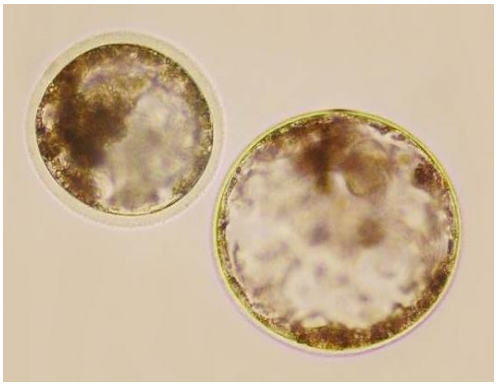
Doctoral Dissertation (Shinshu University)

Establishment of Blastocyst Production System with Freeze-dried Bull Spermatozoa



A Thesis

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Cover page photographs:

(Left) Bovine blastocysts derived from ICSI using freeze-dried spermatozoa.

(Right) Freeze-dried cake of bull sperm suspension and programmable freeze-dryer.

Contents

Abbreviations

AI	artificial insemination
ART	assisted reproductive technologies
β-ME	beta-mercaptoethanol
BSA	bovine serum albumin
COC	cumulus-oocyte complex
CPA	cryoprotective agent
Cys	L-cysteine
DAPI	4'6-diamidino-2-phenylindole
6-DMAP	6-dimethylaminopurine
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EG	ethylene glycol
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
ExB	Expanding blastocysts
FBS	fetal bovine serum
FEB	fully-expanded blastocysts
FITC	fluorescein isothiocyanate
GSH	glutathione (L-γ-glutamyl-L-cysteinyl-glycine)
ICM	inner cell mass
ICSI	Intracytoplasmic sperm injection
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro embryo production
LN_2	liquid nitrogen
mBO	modified Brackett and Oliphant
mSOF	modified synthetic oviductal fluid

MOET	multiple ovulations and embryo transfer
MTOC	microtubule-organizing center
MVC	minimum volume cooling
OPS	open-pulled straw
PBS	phosphate buffered saline
PI	propidium iodide
PN	pronucleus
PVP	polyvinyl pyrrolidone
ROS	reactive oxygen species
TCM	tissue culture medium
TE	trophectoderm
T_e	eutectic temperature
TEM	transmission electron microscope
T_g	glass transition temperature
T_g'	T_g of the maximally freeze-concentrated phase
T_{max}	maximum allowable product temperature

Abbreviations used without definition

ANOVA	analysis of variance
DNA	deoxyribonucleic acid
FSH	follicle stimulating hormone
GV	germinal vesicle
LH	lutenizing hormone
SD	standard deviation
SE	standard error

Chapter 1: General introduction

Introduction

Efficient production and selective breeding of large domestic animals with high economic value in terms of milk, meat and leather production have been enhanced by applying assisted reproductive technologies (ART) such as artificial insemination (AI) and multiple ovulations and embryo transfer (MOET) during 1950's to 2000. In cattle, successful cryopreservation of spermatozoa (Polge & Rowson 1952) and embryos (Wilmut & Rowson 1973) in liquid nitrogen (LN_2) allowed transportation of the genetic resources, and made the AI and MOET technologies more practical because estrous synchronization of recipients is no longer necessary. Discovery of "sperm capacitation" by Chang et al. (1959) activated the research field of in vitro fertilization (IVF), and Brackett et al. (1982) first obtained the IVF-derived calves. The IVF technique was successfully combined with in vitro maturation (IVM) of immature oocytes retrieved from abattoir-derived ovaries and in vitro culture (IVC) of the presumptive zygotes (Hanada et al. 1986, Lu et al. 1988). Thus, large amounts of transferable bovine blastocysts can be produced under in vitro conditions. Recently, freeze-drying has been proposed as an alternative method for sperm preservation. The LN_2 is not necessary for the storage of freeze-dried spermatozoa, resulting in the lower maintenance cost and risk of accidental loss of frozen cell stock. Since freeze-dried and rehydrated spermatozoa completely lose their motility however, an advanced technique such as intracytoplasmic sperm injection (ICSI) must be applied to produce the fertilized zygotes. Although blastocysts have been produced by ICSI with freeze-dried spermatozoa in large domestic species including cattle and pigs (Keskintepe et al. 2002, Kwon et al. 2004, Martins et al. 2007), the practically acceptable offspring rates were achieved only in rodents including mice, rats and hamsters (Wakayama & Yanagimachi 1998, Hirabayashi et al. 2005, Muneto & Horiuchi 2011).

In this chapter, research history and background regarding in vitro production and cryopreservation of bovine embryos, and freeze-drying of bull spermatozoa are overviewed, followed by objective of this research thesis.

In vitro production of bovine embryos

Well-established MOET technology still accounts for the majority of bovine embryos produced worldwide, while no progress has been made in the latest decade to increase the number of transferable embryos and to reduce the side effects on the reproductive performance of the donors. In vitro embryo production (IVP), consisting from IVM, IVF or ICSI, and IVC, was developed initially as a research tool and was applied to rescue follicular oocytes of slaughtered donors. The IVP system in cattle has become important for the production of embryos as an alternative to MOET because of the advantages and flexibility that it offers.

In cyclic cows, embryos develop after fertilization of oocytes that originate from an ovulatory follicle. These follicles have undergone the processes of selection, growth and dominance until oestrus, and then final maturation have been initiated following LH surge approximately 24 h prior to ovulation (Dieleman et al. 1983, Fortune 1994). IVM technique allows us to use large number of matured oocytes derived from slaughtered animals. Since mouse oocytes can be matured and fertilized in vitro and developed to term (Mukherjee 1972), there have been intensive attempts in cattle. At first to investigate the ability of IVM oocytes to be fertilized, IVM oocytes were fertilized in vivo in cattle (Hunter et al. 1972). Finally, Hanada et al. (1986) succeeded in getting calves from IVF oocytes that were matured in vitro. However, it is known that IVM oocytes have lower developmental capacity than in vivo matured oocytes (Rizos et al. 2002). There have been many studies to improve developmental capacity of IVM oocytes by increasing level of glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) in oocytes (de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002, Balasubramanian & Rho 2007), addition of hormone into IVM medium (Harper & Brackett 1993, Luciano et al. 2005), prolonging IVM period (Thomas et al. 2004, Albuz et al. 2010) and decreasing oxygen tension (Thompson et al. 1990).

Attempts to fertilize mammalian oocytes in vitro go back to 19th Century when Schenk (1878) first reported cleavage of guinea pig oocytes after IVF using epididymal spermatozoa. Although Pincus & Enzmann (1934) and Venge (1953) reported the birth of IVF-generated rabbit pups, it was not until 1954 when Thibault et al. (1954) and Chang

(1959) presented unequivocal evidence of successful IVF, again using the rabbit. The use of capacitated spermatozoa collected from the uterus of mated females was the key to their success. Sperm capacitation in vitro has been reported to occur in hamster (Yanagimachi & Chang 1963). In cattle, the first birth of normal offspring after IVF was reported using in vivo-matured oocytes (Brackett et al. 1982). Thereafter, reliable IVC system to produce transferable blastocysts from IVF zygotes has been established (Lu et al. 1988).

Since Uehara & Yanagimachi (1976) reported that hamster oocytes injected with spermatozoa started pronuclear development, the "ICSI" technique can be used not only as a powerful tool for analytical studies of fertilization process but also as the ART which allows immobile sperm to fertilize. The first ICSI offspring were born using the rabbit (Iritani & Hosoi 1989) followed by the cattle (Goto et al. 1990). In mouse, although it was believed that application of ICSI is difficult because of their fragile membrane, mouse offspring was derived from ICSI oocytes using piezo-actuated micromanipulation (Kimura & Yanagimachi 1995). Piezo-actuated micromanipulation harnesses the piezo-electric effect to transmit a small crystal lattice distortion to the tip of a pipette, driving it forward in a precise and controlled manner. The system is used for many mammalian species and gained high developmental rates especially in mouse. On the other hand, ICSI has not been very successful in large domestic species such as cattle and pigs, probably due to failure of oocytes activation, compromised sperm chromatin remodeling, and technical difficulties. To overcome these problems, many studies have been conducted to add mechanical or chemical stimuli to ICSI oocytes (Rho et al. 1998, Hwang et al. 2000, Abdalla et al. 2009c), to treat spermatozoa with reducing agents such as dithiothreitol (DTT) (Rho et al. 1998, Galli et al. 2003) and to visualize the tip of injection pipette by oocyte centrifugation (Rho et al. 1998, Wei & Fukui 2000).

Embryo cryopreservation is the key technique for efficient use of transferable blastocysts. Wilmut & Rowson (1973) first succeeded to obtain pregnancies from cryopreserved bovine embryos. Willadsen (1977) reported a two-step freezing method consisting of slow cooling followed by rapid cooling to -196 ºC, using sheep and cattle embryos. Pregnancy rates following transfer of bovine embryos frozen in this way range from 50 to 60% (Niemann et al. 1985). Vitrification method is an alternative approach for cryopreservation of mouse embryos developed by Rall & Fahy (1985). In this protocol,

dehydration of the embryos was conducted by exposing them to highly concentrated cryoprotective agents (CPA), and then cooled rapidly by direct plunging into LN_2. Recently, new devices for vitrification (electron microscope grid, Cryoloop, and Cryotop, etc.) have been reported to increase the cooling rate which can allow to decrease concentration of CPA. The application of vitrification as an alternative to conventional slow/two-step freezing reduces the operation time and equipment required.

Freeze-drying for sperm preservation

First successful preservation of mammalian spermatozoa has been achieved under subzero-temperature, due to the serendipitous discovery of glycerol as CPA by Polge et al. (1949). Recently, attention has been paid to freeze-drying as an alternative method for sperm preservation because the freeze-dried spermatozoa can be stored without LN_2.

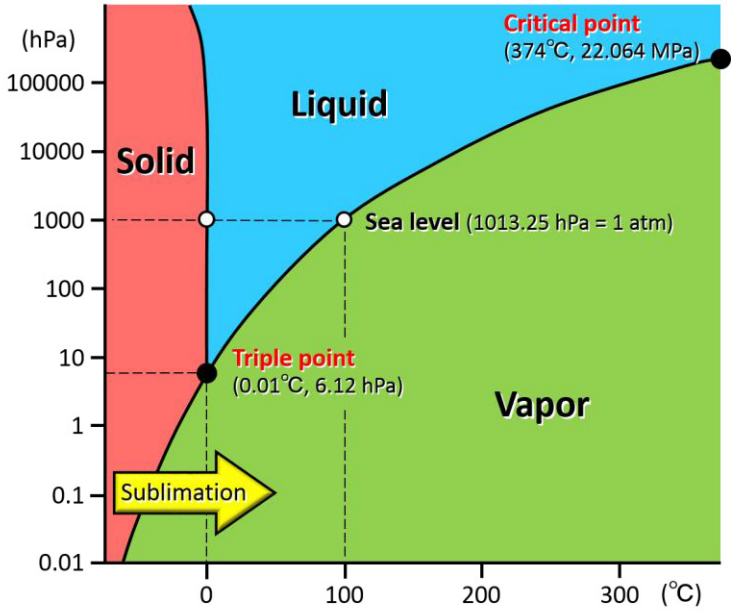

Figure 1-1. Phase diagram of water. At the sea level (1,013 hPa = 1 atm), water changes from solid ice to liquid (melting) when its temperature increases above the freezing point (0 ºC), and then changes to vapor (evaporation) when its temperature increases above boiling point (100 ºC). When the water temperature is higher than the freezing point keeping below 6.12 hPa, the water directly changes to the vapor phase without passing the intermediate liquid phase, which is known as sublimation phenomenon.

Principle of freeze-drying

In contrary to the conventional dehydration which depends on evaporation phenomenon, the freeze-drying depends on direct transition from solid (ice) to vapor (gas), known as sublimation phenomenon (**Figure 1-1**). The prerequisite for a freeze-drying process is the complete solidification of the liquid before initiating drying. The solute phase behavior in freeze-drying is shown by a schematic diagram of the binary system water-sucrose in **Figure 1-2** (MacKenzie 1977). When the solution is cooled, ice crystallization results in freeze-concentration after supercooling. Despite the prediction of classical phase equilibria in which water and solute form eutectic at the eutectic temperature (T_e), freeze-concentrate of the water-sucrose is subjected to progressive supersaturation beyond T_e. The supersaturated solution is thermodynamically unstable and relies for its apparent stability on the extremely high viscosity. Rubber-like freeze-concentrate is retained amorphous at the glass transition temperature of the maximally freeze-concentrated phase (T_g').

Primary drying has been defined as the phase in which ice crystal is sublimated. In order for sublimation to take place, energy is provided in a quantity equal to the heat of ice $\Delta_{sub}H$, which is supplied mostly by the conduction instead of convection (Dushman & Lafferty 1962) and radiation (Nail 1980) from the freeze-drying shelf to the ice at the sublimation front. Following the solid-to-vapor phase change, water vapor must be removed by flow through the porous structure of partially dried product (without melting) and is held by ice condenser. Temperature during primary drying has been believed to be set below T_g'. If product temperature is higher than T_g' during primary drying, the glassy matrix changes to rubber state, resulting in loss of the porous structure. This loss of porous structure of freeze-dried cake is defined as collapse phenomenon (Nail et al. 2002, Sundaramurthi & Suryanarayanan 2012). However, maintaining the product temperature too far below the T_g' results in unacceptably low sublimation rate (Pical 1985). Selection of an optimum chamber pressure involves a trade-off between heat transfer and mass transfer considerations. The pressure in the chamber, which consists almost entirely of water vapor during primary drying (Nail & Johnson 1992), must be lower than the vapor pressure of ice in the product for sublimation., but too low pressure causes extremely low sublimation rate (Franks 1998, Nail et al. 2002).

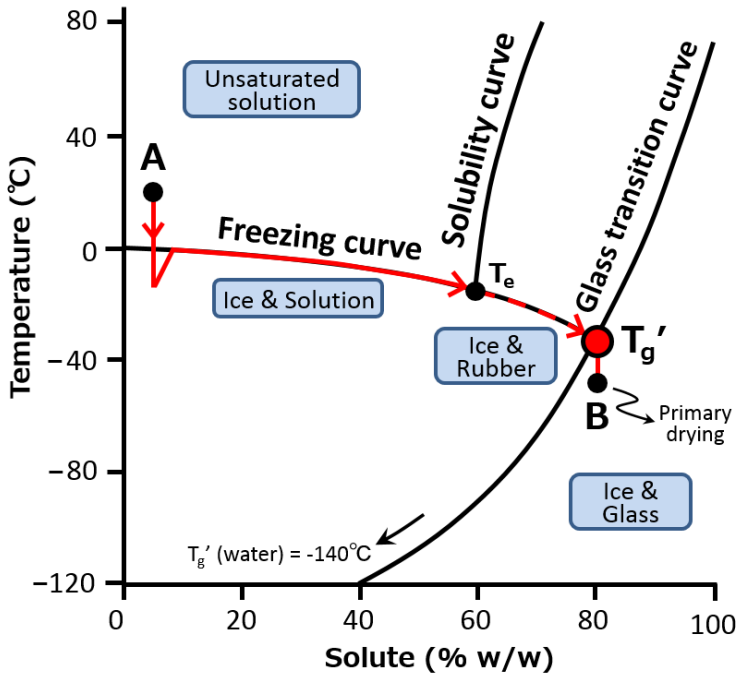

Figure 1-2. Schematic temperature-composition state diagram for the sucrose-water system. A sucrose concentration of initially dilute solution (point A) is changed by freeze concentration during freezing. The important point to note is that, despite the predictions of classical phase equilibria, sucrose does not precipitate as a crystal phase when its solution is cooled to the eutectic temperature (T_e) but becomes subject to progressive supersaturation with high viscosity like rubber. The freeze-concentrate is finally retained amorphous at the glass transition temperature of the maximally freeze-concentrated phase (T_g'). Temperature during primary drying has been believed to be set slightly below T_g' (point B) in order to avoid cake collapse and unacceptable low drying rate. Adopted from MacKenzie (1977).

Secondary drying is conducted if drying status after primary drying is not enough for the subsequent storage. If glass transition temperature (T_g) of freeze-dried cake is lower than the storage temperature, the increased molecular mobility would disturb the stability of the product (Buitink et al. 1998). Excess drying also causes decreased stability of the products (Buitink et al. 1998, Jiang & Nail 1998). Kinetic analysis of secondary drying as a function of temperature and pressure revealed that residual water decreases rapidly during the first few hours of secondary drying, then slows considerably, and reaches a plateau which exceeds the equilibrium water content of the cake (Pikal et al. 1990).

Research history on sperm freeze-drying

Successful recovery of motile bull spermatozoa after freeze-drying was first reported by Leidl (1954), followed by several groups (Bialy & Smith 1957, Albright et al. 1958, Meryman & Kafig 1959, Nei & Nagase 1961). However, reproducibility of these earlier reports was questionable (Sherman, 1957, Saacke & Almquist 1961, Meryman & Kafig 1963). Although application of AI with freeze-dried bull spermatozoa resulted in successful pregnancy (Meryman & Kafig 1959), the fact that freeze-dried spermatozoa lost their motility after short-term storage or further dehydration (Leidl 1954, Bialy & Smith 1957, Larson & Graham 1976) may imply the insufficient dehydration of these sperm for preservation. Wakayama & Yanagimachi (1998) reported successful production of mouse offspring derived from freeze-dried sperm stored at 4 ºC for 3 month. At the present time, it is believed that spermatozoa completely lost their motility after sufficient dehydration for preservation. Therefore, ICSI must be applied for fertilization of immotile freeze-dried spermatozoa. Until now, production of live offspring derived from ICSI of freeze-dried spermatozoa has been reported in mouse (Wakayama & Yanagimachi 1998, Kusakabe et al. 2001, Kaneko et al. 2003, Kaneko et al. 2006, Kusakabe et al. 2008), rat (Hirabayashi et al. 2005, Hochi et al. 2008), hamster (Muneto & Horiuchi 2011) and rabbit (Liu et al. 2004). As to large domestic species including cattle and pigs, blastocysts have been harvested from ICSI oocytes with freeze-dried spermatozoa (Keskintepe et al. 2002, Kwon et al. 2004, Martins et al. 2007).

Some factors are involved in functional integrity of freeze-dried spermatozoa. (1) Composition of freeze-drying buffer: Two conventional culture media (CZB and DMEM) were used for the freeze-drying buffer (Wakayama & Yanagimachi 1998). However, Kusakabe et al. (2001) recommended the use of an unphysiological solution composed of 10 mM Tris-HCl, 50 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and 50 mM NaCl (defined hereafter as EGTA buffer). Addition of the divalent cation chelator is expected to prevent the endonuclease-mediated chromosomal damage by repressing the activity of the Ca^{2+}-dependent enzyme. Increasing the pH of this buffer to 8.0 improved its protective ability (Kaneko et al. 2003). Preincubation of mouse spermatozoa in similar solution for several days at 4 ºC increased the resistance of the sperm to the freeze-drying (Kusakabe et al. 2008). A solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) was also found to maintain the chromosomal

integrity and participation into embryonic development of the mouse and rat freeze-dried sperm stored for 1 year at 4 ºC (Kaneko & Nakagata 2006, Kaneko et al. 2007). Addition of EDTA or EGTA to freeze-drying buffer was efficient to maintain the chromosomal integrity and participation into embryonic development of boar freeze-dried sperm for up to 2 h after rehydration (Nakai et al. 2007). Supplementation of trehalose, a disaccharide accumulated in numerous anhydrobiotic animals during the drying stage, showed a similar protective function to that of EGTA (Martins et al. 2007). Trehalose may make sperm membrane and proteins more stable by replacing the bound water, or the higher glass stability of trehalose may make the dried cells in a static state that inhibits the cellular, chemical, biological and physical processes (Meyers 2006). (2) Drying condition: Drying condition is composed of temperature, chamber pressure and drying period. Kawase et al. (2007) reported that the ability of mouse spermatozoa to participate in full-term development was better maintained when the sperm were dried under a chamber pressure of 0.37 hPa vs 1.03 and 0.04 hPa. Prolongation of freeze-drying period for boar spermatozoa from 4 to 24 h gradually reduced the ability of freeze-dried sperm to support embryonic development in vitro, particularly when the sperm were stored at 25 ºC (Kwon et al. 2004). (3) Storage temperature and period: Although Wakayama & Yanagimachi (1998) reported that the ability of mouse freeze-dried sperm to support embryonic development in vitro or in vivo was not affected by storage either at 4 or 25 ºC for up to 3 months, Kwon et al. (2004) observed that the ability of boar freeze-dried sperm to support embryonic development in vitro was more hampered after 1 month storage at 25 ºC than 4 ºC and that porcine oocytes injected with freeze-dried sperm stored for 3 months at the 4 ºC did not develop in vitro. Rat freeze-dried sperm stored for 1 year at 25 ºC exhibited more frequent chromosomal abnormalities than those stored at 4 ºC (Hochi et al. 2008). Although the successful offspring were produced from freeze-dried sperm stored for 3 years at 4 ºC in mouse (Kaneko & Serikawa 2012a) and for 5 years in rat (Kaneko & Serikawa 2012b), it is reported that freeze-dried mouse spermatozoa must keep at lower than -80 ºC for the prolonged storage, which is determined by the accelerated degradation kinetics (Kawase et al. 2005).

Objective

The main objective of the present study was to establish the system of blastocyst production for freeze-dried bovine sperm. Concentration of intracellular GSH was increased by supplementation of β-ME and cysteine into IVM medium in order to increase developmental ability of IVM oocytes (Chapter 2), and the effects of developmental stage of and/or harvested age of blastocysts on post-vitrification in vitro survival of bovine blastocysts derived from ICSI was investigated (Chapter 3), considering future ET attempts. Then, MTOC function of freeze-dried bull spermatozoa was investigated after ICSI (Chapter 4), and effect of cake collapse, which means loss of porous structure of freeze-dried solution, on the functional integrity of rehydrated bull spermatozoa was investigated (Chapter 5).

Chapter 2: An attempt at improving bovine blastocyst yield by increased intracellular glutathione level prior to ICSI and IVF

Abstract

Although the IVM system allows us to use large number of matured oocytes derived from slaughtered cows, developmental capacity of such in vitro-matured oocytes is lower than that of in vivo-matured oocytes. We have recently reported that some bovine IVM-IVF oocytes exhibited multiple sperm asters by which migration and development of both pronuclei were impaired. Since GSH of bovine IVM-IVF oocytes can stimulate sperm aster formation, the objective of this study was to investigate the effect of GSH content of bovine oocytes on the multiple aster formation and the subsequent in vitro development. Treatment of bovine cumulus-oocyte complex (COC) with β-ME and Cys (β-ME/Cys) during IVM resulted in 2.5-folds higher GSH content when compared with non-treated control oocytes. Proportions of normally fertilized zygotes exhibiting sperm aster(s) were as high as > 95% in the oocytes regardless of β-ME/Cys treatment after IVF. The frequency of multiple aster formation was not affected by the increased level of intracellular GSH with β-ME/Cys. Consequently, there was no effect of increased GSH level on the migration and development of pronuclei, and subsequent development into blastocysts regardless of the method for fertilization (IVF or ICSI).

Introduction

Since Hanada et al. (1986) succeeded in obtaining calves from abattoir-derived IVM oocytes, the technique has been used worldwide for IVP in cattle. However, it is known that the bovine IVM oocytes have lower developmental capacity than in vivo-matured counterparts (Rizos et al. 2002). Many researchers have tried to improve developmental rate of the IVM oocytes by increasing intracellular GSH level of oocytes (de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002, Balasubramanian & Rho 2007), addition

of hormone(s) into the IVM medium (Harper & Brackett 1993, Luciano et al. 2005), extension of the IVM period (Thomas et al. 2004, Albuz et al. 2010) and decreasing oxygen tension (Thompson et al. 1990).

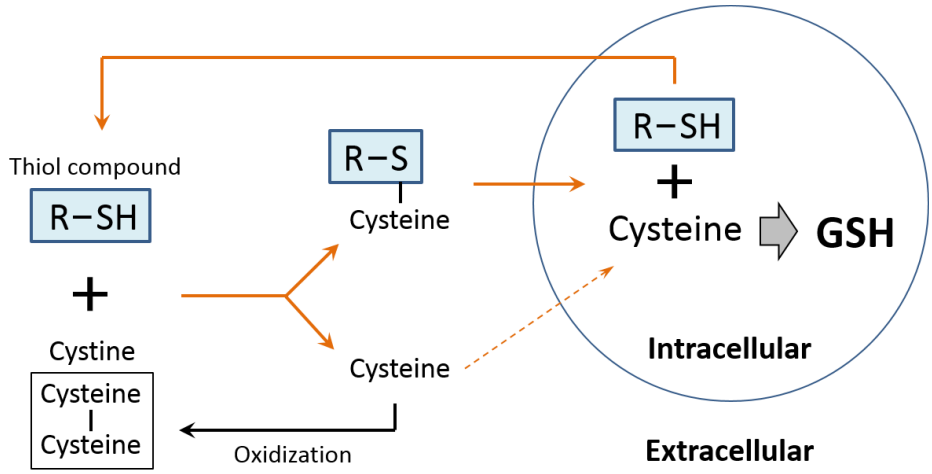

Figure 2-1. Proposed action of thiol compounds on cystine (cysteine-cysteine) and cysteine utilization by oocytes. Thiol compounds such as β-ME and cysteamine are combined to oxidized cysteine 'cystine'. The complex is easily transferred into cell and GSH is synthesized using the transferred cysteine, which is the rate-limiting step in GSH synthesis.

The GSH, a tripeptide thiol composed of glutamate, cysteine and glycine, can be found in virtually all cells and functions to reduce disulfide linkages of proteins and other molecules in the synthesis of the deoxyribonucleotide precursors of DNA (**Figure 2-1**). In addition, the GSH can protect cells against the adverse effects of reactive oxygen species (ROS) that are formed during metabolism (Meister 1983). However, GSH levels of IVM oocytes are lower when compared with those of ovulated oocytes, as reported in some animal species (Brad et al. 2003, Rodríguez-González et al. 2003, Kim et al. 2007, Ge et al. 2008). GSH synthesis is firstly conducted by the enzyme of γ-glutamylcysteine synthetase which synthesizes γ-glutamylcysteine from glutamate and cysteine. Then, glycine is added to the C-terminal of the γ-glutamylcysteine via the enzyme of glutathione synthetase. The first reaction is known as the rate-limiting step in GSH synthesis, which is restricted by amount of cysteine in the medium. However, cysteine in the medium is easily oxidized and changed to cystine within 24 h (de Matos et al. 1997) and the cystine was

taken up by the cultured cells at one-fourth to one-fifth of the cysteine uptake (Ohmori & Yamamoto 1983). It was reported that β-mercaptoethanol (β-ME) enhances cystine uptake four to five times more rapidly (Ishii et al. 1981, Ohmori & Yamamoto 1983). Addition of the β-ME and cysteamine into IVM medium improved the blastocyst yield in cattle and pigs (de Matos et al. 1996, Abeydeera et al. 1998).

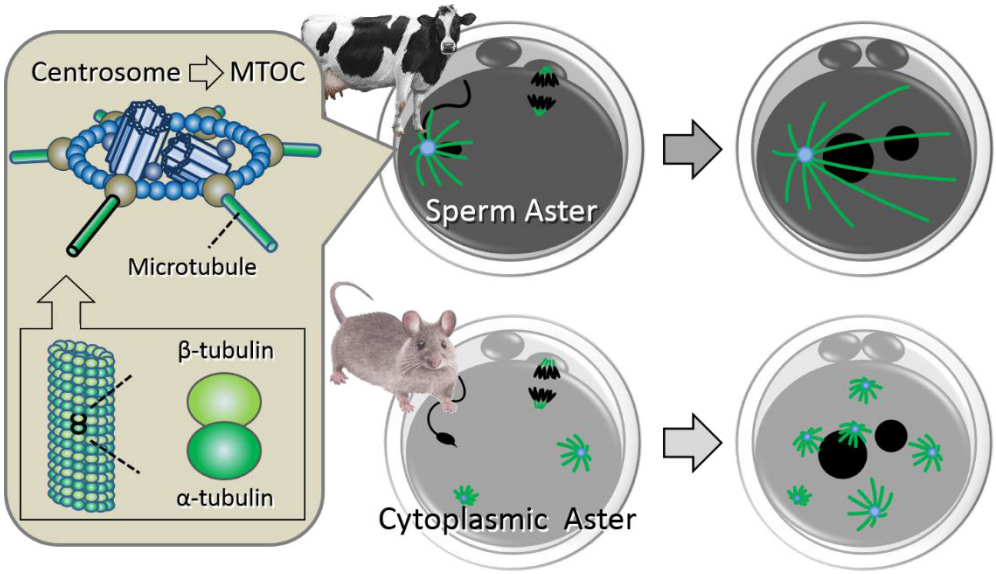

Figure 2-2. Microtubule assembly from sperm/cytoplasmic aster. In most mammals including cattle, a centrosome brought by fertilized spermatozoa acts as MTOC and forms sperm aster which plays a key role in the migration of male and female pronuclei. Conversely in rodents, the oocytes use their own MTOC dispersed in the cytoplasm for formation of cytoplasmic aster because spermatozoa lose their centrioles during spermiogenesis.

During fertilization, a bovine spermatozoon brings a centrosome into an oocyte (Schatten 1994). The centrosome is composed of a pair of centrioles surrounded by the pericentriolar materials, such as γ-tubulin, centrin and pericentrin, and acts as the microtubule-organizing center (MTOC). By polymerization of microtubules (α- and β-tubulin) from the centrosome, a single sperm aster is formed and 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 spermatozoa 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 & Fawcett 1973, Schatten et al. 1985; **Figure 2-2**). Recently, we have reported that cryopreservation of bovine IVM oocytes induces frequent multiple aster formation after IVF and leads to impaired pronuclear migration and development before the first cleavage (Hara et al. 2012). In addition, fresh IVM oocytes also exhibited the multiple aster formation in a certain extent. These results indicates that suppression of multiple aster formation might increase developmental rate of fertilized bovine IVM oocytes.

Since it has been reported that GSH of bovine IVM-IVF oocytes can stimulate sperm aster formation (Sutovsky & Schatten 1997), the effect of GSH content of bovine oocytes on the multiple aster formation and the subsequent in vitro development was investigated in this study.

Materials and methods

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 to 12 ºC) within 24 h after slaughter. The contents of 2 to 8 mm follicles 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 cultured in Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/mL FSH (Kyoritsu Seiyaku Co., Tokyo, Japan), 1 μg/mL 17β-estradiol, and 50 μg/mL gentamycin sulfate for 22 h at 38.5 ºC under 5% CO_2 in air. To increase the intracellular GSH level, 50 μM β-ME (Takahashi et al. 1993) and 1 mM Cys (Choe et al. 2010) (β-ME/Cys) were added to the IVM medium. After the maturation culture, cumulus cells were removed by a brief vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/mL bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1000 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.

Measurement of intracellular GSH

Intracellular GSH content was measured by a 5,5'-dithio-bis(2-nitrobenzoic acid)-GSH reductase recycling assay with a total glutathione quantification kit (Dojin Molecular Technologies Inc., Kumamoto, Japan). Oocytes were washed three times with PBS containing 1 mg/mL polyvinylpyrrolidone (PVP). According to the instructions, pools of 35 to 40 oocytes from each treatment were transferred to 12 μL of 10 mM HCl in a 0.6-mL microfuge tube. Then the oocytes were frozen in LN_2 and were thawed at room temperature. This freeze-thaw procedure was repeated twice and the oocytes were stored at -80 ºC until assay. After final thawing, 3 μL of 5% 5-sulfosalicylic acid was added to the samples and the tubes were centrifuged for 10 min at 8,000 *g*. Ten μL of the supernatant was diluted with 40 μL pure water, and then 20 μL of the sample solution was transferred to each well of 96-well microplate preloaded with 20 μL co-enzyme working solution, 120 μL buffer solution and 20 μL enzyme working solution. After incubation for 10 min at 37.0 ºC, 20 μL of substrate working solution was added to the each well. The absorbance at 405 nm was determined by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) following incubation for 30 min at room temperature. The total GSH content (pmol/oocyte) was calculated by reference to a standard curve prepared with authentic GSH.

In vitro fertilization

Commercially available frozen semen from a Japanese Black bull was used. After thawing in a water bath at 37 ºC 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 1.5×10^7 sperm cells/mL. Ten to 12 matured oocytes in the IVF medium were co-incubated with the above sperm suspension at a final concentration of 3×10^6 sperm cells/mL for 6 h in 100-μL microdrops under mineral oil at 38.5 ºC under 5% CO_2 in air.

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-cysteine	0.50 mM	---	5.55 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.

Immunostaining of pronuclear zygotes

To assess the aster formation of pronuclear zygotes, IVF oocytes were cultured for an additional 4 h in Hepes-buffered TCM-199 supplemented with 5% FBS, 0.2 mM sodium

pyruvate and 50 μg/mL gentamycin sulfate (defined hereafter as TCM-199/5% FBS) at 38.5 ºC under 5% CO_2 in air, and then immunostained according to the method described previously (Hara et al. 2012). The zygotes 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 zygotes 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 1:1000). 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 4',6-diamidino-2-phenylindole (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 (**Figure 2-3**) and assessed with Image-J software (National Institutes of Health, Bethesda, ML, USA; accessed on-line). Zygotes with 2-PN were defined as those fertilized normally, and the larger pronucleus was defined as male pronucleus.

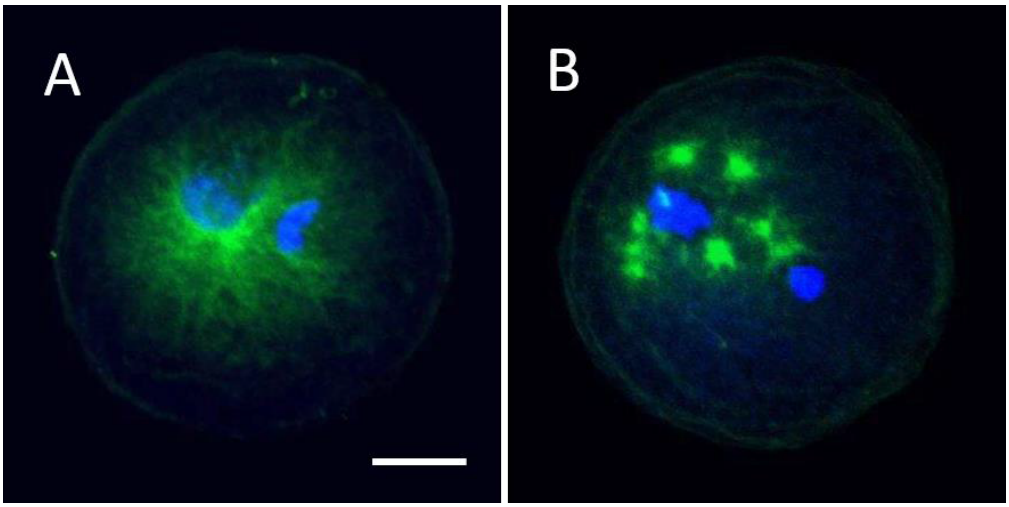

Figure 2-3. Fluorescent images of pronuclear-stage zygotes after immunostaining against α-tubulin and nuclear staining with DAPI. Zygote with (A) single sperm aster and (B) multiple asters. Scale bar represents 30 μm.

Intracytoplasmic sperm injection

A sperm suspension washed by Percoll was treated with 5 mM DTT for 20 min at 38.5 ºC, followed by washing twice with the mBO medium (5 min, 300 *g* each). Then, ICSI was performed using a piezo-driven micromanipulator (PMAS-CT150; PrimeTech, Ibaraki, Japan), as shown in **Figure 2-4**. The ICSI oocytes were first activated with 5 mM ionomycin

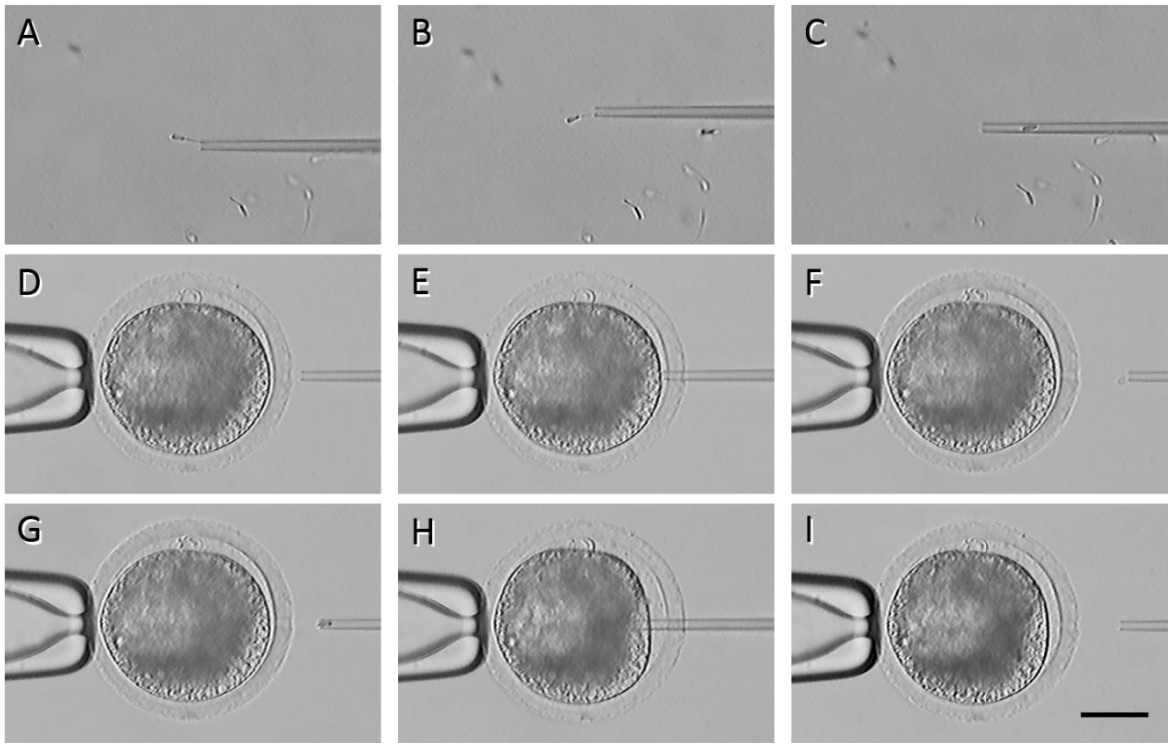

Figure 2-4. Intracytoplasmic sperm injection procedure in the bovine. (A) Catching a motile spermatozoon by the injection pipette. (B) Immobilization of the spermatozoon by several piezo pulses when the midpiece is touching to the tip of the injection pipette. (C) Aspiration of immobilized sperm into the injection pipette and bringing it to the injection droplet containing the oocytes. (D) Holding an oocyte with the first polar body located either at 6 or 12 o'clock and setting injection pipette at the center (Y and Z-axis) of the hold oocytes. (E) Penetration of the zona pellucida by applying a few piezo pulses without any damage to plasma membrane. (F) Removing of the zona core from injection pipette at the droplet. (G) Re-positioning of the aspirated sperm to the tip of injection pipette. (H) Pushing forward the injection pipette to the two thirds of the oocytes from right side and injecting the sperm with minimum amount of medium after applying a single piezo-pulse. (I) Withdrawal of the injection pipette gently. Scale bar = 50 μm.

in PBS for 5 min, and after 4 h culture in 100-μL microdrops of the TCM-199/5% FBS medium at 38.5 ºC under 5% CO_2 in air, a second activation was done with 7% ethanol in Hepes-buffered TCM-199 supplemented with 1% PVP for 5 min.

In vitro culture

Up to 30 presumptive zygotes following IVF or ICSI procedure were cultured in a 250-μL microdrop of modified synthetic oviduct fluid (mSOF) (Holm et al. 1999; **Table 2-1**), supplemented with 30 μL/mL essential amino acids solution (× 50, Gibco-11130), 10 μL/mL non-essential amino acids solution (× 100, Gibco-11140) and 5% FBS (defined hereafter as mSOFaa/5% FBS) at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2 for up to 8 days. Cleavage rate was determined on Day-2 (Day-0 was defined as the day of IVF or ICSI), and number of blastocysts was recorded on Day 7 and Day 8 (**Figure 2-5**).

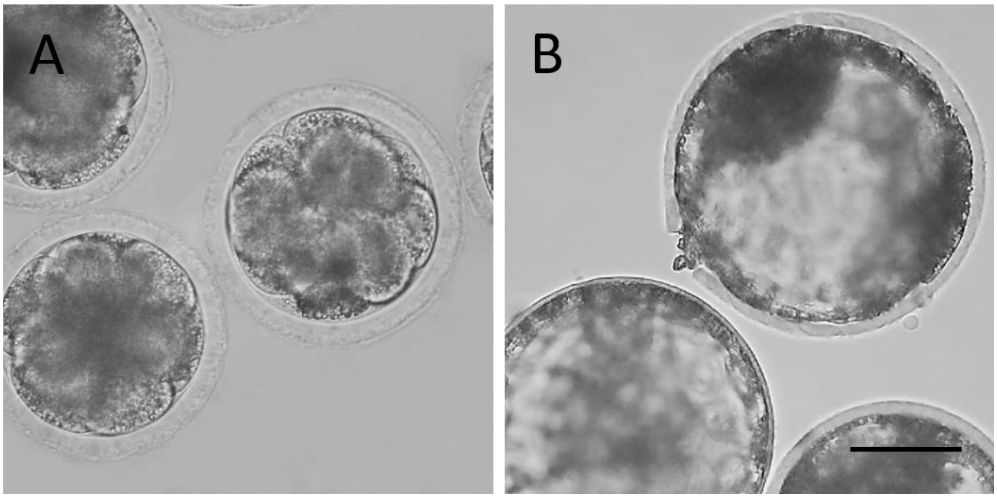

Figure 2-5. Bovine embryos produced by ICSI. (A) Cleaved zygotes on Day 2. (B) Blastocysts on Day 8. Scale bar represents 50 μm.

Statistical analysis

Arcsin-transformed data for maturation rate of COC, fertilization rate, polyspermic penetration rate, aster formation rate and developmental rate of IVF oocytes, as well as data for GSH content were compared between non-treated and β-ME/Cys-treated groups by Student's *t*-test. Data for developmental rate of ICSI oocytes were compared with

chi-square test analysis. Data for distance between male and female pronuclei and pronuclear size were compared by one-way ANOVA. When the ANOVA was significant, differences among means were analyzed by a Tukey's test. A value of $P < 0.05$ was defined as a significant difference.

Results

Effect of β-ME/Cys in IVM medium on intracellular GSH content

As oocyte maturation rate was assessed with the presence of the first polar body, treatment of the COC with β-ME/Cys did not influence the maturation rate (62%, 634/1,029 vs 66%, 658/1,004 in control group, $P > 0.05$). The GSH content of the oocytes matured in the presence of β-ME/Cys was significantly higher than that of control oocytes ($P < 0.05$; **Figure 2-6**).

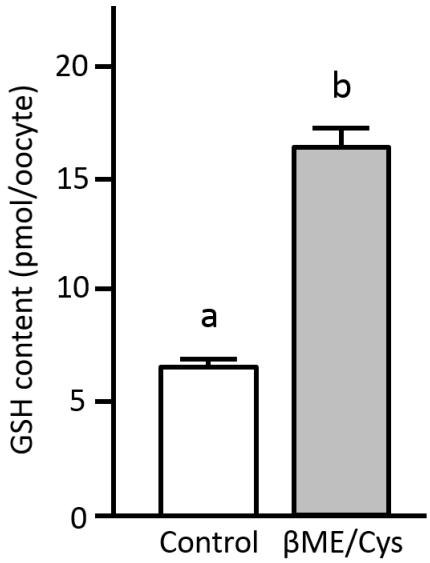

Figure 2-6. Glutathione (GSH) content of bovine oocytes treated with β-mercaptoethanol and L-cysteine (β-ME/Cys) during in vitro maturation. Mean ± SE. Different letters on SE bars denote significant difference ($P < 0.05$).

Effect of increased GSH content on aster formation

Incidences of normal fertilization (2-PN), assessed by DAPI staining, were comparable regardless of β-ME/Cys treatment ($P > 0.05$; **Table 2-2**). Polyspermic

penetration occurred at similar rates between control and β-ME/Cys groups (17 vs 16%, respectively). Immunostaining for α-tubulin indicated that proportions of 2-PN zygotes exhibiting sperm aster(s) were high as > 95% between both groups. Ratios of zygotes exhibiting multiple asters were also comparable regardless of β-ME/Cys treatment ($P > 0.05$).

Pronuclear migration and development of the β-ME/Cys-treated 2-PN zygotes were comparable with those of the control zygotes (**Table 2-3**), as far as zygotes with a single aster were concerned. Zygotes with multiple asters exhibited an impaired migration and development of their pronuclei.

Table 2-2. Aster formation in pronuclear-stage bovine zygotes matured in the presence of β-ME/Cys and fertilized in vitro.

Groups	No. (%) of oocytes		No. (%) of 2PN zygotes	No. (%) of aster-formed zygotes with	
	Inseminated	Fertilized: 2PN	Aster-formed	Single aster	Multiple asters
Control	84	58 (69 ± 3)	57 (98 ± 2)	47 (83 ± 4)	10 (17 ± 4)
β-ME/Cys	87	63 (73 ± 3)	60 (95 ± 2)	50 (83 ± 5)	10 (17 ± 5)

Percentages were expressed as mean ± SE of 6 replicates in each group.

Table 2-3. Migration and development of pronuclei in bovine zygotes with a single aster or multiple asters.

Groups	Aster	Distance between pronuclei (μm)	Pronuclear size (μm^2)	
			Male	Female
Control	Single	27 ± 2^a	259 ± 15^a	130 ± 10^a
	Multiple	50 ± 6^b	159 ± 22^b	83 ± 6^b
β-ME/Cys	Single	29 ± 2^a	264 ± 9^a	142 ± 9^a
	Multiple	47 ± 8^b	139 ± 42^b	77 ± 11^b

Numbers of 2PN-zygotes analyzed correspond to those in Table 2-2.

[a,b] Different superscripts denote significant difference within columns ($P < 0.05$).

Effect of increased GSH content on embryonic development

Cleavage rates of IVF oocytes were comparable between control and β-ME/Cys group ($P > 0.05$; **Table 2-4**). Developmental potential of the oocytes into blastocysts until Day 8 was not improved by increasing intracellular GSH level with β-ME/Cys treatment ($P > 0.05$). Similarly, developmental potential of ICSI oocytes was not also improved by the β-ME/Cys treatment (**Table 2-5**).

Table 2-4. In vitro development of bovine oocytes with an increased level of GSH after in vitro fertilization.

Groups	No. (%) of oocytes			
	Inseminated	Cleaved	Developed to blastocysts on	
			Day 7	Day 7 + 8
Control	104	68 (65 ± 4)	37 (36 ± 6)	43 (41 ± 5)
β-ME/Cys	98	61 (62 ± 6)	38 (39 ± 5)	40 (41 ± 5)

Percentages were expressed as mean ± SE of 4 replicates in each group.

Table 2-5. In vitro development of bovine oocytes with an increased level of GSH after intracytoplasmic sperm injection.

Groups	No. (%) of oocytes			
	Inseminated	Cleaved	Developed to blastocysts on	
			Day 7	Day 7 + 8
Control	23	16 (67)	4 (17)	5 (22)
β-ME/Cys	23	16 (67)	4 (17)	4 (17)

Discussion

Oocyte maturation rate was similar between control and β-ME/Cys-treated groups, but mean GSH level in β-ME/Cys-treated oocytes (16.2 pmol/oocyte) was significantly higher

than that in control oocytes (6.5 pmol/oocyte; **Figure 2-6**). Mizushima & Fukui (2001) reported an enhanced maturation rate of bovine oocytes in the presence of β-ME. The composition of IVM medium, the density of oocytes during IVM and the period of ovary storage were different from those employed in the present study. After IVF, bovine oocytes with increased GSH level exhibited similar incidence of multiple aster formation compared to the oocytes without increased GSH level (**Table 2-2**) and bovine oocytes with multiple asters showed delayed or arrested pronuclear development (**Table 2-3**) following IVF, consistent with our previous study (Hara et al. 2012). Sutovsky & Schatten (1997) reported that sperm aster formation of bovine IVF oocytes was disturbed when the oocytes were treated with buthionine sulfoximine, a specific inhibitor of γ-glutamyl-cysteine synthetase. Yoshida et al. (1993) also reported that intracellular GSH plays an important role in male pronuclear development of porcine IVF oocytes. To make sperm centrosome active as MTOC, reducing activity for disulfide bonds is required (Schatten, 1994). However, our data suggest that GSH level as 6.5 pmol/oocyte is enough to support the function of sperm centrosome as MTOC and to form both pronuclei in our IVF system. Shimizu et al. (2009) reported that knockdown of cyclin G-associated kinase by siRNA in HeLa S3 cells caused multiple aster formation, which was due to abnormal fragmentation of pericentriolar material.

There are several reports dealing with improved yields of bovine blastocysts after treatment of oocytes with thiol compound during IVM (de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002, Balasubramanian & Rho 2007), but our study failed to improve the blastocyst yield by increasing the GSH level regardless of fertilization method (**Table 2-4** and **Table 2-5**). In those reports (de Matos et al. 1995, de Matos et al. 1996, de Matos et al. 2002, Balasubramanian & Rho 2007), the blastocyst yields of IVM oocytes without thiol treatment were all less than 20% of the cultured oocytes. Further study is required to elucidate the mechanism responsible for multiple aster formation in bovine oocytes.

In conclusion, the high content of the GSH in the matured oocytes stimulated by β-ME/Cys treatment did not result in suppression of the incidence of multiple aster formation and improvement of the poor developmental potential of IVM oocytes into blastocyst stage.

Chapter 3: Developmental stage and kinetics affecting cryotolerance of bovine blastocysts produced by ICSI vs IVF

Abstract

The present study was designed to investigate the effects of developmental stage (fully-expanded or expanding blastocysts) and/or kinetics (harvested on Days 7 or 8) on post-vitrification in vitro survival of bovine blastocysts produced by ICSI vs IVF. Post-warming survival (re-expansion of blastocoele within 24 h) of ICSI-derived fully-expanded blastocysts (80%) was similar to that of their IVF-derived counterparts (88%). However, the ability of ICSI-derived expanding blastocysts to survive vitrification procedures (61%) was lower than that of IVF-derived blastocysts (85%), although the ICSI- and IVF-derived fresh blastocysts were of similar quality. The age of the blastocysts before vitrification did not affect cryotolerance for either ICSI-derived (73 and 59% for Days 7 and 8 embryos, respectively) or IVF-derived blastocysts (86% for both Days 7 and 8 embryos). At 24 h of post-warming culture, ICSI-derived blastocysts surviving vitrification contained a higher proportion of dead cells than their IVF-derived counterparts (5-13% vs 2-4%), but these proportions were not different from those of fresh control embryos. There was an adverse effect of vitrification on the ability of blastocysts to hatch within 72 h of culture only in IVF-derived Day 8 blastocysts (41 and 70% in vitrified and fresh control groups, respectively). Thus, the proportion of ICSI-derived blastocysts that survived vitrification procedures was comparable to IVF-derived blastocysts if the former were cultured to the fully-expanded stage prior to vitrification, with no significant difference between embryos harvested on Day 7 vs Day 8.

Introduction

Sensitivity of bovine embryos to cryopreservation is affected by many factors, including the method of embryo production (in vivo- vs in vitro-derived), embryo quality,

and developmental kinetics and stage. Although numerous studies documented that the in vitro-produced embryos, mainly IVF-derived zygotes, have lower cryotolerance than their in vivo-derived counterparts (Massip et al. 1995), the culture conditions used for IVF-derived presumptive zygotes greatly influenced cryotolerance of the resulting embryos (Mucci et al. 2006, Shirazi et al. 2009). Furthermore, faster-developing IVF-derived bovine blastocysts (harvested 7 days after insemination) were tolerated cryopreservation better than slower-developing ones (harvested 8 days after insemination) (Han et al. 1994, Saha et al. 1996, Dinnyés et al. 1999, Gómez et al. 2008). Among blastocysts developed in the same day, embryos reaching more advanced stage or larger diameter were more likely to survive cryopreservation (Han et al. 1994, Dinnyés et al. 1999, Park et al. 1999, George et al. 2008).

The main cryoinjury during the freezing process resulted from formation of intracellular ice crystals (Leibo 1977). Vitrification, which involves an extreme increase in the viscosity of the cryoprotective solution and eliminates ice crystal formation, has become a promising alternative to conventional freezing to improve cryotolerance of IVF-derived embryos (Nedambale et al. 2004, Mucci et al. 2006). The vitrification procedure requires utilization of extremely high concentrations of cryoprotectants and achieving extremely high rates of cooling and warming. The utilization of cryoprotactants in high concentrations may be toxic or result in osmotic stress to embryos (Liebermann et al. 2002). However, the only way to induce vitrification using lower concentrations of cryoprotectants is to increase the cooling rate. Application of various cryodevices that reduced the volume of vitrification solution (minimum volume cooling: MVC) and/or decreasing the temperature of LN_2 (i.e., N_2 slush) has been attempted to increase the rate of cooling (Vajta & Kuwayama 2006). Cryotop technology, an advanced version of the MVC procedure, was originally developed for cryopreservation of human oocytes and embryos (Kuwayama & Kato 2000), and subsequently successfully applied to vitrify oocytes from various species (Liu et al. 2008, Morató et al. 2008), IVF-derived embryos (Kelly et al. 2004, De Rosa et al. 2007), and reconstructed embryos with somatic cell nuclei (Laowtammathron et al. 2005, Du et al. 2007).

Since the first successful production of live calves derived from ICSI (Goto et al. 1990), there were subsequent attempts to improve the yield of ICSI-derived blastocysts

(Horiuchi et al. 2002, Galli et al. 2003). Abdalla et al. (2009c) recently developed an improved activation regimen which increased bovine ICSI blastocyst yield up to 30%, even with oocytes harvested from 1 day-stored ovaries. To our best knowledge, there is only one publication regarding cryopreservation of ICSI-derived bovine embryos (Keskintepe & Brackett 2000); in that study, 75% (12/16) of Day 7 expanded blastocysts and 88% (14/16) of Day 8 hatched blastocysts survived conventional two-step freezing. These cryosurvival rates in ICSI-derived embryos seemed comparable to those in IVF-derived counterparts. However, the ability of ICSI-derived bovine embryos to survive vitrification has apparently not been reported.

The objective of the present study was to investigate the ability of ICSI-derived bovine blastocysts vs IVF derived counterparts to survive vitrification procedures, with special reference to the effect of developmental stage and kinetics.

Materials and methods

Experimental design

A 2 × 2 × 2 factorial designed experiment was conducted (**Figure 3-1**).

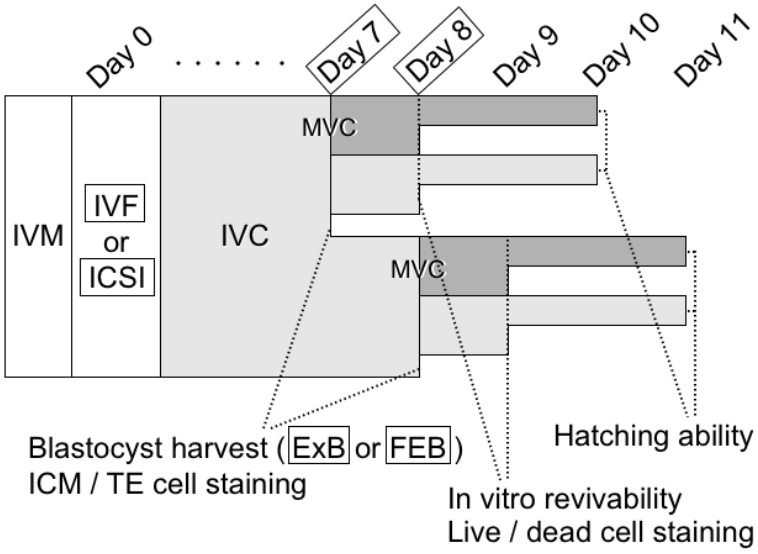

Figure 3-1. Schematic of the experimental design. IVM: in vitro maturation; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; IVC: in vitro culture; MVC: minimum volume cooling; ExB: expanding blastocysts; FEB: fully-expanded blastocysts; ICM: inner cell mass; TE: trophectoderm.

Bovine blastocysts produced either by IVF or ICSI were harvested 7 or 8 days after insemination. After being classified as either expanding or fully-expanded blastocysts (**Figure 3-2A**), some of the embryos were subjected to differential cell staining to assess their quality, based on the total cell number and the inner cell mass (ICM) cell ratio. Most of the remaining blastocysts were vitrified-warmed by Cryotop method, and cultured for 24 h to assess their revivability (re-expansion of the blastocoele; **Figure 3-2B**). Then, blastocysts that survived vitrification were allocated to one of two further analyses; live/dead cell staining, or an additional 48 h culture to assess hatching ability (**Figure 3-2C**). For both assessments, non-vitrified blastocysts were used as fresh controls.

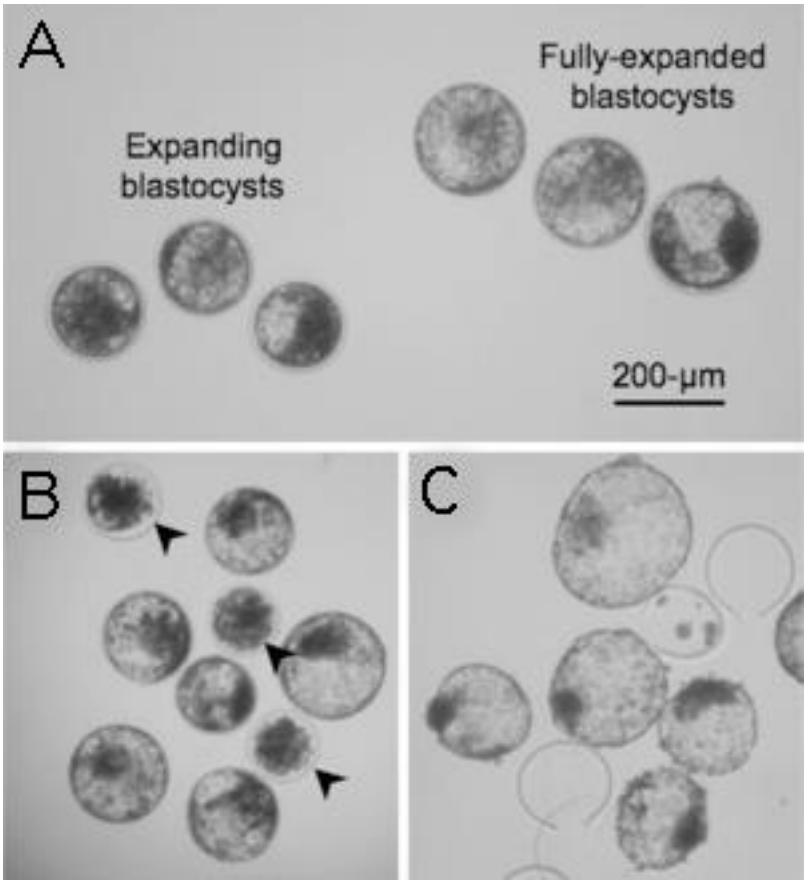

Figure 3-2. Morphology of in vitro-produced bovine blastocysts. (A) Fully-expanded blastocysts (≥ 200-μm in diameter) and expanding blastocysts (140-μm ≤ diameter < 200-μm). (B) Vitrified-warmed blastocysts after 24 h of culture. Re-expanded blastocysts were judged as surviving, while embryos failed to re-expand (arrow heads) were considered as degenerated. (C) Hatched blastocysts after 72 h of post-warm culture. Hatching embryos that more than half of embryonic part was escaped from the zona were defined as hatched.

Production of bovine blastocysts

Preparation of oocytes and spermatozoa for IVF and ICSI have already been described in Materials and methods of Chapter 2 (In vitro maturation / In vitro fertilization / Intracytoplasmic sperm injection / In vitro culture), except for the treatment of β-ME/Cys during IVM.

Assessment of fresh blastocysts

Blastocysts harvested on Day 7 or 8 were classified into one of two developmental stages; fully-expanded blastocysts that were ≥ 200 μm in diameter, or expanding blastocysts that were 140 to 199 μm in diameter. Some of the blastocysts were differentially stained with Hoechst 33342 and propidium iodide (PI) for assessment of ICM cells and trophectoderm (TE) cells, as reported previously (Thouas et al. 2001). Under an epifluorescence microscope, ICM cell nuclei labeled with Hoechst 33342 appeared blue, whereas TE cell nuclei labeled with PI and Hoechst 33342 appeared pink (**Figure 3-3**). Thus, blastocysts' total cell number (TE + ICM) and the ICM ratio (ICM / {TE + ICM} × 100) were determined.

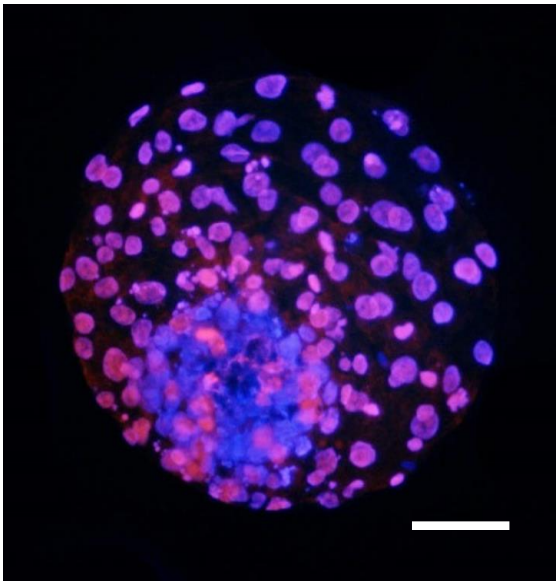

Figure 3-3. A blastocyst differentially stained with Hoechst 33342 and propidium iodide. Cell nuclei of ICM and TE appeared blue and pink, respectively. Scale bar represents 50 μm.

Vitrification and warming

Blastocysts were subjected to vitrification according to the method described previously (Tsujioka et al. 2008), with minor modifications. One to five blastocysts were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries Co., Osaka, Japan) and 7.5% dimethylsulfoxide (DMSO; Wako) in Hepes-buffered TCM-199 containing 20% FBS (defined hereafter as TCM-199/20% FBS) for 3 min at room temperature, and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose in TCM-199/20% FBS for approximately 40 sec at room temperature. Within this 40 sec, blastocysts were loaded onto the top of the polypropylene strip of a Cryotop (**Figure 3-4**; Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of the vitrification solution, and then quickly immersed into LN_2.

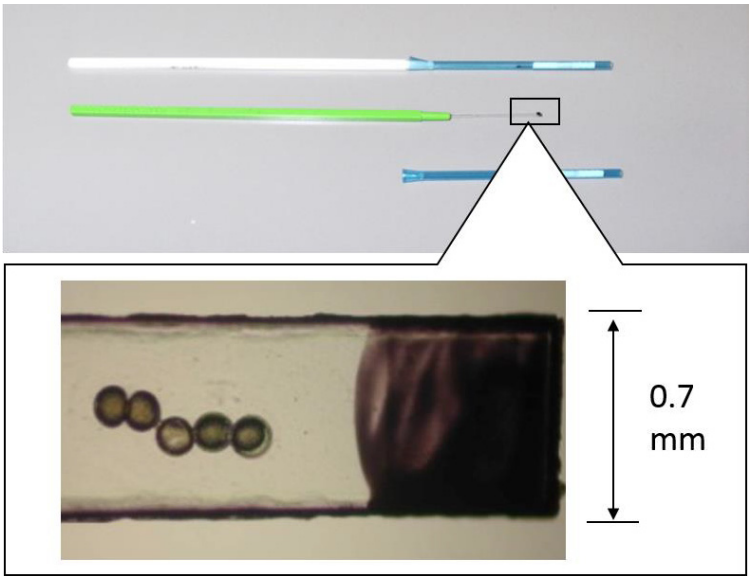

Figure 3-4. Cryotop, a device used for ultra-rapid cooling of bovine blastocysts.

After storage for > 1 day in LN_2 tank, blastocysts were warmed by immersing the polypropylene strip of a Cryotop into 3 mL of TCM-199/20% FBS containing 1 M sucrose at 38.5 ºC, and kept for 1 min. The blastocysts were transferred to TCM-199/20% FBS at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min, respectively). Finally, blastocysts were cultured in mSOFaa /5% FBS at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2.

Post-warming assays

Vitrified-warmed blastocysts were cultured in 250-μL microdrops of the mSOFaa /5% FBS for 24 h at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2, and their cryosurvival was assessed by re-expansion of the blastocoele. Thereafter, surviving embryos, as well as the corresponding fresh control Day 8 or 9 embryos, were randomly allocated either to additional 48 h culture for determination of their ability to hatch, or to live/dead cell staining according to the method described previously (Mucci et al. 2006), with minor modifications. Regarding assessment of hatching ability, embryos with more than half of embryonic part escaped out the zona were defined as hatched. For live/dead cell staining, blastocysts were incubated at 38.5 ºC in PBS supplemented with 3 mg/mL BSA and 20 μg/mL PI for 15 min, fixed in cold ethanol for 5 min, and then stained with 25 μg/mL Hoechst 33342 for 5 min at room temperature. Thereafter, they were washed once with glycerol/1,4-diazabicyclo[2.2.2]octane and mounted on a glass slide with the same medium. Under an epifluorescence microscope, live cells labeled with Hoechst 33342 appeared blue and dead cells labeled with PI and Hoechst 33342 appeared pink. Thus, total cell number of the blastocysts (live + dead cells) and the dead cell ratio (dead / total cells × 100) were determined.

Statistical analysis

Proportions of embryos surviving the vitrification and hatching were compared by Fisher's exact probability test. Proportional data regarding quality analysis of the embryos (ICM ratio and dead cell ratio) were arcsin-transformed and subjected to one-way ANOVA. Differences among groups were identified with Bonferroni's post-hoc multiple comparison test. Mean total cell number of the blastocysts was compared by one-way ANOVA. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Quality of fresh blastocysts

Out of 1,061 ICSI-derived zygotes cultured, 798 (68%) cleaved on Day 2, and 155 (15%) and 87 (8%) developed to expanding or fully-expanded blastocysts on Day 7 and 8, respectively. In addition, out of 1,384 IVF-derived zygotes cultured, 1,101 (80%) cleaved on Day 2, and 406 (29%) and 156 (11%) developed to expanding or fully-expanded blastocysts on Day 7 and 8, respectively. Based on quality analysis of the fresh blastocysts (differential cell staining), the ICM cell ratio (31-35%) was not affected by the method of embryo production (ICSI vs IVF), the day of blastocyst harvest (Day 7 vs Day 8), or developmental stage (expanding vs fully-expanded), as shown in **Figure 3-5**. Fully-expanded blastocysts tended to have higher total cell number (ranged from 125 to 136) than expanding blastocysts (ranged from 96 to 108), regardless of the method of embryo production or day of blastocyst harvest.

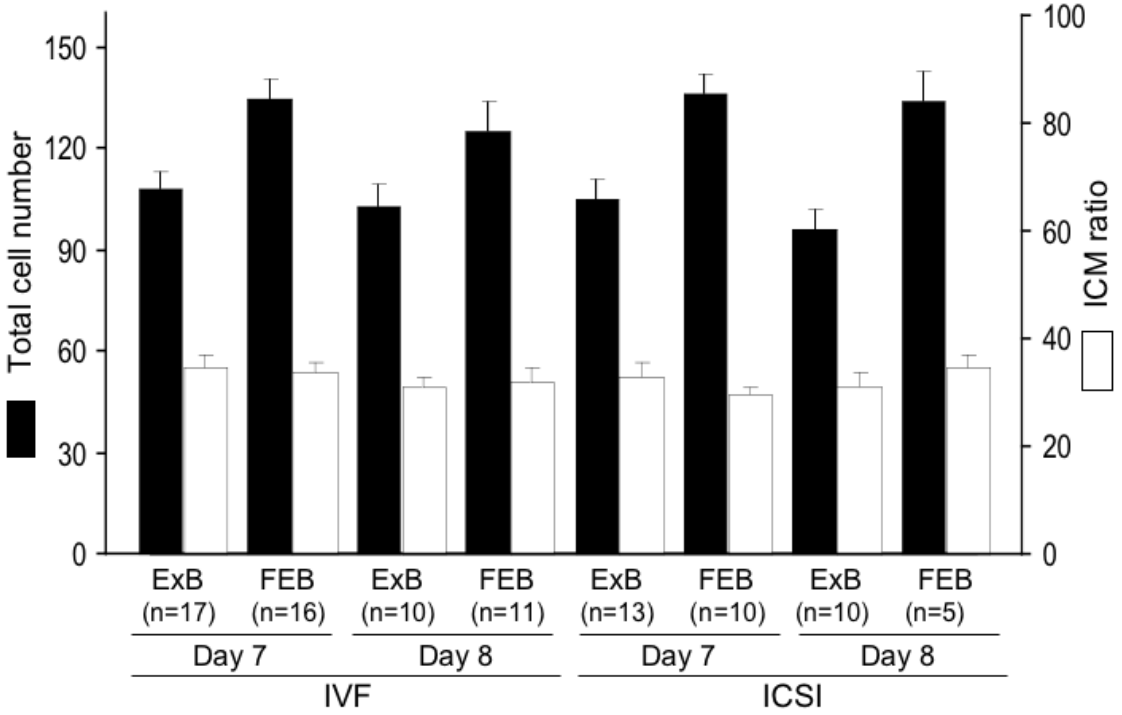

Figure 3-5. Quality analysis of IVF- and ICSI-derived bovine blastocysts by differential staining for ICM/TE cells. ExB: Expanding blastocysts, FEB: Fully-expanded blastocysts. Data are presented as mean number or ratio ± SE.

Assessments of blastocysts after vitrification

In the ICSI group, the ability of fully-expanded Day 7 or 8 blastocysts to survive vitrification procedures tended to be higher than that of expanding-stage counterparts; the difference reached significance when the data were pooled across the day of blastocyst harvest (80 vs 61%; $P < 0.05$). Conversely, the post-warming survival rates of IVF-derived blastocysts (84 to 89%) were not affected by developmental stage or age (**Table 3-1**). Cryosurvival of the ICSI-derived expanding blastocysts were significantly lower than that of the IVF-derived counterparts, regardless of the day of blastocyst harvest. Due to these differences, the overall cryosurvival of ICSI-derived blastocysts was also significantly lower than that of IVF-derived counterparts (69 vs 86%).

Table 3-1. Effect of developmental stage and age on in vitro survival of IVF- and ICSI-derived bovine blastocysts after MVC vitrification.

Origin	Developmental stage	Day of harvest		Subtotal
		Day 7	Day 8	
ICSI	Fully-expanded	25 / 30 (83)	11 / 15 (73)	36 / 45 (80)^{a}
	Expanding	30 / 45 (67)^{x}	8 / 17 (47)^{x}	38 / 62 (61)^{bx}
	Subtotal	55 / 75 (73)^{x}	19 / 32 (59)^{x}	74 / 107 (69)^{x}
IVF	Fully-expanded	58 / 66 (88)	25 / 28 (89)	83 / 94 (88)
	Expanding	63 / 74 (85)^{y}	32 / 38 (84)^{y}	95 / 112 (85)^{y}
	Subtotal	121 / 140 (86)^{y}	57 / 66 (86)^{y}	178 / 206 (86)^{y}

No. re-expanded embryos / No. vitrified-warmed embryos (%).

^{a,b} Different superscripts denote significant difference between the two developmental stages within the IVF or ICSI groups ($P < 0.05$).

^{x,y} Different superscripts denote significant difference between IVF and ICSI groups ($P < 0.05$).

The ICSI-derived blastocysts surviving the vitrification procedures contained a higher proportion of dead cells than IVF-derived counterparts (5-13% vs 2-4%; $P < 0.05$), but these proportions were not different from those of fresh control embryos (ICSI; 5-15%, IVF; 6-8%; **Table 3-2**). Total cell numbers in the vitrified-warmed blastocysts were comparable to those in the fresh control embryos, except in IVF-derived, Day 7 fully-expanded blastocysts.

Table 3-2. Mean ± SE number of total cells and proportion of dead cells in IVF- or ICSI-derived bovine blastocysts that survived MVC vitrification (assessed after 24 h of post-warming culture).

Origin	Day of harvest	Developmental stage	Vitrified blastocysts			Fresh blastocysts		
			N	Total	% Dead	N	Total	% Dead
ICSI	Day 7	Fully-expanded	14	140 ± 12^{a}	9 ± 3^{x}	13	146 ± 14^{x}	15 ± 4
		Expanding	15	102 ± 7^{b}	5 ± 1	11	126 ± 15^{c}	8 ± 2
	Day 8	Fully-expanded	7	102 ± 13	13 ± 4^{x}	4	114 ± 16	5 ± 2
		Expanding	6	94 ± 11	7 ± 2	9	86 ± 5^{d}	7 ± 6
IVF	Day 7	Fully-expanded	16	130 ± 9^{f}	3 ± 1^{y}	15	179 ± 12^{acey}	6 ± 2
		Expanding	15	116 ± 8	4 ± 2	16	104 ± 7^{b}	7 ± 2
	Day 8	Fully-expanded	14	113 ± 10	2 ± 1^{y}	12	118 ± 15^{d}	8 ± 4
		Expanding	16	95 ± 11	3 ± 1	10	95 ± 9	6 ± 2

[a,b] Different superscripts denote significant difference between the two developmental stages within the IVF or ICSI groups ($P < 0.05$).

[c,d] Different superscripts denote significant difference between the 2 d of blastocyst harvest within the IVF or ICSI groups ($P < 0.05$).

[e,f] Different superscripts denote significant difference between vitrified and fresh blastocysts within the IVF or ICSI groups ($P < 0.05$).

[x,y] Different superscripts denote significant difference between IVF and ICSI groups ($P < 0.05$).

The remaining blastocysts that survived vitrification were further cultured for up to 72 h to assess their ability to hatch from zonae pellucidae in vitro. An adverse effect of vitrification on the ability of blastocysts to hatch was found only in IVF-derived, Day 8 blastocysts (hatching rates of 41 and 70% in vitrified and fresh control groups, respectively; **Table 3-3**). The hatching rate of Day 8 vitrified-warmed blastocysts (41%) was also lower than that of Day 7 counterparts (63%). Hatching rate of ICSI-derived, Day 7 expanding blastocysts after vitrification (33%) was lower than that of ICSI-derived, Day 7 fully-expanded blastocysts (73%), as well as that of IVF-derived, Day 7 expanding blastocysts (65%).

Table 3-3. Hatching ability of IVF- or ICSI-derived bovine blastocysts that survived MVC vitrification (assessed after 72 h of post-warming culture).

Origin	Developmental stage	Day 7 blastocysts		Day 8 blastocysts	
		Vitrified	Fresh control	Vitrified	Fresh control
ICSI	Fully-expanded	8 / 11 (73)[a]	5 / 9 (56)	2 / 4 (50)	5 / 9 (56)
	Expanding	5 / 15 (33)[bx]	4 / 10 (40)	0 / 2 (0)	5 / 13 (38)
	Subtotal	13 / 26 (50)	9 / 19 (47)	2 / 6 (33)	10 / 22 (45)
IVF	Fully-expanded	26 / 42 (62)	27 / 36 (75)	6 / 11 (55)	15 / 19 (78)
	Expanding	31 / 48 (65)[cy]	22 / 40 (55)	5 / 16 (31)[d]	13 / 21 (62)
	Subtotal	57 / 90 (63)[c]	49 / 76 (64)	11 / 27 (41)[df]	28 / 40 (70)[e]

No. hatched embryos / No. surviving, cultured embryos (%).

[a,b] Different superscripts denote significant difference between the two developmental stages within the IVF or ICSI groups ($P < 0.05$).

[c,d] Different superscripts denote significant difference between the 2 d of blastocyst harvest within the IVF or ICSI groups ($P < 0.05$).

[e,f] Different superscripts denote significant difference between vitrified and fresh blastocysts within the IVF or ICSI groups ($P < 0.05$).

[x,y] Different superscripts denote significant difference between IVF and ICSI groups ($P < 0.05$).

Discussion

Information regarding cryotolerance of the ICSI-derived bovine embryos is limited to a report by Keskintepe & Brackett (2000); in that study, 16 expanded blastocysts harvested on Day 7, plus 16 blastocysts that hatched after an additional 18 h of culture were exposed to a conventional freezing regimen, resulting in cryosurvival rates of 75 and 88%, respectively. In the present study, ICSI-derived, fully-expanded bovine blastocysts harvested on Day 7 or 8 had similar ability to survive vitrification with IVF-derived counterparts. Conversely, expanding blastocysts derived from ICSI, but not IVF, were more sensitive to vitrification than fully-expanded blastocysts (**Table 3-1**). Based on differential cell staining of fresh blastocysts, we inferred that ICSI-derived expanding blastocysts and their IVF-derived counterparts were of comparable quality (in total cell

number and the ICM ratio; **Figure 3-5**); therefore, differences in embryo quality before vitrification apparently did not account for the higher sensitivity of ICSI-derived expanding blastocysts to vitrification. The relationship between the size of blastocysts and their cryotolerance has been investigated; larger blastocysts tolerated cryopreservation better than the smaller ones (Dinnyés et al. 1996, Keskintepe & Brackett 2000, Berthelot et al. 2007, George et al. 2008), with the exception of one report in cattle (Shirazi et al. 2009). For species-specific reasons, there was opposite size-dependent cryotolerance in human (Vanderwalmen et al. 2002) and horse (Hochi et al. 1995) blastocysts. It has been reported that tolerance to vitrification of in vitro-produced bovine blastocysts was improved when they were larger than 150 to 160 μm in diameter (Dinnyés et al. 1996, Dinnyés et al. 1999, George et al. 2008). Perhaps differential cryotolerance between ICSI- and IVF-derived expanding blastocysts was due to ICSI-derived expanding blastocysts being comparatively smaller than their IVF-derived counterparts, even though the mean total cell number was comparable. Further research is needed to determine why size-dependent cryotolerance was more prominent in ICSI-derived vs IVF-derived blastocysts.

There is also a general agreement that faster-developing bovine embryos are more likely to survive cryopreservation (Han et al. 1994, Saha et al. 1996, Dinnyés et al. 1999, Gómez et al. 2008). Therefore, in most studies of embryo cryopreservation, either all blastocysts harvested on Day 7 were used, or a subset was chosen, based on morphological characteristics. In the present study, the cryosurvival rate of Day 7 IVF-derived blastocysts achieved using the Cryotop (86%) was slightly lower than 94% survival reported using the same cryodevice (Kelly et al. 2004), and was comparable to or even higher than those achieved using other cryodevices for vitrification, e.g., open-pulled straws (OPS; 89-97%) (Vajta et al. 1999), hand-pulled glass micropipettes (84-94%) (Vieira et al. 2007), and electron microscope grids (58-98%) (Park et al. 1999). Moreover, in the present study, Day 8 blastocysts had similar survivability to their Day 7 counterparts (derived from either ICSI or IVF; **Table 3-1**). We have previously reported similar results in domestic cat blastocysts produced in vitro (Tsujioka et al. 2008). Dinnyés et al. (Dinnyés et al. 1996) reported a reasonable cryosurvival rate from IVF-derived Day 8 bovine embryos at the fully-expanded and hatched blastocyst stages (< 90%); however,

those embryos were produced by prolonged culture of Day 7 blastocysts.

A possible adverse effect of the cryopreservation process is to reduce the ability of the post-warming embryos to continue mitotic division (Kaidi et al. 1999), or to induce apoptosis or necrosis in some embryonic cells, especially ICM cells (Gómez et al. 2009). In the present study, except for IVF-derived Day 7 fully-expanded blastocysts, there was no adverse effect of vitrification/warming process on the total cell number of the blastocysts (**Figure 3-5**), which was in agreement with previous results reported for the mouse (Sheehan et al. 2006), cow (George et al. 2006), and cat (Tsujioka et al. 2008). The proportions of dead cells in vitrified/warmed blastocysts (2-13%) were comparable to those in fresh control blastocysts (5-15%), which was contradictory to previous studies with higher proportion of dead cells in vitrified/warmed embryos compared to fresh ones (14 vs 1-2% and 20-45 vs 5-14%) (Mucci et al. 2006, Shirazi et al. 2009). Perhaps these differences were due to better suitability of the Cryotop method compared to the OPS or straw vitrification procedures applied in the previous studies. Based on a higher proportion of dead cells in the ICSI-derived fully-expanded blastocysts after vitrification compared to the IVF-derived counterparts, perhaps embryonic cells in ICSI-derived blastocysts were more sensitive to vitrification procedures than their IVF-derived counterparts. However, non-vitrified control ICSI-derived blastocysts and vitrified-warmed blastocysts contained similar proportions of dead cells.

Although the ability of blastocysts to hatch out of their zonae can be used to assess embryo quality, hatching was greatly influenced by culture conditions (Krisher et al. 1999). In the present study, ICSI-derived expanding blastocysts hatched less than their IVF-derived counterparts (**Table 3-3**), although the initial total cell number and the proportions of the ICM were not significantly different. Perhaps either the culture conditions used in the present study were not optimal to support the development of the ICSI-derived embryos after blastulation, or the quality of the ICSI-derived blastocysts was inferior to those of their IVF-derived counterparts. The absence of a clear relationship between developmental age and hatching ability in the ICSI-derived blastocysts may be due to the limited number of embryos subjected to additional culture. Earlier initiation of hatching in ICSI-derived blastocysts, due to the presence of a piezo-drilled small opening in the zona pellucida, may have contributed to their lower hatching ability. The manner of

the hatching process frequently observed in the ICSI-derived blastocysts (data not shown) was similar to that previously reported in nuclear-transferred bovine zygotes (Laowtammathron et al. 2005).

In conclusion, the present study is apparently the first to document that ICSI-derived fully-expanded (≥ 200 μm in diameter) bovine blastocysts and IVF-derived blastocysts had similar ability to survive Cryotop vitrification. Since ICSI-derived, but not IVF-derived, expanding blastocysts were slightly sensitive to vitrification, it is recommended to prolong in vitro culture of ICSI-derived embryos (until they are fully expanded) prior to cryopreservation.

Chapter 4: Procedure for bovine ICSI, not sperm freeze-drying, impairs the function of the microtubule-organizing center

Abstract

This study was designed to investigate whether freeze-dried bull spermatozoa maintained the function of MTOC after rehydration and ICSI. In a preliminary attempt, the cleavage and blastocyst formation rates of ICSI zygotes derived from freeze-dried spermatozoa (36 and 1%, respectively) were found to be considerably lower than those of ICSI zygotes derived from control spermatozoa (67 and 21%, respectively) or of IVF zygotes (78 and 43%, respectively). In the main experiment, formation of sperm aster in the ICSI zygotes derived freeze-dried spermatozoa 7 h postinsemination occurred at a similar rate when compared with those in the ICSI zygotes derived from control spermatozoa (41 vs 49%). Among the zygotes exhibiting sperm aster formation, the extent of microtubule network assembly was comparable between freeze-dried and control groups. However, the MTOC of the ICSI zygotes was not as functional as that of IVF oocytes in terms of the aster formation rate (97%) and the fluorescent intensity of the microtubule network (2.0 folds). These results suggest that the freeze-drying process per se had no adverse effect on maintaining the MTOC function in bull spermatozoa.

Introduction

A centrosome is composed of a pair of centrioles surrounded by pericentriolar materials such as γ-tubulin, centrin and pericentrin. Since an interphase network of microtubules and the mitotic bipolar spindle are nucleated from the centrosome, the centrosome is considered to be MTOC (Glover et al. 1993). During fertilization in most mammalian species, the centrosome brought into an oocyte by a spermatozoon plays a critical role in sperm aster formation that brings both male and female pronuclei to the center of the newly formed zygote, as reported in humans (Simerly et al. 1995), rhesus

monkeys (Hewitson et al. 1996), rabbits (Pinto-Correia et al. 1994), pigs (Kim et al. 1998) and cattle (Navara et al. 1996). Interestingly, paternal inheritance of the MTOC does not occur in the mouse (Schatten et al. 1985) and rat (Woolley & Fawcett 1973), and the microtubule network developed from multiple cytoplasmic asters, instead of a single sperm aster, is involved in the migration of pronuclei (Manandhar et al. 1998).

Freeze-drying has been proposed as an alternative method to preserve mammalian spermatozoa (Wakayama & Yanagimachi 1998), although freeze-dried spermatozoa after rehydration lose their motility and application of ICSI technique is necessary. Rodent spermatozoa can be stored practically at refrigeration temperatures (Ward et al. 2003, Kaneko & Nakagata 2006, Hochi et al. 2008). However, in large domestic species, in vitro production of blastocysts derived from freeze-dried spermatozoa is still considered to be a challenging endeavor (Keskintepe et al. 2002, Kwon et al. 2004, Nakai et al. 2007). We have recently reported that the freeze-drying protocol slightly reduced the ability of bull spermatozoa to induce calcium oscillations (Abdalla et al. 2009a) and that it had no adverse effect on the active demethylation dynamics of the paternal genome (Abdalla et al. 2009b). Reports on the MTOC function of freeze-dried spermatozoa are available only in rabbits (Liu et al. 2004) and primates (Sánchez-Partida et al. 2008). In those reports, sperm preservation by freeze-drying had no adverse effects on the frequency of ICSI oocytes forming a sperm aster. Since the MTOC function of freeze-dried bull spermatozoa is not well-understood, this study was conducted to investigate whether freeze-dried bull spermatozoa maintained the MTOC function after rehydration and ICSI.

Materials and methods

Freeze-drying of bull spermatozoa

A frozen-thawed semen of Japanese Black bull was washed by Percoll, as described in Materials and methods of Chapter 2 (In vitro fertilization). The sperm pellet washed by Percoll was resuspended in EGTA buffer (10 mM Tris-HCl, Wako, Osaka, Japan; 50 mM EGTA; and 50 mM NaCl, pH 8.0) and washed twice with the same buffer. Aliquots (300 μL) of the sperm suspension (1×10^7 sperm/mL) were divided into 3-mL volume glass vials (No.1; Maruemu, Osaka, Japan) and then frozen at a depth of 1 cm in LN_2. The

frozen samples were transferred onto the shelf (–30 ºC) of a programmable freeze-dryer (ALPHA 2-4; Christ, Harz, Germany). Primary drying of the samples was performed for 14 h at 0.37 hPa with the shelf temperature increased to +30 ºC at the end of this stage, and secondary drying was performed for 3 h at 0.001 hPa (**Figure 4-1**). After filling the vials with inactive N_2 gas and sealing them with rubber caps and aluminum stoppers, the vials were stored in a refrigerator (+4 ºC) for 1 week. The freeze-dried samples were rehydrated by adding 300 µL of ultrapure Milli-Q water and then washed twice with mBO medium at 300 *g* for 5 min each (Abdalla et al. 2009a, Abdalla et al. 2009b).

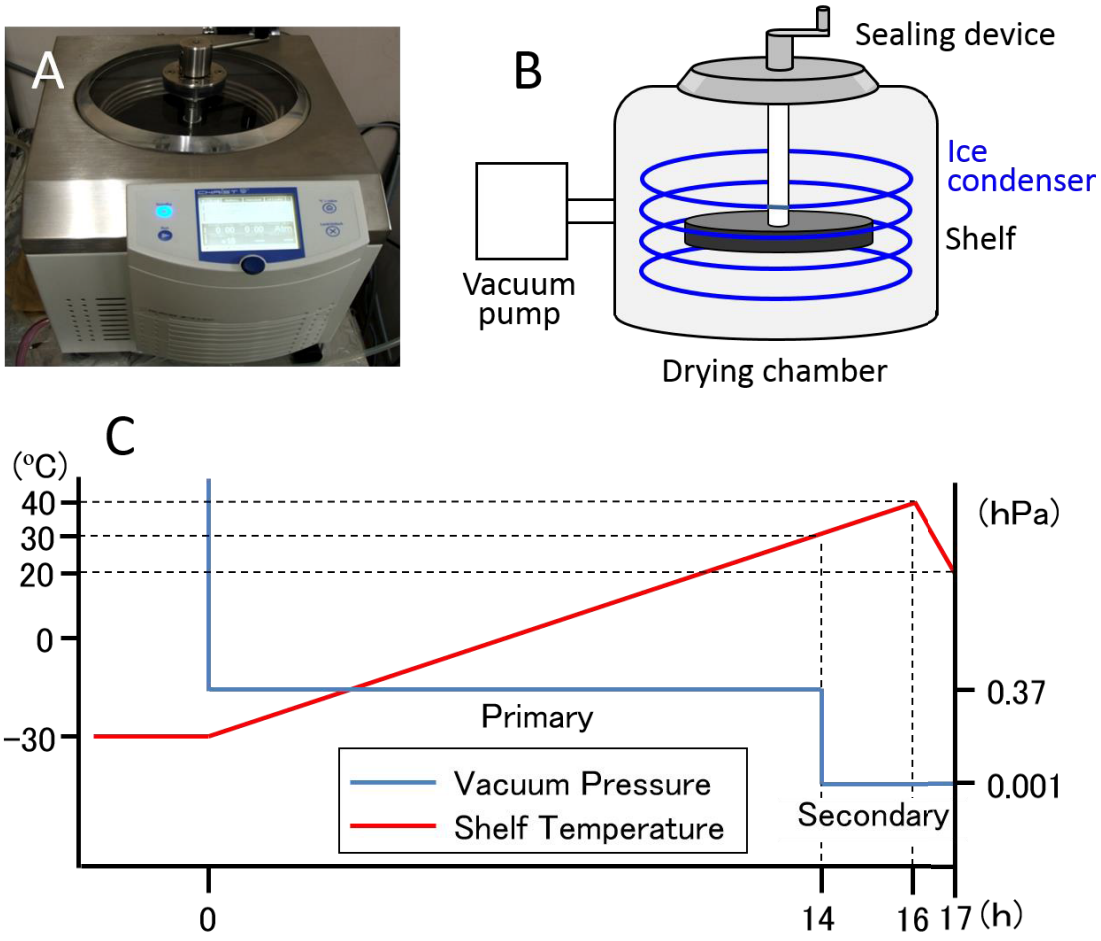

Figure 4-1. Freeze-dryer ALPHA 2-4 employed in this study. (A) Overview, (B) Base system, (C) Program for vacuum pressure and shelf temperature. Drying chamber contains a temperature-controllable shelf and an ice condenser to collect water vapor from the product. Vacuum pumps are usually of the rotary, oil-sealed design, and are capable of maintaining a system pressure of < 0.05 hPa.

Production of bovine blastocysts

Zygotes were produced in vitro either by conventional IVF or ICSI using control or freeze-dried spermatozoa, as described in Materials and methods of Chapter 2 (In vitro fertilization / Intracytoplasmic sperm injection / In vitro culture). However for freeze-dried spermatozoa, sperm suspension in mBO medium was not treated with DTT because aggregation of DTT-treated sperm made it difficult to handle a single spermatozoon.

Alkaline comet assay

An alkaline comet assay (Singh et al. 1988) was used to estimate the DNA damage in freeze-dried spermatozoa in comparison with control spermatozoa. Spermatozoa treated with 10 mM H_2O_2 for 20 min at 4 ºC served as a positive control. According to the operation manual of the CometAssay® kit (Trevigen, Inc., Helgerman, CT, USA), aliquots of spermatozoa (1×10^4 cells/10 μL) were mixed with 0.5% low-melting agarose gel (90 μL), added to agarose slides, treated with lysis solution for 3 h (including 10 mM dithiothreitol for 0.5 h and 4 mM lithium diiodosalicylate for 1.5 h) and then processed with electrophoresis under a pH >13 alkaline condition (15 V, 20 min). The slides were stained with SYBR Green, and the captured BMP images of the comet (50 comets per sample) were analyzed by the Comet Score software (**Figure 4-2**).

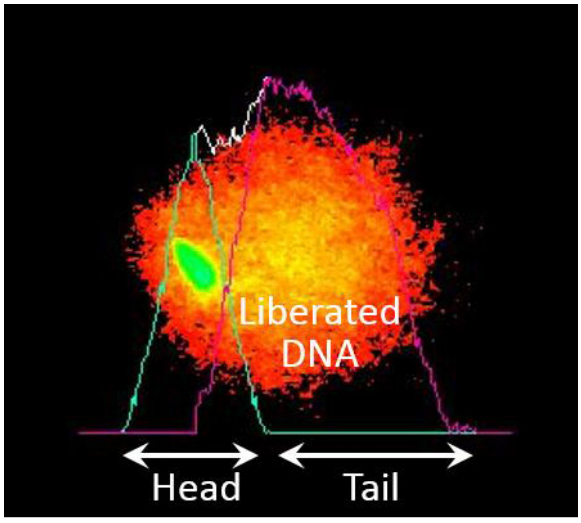

Figure 4-2. Comet image analysis. Moment was calculated as the length of the comet tail × the % DNA liberated.

The length of the comet tail (pixel) was measured from the edge of head, and the DNA content in the comet tail was determined with the relative fluorescent intensity and defined as % DNA liberated. The DNA fragmentation index (Moment) was calculated as the length of the comet tail × the % DNA liberated.

Immunostaining of pronuclear zygotes

To assess MTOC function of spermatozoa in pronuclear zygotes, the oocytes injected with control and freeze-dried spermatozoa as well as IVF derived ones were subjected to immunostaining. For control and freeze-dried groups, activated oocytes following ICSI were further cultured in TCM-199/5% FBS for 3 h at 38.5 ºC under 5% CO_2 in air. Then, the zygotes were sampled for immunostaining (time equivalent to 7 h after insemination). For IVF group, the period for coincubation of oocytes with spermatozoa (preincubated for 2 h in the fertilization medium) was shortened to 2 h in order to restrict the fertilization window. The inseminated oocytes were further cultured in TCM-199/5% FBS for 6 h (6-8 h after sperm penetration). As it was defined that sperm penetration occurred in the middle of the coincubation period, oocytes were sampled 8 h after initiation of coincubation. All the samples at 7 h postfertilization were subjected to immunostaining against α-tubulin, as described in Materials and methods of Chapter 2 (Immunostaining of pronuclear zygotes).

Statistical analysis

Experiments were repeated at least 4 times in each group. Percentage data in each replicate were arcsin-transformed and subjected to one-way ANOVA. Differences among means in the three groups were analyzed by a Bonferroni test. Values for size of sperm-aster were compared by the Student's *t*-test. A value of $P<0.05$ was chosen as an indication of statistical significance.

Results

In a preliminary part of this study, developmental potential of bovine ICSI zygotes derived from freeze-dried and control spermatozoa as well as that of IVF zygotes was

compared (**Table 4-1**). The morphology of freeze-dried bull spermatozoa was shown in **Figure 4-3**. There was no significant difference in the proportion of oocytes extruding the second polar body between the freeze-drying and control groups (74%, 130/176, vs 73%, 98/135). Culture of presumptive zygotes that survived ICSI of freeze-dried spermatozoa resulted in a lower cleavage rate (36%) when compared with the cleavage rates of zygotes derived from control spermatozoa (67%) and conventional IVF (78%). Only one blastocyst (**Figure 4-4**) was obtained from culture of 176 zygotes in freeze-dried group (< 1%), while 21 and 43% of the control sperm- and IVF-derived zygotes developed into blastocysts during the culture period, respectively.

Table 4-1. Developmental potential of bovine oocytes injected with bull spermatozoa freeze-dried and rehydrated.

	No. (%) of oocytes			
Groups	Inseminated	Survived	Cleaved	Developed to blastocysts
Control	161	135 (84)[a]	91 (67)[b]	29 (21)[b]
Freeze-dried	193	176 (91)[a]	63 (36)[a]	1 (1)[a]
Conventional IVF	145	145 (100)[b]	113 (78)[b]	62 (43)[c]

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

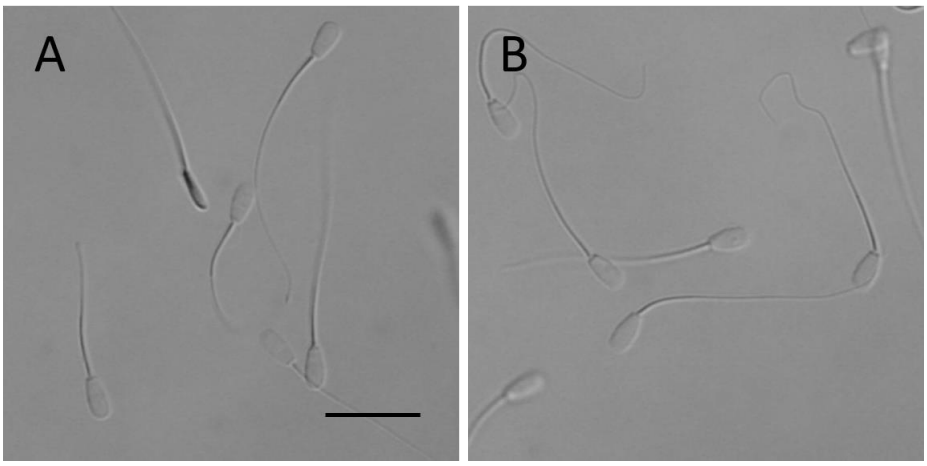

Figure 4-3. No morphological difference between frozen-thawed control (A) and freeze-dried / rehydrated bull spermatozoa. Scale bar represents 30 μm.

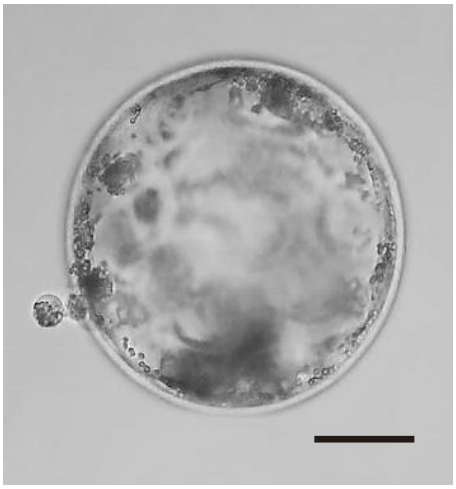

Figure 4-4. A bovine expanding blastocyst produced by intracytoplasmic injection of freeze-dried spermatozoon. Debris can be seen near the pin-hole made by ICSI. Scale bar represents 50 μm.

An alkaline comet assay indicated that the DNA fragmentation index (moment: length of comet tail × % DNA liberated) was not significantly different between control and freeze-dried spermatozoa, while a significant increase of the index was detected in H_2O_2-treated positive control spermatozoa (**Table 4-2**, further discussion at Chapter 5).

Table 4-2. DNA damage in freeze-dried bull spermatozoa estimated by an alkaline comet assay

	Comet parameters		
Spermatozoa	Tail length (pixel)	DNA liberated (%)	Moment[1]
Fresh	12.1 ± 2.6[a]	23.3 ± 6.6[a]	4.6 ± 0.6[a]
Freeze-dried	16.4 ± 6.8[a]	21.7 ± 6.3[a]	5.4 ± 2.8[a]
H_2O_2-treated	404.8 ± 64.0[b]	63.7 ± 5.0[b]	282.9 ± 62.9[b]

[1]Moment = Comet tail length × % DNA liberated / 100.

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

The proportion of zygotes exhibiting sperm aster formation was comparable between freeze-dried and control groups (41%, 14/34, vs 49%, 16/33), both of which were significantly lower than that of IVF zygotes (97%, 31/32), as shown in **Figure 4-5A**. Relative fluorescent intensity of the aster network (defined the mean value in control group as 1.0) was calculated using a stacked image taken by confocal microscope. The fluorescent intensity of the aster network was highly variable but did not differ between the freeze-dried (relative value, 0.9) and control groups, as shown in **Figure 4-5B**. On the other hand, the relative fluorescent intensity of the aster network in the IVF group was 2.0-folds higher than that in the control group.

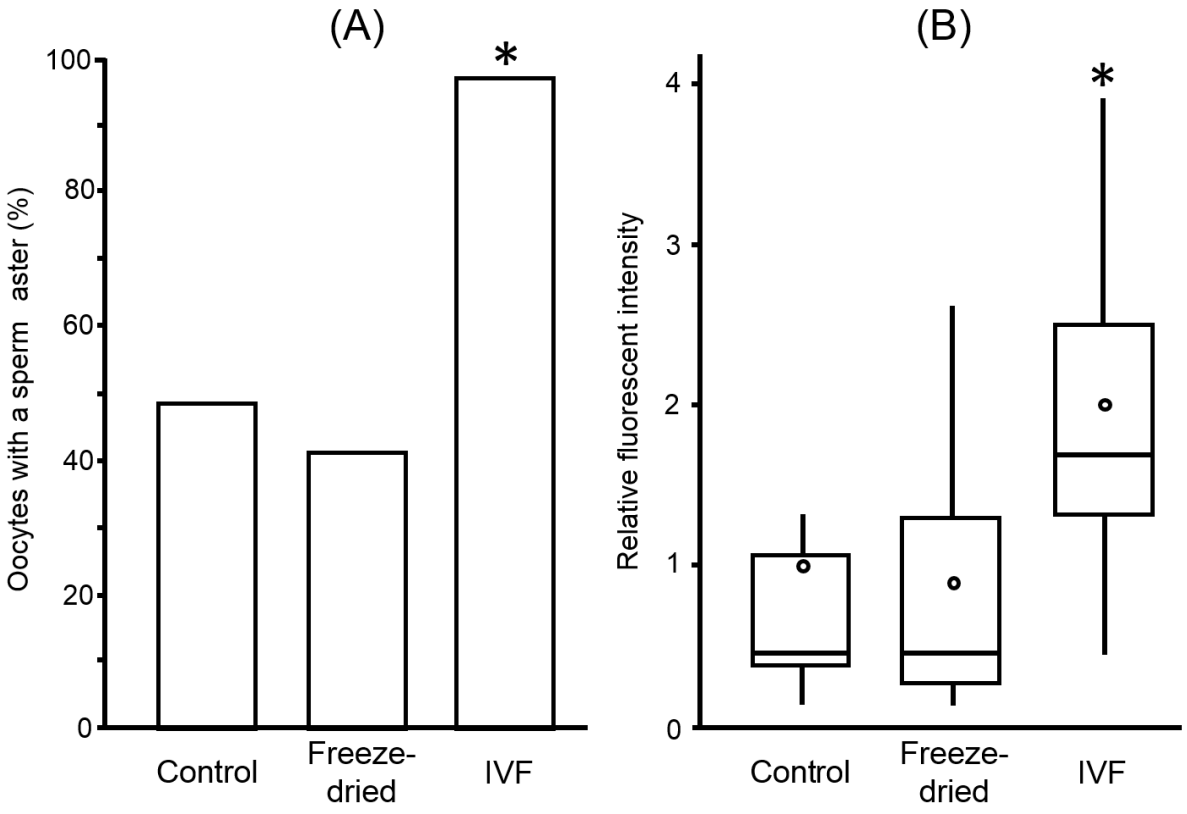

Figure 4-5. MTOC function in freeze-dried bull spermatozoa. (A) Proportions of sperm aster-formed ICSI zygotes derived from control and freeze-dried spermatozoa as well as conventional IVF. The asterisk shows a significant difference from the value in the control ICSI group ($P < 0.05$). (B) Boxplots for overall fluorescent intensities of the MTOC network in the aster-forming zygotes. The asterisk shows a significant difference from the value in the control ICSI group ($P < 0.05$).

Discussion

Firstly, we tried to investigate the ability of freeze-dried bull sperm to support blastocyst development using the protocol proven effective for rodent spermatozoa (Hochi et al. 2008). However, the blastocyst yield of freeze-dried group was extremely low compared with control group (**Table 4-1**). Although no analyses for karyotype or ploidy were performed with these blastocysts, the supplementary activation regimen for ICSI oocytes employed here (ionomycin plus ethanol at a 4 h interval) was unlikely to produce parthenogenetically developing blastocysts (Abdalla et al. 2009c). Considering the poor developmental potential of oocytes injected with freeze-dried spermatozoa, it was investigated whether the centrosome in bull spermatozoa could be a possible target for detrimental injuries induced by the freeze-drying process.

Functional analysis of the MTOC in the freeze-dried bull spermatozoa revealed that the poor developmental potential of oocytes injected with freeze-dried bovine spermatozoa to the early cleavage or blastocyst stage was unlikely to be caused by detrimental damage in the centrosome of the spermatozoa (**Figure 4-5**). The target of the freeze-drying damage that is more responsible for the poor developmental competence of oocytes injected with freeze-dried spermatozoa may be DNA and/or related proteins (Kusakabe et al. 2001, Kawase et al, 2007, Hochi et al. 2008, Kusakabe et al. 2008, Kawase et al. 2009), although it is possible that several small amounts of functional damage may have accumulated to disturb a series of postfertilization events. Therefore, overall improvement of the freeze-drying protocol may be required for bull spermatozoa. On the other hand, the difference in the MTOC function between ICSI- and IVF-derived zygotes may explain the difference in their developmental competence, suggesting a further requirement of improving the bovine ICSI protocol.

Hamano et al. (1999) reported successful calf production following ooplasmic injection of flow cytometrically sorted sperm heads. Ultrasound sonication was applied to prepare the sperm heads in the above study, but it was unclear whether the spermatozoal centrosome was dissociated from the heads by this treatment. Goto et al. (1991) reported in cattle that blastocyst yields from oocytes injected with sperm heads were lower than those with whole sperm. Even though a sperm aster failed to function as the MTOC in the

oocytes injected with sperm heads, a microtubule network developed from multiple cytoplasmic asters may be involved in the migration of pronuclei (Manandhar et al. 1998). A few weak points of our experimental design regarding the MTOC functional analysis include the injection of freeze-dried spermatozoa without DTT treatment and the penetration of capacitated / acrosome-reacted spermatozoa into IVF oocytes, both of which were impossible to control. The DTT facilitates not only destabilization of nuclear packaging in the sperm head but also organization of α-tubulin in the sperm centrosome, from which microtubules are nucleated (Schatten 1994). However, ICSI using bull spermatozoa with or without DTT pretreatment resulted in similar blastocyst yields (14 vs 11%; our unpublished data). Since the effect of acrosomal contents brought nonphysiologically into the oocyte on MTOC function remains unclear, further investigation is required to clarify this interaction.

In conclusion, the process of freeze-drying and rehydration for bull spermatozoa had no adverse effect on MTOC function (sperm aster formation and microtubule network assembly), suggesting that the poor blastocyst yield from the oocytes injected with freeze-dried spermatozoa was not due to impaired MTOC function in the spermatozoa. On the other hand, the significant difference in MTOC function observed in the ICSI and IVF zygotes may be responsible for the lower yield of blastocysts from ICSI-derived oocytes compared with IVF-derived ones.

Chapter 5: Adverse effect of cake collapse on the functional integrity of freeze-dried bull spermatozoa

Abstract

Under optimal freeze-drying conditions, solutions exhibit a cake-like porous structure and become amorphous at T_g'. However, if the solution temperature is higher than the drying-phase T_g', the glassy matrix undergoes viscous flow, resulting in cake collapse. The purpose of the present study was to investigate the effect of cake collapse on the integrity of freeze-dried bull spermatozoa. In a preliminary experiment, factors affecting the T_g' of conventional EGTA buffer (10 mM Tris-HCl, 50 mM EGTA and 50 mM NaCl) were investigated in order to establish the main experimental protocol because EGTA buffer T_g' was too low (-45.0 ºC) to suppress collapse. Modification of the EGTA buffer composition by complete removal of NaCl and addition of 0.5 M trehalose (mEGTA buffer) resulted in an increase of T_g' up to -27.7 ºC. In the main experiment, blastocyst yields after ooplasmic injection of freeze-dried sperm preserved in collapsed cakes (drying temperature: 0 or -15 ºC) were significantly lower than those of sperm preserved in non-collapsed cake (drying temperature: -30 ºC). In conclusion, freeze-dried cake collapse may be undesirable for maintaining sperm functions to support embryonic development, and can be inhibited by controlling both T_g' of freeze-drying buffer and temperature during the drying phase.

Introduction

Although cryopreservation is the most common method for preservation of bull spermatozoa (Curry 2000), LN_2 use is associated with various problems such as high maintenance cost and risk of accidental loss of frozen cell stock. To overcome these problems, freeze-drying has been proposed as an alternative method for sperm preservation. Wakayama & Yanagimachi (1998) reported that freeze-dried mouse sperm

stored at refrigerator temperature could support full-term development although application of ICSI is needed for their fertilization because all of rehydrated spermatozoa were dead and lose their motility. Until now, successful birth of live offspring derived from freeze-dried spermatozoa has been effectively reported only in rodents including mouse (Wakayama & Yanagimachi 1998, Kusakabe et al. 2001, Kaneko et al. 2003, Kaneko & Nakagata 2006, Kusakabe et al. 2008), rat (Hirabayashi et al. 2005, Hochi et al. 2008) and hamster (Muneto & Horiuchi 2011) using the ICSI technique, in addition to rabbit with very low offspring rate as 0.4% (Liu et al. 2004). For large domestic species including cattle and pigs, blastocysts have been harvested from ICSI oocytes with freeze-dried sperm cells (Keskintepe et al. 2002, Kwon et al. 2004, Martins et al. 2007). Several studies have been conducted to improve the developmental ability of oocytes injected with freeze-dried spermatozoa, paying special attention to buffer composition (Kusakabe et al. 2001, Kaneko et al. 2003, Kaneko & Nakagata 2006, Martins et al. 2007). A relatively simple buffer consisting of Tris-HCl, EGTA and NaCl (defined hereafter as EGTA buffer) has been used for suspending and freeze-drying spermatozoa (Kusakabe et al. 2001, Kaneko et al. 2003).

In addition to the buffer composition, the drying conditions are also important for production of pharmaceutical proteins (Jiang & Nail 1998). Frozen samples are generally dried at the maximum allowable product temperature (T_{max}) to acquire the highest dehydration speed (Pikal 1985). For a solute system which forms amorphously after freezing, the theoretically optimal value of T_{max} was restricted by T_g'. The solution exhibits a cake-like porous structure under the optimal drying conditions. But, if the product temperature is higher than the T_g' during the drying phase, the glassy matrix undergoes viscous flow, resulting in loss of its porous structure. This loss is defined as a collapse phenomenon (Nail et al. 2002, Sundaramurthi & Suryanarayanan 2012). Collapsed cakes contain high amounts of residual water (Wang et al. 2004), and prolonged reconstitution time may be required during rehydration due to reduced surface area (Tsourouflis et al. 1976, Adams & Irons 1993). In freeze-drying of pharmaceutical proteins, collapse can adversely affect acellular product stability (Jiang & Nail 1998, Lueckel et al. 1998, Passot et al. 2007). Therefore, T_{max} is set to be the temperature which is slightly lower than T_g'. However, it remains unclear whether collapse incidence in

freeze-dried sperm suspensions has a detrimental effect on rehydrated cell function. In previous studies, we used freeze-drying conditions for rat and bull spermatozoa, but we did not consider this phenomenon, and we recently observed that those conditions can produce cake collapse (Hara et al. 2014). Additionally in somatic cell freeze-drying for nuclear transfer in sheep, collapse can be confirmed qualitatively in figures which depict its typical characteristics (Loi et al. 2008).

The purpose of the present study was to investigate the effect of cake collapse on the functional integrity of freeze-dried and rehydrated bull spermatozoa. Since it was found that T_g' of the EGTA buffer was too low to suppress collapse, in a preliminary experiment (Experiment 1), factors affecting T_g' (including composition of the EGTA buffer and cooling rate during freezing) were examined using calorimetric measurements in order to establish the subsequent experimental protocol. In the main experiment (Experiment 2), collapsed and non-collapsed cakes were produced by drying bull sperm suspensions in modified EGTA buffers at different temperatures. T_g of sperm suspension cakes was measured to confirm freeze-drying completeness, and the functional integrity of the rehydrated sperm cells was assessed by ICSI, alkaline comet assay and transmission electron microscopic observation (TEM).

Materials and methods

Experimental design

In Experiment 1, the T_g' of the EGTA buffer conventionally used for sperm freeze-drying was determined by differential scanning calorimetry (DSC), and the effect of buffer composition modification (removal of NaCl, and addition of trehalose at different concentrations) on T_g' was investigated. Effects of three different cooling rates were also investigated.

In Experiment 2, bull spermatozoa were resuspended in the modified EGTA buffer and freeze-dried under three different temperatures. Both collapsed and non-collapsed cakes were used for measurements of residual water content and T_g. The functional integrity of the rehydrated sperm cells was assessed by blastocyst yield 8 days after ICSI, followed by alkaline comet assay and TEM.

Measurement of T_g' and T_g by DSC

To investigate the effect of buffer composition on T_g', the EGTA buffer consisting of 10 mM Tris-HCl, 50 mM EGTA and 50 mM NaCl (pH 8.0) was modified by complete removal of NaCl and/or addition of trehalose to give a final concentration of 0.01, 0.05, 0.1, 0.5 and 1.0 M. Fifteen μL of the EGTA buffer with or without the above-mentioned modifications was transferred to an aluminum pan and cooled to -80 ºC in DSC (Seiko Instruments Inc., Chiba, Japan). The aluminum pan was then scanned at a rate of 10 ºC/min until it reached 20 ºC, using an empty aluminum pan as a reference. Otherwise, the cooling rate was adjusted to be -1 or -20 ºC/min in DSC, or a presumed < -1,000 ºC/min by direct plunging into LN_2 (5 min). In this series, NaCl-free EGTA buffer supplemented with 0.5 M trehalose, defined hereafter as "mEGTA buffer", was used.

For T_g measurements of sperm suspension cakes, the cakes (> 1 mg) were collected and transferred to aluminum pans in a glove box filled with nitrogen gas and were then hermetically sealed. Cooling rate of the cakes in DSC was -20 ºC/min and the scanning rate was 10 ºC/min. The second scanning was conducted after annealing at 110-130 ºC for 5 min to eliminate interference from enthalpy relaxation (Tant & Wilkes 1981), because the first DSC thermogram of the cake was too unclear to determine T_g.

Freeze-drying of bull sperm

A frozen-thawed semen of Japanese Black bull was washed by Percoll, as described in Materials and methods of Chapter 2 (In vitro fertilization). The sperm pellet was re-suspended in 4 mL of mEGTA buffer and then washed twice (5 min at 300 *g* each). Fifteen μL of sperm suspension at a concentration of 2-4 × 10^7 sperm/mL was placed in a 5-mL volume glass vial and transferred onto shelf (pre-cooled to -30 ºC) of a programmable freeze-dryer (ALPHA 2-4; Christ, Harz, Germany). After freezing for 30 min (the cooling rate between +25 and -25 ºC was estimated as -20 ºC/min), the sperm suspension was dried for 6 h at 0, -15 or -30 ºC relative to the shelf temperature. The chamber pressures employed during the drying phase were determined as one-third of the vapor pressure of ice at each temperature (Nail et al. 2002); they were 1.98, 0.57 and 0.12 hPa for drying at 0, -15 and -30 ºC, respectively. After sealing of vials with rubber caps and aluminum stoppers, the samples were stored overnight at -20 ºC. Residual water

contents in freeze-dried cakes were calculated gravimetrically, using reference weights of sperm suspension before freeze-drying (=100% content) and after heating in oven at 96 ºC for 24 h (=0% content).

Blastocyst production by ICSI

Freeze-dried spermatozoa were rehydrated with 15 μL of ultrapure water immediately before ICSI, and non-dried control spermatozoa were those after Percoll-washing. Each sperm suspension was washed twice with mBO medium at 300 *g* for 5 min. ICSI was performed as described in Materials and methods of Chapter 2 (Intracytoplasmic sperm injection). The ICSI oocytes prepared within 1 h of rehydration were treated with 5 μM ionomycin in Ca^{2+}/Mg^{2+}-free PBS for 5 min and incubated in TCM-199/5% FBS at 38.5 ºC under 5% CO_2 in air for 3 h. Next, the oocytes were treated with 7% ethanol in Hepes-buffered TCM-199 supplemented 1 mg/ml PVP for 5 min and subsequently 2 mM 6-dimethylaminopurine (6-DMAP) in mSOFaa /5% FBS at 38.5 ºC under 5% CO_2 in air for 3 h. IVC was conducted as described in Materials and methods of Chapter 2 (In vitro culture).

Alkaline comet assay

Alkaline comet assay was conducted as described in Materials and methods of Chapter 4 (Alkaline comet assay), with a few modifications in the concentration of agarose gel (1% instead of 0.5%) and the voltage during electrophoresis (10 V instead of 15 V). Half of the sperm suspension was treated with 10 mM H_2O_2 for 20 min at 4 ºC before being mixed with low-melting agarose gel.

Ultrastructural analysis

TEM was applied to the sperm freeze-dried at 0 and -30 ºC and non-dried control spermatozoa. Sperm cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4 ºC. After centrifugation at 300 *g* for 5 min, the sperm pellets were post-fixed with 2% osmium tetraoxide in the same buffer at 4 ºC for 1 h. The sperm cells were subsequently dehydrated in the graded series of ethanol, substituted with the propylene oxide and embedded in epoxy resin (Okenshoji Co., Tokyo, Japan). Ultrathin sections were cut on an

ultra-microtome (Leica Microsystems, Wetzlar, Germany) at 0.1 μm thicknesses, mounted on copper grids and stained with 1% uranyl acetate and lead citrate. Electron micrographs were taken with a transmission electron microscope (JEOL Ltd., Tokyo, Japan). The number of sperm with membrane gaps was recorded.

Statistical analysis

Arcsin-transformed percentage data in residual water content, cleavage and blastocyst yield were compared using one-way ANOVA. Values of T_g' in freeze-drying buffers, T_g of freeze-dried cakes and tail moment were also compared using one-way ANOVA. When ANOVA reached significance, differences among means were analyzed using *post hoc* Tukey's tests. Incidences of membrane damage in TEM were compared using a chi-square test with a Bonferroni correction. Values of $P < 0.05$ were considered significant.

Results

Experiment 1

T_g' of the conventional EGTA buffer was as low as -45.0 ± 0.61 ºC (mean ± SD) in DSC measurement (**Figure 5-1** and **Figure 5-2**). Complete removal of NaCl from the EGTA buffer resulted in a T_g' increase to -41.0 ± 0.59 ºC (**Figure 5-2**). Supplementation of trehalose to the NaCl-free EGTA buffer with trehalose (0.01 to 0.5 M) made it possible to further increase the T_g' (up to -27.7 ± 0.36 ºC; **Figure 5-1** and **Figure 5-2**), but supplementation with 1.0 M trehalose had no further effect on T_g' increase and rendered post-centrifugation sperm retrieval difficult due to the high specific gravity. The T_g' of mEGTA buffer frozen rapidly in LN_2 (-29.4 ± 0.08 ºC) was significantly lower than when frozen slowly (-27.2 ± 0.94 and -27.6 ± 0.05 ºC at -1 and -20 ºC/min, respectively).

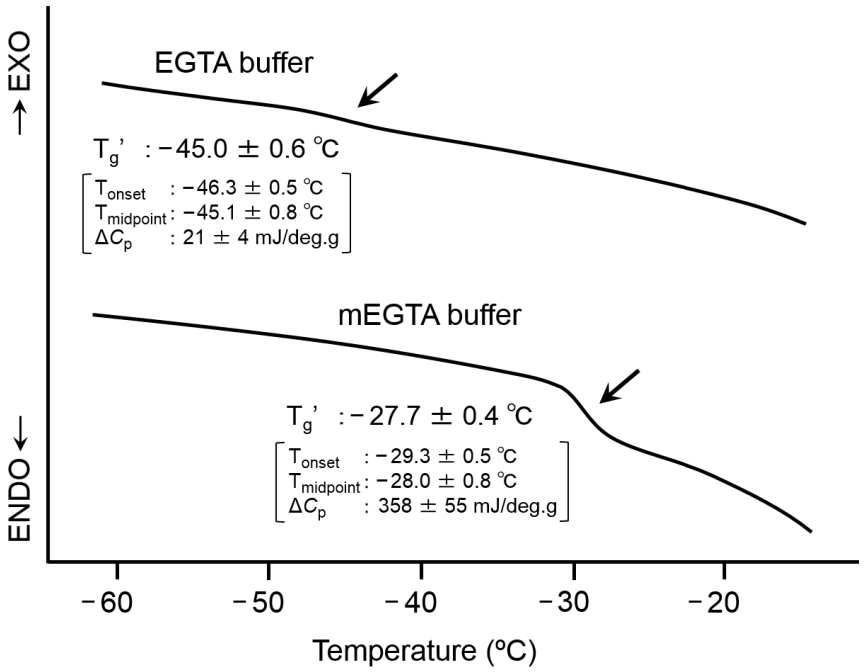

Figure 5-1. DSC thermogram of EGTA buffer and mEGTA buffer. The arrow indicates the glass transition temperature of the maximally freeze-concentrated phase (T_g') of the buffer. Data are expressed as the mean ± SD of 3 replicates in each group. EXO and ENDO indicate exothermic heat flow and endothermic heat flow, respectively.

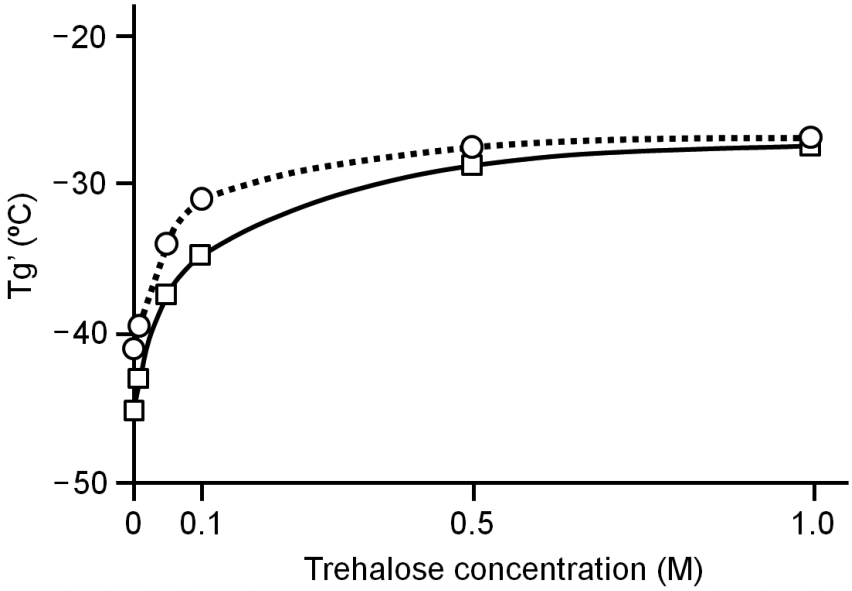

Figure 5-2. Effect of EGTA buffer composition on the glass transition temperature of the maximally freeze-concentrated phase (T_g'). Squares with solid lines represent EGTA buffers, and circles with dotted lines represent NaCl-free EGTA buffers.

Experiment 2

Freeze-dried cake collapse was observed when the drying phase temperature was higher than the mEGTA buffer T_g' (**Figure 5-3A** and **Figure 5-3B**). On the other hand, when processed at -30 ºC the sperm suspension cake exhibited a silky porous structure without any signs of collapse (**Figure 5-3C**). Collapsed cakes contained a higher amount of residual water when compared with non-collapsed cakes (3.3-3.6% vs 0.7%), as shown in **Table 5-1**. T_g values of collapsed cakes were significantly lower than that in their non-collapsed counterparts (13.5-14.1 ºC vs 50.1 ºC; **Table 5-1**).

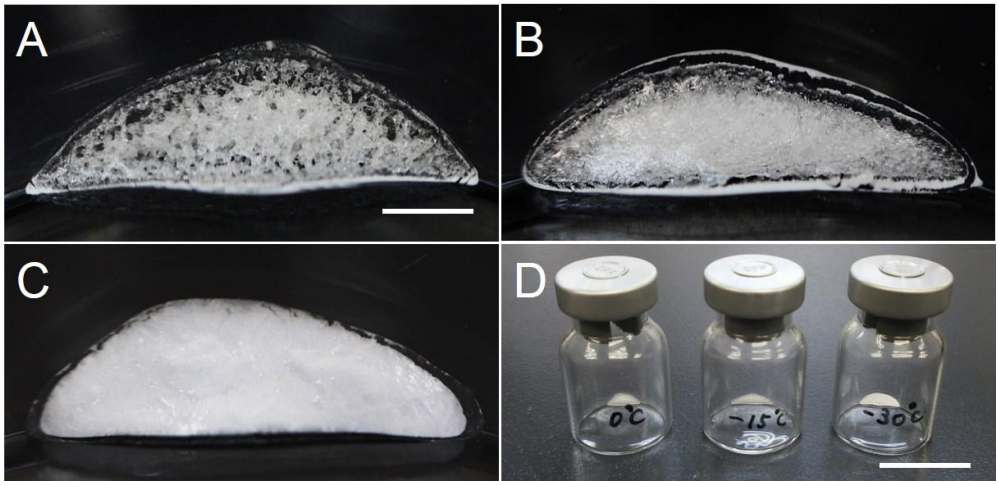

Figure 5-3. Appearance of freeze-dried cakes. (A) Collapsed cake dried at 0 ºC, (B) Collapsed cake dried at -15 ºC, (C) Non-collapsed cake dried at -30 ºC, (D) Zoomed-out view of the glass vial containing a cake. Scale bars represent 2 mm (A to C) and 2 cm (D).

Table 5-1. Residual water contents and T_g of freeze-dried cakes.

Temperature during drying phase (ºC)	Residual water contents (%)	T_g (ºC)
0	3.6 ± 0.1^a	14.1 ± 3.3^a
-15	3.3 ± 0.2^a	13.5 ± 2.1^a
-30	0.7 ± 0.1^b	50.1 ± 1.0^b

Data are expressed as the mean ± SD of 3 replicates in each group.

[a,b] Different superscripts denote significant difference within columns ($P < 0.05$).

Embryonic cleavage ability with freeze-dried spermatozoa was comparable regardless of collapse incidence, but blastocyst yields after injection of sperm dried at 0 and -15 ºC were lower than those dried at -30 ºC (0.7-3.7% vs 14.2%; **Table 5-2**). Both cleavage rate and blastocyst yield in the non-dried control group were significantly higher than in freeze-dried groups. Alkaline comet assay revealed that the freeze-drying process did not induce sperm DNA damage when the baseline of tail moment was not stimulated by H_2O_2 treatment (**Table 5-3**). After application of the H_2O_2 treatment, harmful effects of freeze-drying on sperm DNA integrity were clearly detected (almost double with respect to control), but there was no significant difference in the values of tail moment between sperm samples dried at 0 and -30 ºC. Analysis of TEM images revealed that sperm with severe or moderate membrane gaps (**Figure 5-4A** and **Figure 5-4B**) were frequently observed after drying at 0 ºC vs -30 ºC (43.6%, 140/321 vs 28.7%, 90/314; $P < 0.05$). These proportions were significantly higher than those in non-dried control sperm (10.9%, 36/330). All acrosome membranes of freeze-dried spermatozoa appeared to be damaged regardless of drying temperature.

Table 5-2. Developmental potential of bovine oocytes injected with bull spermatozoa dried at different temperatures.

Freeze-drying	Temperature during drying phase (ºC)	Oocytes (n [%])*		
		Sperm-injected	Cleaved	Developed to blastocysts
+	0	173	67 (37.9 ± 5.3)[a]	1 (0.7 ± 0.6)[a]
+	-15	173	70 (39.1 ± 4.0)[a]	3 (3.7 ± 1.6)[a]
+	-30	172	68 (41.5 ± 3.1)[a]	9 (14.2 ± 2.5)[b]
-	-	149	89 (60.5 ± 2.4)[b]	27 (30.5 ± 4.3)[c]

* Percentages are expressed as the mean ± SEM of at least 4 replicates in each group. Blastocyst yields are calculated from the number of cleaved oocytes.

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

Table 5-3. DNA damage of freeze-dried bull sperm detected by alkaline comet assay.

Freeze-drying	Temperature during drying phase (ºC)	Values of tail moment	
		Standard protocol	H_2O_2-treated
+	0	6.8 ± 0.5	65.4 ± 1.3[a]
+	-30	6.3 ± 0.7	65.6 ± 1.6[a]
-	-	6.9 ± 0.3	37.1 ± 2.0[b]

Data are expressed as the mean ± SD of 3 replicates in each group.
[a,b] Different superscripts denote significant difference within columns ($P < 0.05$).

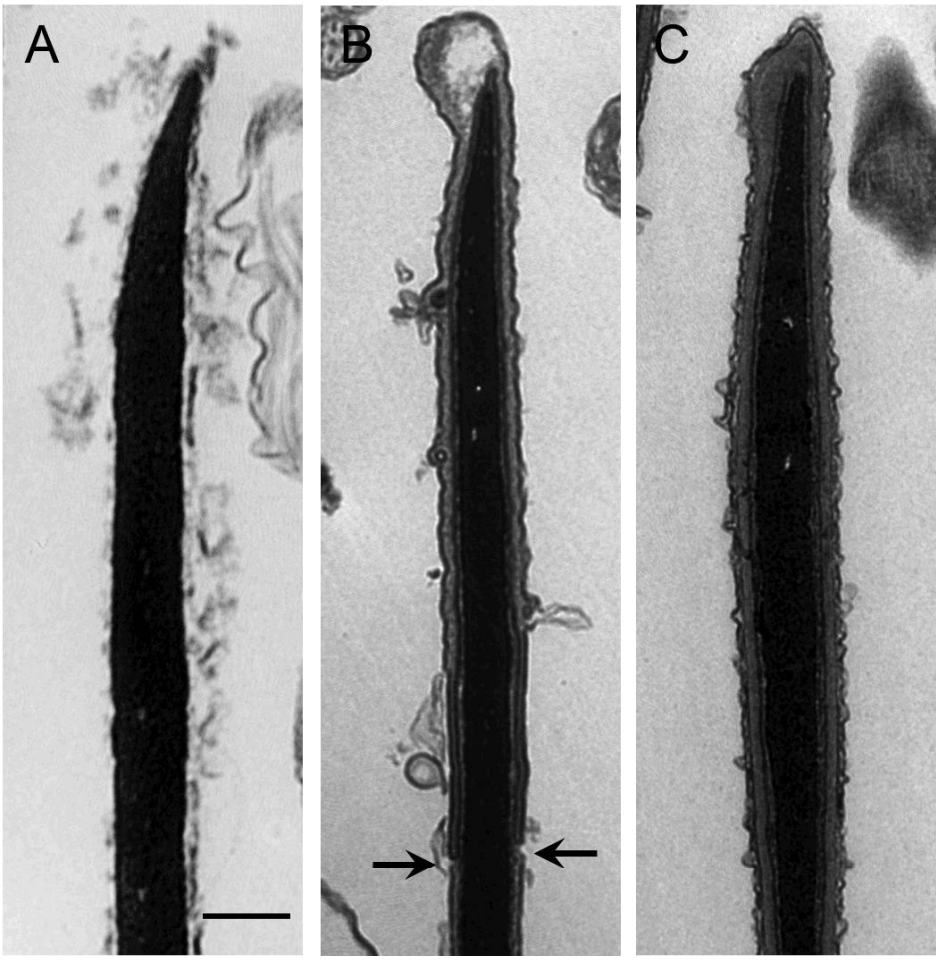

Figure. 5-4. Ultrastructural damage observed in sperm plasma membrane. (A) Sperm with severe membrane gaps, (B) Sperm with moderate membrane gaps, and (C) Intact spermatozoa. Arrows indicate break of sperm plasma membranes. Scale bar represents 0.4 μm.

Discussion

In sperm preservation by freeze-drying, maintenance of cellular function can be influenced by drying conditions. However, few studies have focused on the drying condition for mammalian sperm freeze-drying. Kawase et al. (2007) reported that the ability of mouse spermatozoa to support full-term development was better maintained when the sperm were dried under a chamber pressure of 0.37 hPa vs 1.03 and 0.04 hPa, but did not include information regarding the drying temperature. In freeze-drying of macroscopic samples, it is known that chamber pressure is associated with drying temperature (Kochs et al. 1993). Dehydration speed is dramatically decreased if the chamber pressure is higher than the vapor pressure of ice at the product temperature; on the other hand, excessively low chamber pressure induces low product temperature, resulting in dehydration speed decreases (Franks 1998, Nail et al. 2002). It has therefore been recommended that the chamber pressure should be set to the one-quarter to one-half of the vapor pressure of ice at the product temperature (Nail et al. 2002). In the present study, chamber pressures were fixed to one-third of the vapor pressure at the shelf temperature in order to clarify the effect of collapse on sperm function, regardless of chamber pressure. Because the chamber pressure during primary drying were very variable from 0.001 hPa (Wakayama & Yanagimachi 1998) to 0.37 hPa (Kawase et al. 2007) in the previous studies, the relationship between chamber pressure and drying temperature should be investigated for sperm freeze-drying.

Collapse of freeze-dried cake is believed to harm the stability of acellular pharmaceutical protein products (Jiang & Nail 1998, Lueckel et al. 1998, Passot et al. 2007). Since the conventional EGTA buffer has very low T_g' value (-45 ºC; **Figure 5-1** and **Figure 5-2**), it may be reasonable to assume that conventional freeze-dryers (without drying temperature control) and even programmable freeze-dryers (the lowest shelf temperature of our programmable freeze-dryer was -30 ºC) could have produced collapsed cake in previous studies. In order to suppress collapse, the EGTA buffer composition was modified to yield a higher T_g'. NaCl removal and trehalose addition resulted in T_g' increases from -45 ºC to -28 ºC (**Figure 5-1** and **Figure 5-2**). Electrolytes such as NaCl increase the quantity of unfrozen water in the freeze-concentrate, where the unfrozen

water acts as a plasticizer (Her et al. 1995). Trehalose is a disaccharide which is known to facilitate glass formation, and trehalose solution T_g' has been reported to be between -22 and -32 ºC (Chen et al. 2000). Rapid cooling with LN_2, routinely employed for sperm suspension freezing prior to drying (Wakayama & Yanagimachi 1998, Kaneko et al. 2003), was also found to affect the T_g' of mEGTA buffer. Such small decreases in T_g' may be the result of glass transition prior to sufficient freeze-concentration during rapid cooling. In viral vaccine freeze-drying, the importance of freezing conditions has been described (Zhai et al. 2004).

Collapse of sperm suspension cake was dependent on drying temperatures, as expected from a correlation between T_g' of mEGTA buffer and drying temperature (**Figure 5-3**). This allowed an empirical investigation of the correlation between collapse incidence of freeze-dried sperm suspension and function of rehydrated sperm cell. Drying the sperm samples at -40 ºC was not impossible if our programmable freeze-dryer was run in a cold room (+4 ºC), but the drying process seemed to be insufficient after the scheduled 6 h running (data not shown). The ability of rehydrated sperm to support blastocyst development was impaired when the sperm were preserved in collapsed cakes (**Table 5-2**). High residual water content was characteristic of collapsed cakes (**Table 5-1**), consistent with previous results (Wang et al. 2004). It is known that high residual water content decreases the T_g of freeze-dried cake. If T_g is lower than the storage temperature, the sample stability will decrease with residual water content due to increased molecular mobility (Buitink et al. 1998). However, the residual water would not be responsible for the lower blastocyst yields in the present study, because T_g of collapsed cake (approximately +14 ºC) was higher than the storage temperature (-20 ºC). Since collapse leads to increased product resistance to water vapor flow and decreased sublimation rates, collapse may cause loss of sperm function during the drying phase. These results suggest that collapse should be avoided in order to recover the functional sperm cells after rehydration.

In standard procedure of alkaline comet assay, H_2O_2 treatment serves as positive control reference (Enciso et al. 2009, Berthelot-Ricou et al. 2013). In this study, this standard procedure failed to detect differences in DNA damage, both between freeze-dried and non-dried spermatozoa, and between spermatozoa dried at 0 and -30 ºC (**Table 5-3**).

However, when the baseline of tail moment was stimulated by H_2O_2 treatment, freeze-drying was found to harm sperm DNA integrity. Nevertheless, there was no significant difference in the moment values between spermatozoa dried at 0 and -30 ºC. Since membrane permeability to H_2O_2 is enough to neglect influence (Chance et al. 1979), the present results suggest the higher sensitivity of freeze-dried sperm to oxidative stress, which is likely to increase the sperm DNA damage caused by the reactive oxygen species present in oocytes.

TEM image analysis indicated that freeze-dried sperm damage was localized to their plasma membrane, especially in sperm dried at 0 vs -30 ºC (**Figure 5-4A** and **Figure 5-4B**). Comparable cleavage rates after ICSI of sperm dried at 0 and -30 ºC contradict the hypothesis that active sperm-borne oocyte activating factor flows out through damaged plasma membranes. The damaged membrane resulted in the increase of sperm stickness and rendered the ICSI operation difficult (data not shown). Although TEM unexpectedly failed to detect other ultrastructural damage, the nuclear matrix was reported to be essential for paternal DNA replication (Shaman et al. 2007). Lower blastocyst yield after ICSI with freeze-dried bull sperm, compared with previous reports (Keskintepe et al. 2002, Martins et al. 2007), may be caused by low quality of oocytes retrieved from 1-day-stored ovaries (Matsukawa et al. 2007) and/or suboptimal method to activate ICSI oocytes with freeze-dried spermatozoa. Blastocyst-to-calf developmental potential needs to be further investigated by transfer to recipients, following proof of normal karyotype (Keskintepe et al. 2002).

In conclusion, freeze-dried cake collapse may adversely affect sperm function. Since temperatures higher than the T_g' of freeze-drying buffer induce cake collapse, controlling both the buffer T_g' and the drying-phase temperature can reduce collapse incidence.

Chapter 6: Conclusive summary

Genetic improvement has been drastically advanced by practical adaptation of AI and MOET technologies in cattle. Especially, cryopreservation of bull semen contributed to the widespread use of the AI technology because of the semen availability whenever heifers / cows showed the heat. However, the conventional semen cryopreservation requiring the use of LN_2 in storage faces to some problems such as the high maintenance cost, the restriction of transportation and the accidental total loss of frozen cell stock. Freeze-drying has been recently focused as a new tool for sperm preservation, which allows preservation at ambient or refrigerator temperature. On the other hand, application of ICSI is needed for fertilization of freeze-dried and rehydrated spermatozoa due to the loss of their motility. Blastocyst production system with freeze-dried sperm has not been established in cattle, due to the low developmental ability of ICSI oocytes in vitro as well as the suboptimal freeze-drying condition.

Transferable bovine blastocysts can be produced by in vitro system (IVM, IVF and IVC) using immature GV-stage oocytes retrieved from slaughterhouse ovaries, but developmental rate of the IVM oocytes into blastocyst stage was lower when compared with the in vivo-matured counterparts. Addition of oogenesis-related hormone(s) into the IVM medium, extension of the IVM period and reduction of oxygen concentration during the IVM have been attempted to improve the quality of the IVM oocytes. In the first series of this study, intracellular glutathione, which protects cells against oxidative stress, has been focused. Supplementation of β-mercaptoethanol and cysteine (β-ME/Cys) to the IVM medium resulted in a 2.5-fold increase of the intracellular glutathione level when compared with the non-treated control group. However, this treatment was not effective to improve cleavage rate and blastocyst yield after ICSI as well as IVF.

As same as sperm cryopreservation rendered the AI technology practical, cryopreservation of blastocysts can increase the practical availability of recipient females. In the second series of this study, cryotolerance of the ICSI-derived bovine blastocysts was compared with that of the IVF-derived counterparts. Base on the developmental stage, blastocysts harvested on Day 7 or 8 were classified into either fully-expanded or expanding blastocysts. The blastocysts were vitrified-warmed using Cryotop as a device,

and assessed for their post-warming survival by re-expansion of blastocoele within 24 h. Post-warming survival of ICSI-derived fully-expanded blastocysts was similar to that of their IVF-derived counterparts. However, the ability of ICSI-derived expanding blastocysts to survive vitrification procedures was lower than that of IVF-derived blastocysts, although the ICSI- and IVF-derived fresh blastocysts were of similar quality. The age of the blastocysts before vitrification did not affect cryotolerance for either ICSI-derived or IVF-derived blastocysts. Thus, prolonged in vitro culture prior to the Cryotop vitrification was recommended for the ICSI-derived expanding blastocysts.

Freeze-dried rodent spermatozoa can support full-term development even after long-term preservation at 4 ºC. The third series of this study was conducted to investigate whether freeze-dried bull spermatozoa maintained the function of MTOC after rehydration and ICSI. When a freeze-drying condition established for rat spermatozoa was applied to the bull spermatozoa, the blastocyst formation rates of ICSI zygotes produced with freeze-dried spermatozoa were found to be considerably lower than those of ICSI and IVF zygotes produced with control spermatozoa. Formation of sperm aster, which facilitates the migration of both pronuclei to the center of ooplasm and the subsequent fusion of the pronuclei, occurred at similar rates between the ICSI zygotes produced with freeze-dried spermatozoa and control spermatozoa. Among the zygotes exhibiting sperm aster formation, the extent of microtubule network assembly was comparable between freeze-dried and control groups, but the MTOC of the ICSI zygotes was not as functional as that of IVF oocytes. These results suggest that the freeze-drying process per se had no adverse effect on maintaining the MTOC function in bull spermatozoa.

Under the above freeze-drying condition, the solution temperature was higher than the drying-phase T_g' and the glassy matrix with viscous flow resulted in collapse of cake-like porous structure. The purpose of the last experiment was to investigate the effect of cake collapse on the integrity of freeze-dried bull spermatozoa. Composition of freeze-drying EGTA buffer was modified to have a T_g' of -28 ºC, by complete removal of NaCl and addition of 0.5 M trehalose. The effects of different drying temperatures (0, -15 or -30 ºC) on cake collapse and bull sperm quality were investigated using the above-modified EGTA buffer. While drying at -30 ºC suppressed the incidence of cake collapse, drying at -15 or 0 ºC produced the collapsed cake. Blastocyst yields after ooplasmic injection of the sperm

dried at 0 or -15 ºC were significantly lower than those of the sperm dried at -30 ºC. Thus, freeze-dried cake collapse may be undesirable phenomenon for maintaining sperm's functionality for embryonic development, and can be inhibited by controlling both freeze-drying buffer T_g' and drying-phase temperature.

These findings would contribute to establish the blastocyst production system available for freeze-dried bull spermatozoa, and to propose a new promising research field for gamete preservation in large domestic species.

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List of publications

Original articles, directly related to this thesis

1. Abdalla H, Shimoda M, **Hara H**, Morita H, Kuwayama M, Hirabayashi M, Hochi S. Vitrification of ICSI- and IVF-derived bovine blastocysts by minimum volume cooling procedure: effect of developmental stage and age. Theriogenology 2010; 74: 1028–1035.
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1. Hochi S, Abdalla H, **Hara H**, Shimoda M, Morita H, Kuwayama M, Hirabayashi M. Stimulatory effect of Rho-associated coiled-coil kinase (ROCK) inhibitor on revivability of in vitro-produced bovine blastocysts after vitrification. Theriogenology 2010; 73: 1139–1145.
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Review articles

1. Hochi S, Abdalla H, **Hara H**, Hirabayashi M. Challenging endeavor for preservation of freeze-dried mammalian spermatozoa. J Reprod Dev 2011; 57: 557–563.

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