cell nuclei that can develop to the blastocyst stage.

In conclusion, this study showed that porcine zygote could be stored by TCM-199, pFF, or FBS medium at 25 °C for 24 hours. In which the supplementation of 50 μM CGA to FBS has favorable outcomes on the post-storage development of zygotes. The porcine zygote could develop with the removal of the ZP before vitrification. The pig oocytes used in this study supported the remodeling and reprogramming of the elephant somatic cell nuclei. The SCNT technique developed in this study may soon be used for the mass production of cloned elephant embryos.

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APPENDIX

List of publications and proceedings

Publications

1. **Nhien Thi Nguyen**, Maki Hirata, Fuminori Tanihara, Takayuki Hirano, Quynh Anh Le, Masahiro Nii, Takeshige Otoi (2019). Hypothermic storage of porcine zygotes in serum supplemented with chlorogenic acid. *Reproduction in Domestic Animals*, 54 (5): 750-755.
2. **Nhien Thi Nguyen**, Maki Hirata, Fuminori Tanihara, Yoko Sato, Zhao Namula, Quynh Anh Le, Manita Wittayarat, Mokhamad Fahrudin and Takeshige Otoi (2020). In vitro development of zona pellucida-free porcine zygotes individually cultured after vitrification. *CryoLetters*. In printing.
3. Maki Hirata, Manita Wittayarat , Takayuki Hirano, **Nhien Thi Nguyen,** Quynh Anh Le, Zhao Namula, Mokhamad Fahrudin, Fuminori Tanihara and Takeshige Otoi (2019). The Relationship between Embryonic Development and the Efficiency of Target Mutations in Porcine Endogenous Retroviruses (PERVs) *Pol* Genes in Porcine Embryos. *Animals*, *9 (9)*: E593.
4. Zhao Namula, Manita Wittayarat, Maki Hirata, Takayuki Hirano, **Nhien Thi Nguyen**, Quynh Anh Le, Mokhamad Fahrudin, Fuminori Tanihara,

Takeshige Otoi. (2019). Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes. *In Vitro Cellular & Developmental Biology - Animal*, 55(8): 598–603.

5. Maki Hirata, Fuminori Tanihara, Manita Wittayarat, Takayuki Hirano, **Nhien Thi Nguyen**, Quynh Anh Le, Zhao Namula, Masahiro Nii, Takeshige Otoi (2019). Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes**.** *In Vitro Cellular & Developmental Biology - Animal*, 55 (4): 237–242.
6. Fuminori Tanihara, Maki Hirata , Shigeki Morikawa, **Nhien Thi Nguyen**, Quynh Anh Le, Takayuki Hirano, Yoshiyuki Fukumi, Toshiaki Abe, Takeshige Otoi (2019). The effects of electroporation on viability and quality of *in vivo*-derived bovine blastocysts. *Journal of Reproduction and Development*, 65 (5): 475 – 479.
7. Tanihara, F., Hirata, M., **Nguyen, T.N.,** Le A.Q, Hirano, T., Otoi T (2019). Effects of the concentration of CRISPR/Cas9 components on genetic mosaicism in cytoplasmic microinjected porcine embryos. *Journal of Reproduction and Development*, 65(3): 209-214.
8. Fuminori Tanihara, Maki Hirata, Satoru Iizuka, Shinya Sairiki, Masahiro Nii, **Nhien Thi Nguyen**, Quynh Anh Le, Takayuki Hirano, Takeshige Otoi (2019). Relationship among ovarian follicular status, developmental

competence of oocytes, and anti ‐ Müllerian hormone levels: A comparative study in Japanese wild boar crossbred gilts and Large White gilts. *Animal Science Journal,* 90 (6): 712–718.

9. Namula, Z., Tanihara, F., Wittayarat, M., Hirata, M., **Nguyen, T.N.**, Hirano, T., Le A.Q., Nii, M. and Otoi, T (2019). Effects of tris (hydroxymethyl) aminomethane on the quality of frozen-thawed boar spermatozoa. *Acta Veterinarian Hungarica,* 67(1): 106-114.
10. Tanihara, F., Hirata, M., **Nguyen, T.N.**, Le A.Q, Hirano, T., Takemoto, T., Nakai, M., Fuchimoto, D., Otoi T (2019). Generation of PDX-1 mutant porcine blastocysts by introducing CRISPR/Cas9-system into porcine zygotes via electroporation. *Animal Science Journal,* 90 (1): 55-61.
11. Tanihara, F., Hirata, M., **Nguyen, T.N.,** Le A.Q, Hirano, T., Takemoto, T., Nakai, M., Fuchimoto, D. and Otoi T (2018). Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation. *PLOS ONE,* 13(10): e0206360.
12. Namula, Z., Hirata, M., Wittayarat, M., Tanihara, F., **Nguyen, T.N.**, Hirano, T., Nii, M. and Otoi, T (2018). Effects of chlorogenic acid and caffeic acid on the quality of frozen-thawed boar sperm. *Reproduction in Domestic Animals,* 53 (6): 1600-1604.

13. Tanihara, F., Hirata, M., **Nguyen, T.N.,** Hirano, T., Kunihara, T. and Otoi, T (2018). Effect of ferulic acid supplementation on the developmental competence of porcine embryos during in vitro maturation. *The Journal of Veterinary Medical Science,* 80 (6): 1007–1011.

Proceedings

1. **Nhien Thi Nguyen**, Fuminori Tanihara, Maki Hirata, Takayuki Hirano, Quynh Anh Le, Takeshige Otoi. Efficiency of gene editing by electroporation of Cas9 protein (GEEP) to generate GGTA1-modified pigs. The 15th Transgenic Technology meeting (TT2019) 2019, Kobe, Japan.
2. **Nhien Thi Nguyen**, Fuminori Tanihara, Maki Hirata, Takayuki Hirano, Quynh Anh Le, Masahiro Nii, Takeshige Otoi. Hypothermic storage of porcine zygotes in serum supplemented with chlorogenic acid. The 6th Japan Society for Advanced Medical Engineering Pig Study Meeting 2018, Shizuoka, Japan.

BIOGRAPHY

Mrs. NGUYEN THI NHIEN was born on March 2nd, 1989, in Hai Duong province, Vietnam. She graduated with Degree of Doctor of Veterinary Medicine (DVM) from Faculty of Veterinary Medicine, Vietnam National University of Agriculture, in 2012. Because of her strong academic records, she was offered a lecturer position at the same university. At VNUA, along with teaching and research activities, she completed her Master's degree in Veterinary Science, in January 2017. In April 2017, she received a scholarship from The Japan International Cooperation Agency (JICA) for Science and Technology Research Partnership for Sustainable Development (SATREPS) project for the joint Ph.D. course at Tokushima University, Japan. Her focus research is about studies on the preservation of porcine zygotes for embryo production by interspecies somatic cell nuclear transfer.