MICROTUBULE ASSEMBLY AND IN VITRO DEVELOPMENT OF VITRIFIED BOVINE OOCYTES AFTER IN VITRO FERTILIZATION

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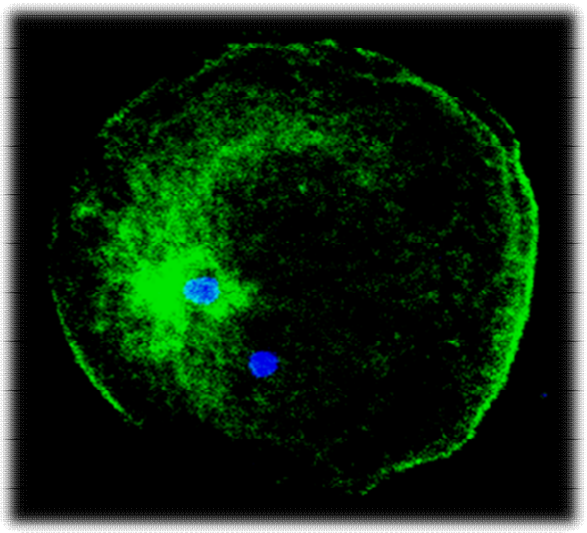

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ABBREVIATIONS

Abbreviations used without definition

ANOVA	analysis of variance
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneguaninetetraacetic acid
FSH	follicle stimulating hormone
GTP	guanosine triphosphate
IgG	immunoglobrin G
SD	standard deviation
SEM	standard error of means

Abbreviations used with definition at the first appearance

AGC	A-kinase, cGMP-kinase, C-kinase
AI	artificial insemination
BSA	bovine serum albumin
CG	cortical granule
COC	cumulus-oocyte complex
CPA	cryoprotective agent
DAPI	4’,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EG	ethylene glycol
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GEFs	guanine exchange factors
GV	germinal vesicle
hpi	hours post-insemination
IVC	in vitro culture
IVF	in vitro fertilization

IVM	in vitro maturation
LIM	Lin11, Isl-1 and Mec-3
LN_2	liquid nitrogen
mBO	modified Brackett and Oliphant
MII	metaphase of the second meiotic division
MLC	myosin light chain
MOET	multiple ovulations and embryo transfer
MPF	metaphase promoting factor
mSOF	modified synthetic oviductal fluid
MTOC	microtubule-organizing center
MYPT	myosin phospatase targeting protein
OPS	open-pulled straw
PBS	phosphate buffered saline
PN	pronucleus / pronuclei
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
ROCK	rho-associated coiled-coil kinase
TCM	tissue culture medium
TUNEL	TdT-mediated dUTP nick end labeling
ZP	zona pellucida

CHAPTER I: GENERAL INTRODUCTION

Many reproductive biotechnologies have been applied to efficient production of large domestic animals, such as pigs and cattle with the high economic importance. In cattle, those originally developed in the decades of 1950's to 1970's include artificial insemination (AI) and multiple ovulations and embryo transfer (MOET) with or without cryopreservation of spermatozoa and preimplantation-stage embryos, respectively. Successful cryopreservation of spermatozoa and embryos made these AI and MOET technologies more practical and available for commercial use, because of their potential advantages to allow long-distance transportation and to omit estrous synchronization, thus reducing the number of recipient female population to be maintained. Thereafter, embryo production by in vitro fertilization (IVF) using in vitro-matured oocytes from abattoir-derived ovaries and frozen-thawed spermatozoa became more or less routine since the decade of 1980's, and production of cloned embryos has been promising with the progress of somatic cell nuclear transplantation (late 1990's and 2000's). Cryopreservation of unfertilized oocytes can be combined with these advanced reproductive technologies, in addition to its potential advantage as oocyte banking for preserving female genetic resources. Revivability of cryopreserved oocytes from small rodents (such as mice or rats) and humans is extremely high, adapting well to the maintenance of the huge numbers of gene-modified transgenic strains and the efficient use in therapies for human infertility. However, the low fertilization rates and developmental competence of cryopreserved bovine oocytes still need to be improved.

1. HISTORY

The first successful cryopreservation of mammalian embryos has been achieved using slow cooling procedure of mouse 8-cell stage embryos (Whittingham et al., 1972). During the slow cooling, embryonic blastomeres are dehydrated in response to the osmotic pressure that gradually increases with the formation of extracellular ice crystals after ice seeding. Then, a great breakthrough for simple and efficient cryopreservation has been reported by a very high cooling rate of fully dehydrated mouse embryos in highly concentrated solutes (Rall and Fahy, 1985). The characteristics of two potential methods for cryopreservation of mammalian embryos, applicable to oocytes, are summarized in Table 1-1.

Table 1-1. Two potential methods for cryopreservation of mammalian embryos.

Procedures for cryopreservation	
Freezing and thawing	Vitrification and warming
Tubing such as 0.25-ml straw	Tubing or surface-type devices
Low concentration of a single CPA	High concentration of multiple CPAs
Slow cooling rate (0.1 to 1 °C/min) terminated at -30 to -79 °C, and then plunging into LN_2	Direct plunging into LN_2 Ultra-rapid cooling rate (2,500 to 20,000 °C/min)
Whittingham et al. (1972), and many others	Rall and Fahy (1985), and many others

1.1. Embryos

Following the first successful freezing of preimplantation mouse embryos (Whittingham et al., 1972), pregnancy from a cryopreserved cattle embryo was reported by Wilmut and Rowson (1973). These initial findings were then extended to embryos from several mammalian species including rat, rabbit, goat, sheep, horse and human. The protocol most commonly used for successful embryo cryopreservation at that time required slow cooling from upper -7 ºC to below -80 ºC in phosphate buffered saline (PBS) supplemented with dimethyl sulfoxide (DMSO) or glycerol as a permeable cryoprotective agent (CPA). The frozen embryos were warmed very slowly to avoid the rapid influx of extracellular water into the dehydrated cells during warming. This protocol was labor intensive and time-consuming.

In 1977, a two-step freezing method was reported using sheep and cattle embryos (Willadsen, 1977). In this procedure, slow cooling of embryos is interrupted at around -30 ºC to -36 ºC, followed by rapid cooling to -196 ºC. The embryos in liquid nitrogen (LN_2) are believed to contain intracellular ice, although it is not detrimental at this point. But to survive, the frozen embryos must be warmed rapidly to avoid injury caused by re-crystallization of the intracellular ice. This two-step freezing regimen allows the development of a temperature-controlled, programmable freezer, and is still used widely for many mammalian species. In cattle, pregnancy rates following transfer of embryos frozen in this way range from 50 to 60% (Niemann et al., 1985). Additional progress resulted from the use of ethylene glycol (EG) as a CPA for embryos from domestic species. Using sucrose as an osmotic buffer, direct transfer of post-thaw embryos into recipients without expelling them from the straws was reported by Leibo (1984).

In a series of experiments aimed at simplifying the cooling process, Rall and Fahy (1985) developed a novel approach to cryopreserve mouse embryos. This protocol involves dehydration of the embryos by exposing them to highly concentrated CPAs prior to cooling them to low temperature, rather than during the cooling process itself. The dehydrated embryos are rapid cooled by being directly plunged into LN_2. Since the cryoprotective solution can be transformed into a stable glass without ice crystal formation during the rapid cooling process, this extremely rapid method of cryopreservation is referred to as "vitrification", meaning "glass formation". The application of vitrification as an alternative to conventional freezing reduces the technical skills and equipment required. So far, successful vitrification producing pregnancy and/or birth of live offspring has been reported with embryos from mouse, rat, rabbit, pig, goat, sheep, cattle, horse and human. A wide variety of vitrification solutions and protocols have been employed even for the same type of embryo, i.e. the same species and developmental stage.

1.2. Oocytes

Cryopreservation of oocytes is short and less successful history than the other reproductive cells like spermatozoa and embryos. The first successful IVF and birth of live offspring using frozen-thawed mouse oocytes was reported in 1976 by Parkening et al., and followed by Whittingham (1977) and Leibo et al. (1978). Other than the mouse, such a slow freezing procedure was acceptable for species whose oocytes are not sensitive to chilling, such as cat (Wolfe and Wildt, 1996; Cocchia et al., 2010) and human (Chen, 1986). There are a few reports regarding successful pregnancies from frozen-thawed bovine oocytes (Fuku et al., 1992; Otoi et al., 1992). However, oocytes from the large domestic species are rich in cytoplasmic lipid droplets and very sensitive to chilling, resulting in the poor revivability following the slow cooling (Ledda et al., 2001). After the publication of innovative results by Rall and Fahy (1985), vitrification has been attempted to apply to oocytes. Pregnancies or birth of live offspring have been published in mouse (Nakagata, 1989), human (Porcu et al., 1997) and cattle (Hamano et al., 1992), with an increased requirement for improving developmental competence of the vitrified-warmed oocytes.

Martino et al. (1996) reported that 15 % of matured bovine oocytes developed into blastocysts follwing vitrificaton, under in vitro culture (IVC) conditions in which > 40 % of the non-treated fresh oocytes were able to develop to that stage. That protocol is characterized by the extremely rapid cooling rate of oocytes suspended in < 1 µl of a vitrification solution consisting of 30 % EG plus 1.0 M sucrose placed onto electron microscope grids, a procedure derived from methods to cryopreserve Drosophila embryos (Steponkus et al., 1990). The microgrids provide a cooling rate estimated to be < 150,000 ºC/min, in contrast to 2,500 ºC/min with the conventionally used plastic straws. Vajta et al. (1998) reported an alternative way of ultra-rapid cooling for vitrification of

bovine oocytes. When the oocytes were aspirated into open-pulled straws (OPS) and cooled by directly plunging into LN_2, 13 % of the post-warm oocytes could develop into blastocysts after IVF and IVC. The OPS method has been improved to use open-pulled glass capillaries (Kong et al., 2000; Hochi et al., 2001) or commercially available gel-loading tips (Tominaga and Hamada, 2001). Other types of cryodevices so far reported for ultra-rapid cooling are the "Cryoloop" (Lane et al., 1999) and "Cryotop" (Kuwayama and Kato, 2000). Complete containerless methods have also been reported from two independent laboratories (Dinnyes et al., 2000; Papis et al., 2000). Blastocyst yields from frozen-thawed or vitrified-warmed bovine metaphase-II (MII) oocytes, reported during the last two decades, are summarized in Table 1-2. There was no significant improvement on the blastocyst yield from cryopreserved bovine MII oocytes, even after increased cleavage rates as > 60 % by using different cryodevices and vitrification protocols were obtained.

Table 1-2. List of Day-8 blastocyst yields from cryopreserved and in vitro-fertilized bovine oocytes.

Publications (Year)	Method / device for cryopreservation	Cryosurvival of oocytes	
		Cleavage rate	Blastocyst yield
Otoi et al. (1992)	Freezing / French straw	42 %	3 %
Hamano et al. (1992)	Vitrification / French straw	22 %	9 %
Martino et al. (1996)	Vitrification / EM-grid *[1]	40 %	15 %
Vajta et al. (1998)	Vitrification / OPS *[2]	50 %	13 %
Kubota et al. (1998)	Freezing / French straw	60 %	12 % *[4]
Dinnyes et al. (2000)	Vitrification / Microdrop	62 %	11 %
Chian et al. (2004)	Vitrification / Cryotop	70 %	7 %
Tominaga et al. (2005)	Vitrification / GL-tip *[3]	49 %	17 %
Zhou et al. (2010)	Vitrification / Cryotop	76 %	12 %

*[1] EM-grid: Electron microscope grid.

*[2] OPS: Open-pulled straw.

*[3] GL-tip: Gel-loading tip.

*[4] Data on Day-9.

Cryopreservation of immature oocytes at the germinal vesicle (GV) stage is also the subject for challenging endeavour. Vajta et al. (1998) reported that 25 % of bovine oocytes vitrified-warmed using OPS system could develop into the blastocyst stage on Day 8. Although the high revivability of post-warm oocytes remains for reproducibility, birth of calves following transfer of embryos derived from cryopreserved immature oocytes (Papis et al., 2000; Vieira et al., 2002) encouraged such challenges. Abe et al. (2005) reported that 8 % of bovine oocytes developed into blastocysts when they were exposed to EG + Ficoll + sucrose-based solution in a stepwise manner and vitrified-warmed on nylon-mesh holder as a cryodevice, with successful data on a live calf after transfer. Bovine oocytes at the GV stage have homogenous (=less variable in size) lipid droplets that show little change following cooling, but intercellular coupling via gap junctions between cumulus cells and the GV-stage oocytes may be sensitive to osmotic stress. Modifying oocytes to fit cryopreservation procedure has been proposed to improve their cryosurvival (Seidel, 2006). By transferring cholesterol to membrane of bovine oocytes, cleavage and development to the 8-cell stage after IVF were improved (Horvath and Seidel, 2006). Cytoplasmic lipid droplets can be partially removed from bovine oocytes by high magnitude centrifugation, and it was reported that incidence of polyspermic penetration in centrifuged and vitrified-warmed oocytes was significantly inhibited (Otoi et al., 1997).

2. OOCYTE CRYOBIOLOGY

2.1. Physiology of fertilization

Once oocytes resume the first meiotic division, the nuclear envelope (=GV) is disintegrated, allowing the nuclear material to mix into the cytoplasm. Some alterations also occur in organelles such as mitochondria, cytoskeleton and cortical granules. Additionally, cumulus cells are expanded, microfilaments of actin are involved in cell shape modifications and movements, and microtubules form the spindle apparatus (Massip, 2003). Centriole–centrosome organization and duplication from sperm incorporation through mitosis of the first cell cycle is shown in Figure 1-1. The microtubule is a cylindrical bundle, comprised of protofilaments (a heterodimer consisting of α- and β-tubulin). Microtubule assembly begins from microtubule-organizing centers (MTOC) at both poles and anchor chromosomes at the kinetochores (Chen et al., 2003; Schatten et al., 2009). It is important to understand the regulation and function of centriole–centrosome-complex and centrosomes in relation to the failure of fertilization (Schatten et al., 2009). A spermatozoon has two distinct centriolar structures with the proximal centriole located within the connecting piece under the sperm head. The distal centriole is organized vertically to the proximal centriole and aligned with the sperm tail (Sathananthan et al., 1996). Shortly after sperm penetration into the oocyte, the distal is

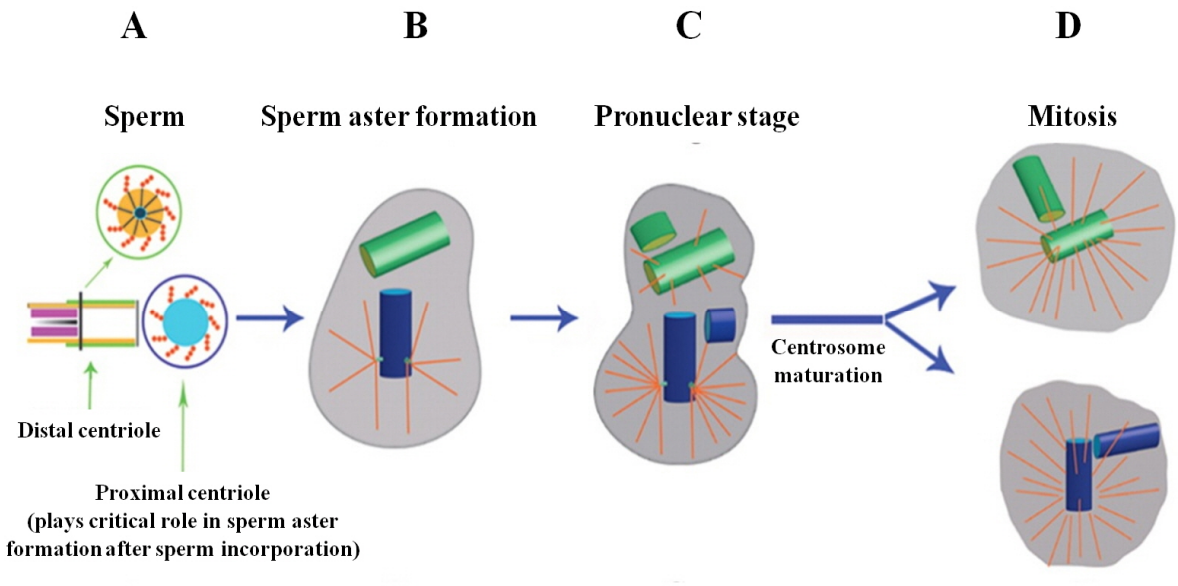

Figure 1-1. Schematic diagram of centriole–centrosome organization and duplication from sperm incorporation through mitosis of the first cell cycle. In most mammalian species (except for rodents), spermatozoal centrioles contribute to the fertilized oocyte, by being duplicated during the pronuclear stage and separated after syngamy to serve as mitotic centers during the first and all the subsequent cell divisions. (A) Prior to fertilization, spermatozoa carry two distinct centriolar structures with the proximal centriole and distal centriole. (B) Shortly after sperm incorporation into the oocyte, a sperm aster is formed from the proximal centriole. The sperm aster has several functions aside from guiding the two pronuclei into close apposition, as it also serves as railroad system for signal transduction molecules and for accumulating centrosomal components from the oocyte to the sperm's centriole–centrosome complex. (C) After pronuclear apposition, the sperm centrioles duplicate during the pronuclear stage. (D) The duplicated centrioles separate and migrate around the zygote nucleus to form the opposite poles of the first mitotic spindle. Adopted from a review article (Schatten and Sun 2009).

centriole removed from main piece of sperm head with its tail and, after sperm incorporation into the oocyte, a sperm aster is formed from the proximal centriole which allows pronuclear apposition. The proximal centriole maintains close connection to the sperm nucleus which gradually develops into a male pronucleus, and the sperm centrioles duplicate. Then, the duplicated centrioles separate and migrate to the zygote nucleus to form the opposite poles of the first mitotic spindle (Sathananthan et al., 1991; Sathananthan et al., 1996). The chromosomes align at the equatorial plane of the meiotic spindles. A recent study in porcine oocytes found that paclitaxel treatment

improved the normality of microtubules by strengthening the bond between α- and β-tubulin and improved the developmental ability of vitrified MII oocytes (Morato et al., 2008a; Ogawa et al., 2010). Successful fertilization is highly dependent on the maintenance of the structural and functional integrity of COC components. Abnormalities of the spindle have been shown to directly correlate with loss of fertilization and developmental abilities, because they are crucial for completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle (Schatten et al., 1985).

2.2. Cryoinjuries

At very low sub-zero temperature in LN_2, biological activity is completely stopped and the cell viability and functional state may be preserved for long terms (Mazur, 1970). However, some physical stresses can damage cells at the various sub-zero low temperatures. Intracellular ice formation is one of the biggest causes to cell damage hence, the freezing protocols use a combination of dehydration, freezing point depression, supercooling and intracellular vitrification in an attempt to avoid cell damages (Saragusty and Arav, 2011). Therefore, it is important to use CPA, such as DMSO, EG or glycerol alone or in combination, when cryopreserving cells in any methods. Due to both of hydorophobic and hydrophilic characteristics, as well as the relatively small molecular weight, these CPAs are permeable to the plasma membrane. On the other hand, use of CPA induces some adverse effects, such as osmotic injury and toxicity of the CPAs.

Incidence of cryoinjures depends on the size and shape of the cell, the permeability of the cell membranes and the quality of the cells. However, these factors differ from species, developmental stage and origin (Vajta and Kuwayama, 2006). Although offspring have been born using frozen-thawed oocytes from various species, the ability to support embryo development following cryopreservation procedures is still low. This may be attributed to the susceptibility of oocytes to damage during cooling and/or freezing and subsequent thawing because of their complex structure. Unfertilized mammalian oocytes are much larger than the blastomeres of an early embryo and therefore have a small surface to volume ratio (Chen et al., 2003). This led to dehydration and penetration of CPA difficult to achieve, which attributes to the difficulty in cryopreservation. Furthermore, the plasma membranes of oocytes differ significantly from those of embryos. There is a rise of intracellular free calcium during fertilization, which makes the ionic strength and membrane potential of the plasma membrane (Gook et al., 1993). The another adverse effects of cryopreservation procedures include the fracture damage in zona pellucida (Lane et al., 1999) and the destruction of intercellular coupling via gap junctions between cumulus cells and the oocyte (Fuku et al., 1995; Hochi et al., 1996).

In general, the primary criteria to assess post-thaw viability of oocytes are the presence or absence of membrane degeneration, cytoplasmic abnormalities and zona pellucida fractures (Ledda et al., 2007). Recent studies in humans have examined the meiotic spindle using a polarized microscope apparatus, which allows the visualization of the polymerization of the meiotic spindle after vitrification and warming. However, this technique is difficult in domestic animal species due to their high cytoplasmic lipid content, which hinders spindle examination. Therefore, such dark oocytes from domestic species must be typically examined through invasive methods, such as fluorescence microscopy and biochemical or molecular analyses (Ledda et al., 2007).

Low fertilization rates of cryopreserved oocytes were reported to be associated with chilling and freezing injuries, including zona hardening due to premature release of cortical granules (Carroll et al., 1990; Fuku et al., 1995) and spindle disorganization and loss or clumping of microtubules (Magistrini and Szollosi, 1980; Aman and Parks, 1994). Briefly, exposure of MII oocytes to CPA and/or chilling procedure induced the transient rise of intracellular free calcium, and prevented the sperm entry via block mechanisms at the level of plasma membrane or zona pellucida (Rojas et al., 2004; Succu et al., 2007; Morato et al., 2008; Pereira and Marques, 2008). These processes also result in damage to the meiotic spindle, actin filaments, chromosomal dispersal and microtubule depolymerization (Massip, 2003; Ogawa et al., 2010). As shown in Figure 1-2, two major hypo-

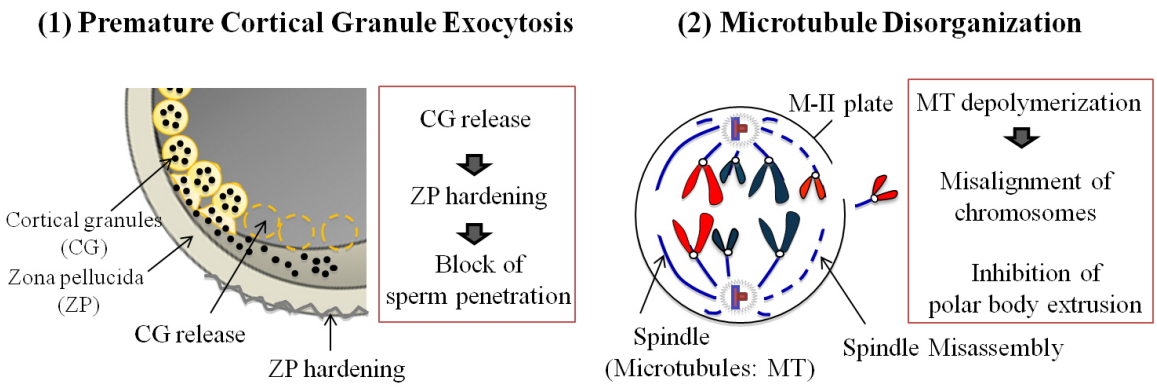

Figure 1.2. Two major hypotheses on cryoinjuries induced in mammalian oocytes; (1) Premature cortical granule exocytosis and (2) Microtubule disorganization. The former causes zona hardening, resulting in block of sperm penetration. The latter means depolymerization of tubulin proteins, leading to misassembly of meiotic spindles, and subsequently resulting in misalignment of chromosomes and inhibition of the second polar body extrusion.

theses on cryoinjuries induced in mammalian oocytes include (1) premature cortical granule exocytosis and (2) microtubule disorganization. The former premature cortical granule exocytosis causes the hardening of zona pellucida, leading to block of sperm penetration. The latter disorganization of the microtubules means depolymerization of tubulin proteins, leading to misassembly of meiotic spindles, misalignment of chromosomes, and inhibition of the second polar body extrusion.

3. OBJECTIVE

The main objective of the present study was to investigate the effect of vitrification and warming on bovine oocytes. In the first series of experiment (Chapter II), profiles of cleavage and blastocyst development were examined for vitrified bovine oocytes, and then function of MTOC/aster(s) in the vitrified oocytes after IVF was analyzed. In the second series of experiment (Chapter III), it was investigated whether short-term treatment of vitrified-warmed bovine oocytes with Rho-associated coiled-coil kinase (ROCK) inhibitor can improve the survival rate and the subsequent developmental competence after IVF. In addition, the mitochondrial activity during the short-term culture with ROCK inhibitor and the function of MTOC after IVF were investigated.

CHAPTER II: HIGH INCIDENCE OF MULTIPLE ASTER FORMATION IN VITRIFIED-WARMED BOVINE OOCYTES

ABSTRACT

In vitro-matured bovine oocytes do not tolerate vitrification as well as mature murine or human oocytes. Delayed first cleavage in vitrified and in vitro-fertilized bovine oocytes may be responsible for the decreased yield of blastocysts in vitro. Because formation of sperm-aster and the subsequent assembly of microtubule network play an important role for migration and fusion of both pronuclei, aster formation in vitrified-warmed oocytes was analyzed by confocal laser-scanning microscopy. At 10 hours post-insemination (hpi), proportions of oocytes fertilized normally were comparable between the vitrified and fresh control groups (67 and 70 %, respectively). Proportions of oocytes that exhibited microtubule assembly were similar between the two groups (95 % each), but the proportion of oocytes with multiple asters was higher in the vitrified group when compared with the fresh control group (68 vs 29 %, $P < 0.05$). Both migration and development of two pronuclei were adversely affected by multiple aster formation. In the next experiment, multiple asters observed in 5.5 vs 8 hpi pronuclear zygotes were located near the male pronucleus, suggesting that those multiple asters were not the cytoplasmic asters of maternal origin. In conclusion, multiple aster formation frequently observed in vitrified-warmed bovine oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly from the sperm-aster.

INTRODUCTION

Although successful pregnancies from frozen-thawed oocytes have been reported in several mammals, including mice (Whittingham, 1977), rabbits (al-Hasani et al., 1989), cattle (Fuku et al., 1992), and humans (Chen, 1986), developmental rate needs to be improved. Proposed reasons for high sensitivity of oocytes to cryopreservation include the large cell size and low permeability of water and CPA. Depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome

misalignment (Shi et al., 2006). Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae (Larman et al., 2006; Fujiwara et al., 2010). Application of vitrification improved the efficacy of oocyte cryopreservation, especially in mice (Nakagata, 1989; Wang et al., 2009) and humans (Kuwayama et al., 2005; Cobo et al., 2008). However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement (Maclellan et al., 2002; Chian et al., 2004; Somfai et al., 2007; Succu et al., 2008; Zhou et al., 2010).

A centrosome is composed of a pair of centrioles surrounded by the pericentriolar materials, such as γ-tubulin, centrin and pericentrin, and acts as the MTOC. In cattle, a sperm brings a centrosome into an oocyte during fertilization (Schatten, 1994) and a single sperm aster is formed by polymerization of microtubules α- and β-tubulin. The microtubule network plays a key role in the migration of male and female pronuclei to the center of a zygote and the subsequent fusion and mitotic cleavage (Kim et al., 1996; Terada et al., 2004). Conversely, rodent sperm lose their centrioles during spermiogenesis (Manandhar et al., 2005). Hence, the oocytes use their own MTOC dispersed in the cytoplasm for aster formation; that is called cytoplasmic aster (Woolley and Fawcett, 1973; Schatten et al., 1985). Timing of first cleavage in IVF-derived bovine oocytes is important for yield and quality of blastocysts, as oocytes cleaving earlier are more likely to become blastocysts (Lonergan et al., 1999; Ward et al., 2001), and the resulting blastocysts have higher cryosurvival potential (Dinnyes et al., 1999) and higher pregnancy rates (Lonergan et al., 1999) than those cleaving later. Thus, developmental kinetics can be used as a proxy of embryo quality.

In the present study, profiles of cleavage and blastocyst development were first examined for vitrified bovine oocytes, and then function of MTOC/aster(s) in the vitrified oocytes after IVF was analyzed.

MATERIALS AND METHODS

Experimental designs

In Experiment 1, cumulus-free bovine matured oocytes were vitrified-warmed, and then fertilized in vitro. The presumptive zygotes were placed in microdrops and cultured to allow development into blastocysts for up to 8 days. Cleavage was checked first at 27 hpi and again at 48 hpi, and the blastocyst yields from earlier-cleaving and delayed-cleaving embryos were separately recorded. Non-vitrified matured oocytes served as fresh controls. In Experiment 2,

pronuclear stage zygotes were produced by IVF of vitrified-warmed and fresh control oocytes (gamete coincubation for 6 h and additional culture for 4 h). The microtubules of the 10 hpi zygotes were immunostained with a monoclonal antibody against α-tubulin and nuclear DNA were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Under a confocal laser-scanning microscopy, aster formation in the normally fertilized zygotes with 2-PN was analyzed. The number of asters, the distance between male and female pronuclei, and the size of both pronuclei were recorded. In Experiment 3, it was investigated whether the observed multiple asters were the cytoplasmic asters (maternal origin) or the fragmentation of sperm-aster (paternal origin). The positioning of multiple asters in the vitrified and IVF oocytes at 5.5 and 8 hpi was compared after the fertilization window was narrowed to 2 h.

In vitro maturation

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Abattoir-derived bovine ovaries were transported to the laboratory in saline (maintained at 10–12 °C) within 24 h after slaughter. The contents of follicles (diameter, 2 to 8 mm) were aspirated with an 18-G needle connected to a 10-ml syringe. Oocytes surrounded with at least two layers of compact cumulus cells were matured in Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA), supplemented with 10 % FBS (SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/ml FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 μg/ml 17β-estradiol, and 50 μg/ml gentamycin sulfate for 22 h at 38.5 °C under 5 % CO_2 in air. Then, cumulus cells were removed by a brief vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/ml BSA, 0.2 mM sodium pyruvate, 1,000 IU/ml hyaluronidase, and 50 μg/ml gentamycin sulfate. Oocytes with an extruded first polar body were defined as matured and were used for experiments.

Vitrification and warming

Matured oocytes were subjected to vitrification according to the method described previously by Tsujioka et al. (Tsujioka et al., 2008), with minor modifications. Briefly, oocytes were equilibrated 7.5 % EG (Wako Pure Chemical, Industries Co., Osaka, Japan) and 7.5 % DMSO (Wako) in Hepes-buffered TCM-199/20 % FBS base medium for 3 min at room temperature (23 ± 2 °C), and then transferred into a vitrification solution consisting of 15 % EG, 15 % DMSO and 0.5 M sucrose in the base medium for approximately 60 sec at room temperature (23 ± 2 °C). Within this 60 sec, up to eight oocytes were loaded onto the top of

the polypropylene strip of a Cryotop (Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of the vitrification solution, and then quickly immersed into LN_2 (Figure 2-1).

After storage for 1 to 10 weeks in LN_2 tank, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 ml of the base medium containing 1 M sucrose at 38.5 °C, and kept for 1 min. The oocytes were transferred to the base medium at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min respectively) (Figure 2-2). They were cultured in Hepes-buffered TCM-199 supplemented with 5 % FBS, 0.2 mM sodium pyruvate and 50 μg/ml gentamycin sulfate (TCM-199/5 % FBS) for 1 to 2 h at 38.5 °C under 5 % CO_2 in air before subjecting to IVF.

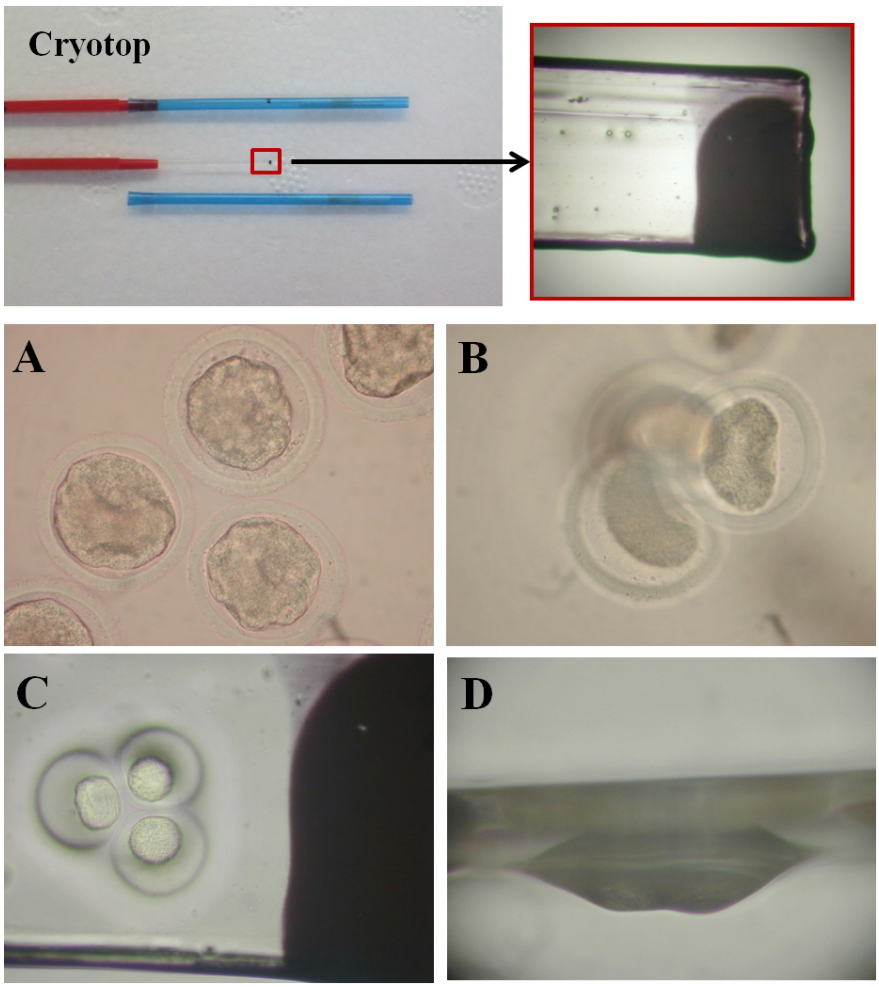

Figure 2-1. Cryotop vitrification of in vitro-matured bovine oocytes. Polar body-extruded mature oocytes were treated with equilibrium solution (A) and vitrification solution (B), and then loaded onto the top of the polypropylene strip of a Cryotop device (C). Plate-D is a side view of the plate-C.

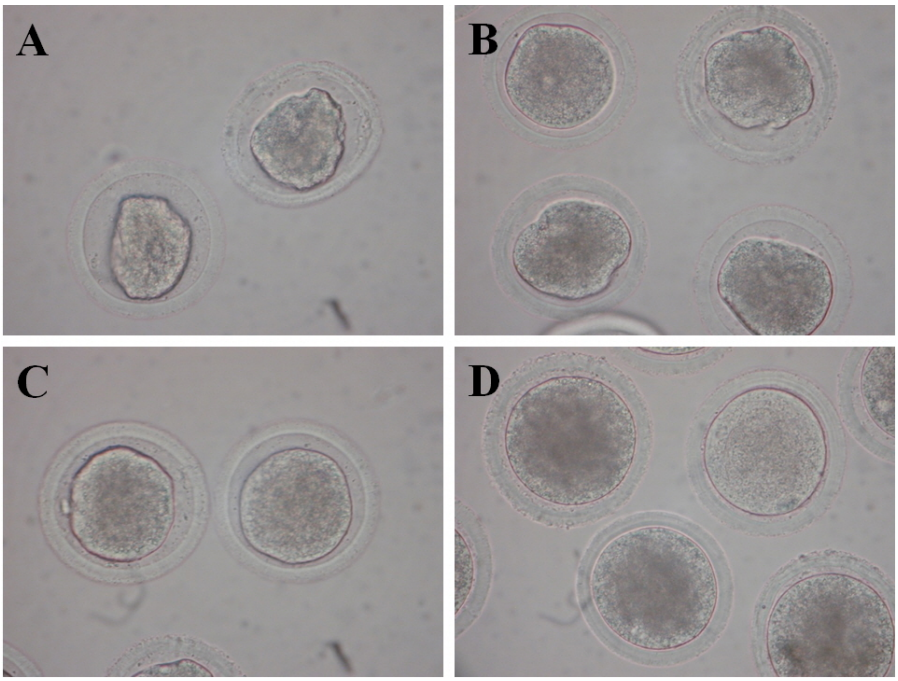

Figure 2-2. Morphological change of vitrified-warmed bovine oocytes during the dilution process. Post-warm oocytes in 1 M sucrose (A), 0.5 M sucrose (B), 0.25 M sucrose (C), and 0 M sucrose (D).

Fertilization and culture in vitro

Commercially available frozen semen from a Japanese Black bull was used. After thawing in a water bath at 37 °C for 30 sec, the contents of a 0.5 ml straw was layered on the top of Percoll density gradient consisting of 2 ml of 45 % Percoll above 2 ml of 90 % Percoll in a 15 ml conical tube, and centrifuged for 20 min at 700 *g*. The pellet was re-suspended in 4 ml of modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan; Table 2-1) supplemented with 5 mM theophylline, washed twice (5 min at 300 *g* each) and then re-suspended in the mBO medium supplemented with 5 mg/ml BSA and 10 μg/ml heparin (IVF medium) to yield a concentration of 4 x 10^7 sperm cells/ml. In Experiments 1 and 2, 10–12 matured oocytes in the IVF medium were coincubated with the above sperm suspension at a final concentration of 8 x 10^6 sperm cells/ml for 6 h in 100-μl microdrops under mineral oil at 38.5 °C under 5 % CO_2 in air. In Experiment 3, a 100 μl sperm suspension at a final concentration of 8 x 10^6 sperm cells/ml in IVF medium was preincubated for 2 h before introduction of the 10 to 12 vitrified oocytes. The period for gamete coincubation was shortened to 2 h for restriction of the fertilization window.

In Experiment 1, up to 30 presumptive zygotes (6 hpi) were cultured in a 250-µl microdrop of modified synthetic oviductal fluid (mSOF; Holm et al., 1999; Table 2-1), supplemented with 30 µl/ml essential amino acids solution (x 50, Gibco-11,130), 10 µl/ml non-essential amino acids solution (x 100, Gibco-11,140) and 5 % FBS at 39.0 °C under 5 % CO_2, 5 % O_2 and 90 % N_2 for 8 days. Cleaving embryos and harvested blastocysts are shown in Figure 2-3.

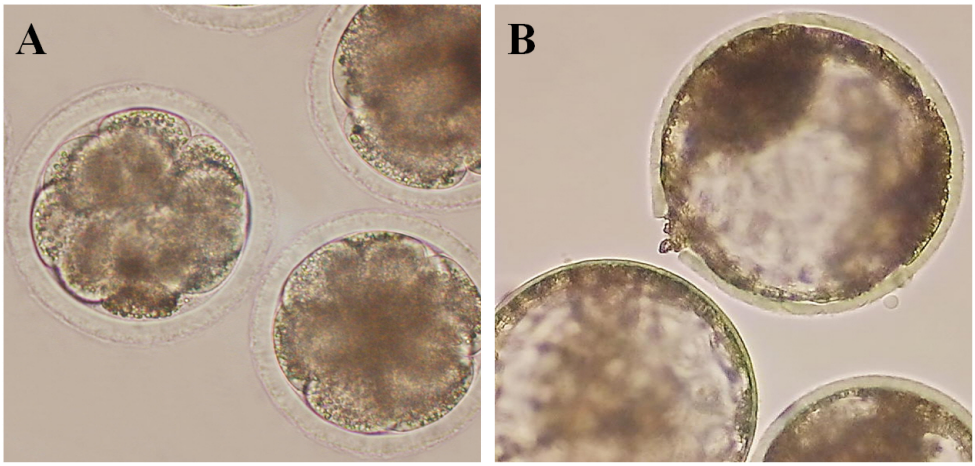

Figure 2-3. Bovine embryos at cleavage-stage (A) and blastocyst-stage (B), produced by IVF.

Immunostaining of pronuclear zygotes

In Experiment 2, fresh or vitrified oocytes after IVF (6 hpi) were cultured for an additional 4 h (10 hpi) in TCM-199/5 % FBS at 38.5 °C under 5 % CO_2 in air, and then immunostained. In Experiment 3, vitrified oocytes after IVF (2 hpi) were cultured for an additional 3.5 and 6 h (5.5 and 8 hpi, respectively), and then immunostained. According to the method described previously (Hara et al., 2011), the oocytes were extracted for 15 min by buffer M (25 % glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) containing 5 % (v/v) methanol and 1 % (v/v) Triton X-100, after zonae pellucidae had been removed with 0.75 % protease in M2 medium (Table 2-1). The oocytes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS containing 0.1 % (v/v) Triton X-100. Microtubules were labeled with a monoclonal antibody against α-tubulin (T5168; diluted 1:1,000). The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F1010; diluted 1:200).

Nuclear DNA was visualized by counterstaining with 2.5 μg/ml DAPI. Preparations were mounted with coverslips in antifade agent, and digital images were collected at 2 μm distance using a confocal laser scanning microscope (FV1000-D, Olympus, Tokyo, Japan). The digital images were stacked and assessed with ImageJ software (National Institutes of Health, Bethesda, ML, USA; accessed on-line). Zygotes with two pronuclei (2-PN) were defined as those fertilized normally, and the larger pronucleus was defined as male pronucleus.

Table 2-1. Composition of different media used in this study.

Components	mBO	mSOF	M2
NaCl	80.08 mM	107.63 mM	94.66 mM
KCl	4.02 mM	7.16 mM	4.77 mM
KH_2PO_4	---	1.19 mM	1.19 mM
NaH_2PO_4	0.83 mM	---	---
$MgCl_2$	0.52 mM	---	---
$MgSO_4$	---	1.51 mM	1.18 mM
$CaCl_2$	2.25 mM	1.78 mM	1.71 mM
$NaHCO_3$	37.00 mM	25.00 mM	4.15 mM
NaOH	7.50 mM	---	---
Hepes	25.00 mM	---	20.85 mM
D-glucose	13.97 mM	---	5.55 mM
L-cystein	0.50 mM	---	---
L-glutamine	---	0.20 mM	---
Sodium pyruvate	1.25 mM	2.27 mM	0.33 mM
Sodium lactate	---	3.20 mM	29.23 mM
Tri-sodium-citrate	---	0.34 mM	---
Myo-inositol	---	2.77 mM	---
Gentamycin	100 μg/mL	50 μg/ml	5 μg/ml
Phenol red	8 μg/ml	10 μg/ml	---
BSA	5 mg/ml	---	---

mBO: modified Brackett and Oliphant medium; mSOF: modified Synthetic Oviductal Fluid.

Statistical analysis

In Experiment 1, cleavage rates at 27 and 48 hpi and blastocyst yields were arcsin-transformed and compared between vitrified and fresh control groups by Student's *t* test. Arcsin-transformed blastocyst yields were compared between subgroups of oocytes cleaved earlier (0–27 hpi) and later (27–48 hpi) by Student's *t* test. In Experiment 2, rates of oocytes for normal fertilization, polyspermic fertilization, overall aster formation, single aster formation and multiple aster formation were arcsin-transformed and compared between vitrified and fresh control groups by Student's *t* test. Mean aster number per zygote exhibiting multiple asters was also analyzed by Student's *t* test. The distance between male and female pronuclei and the pronuclear size were compared by one-way ANOVA. When the ANOVA was significant, differences among means were analyzed by a Tukey's test. In Experiment 3, mean distance from aster to male pronucleus was analyzed between 5.5 and 8 hpi groups by Student's *t* test. Mean aster number per zygote was also analyzed by Student's *t* test. A value of $P < 0.05$ was defined as a significant difference.

RESULTS

Early cleavage and blastocyst yield (Experiment 1)

All 635 post-warm oocytes appeared to be intact (morphologic cryosurvival, 100 %). After IVF and IVC of the presumptive zygotes, the cleavage rate 48 hpi was lower than that of fresh control counterparts (63.1 vs 74.9 %, $P < 0.05$; Table 2-2). The adverse effects of vitrification were more prominent in the developmental rate to blastocyst stage (15.8 and 41.8 % in vitrified and fresh control groups, respectively; $P < 0.05$). The proportion of oocytes cleaving earlier in the fresh control group (62.7 %) was more than twice that in the vitrified group (23.6 %). The earlier-cleaving oocytes developed to blastocysts at a higher rate ($P < 0.05$) when compared to the later-cleaving ones (41.0 %, 16/39 vs 15.4 %, 10/65 and 59.8 %, 61/102 vs 35.0 %, 7/20 in vitrified and fresh control groups, respectively).

Table 2-2. Development into blastocysts of in vitro-matured bovine oocytes after vitrification and IVF.

		No. (%) of oocytes			
		Cleaved		Developed to blastocysts	
Groups	Inseminated	Earlier: 27 hpi	Total: 48 hpi	Earlier: 27 hpi	Total: 48 hpi
Fresh control	163	102 (62.7 ± 3.3) [a]	122 (74.9 ± 1.8) [a]	61 (37.6 ± 3.2) [a]	68 (41.8 ± 3.0) [a]
Vitrified	165	39 (23.6 ± 2.3) [b]	104 (63.1 ± 2.5) [b]	16 (9.7 ± 2.8) [b]	26 (15.8 ± 2.9) [b]

Percentages were expressed as mean ± SEM of four replicates in each group. hpi, h post-insemination.
[a,b] Different superscripts denote significant difference between vitrified and fresh control groups ($P < 0.05$).

Analyses of aster formation (Experiment 2)

Presumptive zygotes 10 hpi with or without vitrification at their metaphase-II stage were assessed for the presence of aster(s), as shown in Table 2-3. Counterstaining with DAPI indicated the comparable incidence of normal fertilization between vitrified and fresh control groups (66.5 and 69.6 %, respectively). Polyspermic penetration occurred at a similar rate between the two groups (20.5 ± 4.3 and 10.7 ± 3.4 %, respectively).

Table 2-3. Formation of single or multiple asters in pronuclear-stage bovine zygotes (10 hpi).

	No. (%) of oocytes		No. (%) of 2-PN zygotes		
Groups	Inseminated	Fertilized: 2-PN	Formed aster(s)	With single aster	With multiple asters
Fresh control	97	68 (69.6 ± 3.6)	64 (95.4 ± 3.2)	45 (66.7 ± 4.7) [a]	19 (28.7 ± 4.1) [a]
Vitrified	86	56 (66.5 ± 4.4)	53 (95.2 ± 2.3)	16 (27.5 ± 4.6) [b]	37 (67.7 ± 5.4) [b]

Percentages were expressed as mean ± SEM of seven replicates in each group. hpi, h post-insemination.
[a,b] Within a column, means without a common superscript differed ($P < 0.05$).

Immunostaining for α-tubulin indicated that proportions of zygotes exhibiting aster formation were also comparable between vitrified and fresh control groups (95.2 and 92.4 % of 2-PN zygotes each). However interestingly, relative ratio of zygotes with a single aster (Figure 2-4A) vs multiple asters (Figure 2-4B) was significantly different between the two groups. Incidence of multiple aster formation in zygotes derived from vitrified oocytes (67.7 %) was more than double that in zygotes derived from fresh control oocytes (28.7 %, $P < 0.05$). Mean aster number per zygote exhibiting multiple asters was 5.4 ± 0.5 and 4.9 ± 0.5 in vitrified and fresh control groups, respectively ($P > 0.05$).

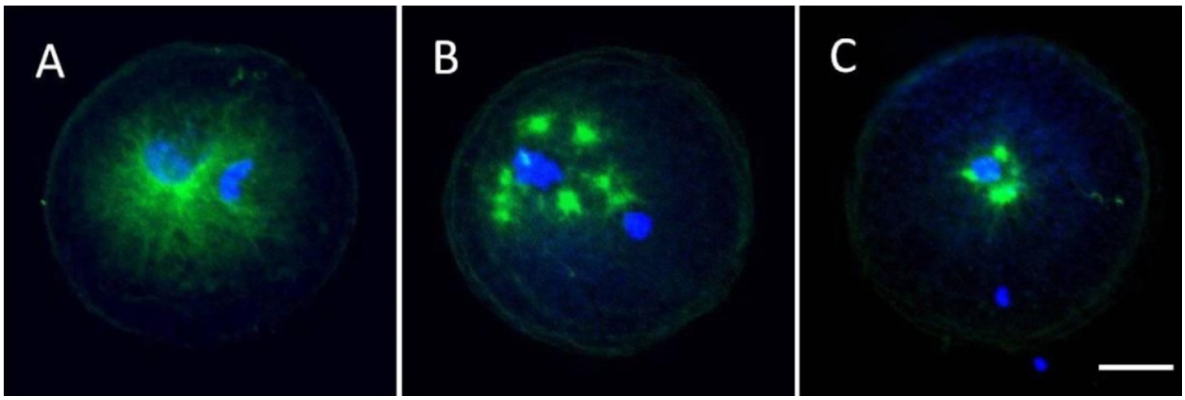

Figure 2-4. (A) A bovine oocyte forming a single sperm aster 10 h post-insemination (hpi). (B) An oocyte forming multiple asters 10 hpi. Note that pronuclear development and migration are not comparable with those in the panel-A. (C) An oocyte with multiple asters 5.5 hpi. These asters were located near the male pronucleus. Green and blue indicate asters and nuclei, respectively. Scale bar = 30 μm.

With regards to pronuclear migration and development, zygotes with multiple asters were retrospectively compared with those with a single aster, as shown in Table 2-4. Distances between male and female pronuclei in the zygotes exhibiting multiple asters (41.7 and 45.1 μm in vitrified and fresh control groups, respectively) were significantly longer than those in the zygotes exhibiting a single aster (30.3 and 27.0 μm in vitrified and fresh control groups, respectively; $P < 0.05$) regardless of vitrification. In addition, areas of both pronuclei in the zygotes exhibiting multiple asters were significantly smaller than those in the zygotes exhibiting a single aster ($P < 0.05$). There were no significant differences in the pronuclear size between vitrified and fresh control groups.

Table 2-4. Migration and development of pronuclei in zygote with a single or multiple asters.

Groups	Aster formation	Distance between pronuclei (μm)	Pronuclear size (μm^2)	
			Male	Female
Fresh control	Single	27.0 ± 3.4 [a]	299.6 ± 18.2 [a]	147.9 ± 6.6 [a]
	Multiple	45.1 ± 4.6 [b]	176.9 ± 26.9 [b]	78.3 ± 5.6 [b]
Vitrified	Single	30.3 ± 3.6 [a]	314.6 ± 45.4 [a]	129.4 ± 19.9 [a]
	Multiple	41.7 ± 3.0 [b]	198.8 ± 22.3 [b]	79.0 ± 6.8 [b]

Number of 2-PN zygotes analyzed corresponds to Table 2-3. Mean ± SEM. [a,b] Within a column, means without a common superscript differed ($P < 0.05$).

Origin of multiple asters (Experiment 3)

Vitrified oocytes were inseminated with a different IVF regimen and multiple asters observed in the presumptive zygotes 5.5 hpi (n = 14) and 8 hpi (n = 24) were analyzed. Mean number of asters per zygote with multiple asters increased from 3.2 ± 0.3 at 5.5 hpi to 5.3 ± 0.6 at 8 hpi. When distances between the center of each aster and the center of male pronucleus were measured, the mean value among the zygotes increased significantly in a time-dependent manner (9.1 ± 1.1 at 5.5 hpi vs 21.7 ± 2.9 at 8 hpi, $P < 0.05$). Because the 5.5-hpi asters were located near the male pronucleus (Figure 2-4C), it was speculated that the multiple asters have not been derived from the cytoplasmic asters of maternal origin.

DISCUSSION

In Experiment 1, cleavage rate of vitrified-warmed bovine oocytes 27 hpi was much lower than that of fresh control oocytes, whereas the difference in cleavage rate at 48 hpi was smaller than that at 27 hpi (Table 2-2). Incidence of delayed cleavage and overall lower cleavage rate in vitrified-warmed oocytes after IVF were also reported in sheep (Succu et al., 2008). Higher potential of oocytes cleaving earlier, compared to those cleaving later, to reach blastocysts (Lonergan et al., 1999; Ward et al., 2001), to survive cryopreservation (Dinnyes et al., 1999) and to achieve pregnancy (Lonergan et al., 1999) have been reported in cattle. Although genetic factor (Warner et

al., 1998), bull individuals for sperm (Ward et al., 2001), chromosomal normality (Yadav et al., 1993), embryonic sex (Avery et al., 1991; Yadav et al., 1993; Lonergan et al., 1999), and culture conditions (Peippo et al., 2001) are involved in the developmental kinetics of embryos, the principle factor that controls the timing of first cleavage remains unclear. Developmental arrest was observed more frequently in vitrified group than fresh control group (Table 2-2), suggesting that oocyte activation by sperm penetration was suboptimal in the vitrified-warmed oocytes. Vitrification induced the reduction of MPF in ovine oocytes before fertilization (Succu et al., 2007), the damage of mitochondria in bovine oocytes (Rho et al., 2002), and of endoplasmic reticulum in mouse oocytes (Lowther et al., 2009). Post-warm oocytes may have been recovered by 2 h culture before IVF in the present study, but the harmful effect of remaining intracellular CPA (EG and/or DMSO) could not be estimated.

In Experiment 2, the influence of oocyte vitrification on aster formation which may be deeply involved in the first cleavage (Kim et al., 1996; Terada et al., 2004), was investigated. Because the normal fertilization rate was comparable between vitrified and fresh control groups (Table 2-3), significant difference in the overall cleavage rate (Table 2-2) was not due to failure of fertilization (Otoi et al., 1997) or polyspermic (abnormal) fertilization (Hochi et al., 1998). A high incidence of oocytes with multiple asters was notable in the vitrified group (Table 2-3, Figure 2-4B). Except for rodents in which multiple cytoplasmic asters function as MTOC (Woolley and Fawcett, 1973; Schatten et al., 1985), each paternal centrosome organizes only a single aster and functions as an MTOC in many mammalian species. Conversely, parthenogenesis (Tremoleda et al., 2003) and taxol treatment to stabilize microtubules (Battaglia et al., 1996; Kim et al., 1996) can induce formation of cytoplasmic asters. Navara et al. (1996) reported the incidence of multiple asters in bovine oocytes after IVF as done in the present study, but no further analysis of these multiple asters was performed. In the bovine zygotes with multiple asters, pronuclear migration and development were disturbed when compared to those with a single aster (Table 2-4), suggesting that multiple asters were not equally functional as a single sperm aster. In addition to the vitrification process, prolonged transportation period (approximately 1 day after slaughter) and storage condition (10–12 °C in saline) of bovine ovaries were potential causes of zygotes with multiple asters.

Experiment 3 was conducted to investigate whether the observed multiple asters were the cytoplasmic asters (maternal origin) or the fragmentation of sperm aster (paternal origin). Based on the comparison of aster positioning between 5.5 and 8 hpi samples, the multiple asters frequently observed in the vitrified oocytes were considered not to be typical cytoplasmic asters of maternal origin reported in rodents oocytes (Woolley and Fawcett, 1973; Schatten et al., 1985), parthenogenetically activated horse oocytes (Tremoleda et al., 2003), and taxol-treated porcine (Kim et al., 1996) and human (Battaglia et al., 1996) oocytes. Because sperm cells lose most of the

pericentriolar materials during spermiogenesis, sperm-derived centrosome can function as MTOC after recruiting centrosomal proteins dispersed in oocytes (Schatten, 1994; Manandhar et al., 2005). Vitrification procedures, including exposure to highly concentrated CPA and ultrarapid cooling in the cryodevice, may adversely affect recruitment of the centrosomal proteins by the sperm centrosome. If participation of oocyte-derived γ-tubulin into MTOC was not completely organized within a limited period after fertilization, multiple asters may be induced near the male pronucleus and be dispersed gradually. A low glutathione concentration reported in vitrified porcine oocytes (Somfai et al., 2007) may also be responsible for multiple aster formation, because a sperm centrosome needs a reducing agent to function (Schatten, 1994). Otherwise, low-quality oocytes may be simply unable to maintain the single sperm aster. Mean number of asters per zygote with multiple asters increased with culture period, suggesting either the time-dependent increase or the difficult counting of overlapping asters.

In conclusion, the present study was apparently the first to document that vitrification of bovine matured oocytes increased formation of multiple sperm asters after IVF, and that the multiple asters contributed to the migration and development of pronuclei to a lesser extent. Thus, formation of multiple asters may be involved in the delayed first cleavage of vitrified-warmed bovine oocytes after IVF and impaired development into blastocysts.

CHAPTER III: RESCUE OF VITRIFIED-WARMED BOVINE OOCYTES BY ROCK INHIBITION

ABSTRACT

Cryotolerance of bovine matured oocytes is not fully practical even though promising vitrification procedure with ultra-rapid cooling rate was applied. The present study was conducted to investigate whether recovery culture of vitrified-warmed bovine oocytes with an inhibitor (Y-27632) of ROCK can improve the developmental potential after IVF and IVC. Immediately after warming, almost all oocytes appeared morphologically normal. Treatment of the post-warm oocytes with 10 μM Y-27632 for 2 h resulted in the significantly higher oocyte survival rate before IVF as well as higher cleavage rate and blastocyst formation rate. Quality analysis of the resultant blastocysts in terms of total cell number and apoptotic cell ratio also showed the positive effect of the Y-27632 treatment. Time-dependent change in mitochondrial activity of the vitrified-warmed oocytes was not influenced by ROCK inhibition during the period of recovery culture. However, the ability of ooplasm to support single-aster formation was improved by the ROCK inhibition. Thus, inhibition of ROCK activity in vitrified-warmed bovine oocytes during a short-term recovery culture can lead to higher developmental competence, probably due to decreased apoptosis and normalized function of MTOC.

INTRODUCTION

Successful pregnancies or birth of offspring derived from frozen-thawed oocytes have been reported in several mammalian species over the last few decades (Wilmut and Rowson, 1973; Hamano et al., 1992; Saragusty and Arav, 2011), with relatively low developmental rates. Application of vitrification, instead of the conventional two-step freezing, improved the efficacy of oocyte cryopreservation, especially in mice (Rall and Fahy, 1985; Rall, 1987; Rall et al., 1987) and humans (Hunter et al., 1991; Hunter, et al. 1995; Hong et al., 1999); thus, oocyte cryopreservation has become an important tool for gamete banking and assisted reproductive technology. Various

cryodevices, such as open-pulled straws (Vajta et al., 1998), cryoloop (Lane et al., 1999), and cryotop (Chian et al., 2004), have been developed to accelerate the cooling rate. However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement (Hara et al., 2005; Ambruosi et al., 2009; Hao et al., 2009). Proposed reasons for the high sensitivity of oocytes to cryopreservation include the large cell size and low permeability of water and CPA (Saragusty and Arav, 2011). Depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome misalignment (Coticchio et al., 2009). Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae (Larman et al., 2006; Kohaya et al., 2011). Recently, we have proposed a hypothesis for cryodamage of bovine oocytes that multiple aster formation frequently observed in vitrified-warmed and fertilized oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly (Hara et al., 2012), as described in CHAPTER II (Figure 3-1).

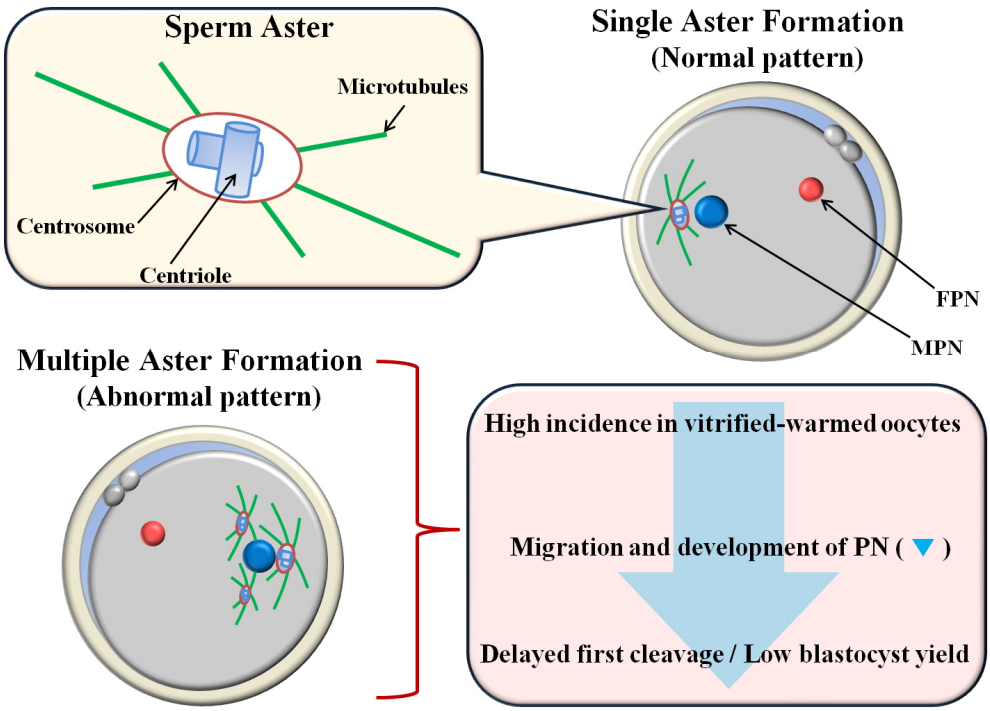

Figure 3-1. A new hypothesis for cryodamage of bovine mature oocytes. Multiple-aster formation is a possible cause of delayed first cleavage and the subsequent low yield of transferable blastocysts.

Increased apoptosis of vitrified-warmed oocytes resulted in reduction of developmental competence (Morato et al., 2010; Li et al., 2012). The ROCK, which belonged to the AGC (PKA, PKG, and PKC) family of serine-threonine kinases, was discovered as a downstream target of the small GTP-binding protein Rho (Matsui et al., 1996), which can regulate cellular growth, adhesion, migration, metabolism, and apoptosis through controlling the actin-cytoskeletal assembly and cell contraction (Riento and Ridley, 2003), as shown in Figure 3-2. The ROCK is comprised of a catalytic domain at the N-terminal, followed by a coiled-coil domain for the Rho protein binding, and a Pleckstrin-homology domain. Target proteins for phosphorylation by ROCK include the regulatory myosin light chain (MLC; Riento and Ridley, 2003) and the Lin11, Isl-1 and Mec-3 (LIM) kinase-1 and kinase-2 (Ott et al., 2007). Inhibition of the ROCK activity was involved in reduction of apoptosis in embryonic stem cell-derived neural cells (Koyanagi et al., 2008). Inhibition of the ROCK activity was also effective to improve the plating efficiency of dissociated human pluripotent stem cells after cryopreservation (Watanabe et al., 2007; Claassen et al., 2009; Li et al., 2009; Gauthaman et al., 2010a; Gauthaman et al., 2010b) and the revivability of in vitro-produced bovine blastocysts after vitrification and warming (Hochi et al., 2010).

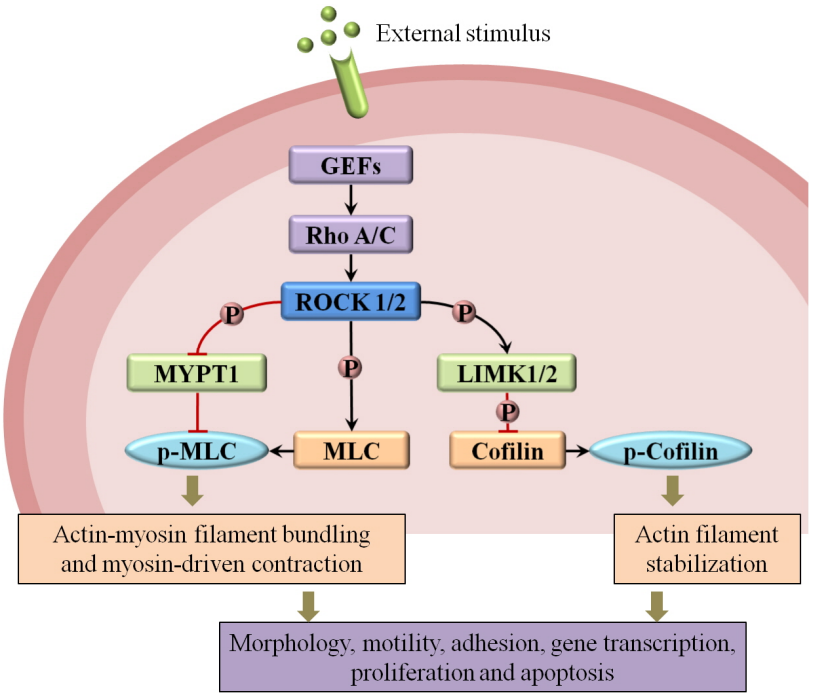

Figure 3-2. Rho/ROCK signaling pathway. GEFs: guanine exchange factors. LIM: Lin11, Isl-1 and Mec-3. MLC: myosin light chain. MYPT: myosin phosphatase targeting protein. Phosphorylation of MLC or Confilin directly or indirectly influences various cellular behaviours (Rath and Olson, 2012).

The present study was designed to investigate whether short-term treatment of vitrified-warmed bovine oocytes with ROCK inhibitor can improve the survival rate and the subsequent developmental competence after IVF. In addition, the mitochondrial activity during the short-term culture with ROCK inhibitor and the function of MTOC after IVF were investigated.

MATERIALS AND METHODS

Experimental design

Vitrified-warmed oocytes with morphologically normal appearance were randomly allocated to recovery culture with or without ROCK inhibitor. Fresh matured oocytes were served as controls. Morphological survival after the recovery culture, cleavage and blastocyst formation after IVF, and cell construction of the resultant blastocysts were investigated in the first series of experiments (Experiment 1). Time-dependent change in activity of mitochondria during the recovery culture and the potential of oocytes to support single-aster formation after IVF were investigated in the second series of experiments (Experiment 2).

In vitro maturation

Abattoir-derived bovine ovaries were transported to the laboratory in cold saline (maintained at 10-12 °C) within 24 h after slaughter. The contents of follicles (diameter, 2-8 mm) were aspirated with an 18-G needle connected to a 10-ml syringe. Oocytes surrounded with at least two layers of compact cumulus cells were matured in 100-μl microdrops of Hepes-buffered TCM-199 supplemented with 10 % FBS, 0.2 mM sodium pyruvate, 0.02 AU/ml FSH, 1 μg/ml 17 β-estradiol, and 50 μg/ml gentamycin sulfate for 22 h at 38.5 °C under 5 % CO_2 in air (10-12 oocytes per microdrop). Then, cumulus cells were removed by vortex-mixing for 3 min in the Hepes-buffered TCM-199 supplemented with 3 mg/ml BSA, 0.2 mM sodium pyruvate, 1,000 IU/ml hyaluronidase, and 50 μg/ml gentamycin sulfate. Oocytes were comprehensively checked for their extrusion of the first polar body, and the oocytes with an extruded first polar body were defined as matured and used for further experiments.

Vitrification and warming

Matured oocytes were subjected to a vitrification procedure according to the method described previously by Tsujioka et al., with minor modifications. Briefly, oocytes were equilibrated with 7.5 % EG and 7.5 % DMSO in Hepes-buffered TCM-199/20 % (v/v) FBS base medium for 3 min at

room temperature (23 ± 2 °C), and then transferred into a vitrification solution consisting of 15 % EG, 15 % DMSO and 0.5 M sucrose in the base medium for approximately 60 sec at room temperature (23 ± 2 °C). Within this 60-sec, up to 15 oocytes were loaded onto the polypropylene strip of a Cryotop with a minimal amount of the vitrification solution (less than 0.1 µl), and then quickly plunged into LN_2.

After storage for more than 1 week in the LN_2, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 ml of the base medium containing 1 M sucrose at 38.5 °C for 1 min. The oocytes were transferred to the base medium at room temperature in a stepwise manner (0.5, 0.25, and 0 M of sucrose for 3, 5, and 5 min, respectively). According to the manufacture's instruction, the predicted cooling and warming rates of the Cryotop procedure are 23,000 and 42,000 °C/min, respectively.

Recovery culture with or without ROCK inhibitor

Post-warm oocytes were cultured in 100-µl microdrops of Hepes-buffered TCM-199 plus 5 % FBS, 0.2 mM sodium pyruvate and 50 µg/ml gentamycin sulfate for 2 h at 38.5 °C under 5 % CO_2 in air (15-30 oocytes per microdrop). The culture medium was supplemented with or without an inhibitor of ROCK, Y-27632 [(R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane-carboxamide dihydrochloride • monohydrate ($C_{14}H_{21}N_3O$•2HCl•H_2O, MW=338.27)], to yield a final concentration of 10 µM.

Fertilization and culture in vitro

Commercially available frozen semen of a Japanese Black bull was used for IVF. After thawing in a water bath at 37 °C for 30 sec, the contents of a 0.5 ml straw was layered on the top of Percoll density gradient consisting of 2 ml of 45 % Percoll above 2 ml of 90 % Percoll in a 15 ml conical tube, and centrifuged for 20 min at 700 *g*. The sperm pellet was re-suspended in 4 ml of mBO medium supplemented with 5 mM theophylline, washed twice (5 min at 300 *g* each) and then re-suspended in the mBO medium supplemented with 5 mg/ml BSA and 10 µg/ml heparin (IVF medium) to yield a concentration of 4 x 10^7 sperm cells/ml. Ten to 12 oocytes in the IVF medium were co-incubated with the above-mentioned sperm suspension at a final concentration of 8 x 10^6 sperm cells/ml for 6 h in a 100-µl microdrop under mineral oil at 38.5 °C under 5 % CO_2 in air.

Up to 30 presumptive zygotes (6 hpi) were cultured in a 250-µl microdrop of mSOF (Holm et al., 1999), supplemented with 30 µl/ml essential amino acids solution, 10 µl/ml non-essential amino acids solution and 5 % FBS at 39.0 °C under 5 % CO_2, 5 % O_2 and 90 % N_2. The cleavage rate was determined on Day-2 (Day-0 = Day of IVF) and appearance of expanded blastocysts was recorded on Day-7 and -8.

In an additional experiment, the mean time until the first cleavage was determined by observation every 6 h of post-warm oocytes treated with or without Y-27632 and fertilized in vitro (from 18 to 48 hpi). The time of the first cleavage in each zygote was defined as the point when the cleavage was initially observed.

Quality analysis of blastocysts

Fully expanded blastocysts harvested on Day-8 (n=20 per each group) were analyzed for apoptotic cell ratio and total cell number by TdT-mediated dUTP nick end labeling (TUNEL) assay and Hoechst staining, respectively. According to the manufacture's manual for In Situ Cell Death Detection Kit (TMR Red, Roche Diagnostics, Mannheim, Germany), blastocysts were washed three times in phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 % PVP and fixed in 4 % paraformaldehyde for 24 h at 4 °C. Membranes of the blastocysts were permeabilized with 0.5 % (v/v) Triton X-100 for 30 min at room temperature. Broken DNA ends were labeled with terminal deoxyribonucleotidyl transferase and fluorecein-dUTP for 1 h at 38.5 °C in the dark. The blastocysts were counterstained with 10 μg/ml Hoechst-33342 for 15 min at room temperature in the dark to determine the total cell number of the blastocyst (Figure 3-3). Then, the blastocysts were washed at least three times in PBS and mounted onto slides with mounting solution (Mount-Quick, Daido Sankyo, Tokyo, Japan). The apoptotic cell number and total cell number in each blastocyst were counted under an epifluorescence microscopy (Nikon, Tokyo, Japan).

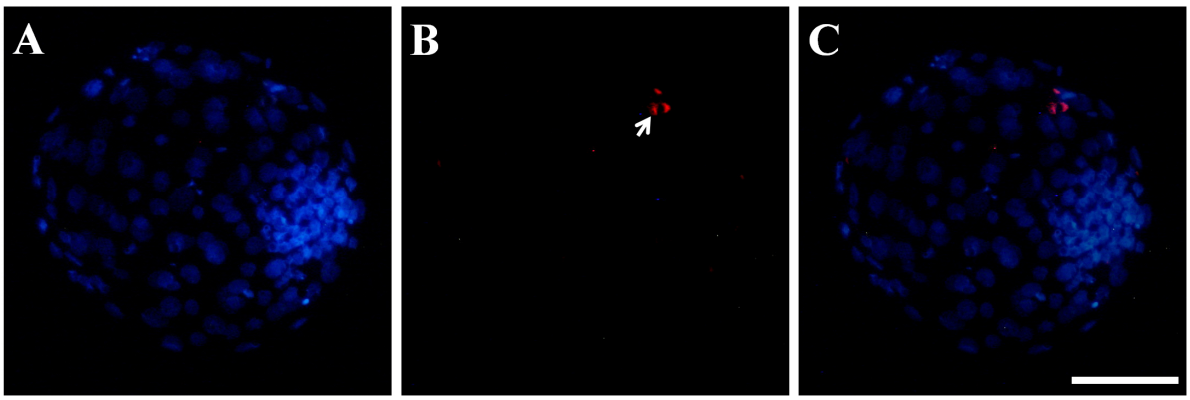

Figure 3-3. Fluorescent images by Hoechst staining and TUNEL assay. (A) Total cells in Day-8 blastocyst with blue signals. (B) Apoptotic cells with red signals (arrow). (C) Merged image. Scale bar represents 100 μm.

Mitochondrial activity during recovery culture

Vitrified-warmed oocytes were harvested at 0, 30, 60 and 120 min of the recovery culture to determine the changes of mitochondrial activity. The oocytes were washed three times with PBS and fixed in 4 % paraformaldehyde suspended in PBS for 15 min at room temperature. The oocytes were incubated for 15 min with 0.1 μg/ml MitoTracker Red CMXRos (Lonza Walkerscille, MD, USA) suspended in PBS under a dark condition. Then, the mitochondria-labeled oocytes were rinsed three times with PBS and mounted on the glass bottom dish (SPL, Seoul, Korea) as a micro-droplet under mineral oil. Each oocyte was observed under a confocal laser scanning microscope, and 10 sections per oocyte were captured and stacked (Figure 3-4). The fluorescence intensity of each oocyte was measured using ImageJ analysis software. In each of the total 4 replicates, the average intensities of 3 oocytes per group were calculated. The value in the fresh control group was defined as 1.0, and the relative values were given for the vitrified-warmed groups.

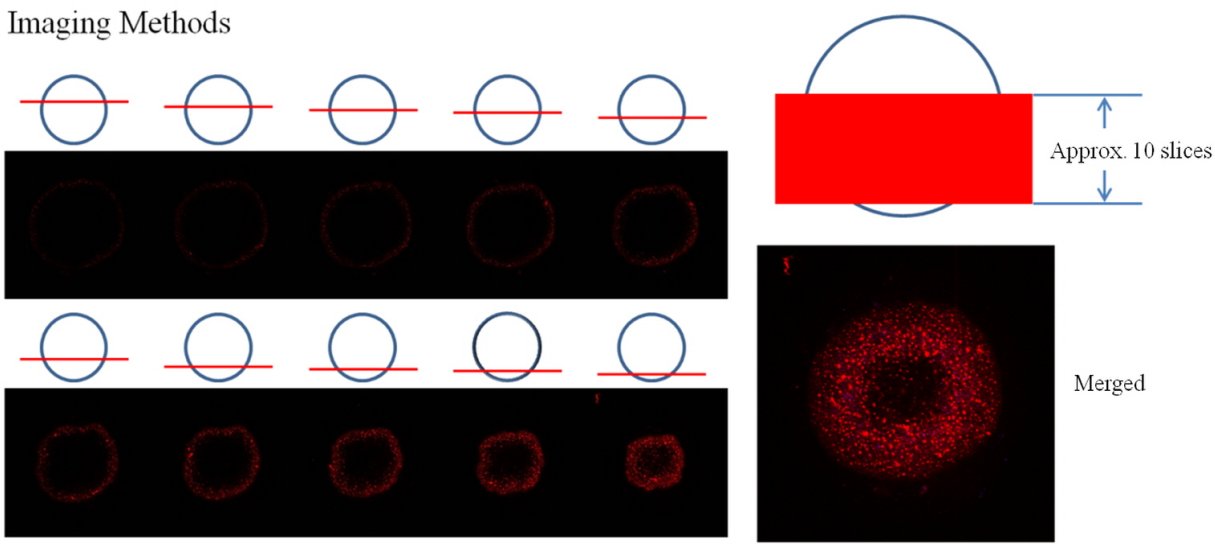

Figure 3-4. Confocal laser scanning microscopy of mitochondrial activity. Approximately 10 slices from each oocyte were taken with confocal laser scanning microscope.

Function of microtubule-organizing center

Presumptive zygotes after IVF (6 hpi) were cultured for an additional 4 h in the Hepes-buffered TCM-199 plus 5 % FBS at 38.5 °C under 5 % CO_2 in air, and then immunostained as described previously (Hara et al., 2012). Briefly, the zygotes at 10 hpi were extracted for 15 min by buffer M (25 % glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) containing 5 % (v/v) methanol and 1 % (v/v) Triton X-100, after zonae

pellucidae had been removed by 0.75 % protease in M2 medium (Quinn and Kerin, 1986). The zygotes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS containing 0.1 % Triton X-100. Microtubules were labeled with a monoclonal antibody against α-tubulin (diluted 1:500). The primary antibodies were detected by FITC-conjugated goat anti-mouse IgG (diluted 1:200). Nuclear DNA was visualized by counterstaining by 2.5 μg/ml DAPI. All samples were mounted onto slides with anti-fade agent, and digital images were taken at 2 μm thickness using a confocal laser scanning microscope. The digital images were stacked and assessed with ImageJ software. Zygotes with 2-PN were defined as those fertilized normally.

Statistical analysis

Experiments were replicated at least 4 times in each group. All the data were analyzed with Origin 8 software (OriginLab Corporation, Northhampton, MA, USA) by applying one-way analysis of variance with Turkey honest significance test. A value of $P < 0.05$ was considered to be a significant difference.

RESULTS

Blastocyst yield and quality (Experiment 1)

All the 371 vitrified-warmed oocytes were harvested and 368 oocytes among them (99.2 %) appeared intact. After recovery culture for 2 h, survival rate of post-warm oocytes cultured with Y-27632 (97.8 %, 181/185) was significantly higher than that of those cultured without Y-27632 (90.2 %, 165/183, $P < 0.05$).

The cleavage rate of post-warm oocytes treated with Y-27632 (72.4 %) was significantly higher than that of those treated without Y-27632 (56.2 %, $P < 0.05$), but comparable to that of non-treated fresh control oocytes (71.4 %), as shown in Table 3-1. Based on a separate series of experiment (three replicates), the mean time until the first cleavage was found almost comparable between the post-warm oocytes treated with Y-27632 (31.3 ± 1.3 hpi, number of cleaved zygotes = 66) and without Y-27632 (32.6 ± 1.6 hpi, number of cleaved zygotes = 55). However, there was a likelihood of the earlier cleaving in the Y-27632-treated post-warm oocytes when compared to the counterpart (Figure 3-5). On Day-8, blastocyst yield from post-warm oocytes treated with Y-27632 (21.4 %) was significantly higher than that of those treated without Y-27632 (13.9 %, $P < 0.05$), but significantly lower than that of fresh control oocytes (34.4 %, $P < 0.05$). No difference was found in the proportion of Day-7 blastocysts versus Day-8 blastocysts among the three groups, ranging from 2.8 to 3.7.

Table 3-1. Effect of ROCK inhibition on developmental competence of vitrified-warmed bovine oocytes.

Groups	Y-27632	No. (%) of oocytes			
			Cleaved on	Developed to blastocysts on	
		Inseminated	Day 2	Day 7	Day 7 + 8
Vitrified-warmed	+	154	112 (72.4 ± 2.4) [a]	26 (16.9 ± 2.0) [b]	33 (21.4 ± 1.6) [b]
Vitrified-warmed	-	162	91 (56.2 ± 2.9) [b]	17 (10.1 ± 1.3) [b]	23 (13.9 ± 1.2) [c]
Fresh control		117	85 (71.4 ± 3.1) [a]	31 (26.7 ± 2.7) [a]	40 (34.4 ± 1.8) [a]

Percentages were expressed as mean ± SEM of six replicates in each group.

[a-c] Different superscripts denote significant differences within columns ($P < 0.05$).

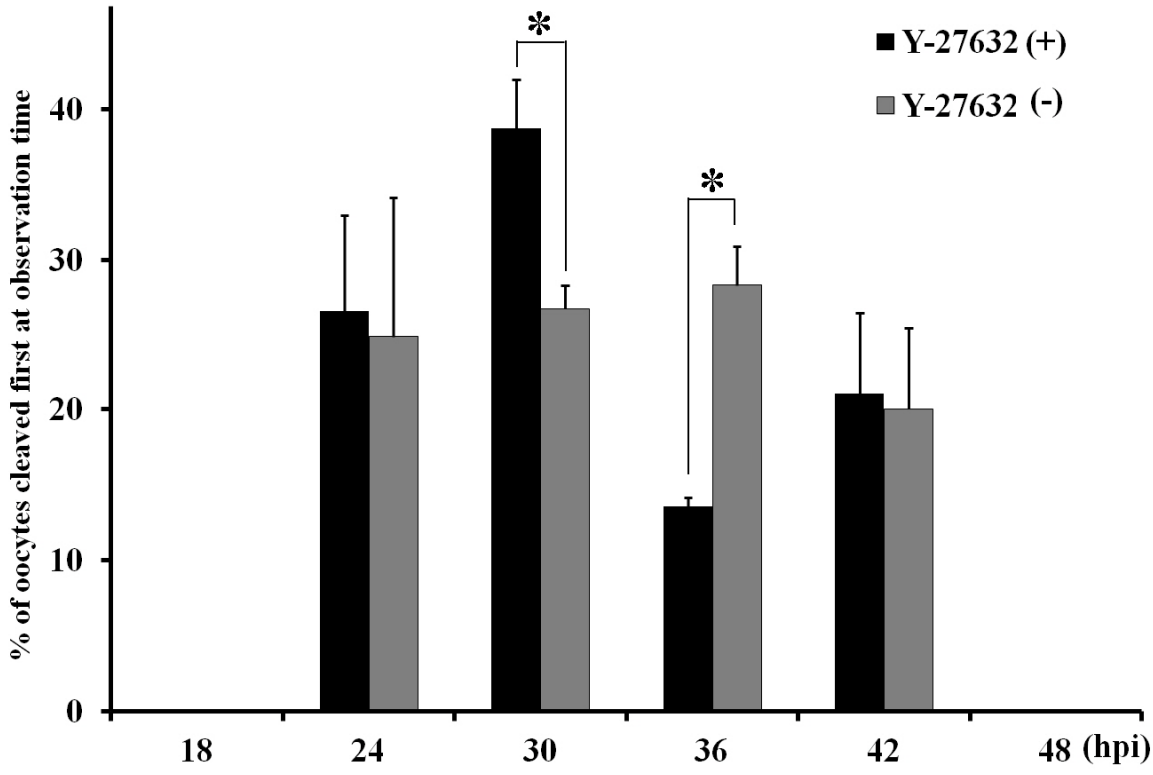

Figure 3-5. Histograms showing the timing of first cleavage in bovine oocytes cultured with or without Y-27632 after vitrification and warming. Proportions were calculated, based on the number of cleaved oocytes in each of 3 replicates. Mean + SD. * $P < 0.05$.

Quality analysis of expanded blastocysts harvested on Day-8 by Hoechst staining showed that mean total cell number of blastocysts in the group cultured with Y-27632 (124.6 cells) was significantly higher than that in the group cultured without Y-27632 (97.5 cells, $P < 0.05$) but comparable to that in fresh control group (135.7 cells), as shown in Figure 3-6A. TUNEL assay also showed that the proportion of apoptotic cells per blastocyst in the group cultured without Y-27632 (4.0 %) was significantly higher than those in the group cultured with Y-27632 (2.2 %) and fresh control group (1.8 %), as shown in Figure 3-6B.

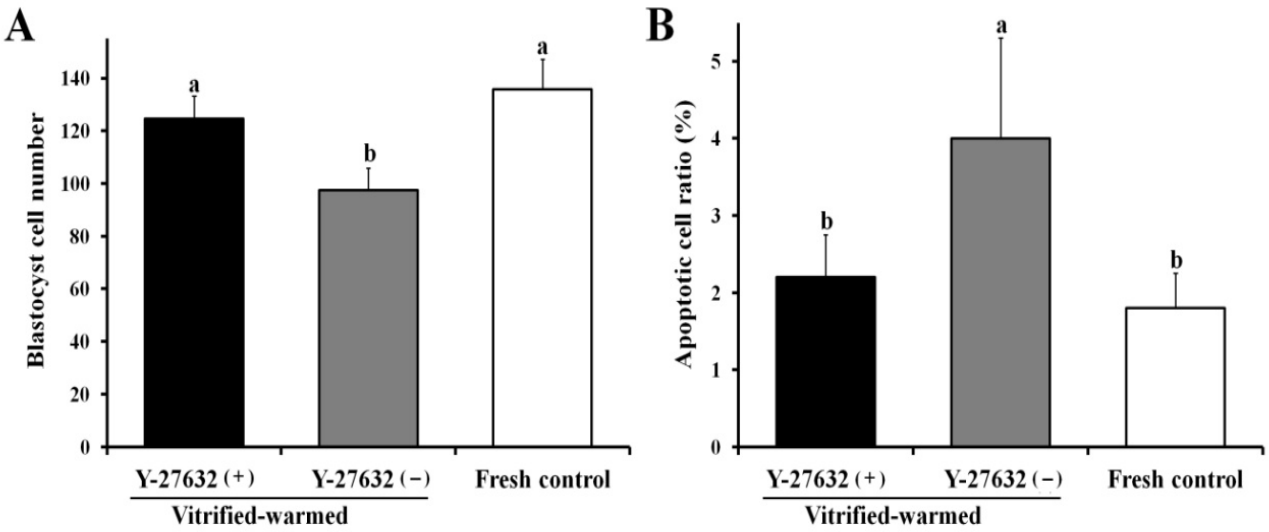

Figure 3-6. Total cell number (A) and apoptotic cell ratio (B) in Day-8 expanded blastocysts derived from vitrified-warmed bovine oocytes. Mean + SD. ^{a,b} $P < 0.05$.

Mitochondrial activity and MTOC function (Experiment 2)

Mitochondrial activity of vitrified-warmed oocytes regained to the original (non-vitrified control) level in a time-dependent manner during the recovery culture for 2 h, as shown in Figures 3-7 and 3-8. Both time course and extent in the recovery of mitochondria activity were not different between post-warm oocytes cultured with or without Y-27632.

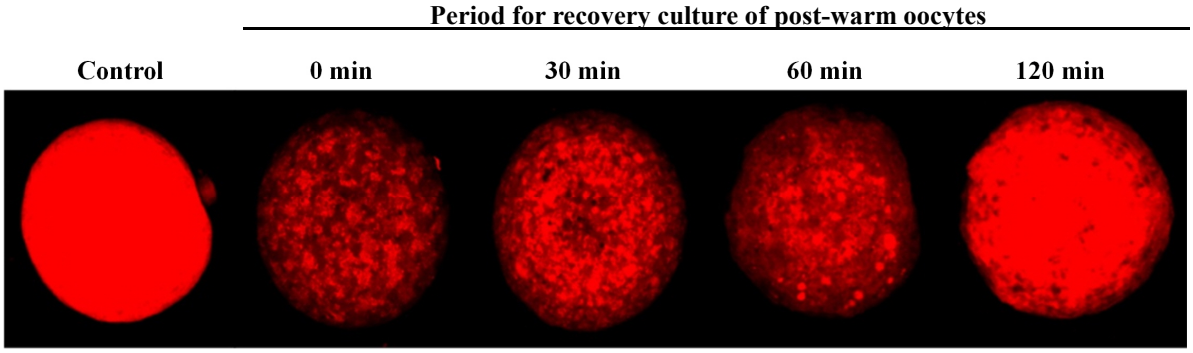

Figure 3-7. Fluorescent images of mitochondria in vitrified-warmed oocytes during recovery culture. The red signal indicates mitochondrial activity by its intensity**.**

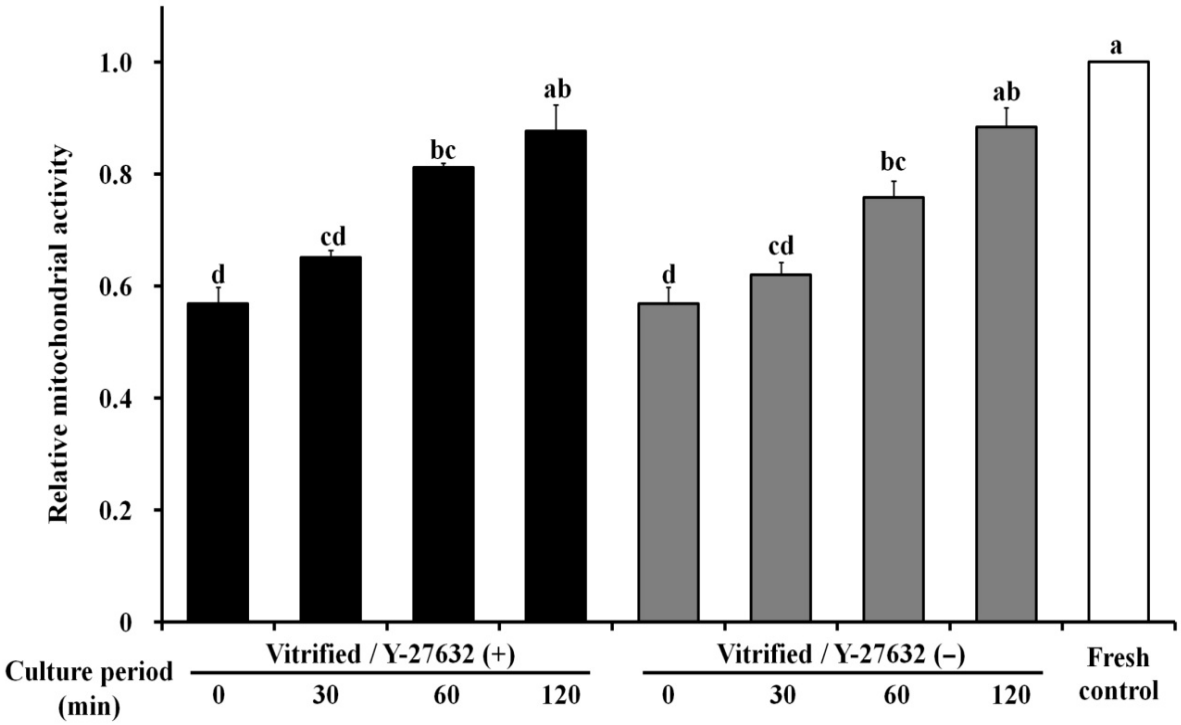

Figure 3-8. Time-dependent change of mitochondrial activity in vitrified-warmed bovine oocytes during 2 h of recovery culture. Mean + SD. $^{a-d}$ $P < 0.05$.

At 10 hpi, proportions of oocytes fertilized normally with 2-PN (73.3-92.6 %) were statistically comparable among the three groups, as shown in Table 3-2. Regardless of vitrification, proportions of zygotes formed aster(s) were also comparable among the groups (80.7-83.3 %). A higher incidence of multiple aster formation in vitrified-warmed oocytes (50.6 versus 30.2 % in fresh

control group, $P < 0.05$) was observed when the oocytes were not treated with Y-27632 during the recovery culture (Table 3-2, Figure 3-9A). However, the multiple aster formation in vitrified-warmed oocytes was inhibited when the oocytes were treated with Y-27632 in the post-warm culture (32.9 %). Mean aster number per zygote exhibiting multiple asters was 2.5 ± 0.2, 2.6 ± 0.1 and 2.5 ± 0.2 in vitrified/Y-27632-treated, vitrified/non-treated and fresh control groups, respectively. Inversely, the single aster formation rate of vitrified-warmed oocytes in the Y-27632 treated group (67.1 %, Figure 3-9B) was similar to that of fresh control oocytes (69.8 %).

Table 3-2. Effect of ROCK inhibition on aster formation of vitrified-warmed bovine oocytes 10 h after IVF.

Groups	Y-27632	No. of oocytes Evaluated	No. (%) of 2-PN zygotes	No. (%) of aster-formed zygotes		
				Total	Single-asters	Multiple-asters
Vitrified-warmed	+	62	50 (78.4 ± 5.4)	40 (80.7 ± 3.2)	26 (67.1 ± 2.8) [a]	14 (32.9 ± 2.8) [b]
Vitrified-warmed	-	58	42 (73.3 ± 6.1)	35 (81.8 ± 4.4)	17 (49.4 ± 2.7) [b]	18 (50.6 ± 2.7) [a]
Fresh control		57	52 (92.6 ± 3.5)	43 (83.3 ± 4.3)	30 (69.8 ± 4.0) [a]	13 (30.2 ± 4.0) [b]

Percentages were expressed as mean ± SEM of five replicates in each group. [a,b] Different superscripts denote significant differences within columns ($P < 0.05$).

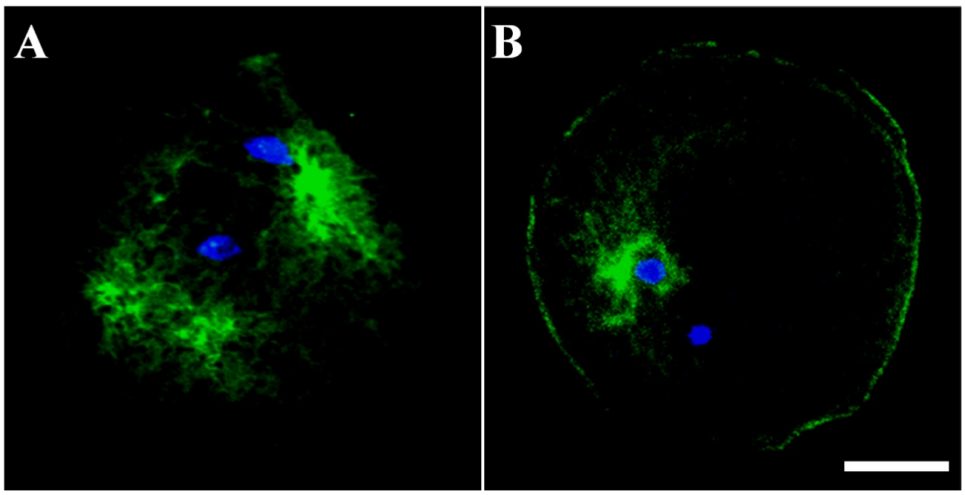

Figure 3-9. Fluorescent images of vitrified-warmed and in vitro-fertilized bovine oocytes after immunostaining against α-tubulin and nuclear staining with DAPI. (A) 2-PN zygote with multiple asters. (B) 2-PN zygote with single aster. . Green and blue indicate asters and nuclei, respectively. Scale bar represents 25 μm.

DISCUSSION

Inhibition of ROCK by a short-term treatment with Y-27632 improved both morphological survival of vitrified-warmed bovine oocytes prior to IVF (97.8 vs. 90.2 %) and apoptotic cell ratio in the Day-8 blastocysts after oocyte vitrification and IVF (2.2 vs. 4.0 %; Figure 3-6B). The ROCK pathway is closely related to induction of cell apoptosis (Coleman et al., 2001; Coleman and Olson, 2002; Chang et al., 2006). Under a stressful situation in which caspase activity is increased, caspase-mediated cleavage and consequent activation of ROCK1 may trigger to accelerate the apoptosis process (Chang et al., 2006; Ark et al., 2010). The ROCK inhibitor, Y-27632, has been used to improve the cryosurvival of human neural stem cells (Kuleshova et al., 2009), human embryonic stem cells (Li et al., 2008; Li et al., 2009), and in vitro-produced bovine blastocysts (Hochi et al., 2010), whereas Bueno et al. (2010) reported the negative effect of Y-27632 on expansion and survival rate of fresh and cryopreserved hematopoietic stem cells. The Y-27632 does not act directly as a protector for anoikis (dissociation-induced apoptosis; Krawetz et al., 2009). An alternative mechanism for ROCK-dependent cell death has been proposed in human embryonic stem cell research. It was demonstrated that actin-myosin contraction is a major mechanism promoting the death of cells (Chen et al., 2010), and that the ROCK-dependent hyperactivation of myosin is involved in the apoptosis of cells (Ohgushi et al., 2010).

Improved developmental competence of vitrified-warmed bovine oocytes by a short-term culture with Y-27632, the main finding of the present study, may be explained by prompt recovery of ooplasmic function to support the formation of single aster (Table 3-2) and the earlier first cleavage (Figure 3-5). Mitochondria in the eukaryotic cells play many roles, including ATP production, redox, calcium homeostasis, and apoptosis (Dumollard et al., 2007). However, the recovery of ooplasm to support the MTOC function did not match with the time-dependent kinetics of mitochondrial activity (Figures 3-7 and 3-8). Except for a subset of rodents in which multiple cytoplasmic asters function as MTOC (Kim et al., 1996; Kim et al., 1998), paternal centrosome organizes only a single sperm aster and functions as an MTOC in many mammalian species including humans and bovine species. Higher incidence of multiple aster formation was observed in vitrified-warmed bovine oocytes after IVF, and pronuclear development and migration were delayed in the zygotes with multiple aster formation (Hara et al., 2012). Very recently, we failed to inhibit the occurrence of multiple aster formation in bovine oocytes by increasing intracellular glutathione level before vitrification and IVF (Hara et al., 2013). Since the ROCK regulates microtubule acetylation via phosphorylation of the tubulin polymerization promoting protein 1, inhibition of the ROCK activity resulted in increased cellular microtubule acetylation (Schofield et al., 2012; Schofield et al., 2013). Molecular mechanisms by which the ROCK inhibition improves the tolerance of bovine oocytes after

vitrification and warming remain to be clarified and need further research.

Some attempts have been made to improve the developmental competence of vitrified-warmed oocytes using chemical reagents. Cyclosporine, an immune suppression reagent, was applied to maintain the potential of mitochondria (ATP contents) and to decrease the level of reactive oxygen species in bovine oocytes, resulting in an improved yield of parthenogenetic blastocysts after oocyte vitrification (Zhao et al., 2011). Pretreatment of bovine oocytes with Taxol (paclitaxel), a mitotic inhibitor, had beneficial effect on early embryonic cleavage of post-warm oocytes through stabilization of spindle configuration (Morato et al., 2008). Recently, the supplementation of L-carnitine, a quaternary ammonium compound, into maturation medium improved the cryotolerance of bovine oocytes through redistribution of lipid droplets and no reduction of ATP contents (Chankitisakul et al., 2013). For mouse oocytes, antifreeze protein-III supplemented directly into vitrification solution resulted in an improved developmental competence through preserving spindle-forming ability and membrane integrity (Jo et al., 2012). On the other hand, the Y-27632 employed in the present study has been used for a short-term treatment after (not before and/or during) vitrification and warming procedure.

In conclusion, we have demonstrated, to our knowledge for the first time the beneficial effects of ROCK inhibitor on developmental competence of vitrified-warmed bovine oocytes. The improved blastocyst yield from vitrified-warmed oocytes may be due to decreased apoptosis and normalized function of MTOC.

CHAPTER IV: CONCLUSIVE SUMMARY

Successful pregnancies or birth of offspring derived from frozen-thawed oocytes have been reported in several mammalian species a few decades ago, with relatively low developmental rates. Application of vitrification, instead of the conventional two-step freezing, improved the efficacy of oocyte cryopreservation, especially in mice and humans. Various cryodevices have been developed to accelerate the cooling rate. However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement. During the vitrification procedures, oocytes can be damaged because of their large cell size and low permeability of water and CPA. Moreover, depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome misalignment. Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae. The objective of the present study was to investigate the effect of vitrification and warming on cryoinjuries of bovine MII oocytes, and to improve their revivability in terms of production of transferable high quality blastocysts by IVF and IVC.

A centrosome is composed of a pair of centrioles surrounded by the pericentriolar materials, such as γ-tubulin, centrin and pericentrin, and acts as the MTOC. In cattle, a sperm brings a centrosome into an oocyte during fertilization and a single sperm aster is formed by polymerization of α- and β-tubulin. The microtubule network plays a key role in the migration of male and female pronuclei to the center of a zygote and the subsequent fusion and mitotic cleavage. Additionally, timing of first cleavage in IVF derived bovine oocytes is important for yield and quality of blastocysts, as oocytes cleaving earlier are more likely to become blastocysts, and the resulting blastocysts have higher cryosurvival potential and higher pregnancy rates than those cleaving later. Thus, developmental kinetics can be used as a proxy of embryo quality. Therefore, profiles of cleavage and blastocyst development were first examined for vitrified bovine oocytes, and then function of MTOC/aster(s) in the vitrified oocytes after IVF was analyzed (CHAPTER II). The oocytes cleaved early can be developed to blastocysts at a higher rate than the oocytes cleaved later. Immunostaining for α-tubulin indicated that proportions of zygotes exhibiting aster formation were comparable between vitrified and fresh control groups. However interestingly, relative ratio of zygotes with a single aster vs multiple asters was significantly different between the two groups. Incidence of multiple aster formation in zygotes derived from vitrified oocytes was more than double that in zygotes derived from fresh control oocytes. Thus, we have proposed a new hypothesis for cryodamage of bovine oocytes that multiple aster formation frequently observed in vitrified-

warmed and fertilized oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly from the sperm-aster.

Increased apoptosis of vitrified-warmed oocytes resulted in reduction of developmental competence. The ROCK was discovered as a downstream target of the small GTP-binding protein Rho, which can regulate cellular growth, adhesion, migration, metabolism, and apoptosis through controlling the actin-cytoskeletal assembly and cell contraction. From stem cell researchers, it has been reported that inhibition of the ROCK activity was involved in reduction of apoptosis in embryonic stem cell-derived neural cells, and that inhibition of the ROCK activity was effective to improve the plating efficiency of dissociated human pluripotent stem cells after cryopreservation. Hochi et al. (2010) found that supplementation of ROCK inhibitor (Y-27632) to post-thaw culture medium for 48 h also significantly improved the revivability of in vitro-produced bovine blastocysts after vitrification and warming. Therefore, it was investigated whether short-term treatment of vitrified-warmed bovine oocytes with the ROCK inhibitor can improve the survival rate and the subsequent developmental competence after IVF (CHAPTER III). Treatment of the post-warm oocytes with 10 μM Y-27632 for 2 h resulted in the significantly higher oocyte survival rate prior to the IVF, cleavage rate and blastocyst formation rate. Quality analysis of the resultant blastocysts in terms of total cell number and apoptotic cell ratio also showed the positive effect of the Y-27632 treatment. Time-dependent change in mitochondrial activity of the vitrified-warmed oocytes was not influenced by ROCK inhibition during the period of recovery culture. However, the ability of ooplasm to support single-aster formation was improved by the ROCK inhibition. Thus, inhibition of ROCK activity in vitrified-warmed bovine oocytes during a short-term recovery culture can lead to the higher developmental competence, probably due to decreased apoptosis and normalized function of MTOC.

In conclusion, vitrification of bovine matured oocytes increased the formation of multiple sperm asters after IVF which less contributed to the migration and development of pronuclei. This is a new hypothesis for injuries induced in the vitrified-warmed bovine oocytes. Interestingly, inhibition of ROCK activity during a short-term recovery culture prior to IVF had a beneficial effect on the developmental competence (both blastocyst yield and quality) of the vitrified-warmed bovine oocytes. These results would push forward the cryo-banking of unfertilized oocytes in the bovine species.

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LIST OF PUBLICATIONS

Original articles, directly related to this thesis

(1) Hara H, **IS Hwang**, N Kagawa, M Kuwayama, M Hirabayashi, and S. Hochi (2012) High incidence of multiple aster formation in vitrified-warmed bovine oocytes after in vitro fertilization. *Theriogenology* 77 (5): 908-915.

(2) **Hwang IS**, H Hara, HJ Chung, M Hirabayashi, and S. Hochi (2013) Rescue of vitrified-warmed bovine oocytes with Rho-associated coiled-coil kinase inhibitor. *Biol Reprod* 89 (2): 26 1-6.

Reference articles

(1) Lee HG, HC Lee, HJ Chung, **IS Hwang**, MS Choi, SJ Byun, MJ Kim, JS Woo, WK Chang, P Lee, HT Lee, and JK Park (2008) Study on the reproductive function in transgenic pig harboring human erythropoietin (EPO) gene. *Reprod Dev Biol* 32: 117-121.

(2) Lee HG, HC Lee, SW Kim, P Lee, HJ Chung, YK Lee, JH Han, **IS Hwang**, JI Yoo, YK Kim, HT Kim, HT Lee, WK Chang, and JK Park (2009) Production of recombinant human von Willebrand factor in the milk of transgenic pigs. *J Reprod Dev* 55: 484-490.

(3) Byun SJ, SW Kim, KW Kim, JS Kim, **IS Hwang**, HK Chung, IS Kan, IS Jeon, WK Chang, SB Park, and JG. Yoo (2011) Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. *Biosci Biotech Biochem* 75: 646-649.

(4) **Hwang IS**, SW Kim, SJ Byun, KW Kim, HK Chung, JJ Park, K Gobianand, JK Park, and JG Yoo (2012) The effect of horse serum on in vitro development of porcine parthenogenetic embryos. *Reprod Biol* 12: 25-39.

(5) Choi MS, MR Shim, MY Oh, KW Kim, HC Lee, BC Yang, HK Chung, JH Kim, HT Lee, **IS Hwang**, S Hochi, YT Heo, NH Kim, SJ Uhm, JK Park, WK Chang, and HJ Chung (2012) Proteins associated with reproductive disorders in testes of human erythropoietin gene-harboring transgenic boars. *Theriogenology* 78: 1020-1029.

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