CLONING OF BOVINE EMBRYOS BY USING EAR FIBROBLASTS AS DONOR CELL : COMPARISON OF SURVIVAL RATE AFTER FREEZING IN SEVERAL MEDIA

Mr. Chuti Laowtammathron

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

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การโคลนนิ่งตัวอ่อนโคโดยใช้เซลล์ไฟโบรบลาสจากใบหูเป็นเซลล์ต้นแบบ : เปรียบเทียบอัตราการอยู่รอดหลังจากแช่แข็งในน้ำยาชนิดต่างๆ

นายชุติ เหล่าธรรมธร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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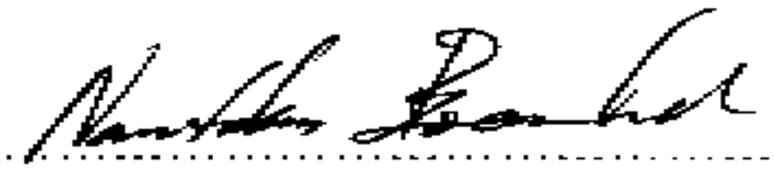

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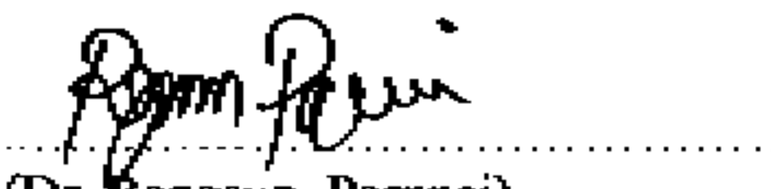

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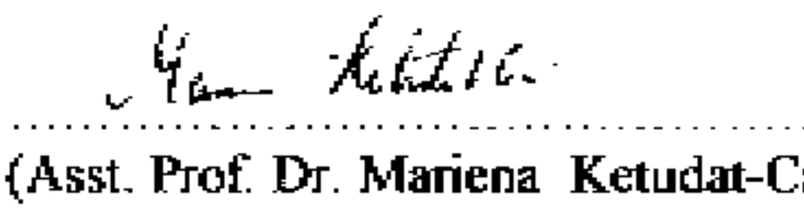

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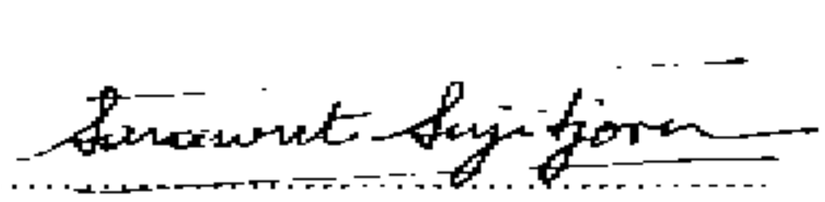

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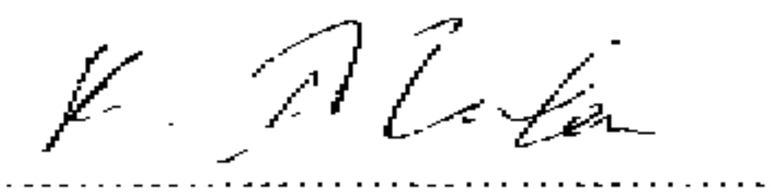

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ชุดิ เหล่าธรรมธร : การโคลนนิ่งตัวอ่อนโคโดยใช้เซลล์ไฟโบรบลาสจากใบหูเป็นเซลล์ต้นแบบ : เปรียบเทียบอัตราการอยู่รอดหลังจากแช่แข็งในน้ำยาชนิดต่างๆ (CLONING OF BOVINE EMBRYOS BY USING EAR FIBROBLASTS AS DONOR CELL : COMPARISON OF SURVIVAL RATE AFTER FREEZING IN SEVERAL MEDIA) อาจารย์ที่ปรึกษา : ดร. รังสรรค์ พาลพ่าย, 83 หน้า ISBN 974-533-324-7

การทดลองนี้ได้ศึกษาผลของน้ำยาแช่แข็งที่มีและไม่มี 10% Ficoll และผลของ hatching status ต่ออัตรารอดของตัวอ่อนโคโคลนนิ่งอายุ 7 วัน หลังจากแช่แข็งด้วยวิธี Vitrification ตัวอ่อนระยะ hatching blastocyst จะถูกแบ่งออกเป็นสามกลุ่ม จากอัตราส่วนของเส้นผ่าศูนย์กลางของตัวอ่อนภายนอก zona pellucida ต่อเส้นผ่าศูนย์กลางของตัวอ่อนภายใน zona pellucida จากนั้นนำตัวอ่อนไปแช่ในน้ำยาที่มี 10% EG + 10% DMSO นาน 2 นาทีแล้วย้ายตัวอ่อนไปไว้ในน้ำยา ที่มี 20% EG + 20% DMSO + 0.5M sucrose ที่มีและไม่มี Ficoll นาน 30 วินาที นำตัวอ่อนไปวางที่ปลายของ Cryotop แล้วจุ่มลงในไนโตรเจนเหลวทันที หลังจากละลายพบว่าตัวอ่อนทั้ง 3 กลุ่มที่แช่แข็งในน้ำยาที่มีและไม่มี Ficoll มีรูปร่างปกติและมีอัตรารอดที่ไม่มีความแตกต่างทางสถิติ หลังจากเลี้ยงในหลอดแก้ว 24 ชั่วโมง พบว่าตัวอ่อนที่แช่แข็งในน้ำยาที่ไม่มี Ficoll มีอัตรารอดสูงกว่ากลุ่มที่มี Ficoll และพบว่าตัวอ่อนกลุ่ม C มีอัตรารอดสูงสุด (80%, 24/30) แต่ไม่มีความแตกต่างทางสถิติ จากการทดลองสามารถสรุปได้ว่า Ficoll และ hatching status ไม่มีผลต่ออัตรารอดของตัวอ่อนโคโคลนนิ่งที่แช่แข็งโดยวิธี Vitrification

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

CHUTI LAOWTAMMATHRON : CLONING OF BOVINE EMBRYOS BY USING EAR FIBROBLASTS AS DONOR CELL : COMPARISON OF SURVIVAL RATE AFTER FREEZING IN SEVERAL MEDIA

THESIS ADVISOR : RANGSUN PARNPAI, Ph.D. 83 PP.

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This study examined the effect of vitrification solution with and without 10% Ficoll and hatching status of day 7 cloned bovine hatching blastocysts on the survival rate after vitrification and warming. Hatching blastocysts were divided into three groups according to the ratio of extruding embryonic cells diameter and embryonic cells diameter inside the zona pellucida. The embryos were exposed to 10% EG + 10% DMSO for 2 minutes then moved to 20% EG + 20% DMSO + 0.5M sucrose with and without 10% Ficoll for 30 seconds. The embryos were placed on the sheet of Cryotop and immediately submerged in liquid nitrogen. Immediately after warming, morphology and survival rate of embryos in all groups in vitrification solution with and without Ficoll were not significantly different. After 24 h of *in vitro* cultured, the embryos in vitrification solution without Ficoll gave higher survival rate than the embryos in vitrification solution containing 10% Ficoll and the embryos in group C gave highest survival rate (80%, 24/30), however, it was not significantly different. In conclusion, Ficoll and hatching status of bovine cloned embryos did not effect the survival rate after vitrification and warming.

School of Biotechnology

Academic Year 2003

Student's Signature.....

Advisor's Signature.....

Co-advisor's Signature.....

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Chuti Laowtammathron

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LIST OF ABBREVIATIONS

cm	=	centimeter
°C	=	degree celsius
∅	=	diameter
DC	=	direct current
g	=	gram
iu	=	international unit
kg	=	kilogram
l	=	liter
µg	=	microgram
µl	=	microliter
µsec	=	microsecond
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
min	=	minute
rpm	=	round per minute
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Cattle are separated to families of *Bos taurus* and *Bos indicus.* The cattle genome composed of 58 autosomes and two sex chromosomes (total of 60 chromosomes). All autosomes are acrocentric whereas two sex chromosomes, X and Y, are submetacentric.

The Zoological Scheme of Cattle

Kingdom	***Animalia***
Phylum	***Chordata***
Class	***Mammalia***
Order	***Artiodata***
Family	***Bovidae***
Genus	***Bos***
Species	***taurus* and *indicus***

1.1 The overview of cloning technology

1.1.1 The history of animal cloning

Normally, embryos (2n) are produced by fertilization of sperm (n) and egg (n). The genetics of embryo would be the sharing of paternal and maternal genetics in the ratio of 50 : 50. On the other hand, cloning is a technique that can produce new animals by using only donor cell (2n) as a source of all embryo's genetic materials. The donor cell can be derived from all part of the body such as mammary epithelial

cells (Wilmut et al., 1997), cumulus cells (Wakayama et al, 1998), oviduct epithelial cells (Kato et al., 1998), leukocytes (Galli et al., 1999), genital ridge cells (Betthauser et al., 2000), gonad (Wakayama and Yanagimachi, 2001) and others.

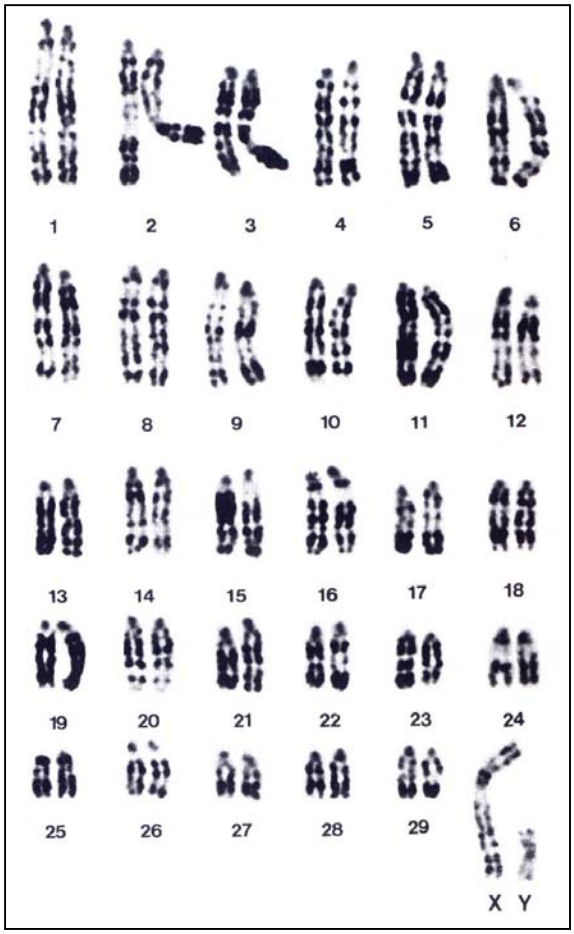

Figure 1. Bovine chromosomes consist of 58 autosomes and 2 sex chromosomes.

The word "Clone" derived from "Klon" of Greek means twig. Cloning or nuclear transfer was first studied by Spemann (1938), who demonstrated that nuclei of newt salamander (Figure 2) are pluripotent upto 8-cell stage. The study of Briggs and King (1952) and King and Briggs (1955) demonstrated that blastula nuclei of *Rana pipients* (Leopard frog) could be developed to mature

frog after injected into oocytes. However, gastrula nuclei from mesoderm or endoderm injected into oocytes were unable to develop to mature frog (Briggs and King, 1960). In 1962, Gurdon reported the using of nuclei from intestine of tadpoles to produce fertile adult frogs.

Figure 2. Newt salamander was used for nuclear transplantation.

In mammals, nuclear transfer by direct microinjection of mouse inner cell mass (ICM) cells and trophoblast cells into enucleated pronuclear mouse zygotes was performed by Illmensee and Hoppe (1981). Three mice were born from ICM cells whereas trophoblast cells produced neither blastocyst nor live offspring. This report was met with skepticism because of other researchers were unable to repeat this results. A more efficient system for introduce of nuclei by using the cell fusion was developed by McGrath and Solter (1983). In other species, offspring have resulted from the fusion of embryonic cells into enucleated oocytes in sheep (Willadsen et al., 1986; Smith and Wilmut, 1989), cattle (Prather et al., 1987; Bondioli et al., 1990), pig (Prather et al., 1989), rabbit (Stice and Robl, 1989), rhesus monkey (Meng et al., 1997).

The success of somatic cell cloning verified by the birth of the cloned sheep(Wilmut et al., 1997), Dolly (Figure 3), gave rise to widely studies in many laboratories. Viable cloned animals from somatic cell cloning have been produced in cattle (Cibelli et al., 1998; Kato et al., 1998), mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999) and pig (Polejaeva et al., 2000; Onishi et al., 2000; Betthauser et al., 2000). The success of live offspring produced from somatic cell cloning is show in Table1.

In general, cloning needs donor cells from the organ or tissue of interested animal and recipient cytoplasm. The donor cells were cultured for several passages and storage by freezing (i.e. mammary epithelial cells : Wilmut, et al. 1997, oviduct epithelial cells : Kato et al. 1998, fetal genital ridge cells : Betthauser et al. 2000, fetal skin fibroblasts : Onishi et al. 2000, adult skin fibroblasts : Parnpai et al. 2000, granulosa cells : Polejaeva et al. 2000). The recipient cytoplasm could be derived from *in vivo* or *in vitro* matured oocytes. The nucleus of matured oocyte was removed and replaced by nucleus of donor cell.

Figure 3. Dolly, the first mammalian species produced by somatic cell cloning.

The somatic cell - cytoplast complexes (SCCCs) needed to be fuse together by using electric pulses (Wilmut et al., 1997; Kato et al., 1998; Polejaeva et al., 2000). On the other hand, in case of the donor cell or nucleus directly injected into cytoplasm of recipient cytoplast, the fusion need not to be done (Wakayama et al., 1998; Zhou et al., 2000, 2001). In cloning, the lack of sperm-induced fertilization steps necessitates the application of an artificial activation in order to trigger further development. Various chemicals have been employed for activation including ethanol (Kishikawa et al., 1999), calcium ionophore (Koo et al., 2001), ionomycin (Rho et al., 1998) and strontium (Wakayama and Yanagimachi, 1999), moreover, electric pulses (Nagashima et al., 1997) had also been used for activation.

1.2 The overview of cryopreservation

The cryopreservation procedures have been developed to maintain spermatozoa, oocytes and embryos of various animals in frozen state. Spallanzani and his colleagues (1776) was the first team who found that the horse sperm and egg silk could be preserved at low temperature of snow. The obvious development just began after discovery of glycerol, a cryoprotectant, could prevent fowl sperm cell injury from freezing method and improve the survival rate after warming (Polge et al., 1949). Then glycerol was employed to use as cryoprotectant in freezing of fowl and bull semen (Polge, 1951, Polge and Lovelock, 1952). Whittingham and colleagues (1972) reported the first success in frozen and thaw mouse embryos at 8-cell stage. Sixteen years later, Friedler and colleagues (1988) reported the frozen of matured oocytes. The procedures which have been used in early experiments were also used in several mammalian species.

Table 1. Live offspring produced by using somatic cell nuclear transfer*

Species	Donor cell	Offspring	Reference
Sheep	Mammary epithelial cells	Died in the year 2003	Wilmut et al., Nature 1997, 358: 810-813.
	Transfected fetal fibroblasts	Alive	Schnieke et al., Science 1997, 278: 2130-2133.
	Gene-targeted fetal fibroblasts	Alive	McCreath et al., Nature 2000, 405: 1066-1069.
	Gene-targeted fetal fibroblasts	Survived 12 days	Denning et al., Nat. Biotechnol. 2001, 19:559-562.
Cattle	Transfected fetal fibroblasts	Alive	Cibelli et al., Science 1998, 280: 1256-1258.
	Cumulus cells	Alive	Kato et al., Science 1998, 282: 2095-2098.
	Oviduct epithelial cells	Alive	Kato et al., Science 1998, 282: 2095-2098.
	Leukocytes	Alive	Galli et al., Cloning 1999, 1: 161-170.
	Mural granulosa cells	Alive	Wells et al., Biol. Reprod. 1999, 60: 996-1005.
	Mammary epithelial cells	Alive	Zakharchenko et al., Mol.Reprod.Dev. 1999a, 54: 264-272.
	Fetal germ cells	Died within 24h	Zakharchenko et al., Mol.Reprod.Dev. 1999b, 52: 421-426.
	Transfected fetal fibroblasts	Alive	Zakharchenko et al., Mol.Reprod.Dev. 2001, 60: 362-369.
	Ear fibroblasts	Alive	Parnpai et al., Theriogenology 2002, 57: 443.

Table 1. Live offspring produced by using somatic cell nuclear transfer (continue)*

Species	Donor cell	Offspring	Reference
Goat	Transgenic fetal fibroblasts	Alive	Baguisi et al., Nat. Biotechnol. 1999, 17: 456-461.
	Transfected fetal fibroblasts	Alive	Keefer et al., Biol. Reprod. 2001, 64: 849-856.
Pig	Granulosa cells	Alive	Polejaeva et al., Nature 2000, 407: 86-90.
	Fetal fibroblasts	Alive	Onishi et al., Science 2000, 289: 1188-1190.
	Fetal fibroblasts	Alive	Betthauser et al., Nat. Biotechnol. 2000, 18: 1055-1059.
	Genital ridge cells	Alive	Betthauser et al., Nat. Biotechnol. 2000, 18: 1055-1059.
Mouse	Cumulus cells	Alive	Wakayama et al., Nature 1998, 394: 369-374.
	Tail tip cells	Alive	Ogura et al., Mol.Reprod.Dev. 2000, 57: 55-59.
	Fetal ovarian cells	Alive	Wakayama et al., Mol.Reprod.Dev. 2001, 58: 376-383.
	Fetal testicular cells	Alive	Wakayama et al., Mol.Reprod.Dev. 2001, 58: 376-383.
	ES cells	Alive	Humpherys et al., Science 2001, 293: 95-97.
Cat	Cumulus cells	Alive	Shin et al., Nature 2002, 415: 859.

Table 1. Live offspring produced by using somatic cell nuclear transfer (continue)*

Species	Donor cell	Offspring	Reference
Mule	Fetal fibroblasts	Alive	Wood et al., Science 2003, 301: 1063.
Horse	Skin fibroblasts	Alive	Galli et al., Nature 2003, 424: 635.
Rat	Fetal fibroblasts	Alive	Zhou et al., Science 2003, 302: 1179.
Deer	Skin fibroblasts	Alive	Kraemer and Long. 2003, http://www.eurekalert.org/pub_releases/2003-12/tau-tas122203.php.

*modified from Brem and Kuhholzer, 2002.

There are 2 methods for embryo cryopreservation.

1. Slow freezing (Conventional freezing)
2. Ultra-rapid freezing (Vitrification)

1.2.1 Slow freezing (Conventional freezing)

Whittingham and colleagues issued the first successful of slow freezing embryos in 1972. The cryopreserved mouse embryos at 8-cell stage were transferred into foster mothers resulting in healthy live offspring. Therefore, this technique has been widely used in several animal species (Table 2). The cryopreservation procedures of cattle embryos were shown in Figure 4. Briefly, the embryos were equilibrated in freezing solution by using glycerol or ethylene glycol as cryoprotectant. After that embryos were loaded into straw and transfer into programmable freezing machine (Figure5).The cooling rate around 1°C/min to 15°C/min were used to decrease temperature from room temperature to -7°C. Seeding was performed at -7°C and holding at this temperature for 5-10 min. Then temperature was decreased to –35°C by use cooling rate 0.3°C/min. After that the straw was directly plunged into liquid nitrogen tank.

1.2.1.1 The cooling rate of slow freezing

During cooling steps, the cryoprotectants penetrated into the cells to remove water (Figure 6) caused cell dehydration and shrinking. If the cooling rate was too slow, the concentration of cryoprotectants inside cell increased too much making cell injury from the toxic of cryoprotectants, called solution injury. Figure 7 showed the oocytes morphology during cooling steps, A: The suitable cooling rate, the cell was dehydrated and then concentration of cryoprotectants inside the cell increased making

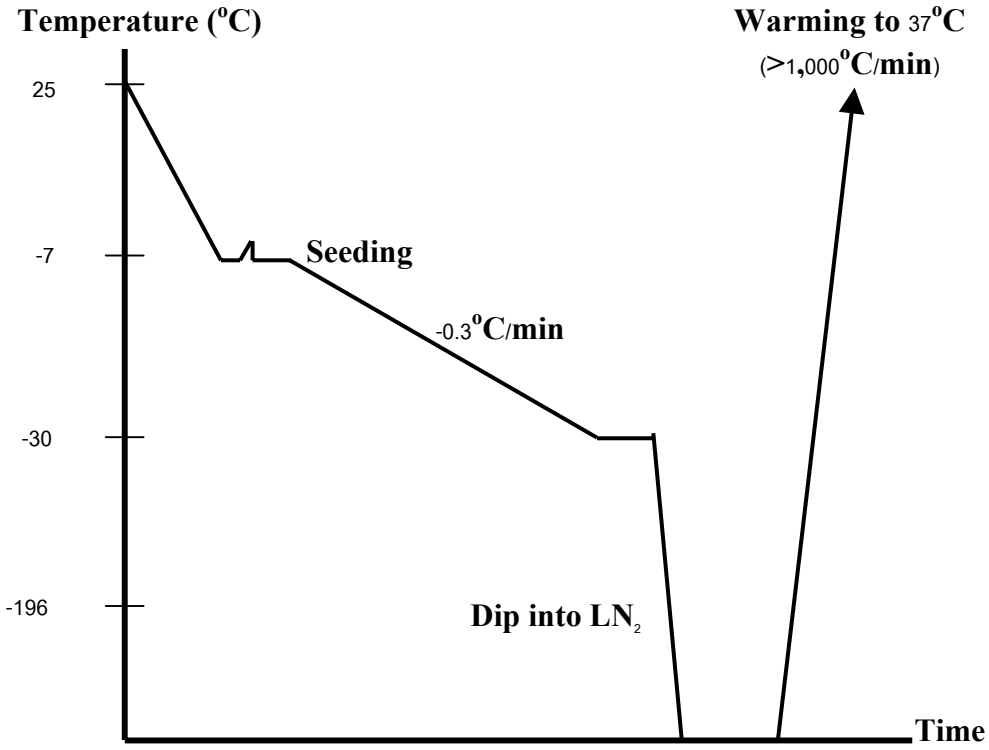

Figure 4. The cryopreservation procedure of cattle embryos by slow freezing.

Figure 5. Programmable freezing machine.

cell injury from the toxic of cryoprotectants; B: rapid cooling rate, not all water was removed from the cell making intracellular ice crystal formation, C: very rapid cooling rate, water level in the cell was higher than in the slow and rapid cooling rate, which generated intracellular ice crystal to destroy the cell.

1.2.1.2 Seeding

The major cause of cell destruction during freezing and thawing was the formation of intracellular ice crystals (Ruffing et al., 1993), as showed in Figure 8. The slow freezing procedure was the introduction of freezing solution to solid stage by seeding. Seeding can protect oocytes or embryos injury from the effect of supercooling. Supercooling is the tendency of water and aqueous solutions that could still being as liquid form even the temperature is cooled down to cool below their melting point before nucleation occurs. The extention of supercooling is the difference between the temperature of unfrozen system and the melting point.

1.2.1.3 Final cooling temperature

Final cooling temperature is the last temperature of embryos before plunging straw into liquid nitrogen. At this temperature, the penetration of water and cryoprotectants from the cell were stopped. The final cooling temperature is depending on stage of embryo or oocyte. The optimum final cooling temperature of oocyte and embryo at 2-8 cells stage and blastocyst stage are -80°C and -35°C, respectively.

1.2.1.4 Warming rate

Warming rate is depending on cooling rate and volume of water in the cell before plunging into liquid nitrogen. Leibo (1982) described that the concentration of cryoprotectants in the cell was higher than warming solution. Therefore, the water was penetrated into cell (Rehydration) and at the same time the cryoprotectants were removed out at the same time until equilibration. If warming rate is too fast, the cell will injury from osmotic swelling, which water was penetrated into the cell faster than the cryoprotectant removed out from cell. Whittingham (1981) examined the effects of final cooling temperatures (Figure 9), -40°C or -80°C, and warming rate, 20°C/min or 500°C/min, of mouse embryos at 8-cell stage. High survival rates were found at -40°C and 500°C/min as well as -80°C and 20°C/min of final temperature and warming rate, respectively.

1.2.2 Ultra-rapid freezing (Vitrification)

In 1985, Rall and Fahy reported the first successful of cryopreserved mouse embryos by vitrification. This technique has also been used to study in several animal species (Table 3). The physical definition of vitrification is the solidification of solution at low temperature. The water will cool by use ultra-rapid cooling rate and it changes into a glassy, vitrify state from the liquid phase. Vitrification technique not created ice crystal by used extreme elevation in viscosity during cooling, (Fahy et al., 1984). The ultra-rapid cooling rate is based on direct contact between the vitrification solution and liquid nitrogen. The vitrification technique requires high concentration of cryoprotectants and supplement with macromolecule to dehydrate the cell such as sucrose, Ficoll70 and Polyvinylpyrolidone (PVP). The vitrification solution will penetrate into the cell for a short time to avoid the toxic of the cryoprotectants after

Table 2. The normal offspring of mammalian species after cryopreserved and transferred embryos to foster mothers.

Years	Species	References
1972	Mouse	Whittingham et al.
1973	Cattle	Wilmut et al.
1974	Rabbit	Bank et al.
1974	Sheep	Willadsen et al.
1975	Rat	Whittingham et al.
1976	Goat	Bilton et al.
1982	Horse	Yamamoto et al.
1984	Human	Zeilmaker et al.
1984	Baboon	Pope et al.
1986	Mamoset	Summers et al.
1987	Cynomolgus macaque	Balmaceda et al.
1988	Cat	Dresser et al.
1989	Rhesus macaque	Wolf et al.
1989	Pig	Dixon et al.
1991	Wapidi	Wenkoff et al.
1992	Hybrid macaque	Cranfield et al.
1993	Swamp buffalo	Kasiraj et al.
1999	Hamster	Lane et al.

Modified from William, 2001

use high extreme cooling rate. Vitrification has more advantages than conventional freezing. For example, in case of economic, vitrification technique is cheaper and much more simple than conventional freezing because programmable freezing machine is not required to decrease the temperatures. For the operating time, vitrification consumed a few seconds while conventional freezing took at least 2 h. Moreover, vitrification technique can get away from the formation of intracellular and extracellular ice crystals which are the major cause of cells damage. The comparison of the advantages and disadvantages of conventional freezing and vitrification were shown in Table 4. During cooling, the embryo diameter was changed because water inside the cell removed out faster than cryoprotectants removed into the cell (Figure 10). The ultra- rapid cooling and warming rate were introduced for vitrification, as showed in Figure 11. Several works achieved in vitrification of mammalian embryos and oocytes, including human (Shaw et al., 2000 ; Park et al., 2000 ; Chung et al., 2000). Dobrinsky and his colleagues (1991) vitrified bovine embryos by incubation embryos in equilibration solution, 10% glycerol + 25% propylene glycol (PG) + 4% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (DPBS), at 20°C for 7 min followed by loading the embryos into vitrification solution (25% Glycerol + 25% Propylene glycol, 4% BSA in DPBS) for 1 min and submerging into liquid nitrogen. The vitrified embryos were warmed by submerged straw in water at 20°C for 5-10 sec which was better than slow cooling since it could prevent the chilling injury during bovine embryos cryopreservation. They concluded that the vitrification procedure might be a technically simple alternative for bovine embryos. There has been suggested that ultra-rapid cooling may be better than slow cooling because it

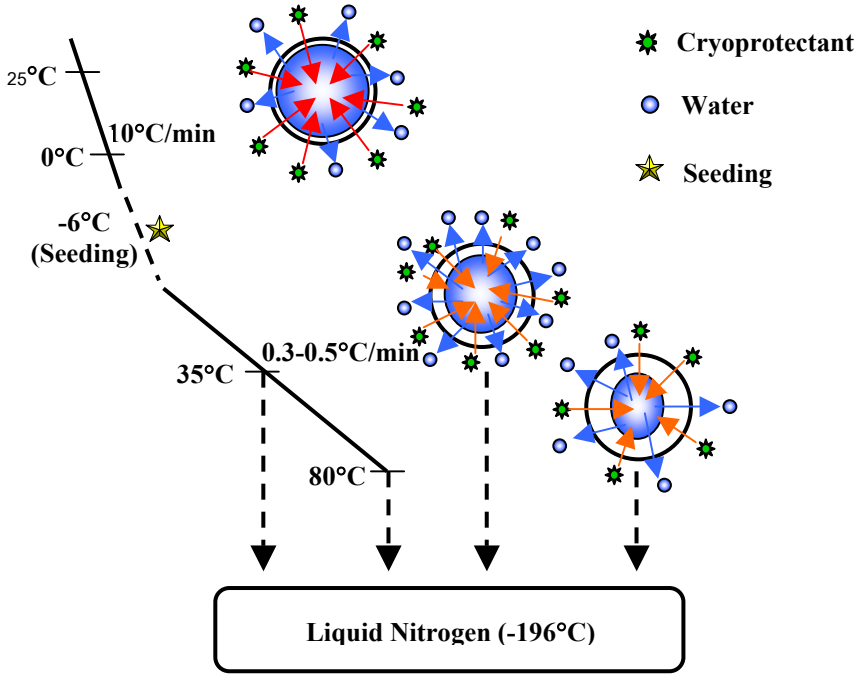

Figure 6. Cell dehydration during slow freezing.

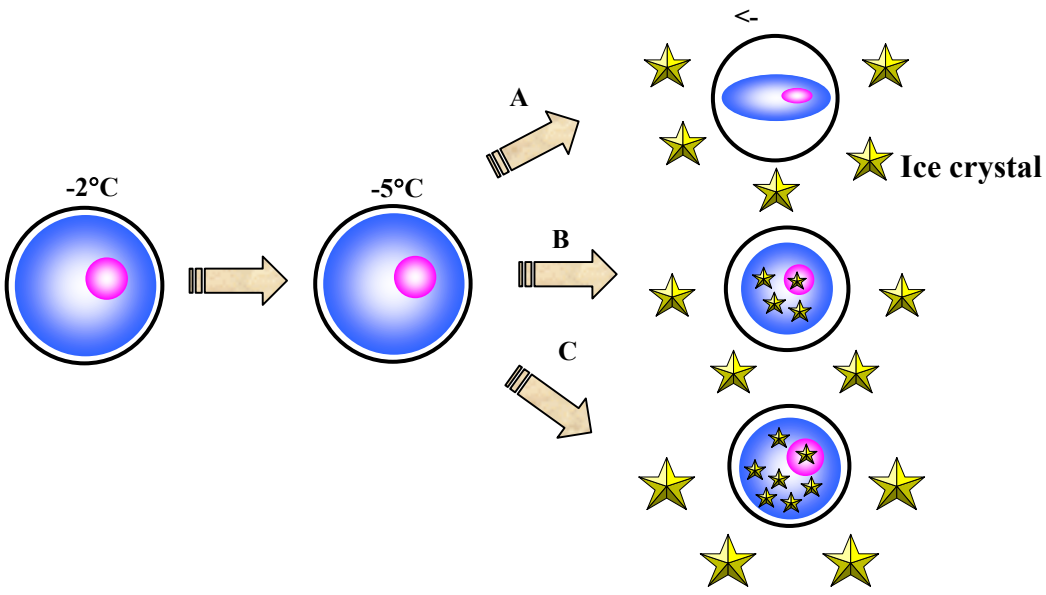

Figure 7. The oocytes morphologies during slow cooling (A), rapid cooling (B) and very rapid cooling (C).

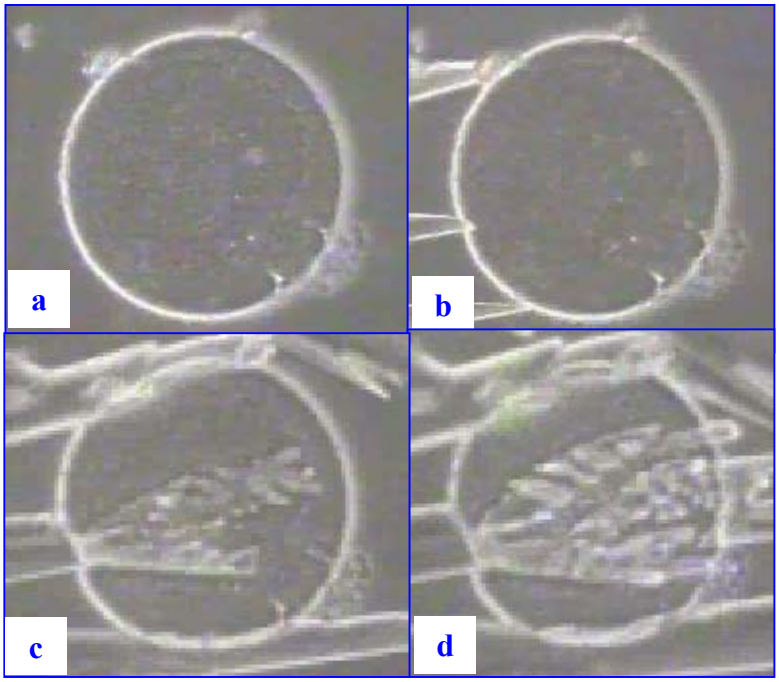

Figure 8. Ice crystal growing through the oocyte, it is a major cause to destroy the oocyte during cooling method.

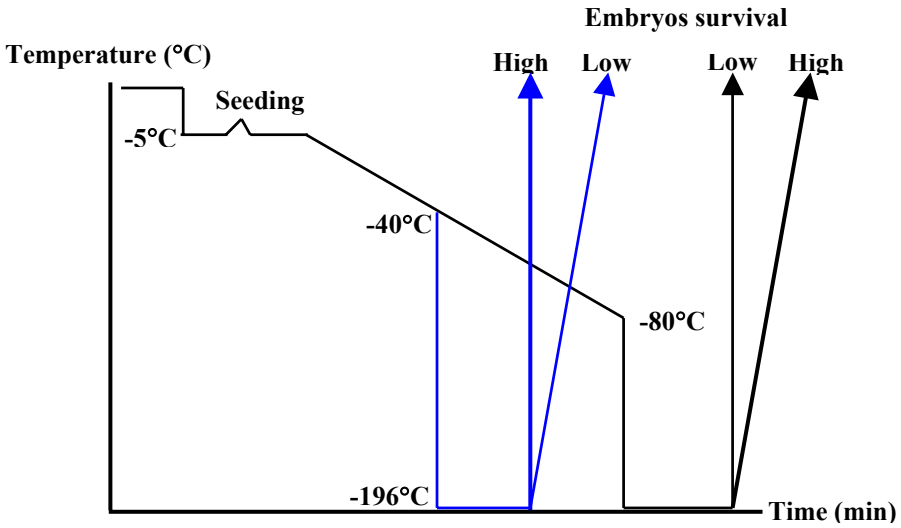

Figure 9. The effect of final cooling temperature and warming rate on embryos survival rate.

could prevent the chilling injury during bovine embryo cryopreservation (Dobrinsky et al., 1991).

1.2.2.1 Vitrification by using straw

There are two types of straw that used as an embryo/oocyte container for vitrification, opened and sealed tip straw. For sealed tip straw, the straw was sealed by using heat seal or powder sealing such as polyvinylalcohol (PVA). For the heat sealing, the effect of increasing temperature of vitrification solution during heating should be considered because it could decrease the survival rate after warming. Powder sealing was performed by put the PVA powder into the tip of straw before vitrification. If straw is not completely sealed, the liquid nitrogen could pass inside the straw and the straw could be broken resulting to embryo damage or loss. Vitrification of cloned bovine embryos using straw as container was reported by Nguyen and colleagues (2000). Cloned bovine embryos were selected at blastocyst stage for vitrification. After warming they found that 93% of embryos survived and 95% of embryos developed to hatching blastocyst. One of the famous techniques, open-pulled straw or OPS was introduced as a new vitrification technology by using open tip of straw (Vajta et al., 1997). OPS have smaller area than insemination straw that caused high cooling rate during vitrification at 20,000°C/min and gave high survival rate after warming.

1.2.2.2 Vitrification by containerless method.

Insemination straw was the first vitrification container. Due to its limitation as mentioned before, the containerless method was developed. Nowadays, many new containersless for oocytes and embryos cryopreservation have been developed such as electron microscope grids (Martino et al, 1996, Arav and Zeron, 1997), open pulled

Table 3. Vitrification of embryos in several species by using difference type of cryoprotectant and container.

Species	Stage	Equilibration solution	Vitrification solution	Container	Reference
Mouse	Ex. Blast.	10% EG: 5 min	40% EG + 18% Ficoll70 + 0.3M sucrose (EFS40): 30 sec	**Straw**	Kasai et al., 1996
Rat	4-, 8-C, Morula	10% EG: 5 min	40% EG + 18% Ficoll70 + 0.3M sucrose (EFS40): 30 sec	**Straw**	Han et al., 2004
Bovine	8-, 16-C, Early morula	10% EG + 10% DMSO: 2 min	20% EG + 20% DMSO + 0.6M sucrose: 10 sec	**Open pulled straw (OPS)**	Vajta et al., 1997
	Blast.	1) 10% Glycerol: 5 min 2) 10% Glycerol + 20% EG: 5 min, RT	25% EG + 25% Glycerol: 30 sec, RT	Straw	Donnay et al., 1998

Table 3. Vitrification of embryos in several species by using difference type of cryoprotectant and container.(Continue.)

Species	Stage	Equilibration solution	Vitrification solution	Container	Reference
Bovine	Blast.	20%EG: 1.5 min	EFS40: 30 sec	Electron microscope grid	Park et al., 1999
	Compacted morula, Blast.	1) 10% Glycerol: 5 min 2) 10% Glycerol + 20% EG: 5 min, RT	25% EG + 25% Glycerol: 30 sec, RT	Straw	Martinez et al., 2002
	Blast.	10% EG + 10% DMSO: 2 min	20% EG + 20% DMSO + 0.6M sucrose: 30 sec	**Gel-tip**	Tominaga et al., 2001.
Rabbit	1-, 2-, 8-, 16- C, Morula, Blast.	10% Gly + 20% 1,2- propanediol 10 min, RT	30% Gly + 30% 1,2-propanediol: 5 min, 0^{o}C	**Glass ampoules**	Smorag et al., 1989

Table 3. Vitrification of embryos in several species by using difference type of cryoprotectant and container.(Continue.)

Species	Stage	Equilibration solution	Vitrification solution	Container	Reference
Rabbit	Morula	20% EG or 5% EG + 5% DMSO: 5 min, 20°C	40% EG or 20% EG + 20% DMSO: 1 min, 20°C	**Straw**	Vicente and Garcia-Ximenez, 1994
	Morula	-	40% EG + 18% Ficoll70 + 0.3M sucrose (EFS): 2 min	**Straw**	Kauffman et al., 1998
	Zygote	10% EG + 10% DMSO: 2 min	20% EG + 20% DMSO + 0.6M sucrose: 30 sec	**Gel-tip,** Cryoloop, Cryotop	Hochi et al., 2004
Human	Blast.	10% EG + 10% DMSO	20% EG + 20% DMSO + 25μM Ficoll+0.75M sucrose	**Hemi-Straw**	Vanderzwalmen et al., 2003

EG: Ethylene glycol, DMSO: Dimethylsulfoxide, RT: Room temperature.

Table 4. Comparison of cryopreservation techniques between vitrification and slow freezing.

Accessibility and regulation	Vitrification	Slow freezing
Can be observed	Yes	No
Can be analyzed	Yes	No
Interaction with the oocytes or embryo	Yes	No
Control of solute penetration	Yes	No
Control of dehydration rate	Yes	No
Maintain of physiological temperature during equilibration procedure	Yes	No
Duration out of incubator	~10 min	~ 3 h
Prolong temperature shock	No	Yes
Interference with oocyte or embryo	Low	High
Fracture of zona pellucida	No	Possible
Capture by growing ice crystal	No	Possible

From Kuleshova and Lopata (2002).

straw (Vatja et al., 1997), cryoloop (Lane et al., 1999), hemi straw (Vanderzwalmen et al., 2000), gel-loading tip (Tominaga and Hamada, 2001) and flexipet-denuding pipette (Liebermann et al., 2002). The successes of vitrification could be increased by ultra-rapid vitrification procedure. The highly increasing of cooling and warming rates can reduce ice crystal formation and chilling injuries (Vanderzwalmen et al., 2003).

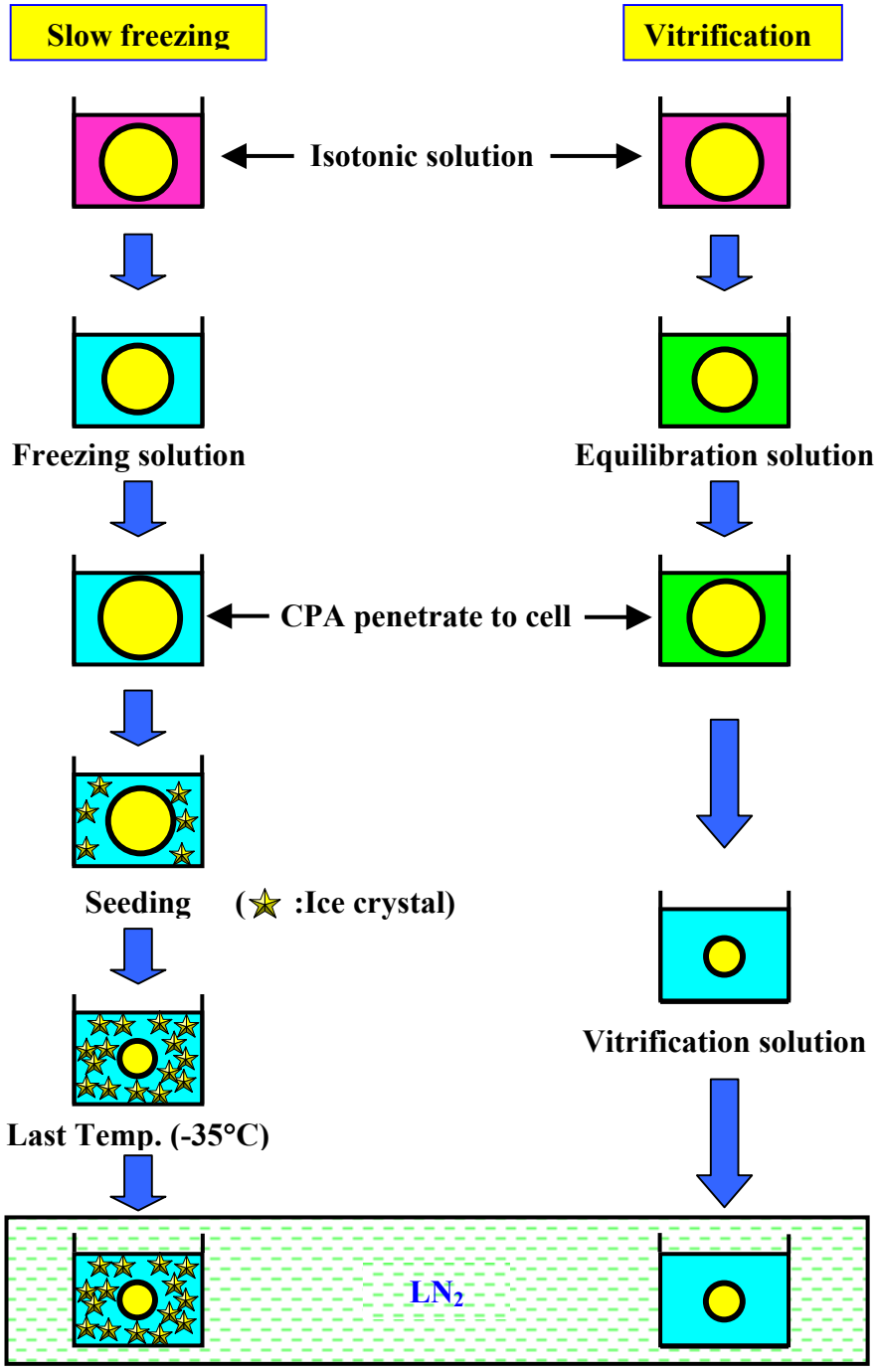

Figure 10. The volume of oocyte during cryopreserved by slow freezing and vitrification.

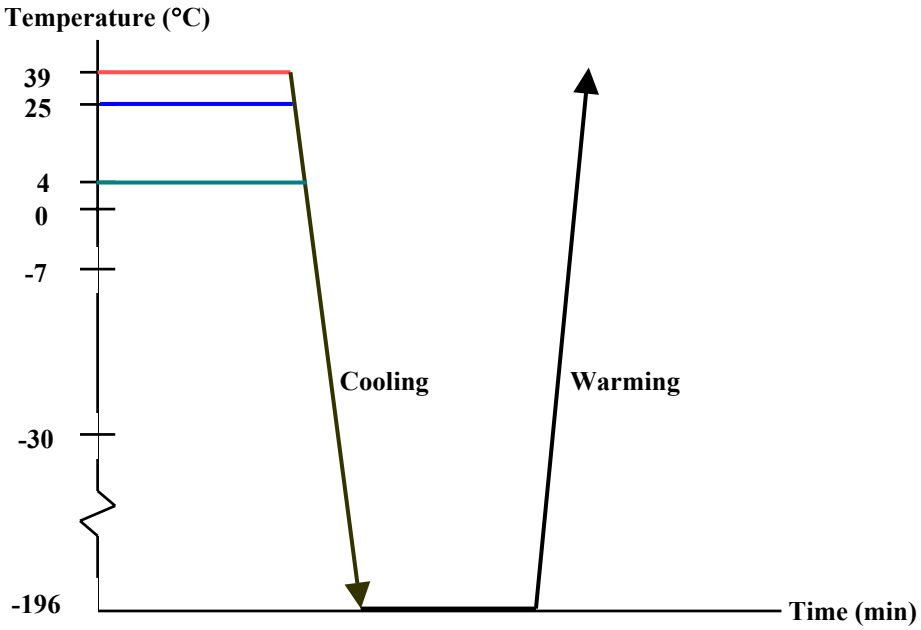

Figure 11. Cooling and warming rates of vitrification technique.

1.2.2.3 Toxicity of vitrification solution

Nowadays, vitrification solution have been tested for oocytes and embryos cryopreservation (Kasai et al., 1990, Mahmoudzadeh et al., 1993, Martino et al., 1996, Rall, 1987, Rall., 1994, Songsasen et al., 1995, Suzuki et al., 1995, Szell et al., 1989) because the toxicity of vitrification solutions is the important key for embryos survival. In the early year, the vitrification solution were more toxic than current time. Researchers have been working on the formulation of nontoxic/less toxic vitrification solution. Some vitrification solutions contained more than one type of cryoprotectant to reduce the concentration of cryoprotectant in the cell. Sometime sugar and/or polymers were used to improve the survival rate after warming.

There are several ways to reduce the toxic of cryoprotectant solution. For example, in 1996 and 1997 Saha and his colleagues reported the high survival rate by equilibration embryos in vitrification solution (40% EG + 11.3% trehalose + 20% PVP) at low temperature (5°C). Once the equilibration temperature was

increased up to 20°C they found that the embryos survival rate was lower than equilibration at 5°C.

Many researchers have studied the comparisons of toxicity of cryoprotectants. The EG was observed as non-toxic compound for mouse embryos (Valdez et al., 1992; Zhu et al., 1996). The toxicity of different cryoprotectants was compared in variation of equilibration periods by Takagi and his colleagues (1993). They found that EG was non-toxic, regardless of the length of the equilibration period. In the among of 3 vitrification solutions, 40% EG, 40% DMSO and 40% Glycerol, the highest survival rate was found after incubation of embryo in 40% EG (Sun and Jiang, 2000).

Low molecular weight of cryoprotectants was reported to give high permeability (Kasai et al., 1990). Molecular weights of EG, glycerol, propylene glycol, DMSO were 62.07, 92.10, 76.10 and 78.13, respectively. Therefore, EG was reported to give higher permeability than other cryoprotectant agents. *In vitro* produced bovine embryos usually vitrified in cryoprotectant solution based on EG and DMSO or EG only. Park and his colleagues (1998) reported the high survival rate of *in vitro* matured bovine oocytes by simple two-step vitrification in EFS40 freezing solution. Blastocysts could be obtained after *in vitro* fertilization (IVF). One and three-steps additions of cryoprotectant were studied by Saha and colleagues (1997). They found that three-step addition could improve the survival rate after warming more than one-step addition.

1.2.2.4 Macromolecule in vitrification solution

The cryoprotectants can stabilize macromolecular structures through the strengthening of hydrophobic forces. It can stabilize the cell or cell membrane by tolerated to the freezing procedure (Meryman et al, 1977). The roles of polymers in vitrification solution were unclear sofar. Fahy and colleagues (1984) reported the promotion of vitrification by macromolecule. The macromolecules reduced the incidence of damage of the zona pellucida and acted like serum for mouse oocytes and embryos by slow freezing procedure (Dumoulin, et al., 1994; Gutierrez et al., 1993; Carroll et al., 1993). Normally, vitrification solution containing basic solutions (Physiological saline, Culture medium), cryoprotectants (EG, glycerol, DMSO, polyethylene glycol), polymers (Ficoll, PVP) serum or BSA and sugars (sucrose, trehalose). Moreover, in some solutions, sugars and polymers can be added together and gave the effective for mouse, sheep and bovine oocytes and embryos (Kasai et al., 1990, Mahmoudzadh et al., 1993, Zhu et al., 1993). Protein supplementation in vitrification solution were benefit for oocytes and embryos (Tsunoda and Sugie, 1977, Shaw and Trounson, 1989). Silvestre and colleagues (2003) studied the supplementation of 20% to 40% fetal calf serum (FCS) in vitrification solution they found that the efficiency of vitrification could not be improved but the incidence of cellular damages was decreased. Pugh and colleagues (1998) found that concentrations of FCS in the rang of 10% to 50% could reduced early survival rate after warming. Similarly, increasing of BSA supplementation from 5-25 mg/ml in cryopreservation solution did not improve survival rate. Vitrification solution contained only sucrose (Luyet et al., 1968; Rasmussen et al., 1969) or PVP can forming glass without crystallisation (Luyet et al., 1967). However, cryoprotectant is required to prevent devitrification during warming. Shaw and colleagues (1997)

reported that there was no evidence of cracking after cooling and warming which found from vitrification solutions consisted of polymer after first cooling and warming. Vitrification solution without any polymer showed evidence of cracks about 75%. So polymers were added in vitrification solutions to improve the survival rates of oocytes and embryos after warming.

1.2.2.5 Stage of embryos for freezing

The cryopreserved embryos at difference developmental stages, morphology quality and day of culture have been demonstrated. Some reports showed that compacted morulae gave high survival rates (Cseh et al, 1995) while many reports showed the best survival rates of embryos at day 7, day 8, expanded blastocyst stage (Cseh et al, 1995; Mahmoudzadeh et al, 1995; Myers et al, 1996; Saha et al, 1997; Takagi et al., 1994; Sommerfeld et al., 1999). Pregnancy rate of fresh cloned bovine blastocyst and vitrified blastocyst by OPS were examined in 2001 by French and colleagues. The results showed that there was no significant difference between fresh (16.5%) and vitrified cloned embryos (19.2%) in the initial pregnancy rate.

Vitrification is a powerful technique to cryopreservation of oocytes and embryos for long term. The vitrification procedures have been developed to find the optimum condition for cryopreserved oocytes and embryos. There are many factors effect to the optimum condition such as animal species, stage of oocytes or quality of embryos, vitrification solution, temperature during equilibration, warming and time of exposure in vitrification solution. Thus, in this experiment the effect of Ficoll in vitrification solution and hatching status of cloned bovine blastocysts will be studied.

1.3. Objectives

1. To compare the effect of hatching status of bovine cloned hatching blastocysts and effect of Ficoll in vitrification solution on survival rate after warming.
2. To investigate the appropriate vitrification solution to vitrified bovine cloning embryos in order to maintain high survival after warming.

CHAPTER II

MATERIALS AND METHODS

2.1 Donor cell preparation

The ear skin was biopsied from female dairy cattle (Figure 12) and kept in modified Dulbecco's phosphate buffer saline (mDPBS) during transport to laboratory. Skin tissues were removed from cartilage and cut into small pieces (about 1x1 mm) before being placed in culture dishes (Nunc, ∅ 60 mm.) and covered with glass slide. Five milliliter of alpha modification minimal essential medium Eagle (α-MEM) + 10% FCS was added into the dishes and cultured under humidified atmosphere of 5% CO_2 in air at 37°C for 8-10 days. The fibroblasts that outgrowth from ear skin tissues (Figure 13) were passaged using 0.0025 g/ml Trypsin and 0.0004 g/ml EDTA in Phosphate Buffer Saline (PBS) without Ca^{++} and Mg^{++} (Trypsin/EDTA) and seeded on 25 cm^2 culture flask (Nunc) in α-MEM + 10% FCS. At sub-confluence, fibroblasts were harvested by standard trypsinization and cultured on 25 cm^2 culture flask in α-MEM + 10% FCS. Ear fibroblasts were frozen with 10% DMSO in tissue culture medium (TCM) 199 + 25mM *N*–2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) + 20% FCS (TCM199H-20) at the third cell culture passage and stored in liquid nitrogen. Frozen-thawed fibroblasts were cultured in α-MEM + 10% FCS and used for cloning between passages 3 and 8 of culture. Few minutes before injection, proliferative donor cells at sub-confluence were harvested by standard trypsinization

Figure 12. Dairy cattle at SUT farm No.346 that gave milk production 8,000 kg/lactation.

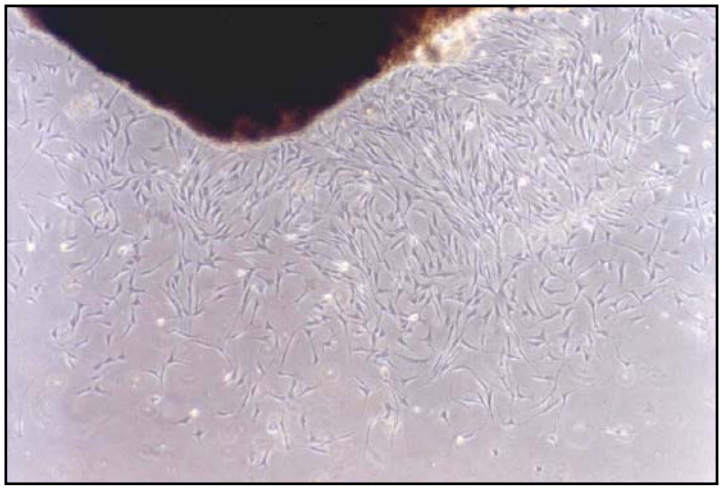

Figure 13. Ear fibroblasts outgrowth from ear skin.

Figure 14. Ear fibroblasts just after trypsinization.

and the cells were pelleted and dissociated to be single cells (Figure 14) suspension in TCM199 + 25mM Hepes + 10% FCS (TCM199H-10).

2.2 Oocytes preparation

Bovine ovaries were collected from the slaughterhouse and kept in 0.9% normal saline then transported to laboratory at room temperature within 2-3 h. Cumulus oocytes complexes (COCs) were obtained by aspiration from follicle diameter 3-6 mm using 18G needle connected with 10 ml syringe. COCs (Figure 15) were selected under stereomicroscope and washed 4 times in DPBS supplemented with 0.1% PVP. Each of 20 COCs were placed in 100 µl droplets of maturation medium covered with mineral oil and cultured under humidified atmosphere of 5% CO_2 in air at 38.5°C for 20 h. The *in vitro* maturation medium (IVM) consisting of TCM199 (Sigma) supplemented with 10% FCS, 50 IU/ml human chorionic

gonadotropin (hCG, Chorulon®, Intervet), 0.02 AU/ml follicular stimulating hormone (FSH, Antrin®, Denka Phamaceutical) and 1 μg/ml 17β estradiol (E_2, Sigma).

2.3 Enucleation

At 20 h of culture (Figure 16), the cumulus cells were mechanically removed by repeated pipetting using a fine-tip pipette in 0.2% hyarulonidase and were subsequently washed 5 times in TCM199H-10. Oocytes at metaphase II (with first polar body, Figure 17) were enucleated by micromanipulator under inverted microscope (Figure 18). Oocytes were placed in TCM199H-10 containing 5 μg/ml cytochalasin B for 15 min. The zona pellucida above the first polar body was cut with glass needle and small volume (about 10%) of cytoplasm laying beneath the first polar body was squeezed out of the zona pellucida. After enucleation, the enucleated oocytes were washed 5 times in TCM199H-10 and kept in this medium until donor cell injection. The successfully enucleated oocyte was confirmed by Hoechst 33342 fluorescent staining (Figure 19) of the squeezed out karyoplasts.

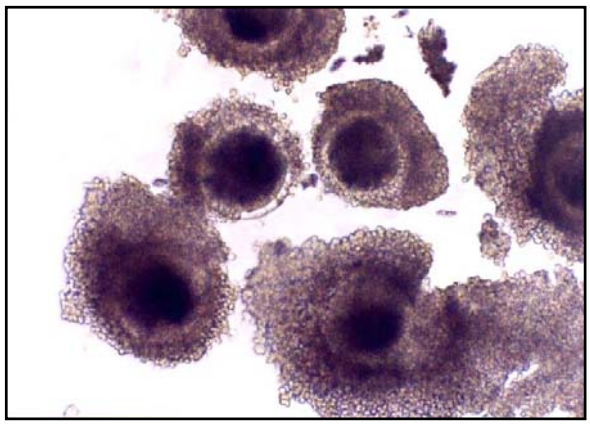

Figure 15. COCs after aspirated from bovine ovaries.

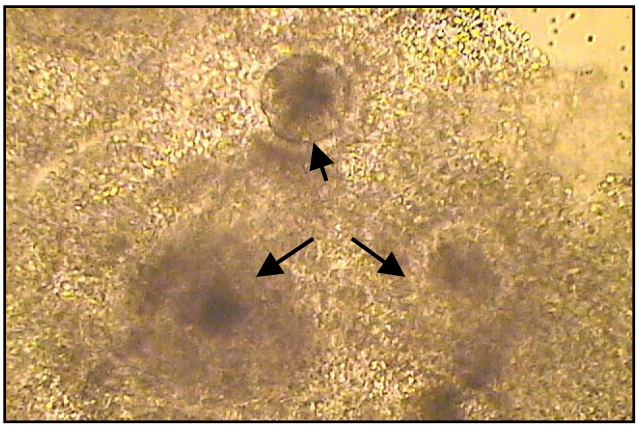

Figure 16. COCs at 20 h after cultured in IVM medium, cumulus surround the oocytes were expanded.

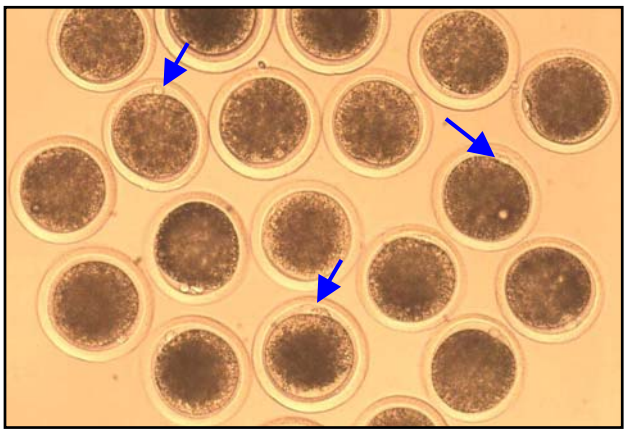

Figure 17. Matured oocytes (MII) with first polar body (arrow)

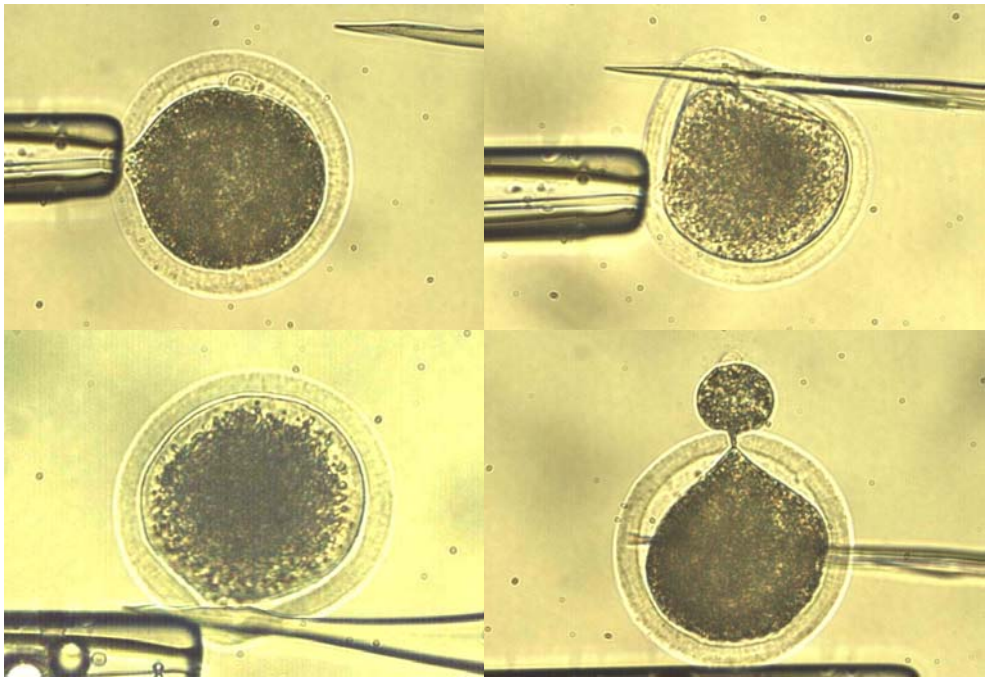

Figure 18. Enucleation of matured oocyte by micromanipulator.

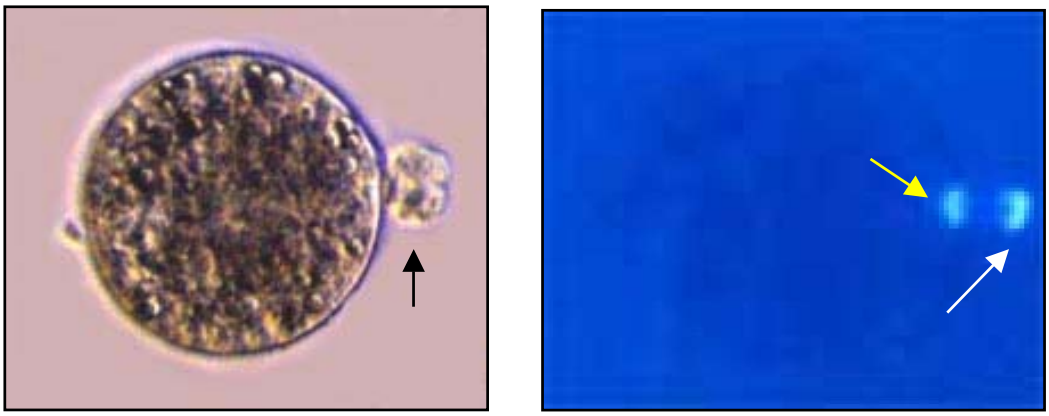

Figure 19. First polar body and cytoplasm under polar body were stained by Hoechst 33342 and examine under fluorescent microscope.

2.4 Nuclear transfer

Each individual donor cell (diameter 14-16 μm) was inserted into perivitelline space of enucleated oocyte (Figure 20). The resulting of somatic cell-cytoplast complexes (SCCCs) were transferred to Zimmermann fusion medium. An individual SCCC was placed between both tips of electrodes to electrostimulate (Figure 21) with double DC pulses of 24V/SCCC for 15 μsec in each time. The DC pulses were generated by fusion machine (SUT F-1, Suranaree University of Technology). After fusion for 1 h, the fusion rate was checked under inverted microscope.

2.5 Activation

The reconstructed embryos were activated by placing in 7% Ethanol in Emcare holding medium (ICP bio) for 5 min. After that, the embryos were incubated in mSOFaa + 10% FCS (mSOF10), which supplemented with 10 μg/ml cycloheximide and 1.25 μg/ml cytochalasin D, under humidified atmosphere of 5% CO_2 in air at 38.5°C for 5 h.

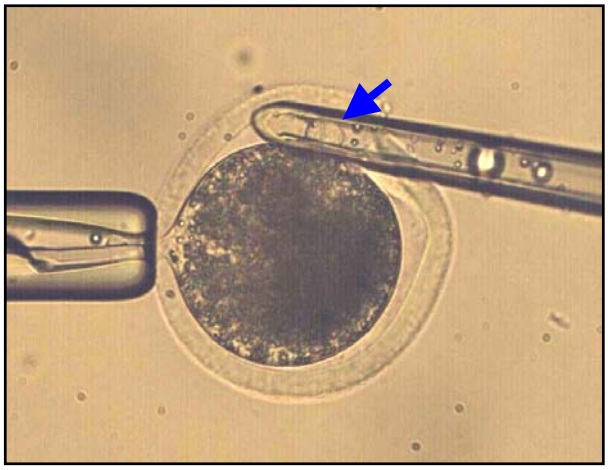

Figure 20. Donor cell was inserted into perivitelline space of enucleated oocyte.

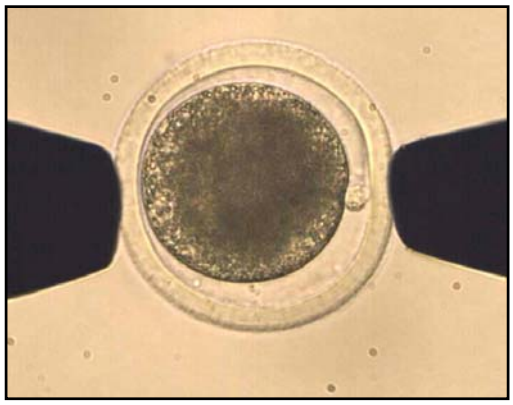

Figure 21. The donor cell was placed between both tips of fusion electrode.

2.6 *In vitro* embryos culture

The activated reconstructed embryos were cultured in mSOFaa medium supplemented with 0.1% fatty acid free BSA (A-6003, Sigma), 0.2% Linoleic acid albumin (LAA, L-8384, Sigma: mSOFaa-LAA). A group of 20 reconstructed embryos was cultured in 100 µl droplets of mSOFaa-LAA under humidified of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C for 2 days. After that, 8-cell stage embryos were selected and co-cultured with bovine oviductal epithelium cells (BOEC) in 100 µl droplets of mSOFaa-LAA at 38.5°C in humidified atmosphere of 5% CO_2 in air for 3 days. The development of embryos was observed and half of the medium was changed daily.

2.7 Vitrification

Grade 1 and 2 of hatching blastocysts at day 7 of cultured were divided into 3 groups (Figure 22) depending on extruded part from zona pellucida (D2) and remaining part inside the zona pellucida (D1), group A: D2/D1 = 0.01-0.70, group B: D2/D1 = 0.70-0.10 and group C: D2/D1 = 1.01-1.7. Hatching blastocysts were

exposed in equilibration solution containing of 10% EG + 10% DMSO in TCM199H-20 at 22^{o}C for 2 minute. After that embryos were equilibrated in vitrification solution containing of 20% EG + 20% DMSO + 0.5M sucrose with and without 10% Ficoll (Sigma) in TCM199H-20 at 22^{o}C for 30 sec. During this time, the 1-3 embryos were loaded onto the tip of cryotop device (Kitazato Supply Co., Tokyo) and immediately submerged into liquid nitrogen.

2.8 Warming

Embryos were warmed by serial dilutions from 0.5M, 0.4M, 0.3M, 0.2M, 0.1M, 0M sucrose at 22^{o}C for 5 minute of each dilution and then the embryos were co-cultured in 100 μl droplets of mSOFaa-LAA with BOEC under humidified atmosphere of 5% CO_2 in air at 38.5°C for 24 h. At 0 h and 24 h post-warmed, the embryos morphologies were examined.

2.8 Statistical analysis

The significant differences were determined by ANOVA using SAS program ($P<0.05$).

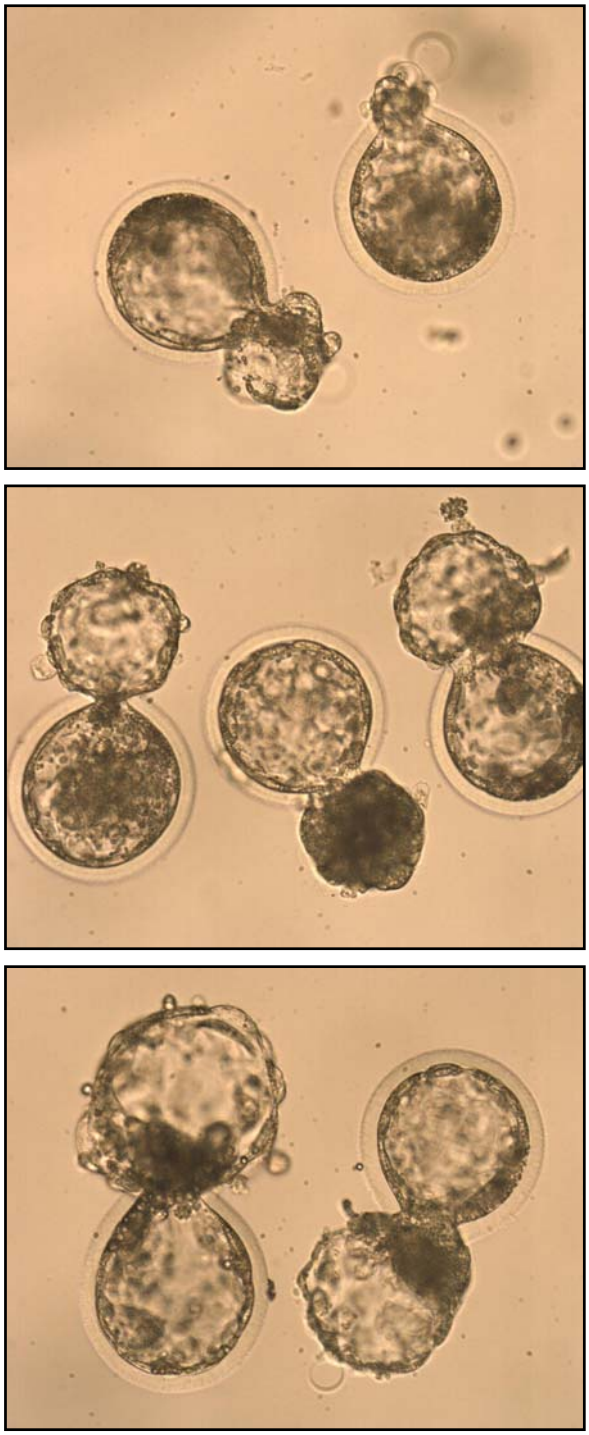

Figure 22. Cloned embryos at day 7 of cultured a: group A, b: group B, c: group C.

CHAPTER III

RESULTS

The preliminary study examined the effect of LAA on the development of cloned bovine embryos by compare culture medium with LAA (+LAA) and without LAA (-LAA). From Table 5, embryos in +LAA group have higher developmental rate than –LAA group in every stage, however, it was not significantly different (P>0.05).

Table 5. *In vitro* development of cloned bovine embryos in culture medium with LAA and without LAA.

Culture medium	Fused (%)	Cultured	Cleaved (%)	8-cell (%)	Mor. at day 5 (%)	Blast. at day 7 (%)
(+) LAA	453/532 (85.1)	452	419/452 (92.7)	333/452 (73.7)	206/419 (49.2)	178/419 (42.5)
(-) LAA	465/548 (84.7)	451	393/541 (87.1)	292/451 (64.8)	173/393 (44.0)	125/393 (31.8)

The vitrified embryos in solution without Ficoll were warmed and the morphology were also observed. From Table 6, immediately after warming (0 h), the normal morphology of embryos in group B (100%, 27/27) and C (100%, 30/30) were higher than those in group A (93%, 28/30), however, it was not significantly different (P<0.05). The survival rate observed at 24 h after cultured

***in vitro* (24 h) of group C (80%, 24/30) was higher than those in group A (77%, 23/30) and B (74%, 20/27), but it was not significantly different.**

Table 6. The post warmed normal morphology at 0 h and survival rate at 24 h after cultured *in vitro* of vitrified embryos in solution with and without Ficoll.

Vitrification solution	Group	0 h (%)	24 h (%)
Without Ficoll	**A**	28/30 (93.3)	23/30 (76.7)
	B	27/27 (100.0)	20/27 (74.1)
	C	30/30 (100.0)	24/30 (80.0)
With 10%Ficoll	**A**	32/34 (94.1)	22/34 (64.7)
	B	28/28 (100.0)	15/28 (53.6)
	C	32/32 (100.0)	19/32 (59.4)

From Table 6, the normal morphology of vitrified embryos in solution with 10% Ficoll at 0 h after warming in group A (94%; 32/34) was lower than those in group B (100%; 28/28) and C (100%; 32/32), but it was not significantly different. The survival rate observed at 24 h after cultured *in vitro* of embryos in group A (65%, 22/34) was higher than those in group B (54%, 15/28) and C (59%, 19/32).

The overall normal morphology at 0 h between embryos vitrified in solution with and without 10% Ficoll were not significantly different in all groups.

The survival rate at 24 h after cultured *in vitro* (Figure 23) of embryos which vitrified in solution without Ficoll (Figure 24) in all groups were higher than those vitrified in solution with 10% Ficoll (Figure 25), however, it was not significantly different (P>0.05).

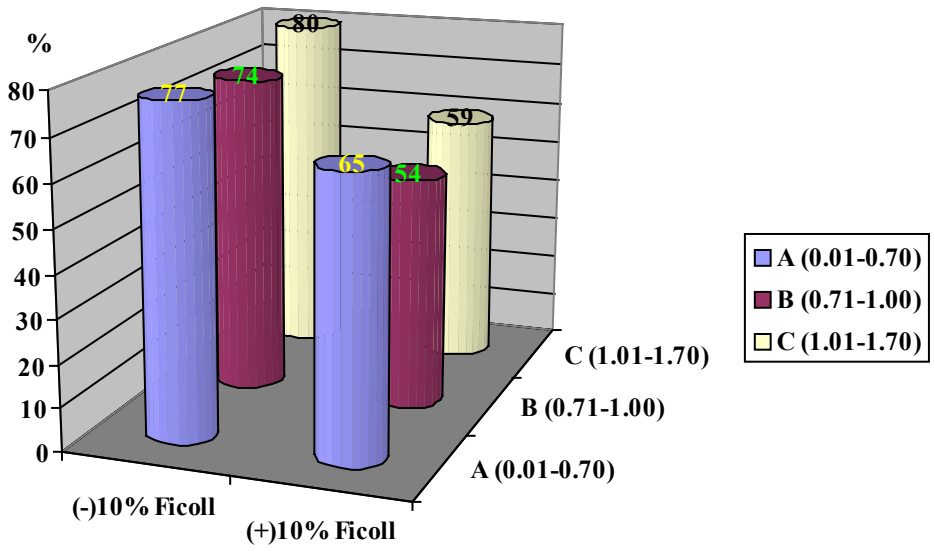

Figure 23. Survival rate at 24 h of bovine hatching blastocysts which vitrified in solution with and without 10% Ficoll.

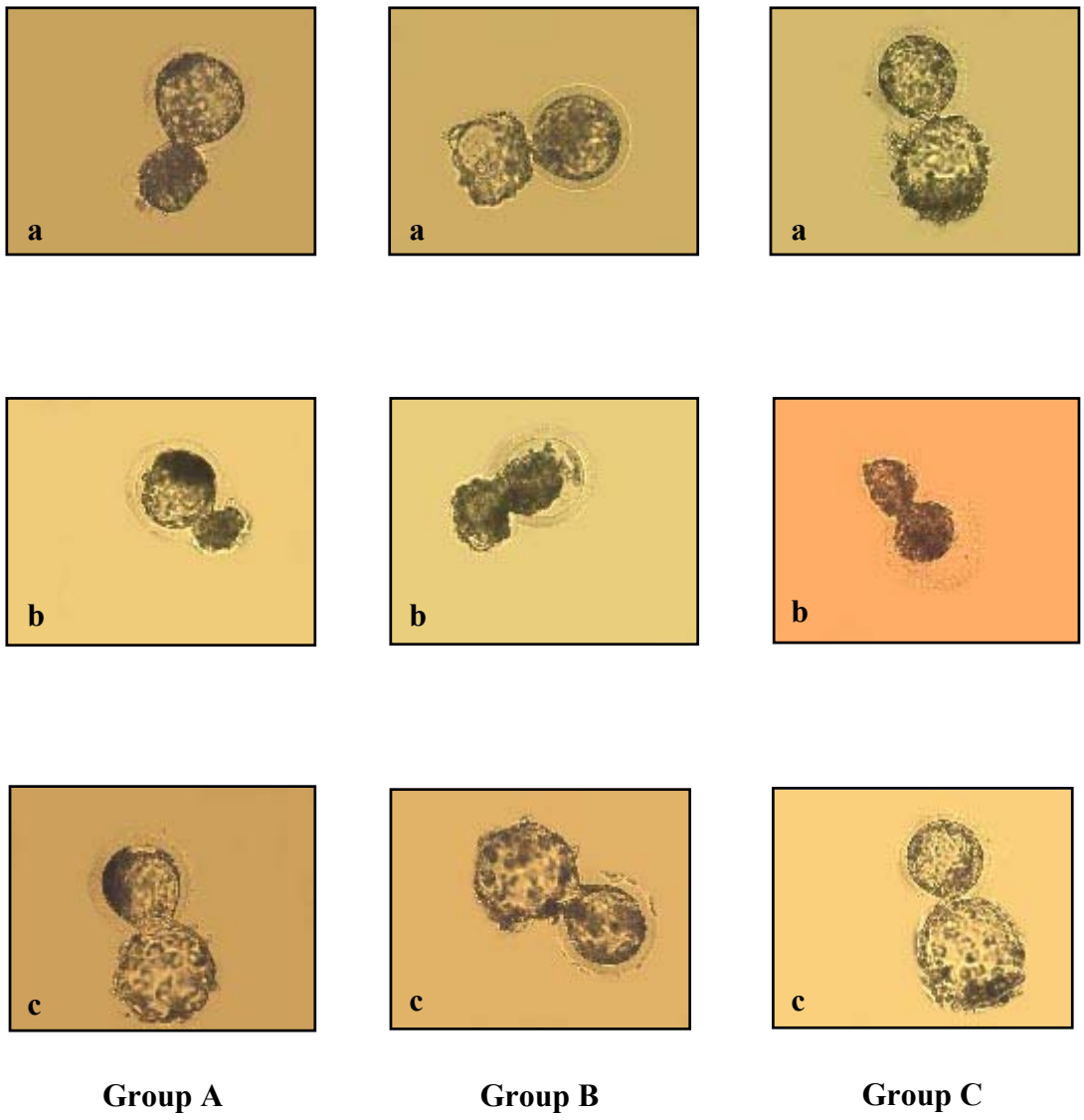

Figure 24. Morphology of hatching blastocysts vitrified in solution without Ficoll. a: before vitrification, b: 0 h post-warmed and c: 24 h after cultured *in vitro*.

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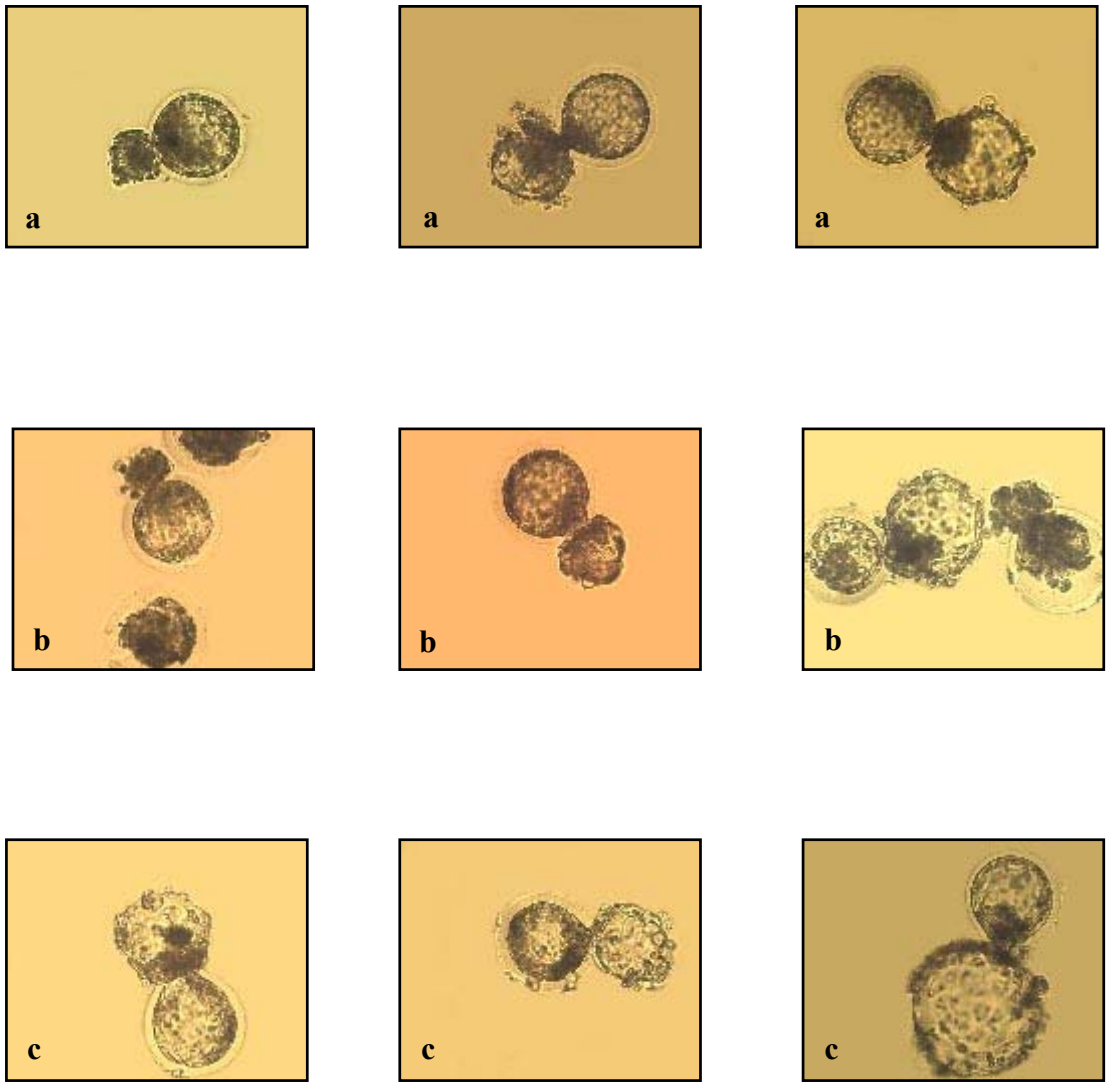

Figure 25. Morphology of hatching blastocysts vitrified in solution with Ficoll. a: before vitrification, b: 0 h post-warmed and c: 24 h after cultured *in vitro*.

CHAPTER IV

DISCUSSION AND CONCLUSION

The effect of hatching status of cloned bovine blastocysts and macromolecule, Ficoll, in vitrification solution on survival rate after warming were studied. At the first era of vitrification technique, standard French mini-straws (0.25 ml) was used for storages of oocytes and embryos. However, heat transfer of the straws is low due to the thickness of the wall of the straw. Moreover, straw needs to use large volume of vitrification solution, resulting in the decreasing of cooling and warming rates. Pollard and Leibo (1994) suggested that rapid cooling may help prevent chilling injury during cooling. In this experiment, cryotop was used as a container. Cryotop can give higher cooling and warming rates when compared to straw because the volume of vitrification solution was less than 1 μl. Furthermore, the plastic sheet of cryotop is thinner than that of the straw, resulting in the increasing of cooling rate during vitrification. Martino and colleagues (1996) suggested that the high cooling rate could prevent the sensitivity of cells from chilling injury.

Bovine embryos produced *in vitro* are more sensitive to cryopreservation than the embryos produced *in vivo* (Leibo et al., 1993), because they contained high lipid inside of their cytoplasm. The higher content of intracytoplasmic lipid droplets gave more sensitivity of bovine embryos to cryopreservation (Liebermann et al., 2002). The addition of LAA in culture medium could improve the survival rates after vitrification (Imai et al, 1997; Hochi et al, 1999a, Hochi et al, 1999b; Hochi et al.,

2000). Drobnis and colleagues (1993) suggested that the membrane fatty acid composition effect the fluidity of the membrane lipid bilayer that influence freezing sensitivity. Hochi and colleagues (1999b) explained the role of LAA that it carried to the membrane site by the bounded albumin directly incorporated into the lipid bilayer, and thus increases the membrane fluidity in order to facilitate water loss from the zygotes during cooling. The depletion of membrane cholesterol is also related to the increased membrane fluidity. It has been reported that incubation of adenocarcinoma cells with polyunsaturated fatty acids (linoleic acid, linolenic acid, eicosapentaenoic acid: EPA etc.) resulted in the modification of membrane fatty acid composition and decrease the rates of synthesis and esterification of cholesterol (Murthy et al.,1988). Therefore, in this experiment LAA was supplemented into culture medium to support embryos tolerate the vitrification procedure. Moreover, LAA also supported embryos development as result showed in Table 5.

The survival rate at 0 h after warming of cloned hatching blastocysts vitrified in solution with or without Ficoll from 3 groups was not different. Survival rate of embryos from group A of both vitrification solutions was slightly lower (94.1% with Ficoll and 93.3% without Ficoll, respectively) than that of group B and C. The results showed that the embryos from group A were slightly more sensitive than group B and C. Normally, embryos from IVF have intact zona pellucida, so they will not start hatching at day 7 of *in vitro* culture. The diameter of embryos gradually expanded until zona pellucida lysis, then the embryos will start hatching. On the other hand, expanded blastocyst stage was not found in cloned embryos because there were a slit on zona pellucida occurred during enucleation process. Hence, cloned embryos will start to hatch faster than embryos from IVF technique (Figure 26).

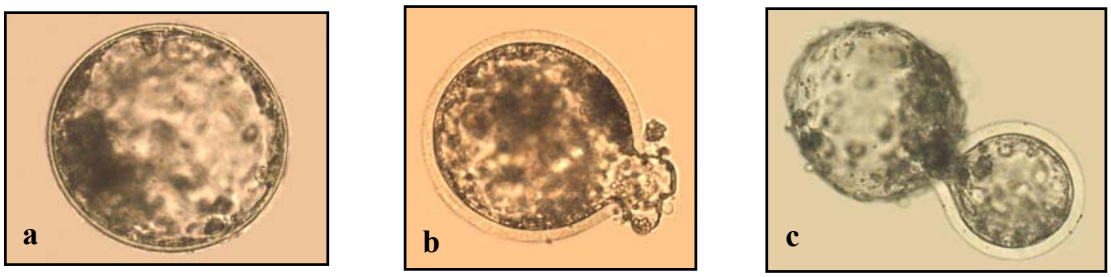

Figure 26. a: Expanded blastocyst stage from IVF technique at day 7. b: Cloned bovine embryos start hatching blastocyst stage at day 5.5. c: Embryonic cells of cloned bovine embryos more hatching at day 7.

The results showed that Ficoll did not improve the survival rate after vitrification and thawing. There are many macromolecules added in vitrification solution such as Ficoll and PVP. Macromolecule are believed to promote vitrification and may protect the cellular membrane and the zona pellucida from damage during the cooling and warming procedure. (Fahy,G.M et al., 1984). Kasai and colleagues (1996) reported that polymers might reduce the tendency of zona pellucida crack. However, the role of polymers to reduce the tendency of zona pellucida crack has not been reported clearly. They suggest that size of polymers or configuration of the molecules may be important in this role. Dumoulin and colleagues (1994) reported that macromolecule can protect embryos from the mechanical stresses during vitrifcation. In this experiment, the results showed lower survival rate of embryos at 24 h after *in vitro* culture after warming. It might be dued to the concentration of cryoprotectant in the cell was not enough to protect the embryos from the cryoinjury during vitrification. The macromolecule played role at the cell membrane that could limit the concentration of cryoprotectant passing into the cell. So after warming it was

found that the embryos in vitrification solution with Ficoll had lower number of survival than vitrification solution without Ficoll.

The temperature of vitrification solution during vitrification affected on penetration rate of cryoprotectant and its toxicity. The permeation rate of cryoprotectant into the cell will increased if the temperature increased. Embryos could expose to cryoprotectant at low temperature longer time than at high temperature of solution. This experiment used high concentration of cryoprotectant, 40% CPA, 20% EG + 20% DMSO + 0.5M sucrose with and without Ficoll. So during vitrification and warming, the temperature was controlled at 22-24°C to reduce the toxicity of cryoprotectant. The results showed that survival rate of embryos in vitrification solution without Ficoll was higher than that of embryos in the solution with Ficoll (Table 6). It may be dued to equilibration time of embryos in vitrification solution with Ficoll at this temperature was not enough. Because at the temperature during 22-24°C the cryoprotectant had low penetration rate when compare to 39°C. Moreover, as suggested in above that Ficoll in vitrification solution decreased the penetration rate of cryoprotectant into the cell, thus the concentration of cryoprotectant in the cell might be too low which is not enough to protect the embryos from cryoinjury during vitrification, resulting in the survival of embryos decreased.

In this experiment, zona pellucida fracture after vitrification and warming was not found. Sometime, freezing procedure caused cracking of zona pellucida by mechanical stresses that produced from volume change of the suspending medium during phase change. Kasai (1996) reported that zona pellucida damage, fracture damage, was caused by non-uniform volume change of freezing solution during rapid cooling or presence of ice crystal during freezing. In conventional freezing, fracture

damage was found more than 50% (Landa et al., 1982). Previous studies expected the fracture damage occurred at the temperature range –110°C to -135°C because of the phase changing during freezing between solid and liquid (Luyet et al. 1967, Rall et al. 1987 and 1984, Yoshino et al. 1993). In conventional freezing of bovine embryos, when the solution temperature reduce to –30°C or -60°C before dipping into liquid nitrogen, zona pellucida damage was found at –60°C rather than -30°C (Lehn-Jensen and Rall, 1983). In addition, the flexible of container affected on zona pellucida damage. Polypropylene cryotube or plastic straw was more flexible than glass ampule or glass test tube so it could reduce of fracture damage during freezing (Landa, 1982; Rall and Meyer, 1989). Vitrification by using cryotop as a container used less than 1 μl of the solution, so this method was not effected from the volume change during cooling which cause zona pellucida damage. Also, it would be suggested that the slit on zona pellucida of cloned embryos occurred during enucleation process, might decrease the effect of volume change during vitrification. Then zona pellucida will not destroyed because the slit on zona pellucida improved its flexibility.

In this experiment, it was found that size of hatching blastocysts at day 7 did not effect to the survival rate after vitrification and warming. However, the embryo at group C had higher survival rate than group A and B. Donnay and colleagues (1998) reported that the vitrifications of embryos at day 7, which had different diameter did not effect on survival rate of those embryos. However after warming, blastocysts which diameter bigger than 180 μm were more efficient to develop themselves to hatching stage than smaller diameter. The size of blastocysts did not influence to the survival of blastocyst or cell number.

From the results of this experiment we can concluded that the Ficoll in vitrification solution did not improve the survival rate after vitrification and warming. Hatching status of bovine cloning embryos did not effect to the survival rate after vitrification and warming, however, embryos at group C which vitrified in solution without Ficoll had higher survival rate than group A and B.

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APPENDIX

Mediums and Solutions

P-S (Stock)

1. Penicillin G (Sigma, P-3032)	**0.6000**	**g**
2. Streptomysin Sulfate (Sigma, S-9137)	**1**	**g**
3. PBS (-)	**10**	**ml**

Filter sterile and storage at -20°C for upto 3 months.

TCM 199 (Stock)

1. TCM 199 (Sigma, M-5017)	0.9990	g
2. Na pyruvate (Sigma, P-5280)	0.0056	g
3. $NaHCO_3$ (Sigma, S-5761)	0.2200	g
4. Ultra pure water to	100	ml

Storage at 4°C for upto 1 week.

TCM199 + 10% Fetal Bovine Serum (FBS)

1. TCM199 (Stock)	9	ml
2. FBS (Gibco-10270-098)	1	ml
3. P-S (Stock)	10	µl

Filter sterile and storage at 4°C for upto 1 week.

Maturation medium

1. FBS	**1 ml**
2. HCG (Chlorulon®, Intervet)	**10 iu/ml**
3. FSH(Antrin®, Denka Phamaceutical)	**0.02 AU/ml**
4. P-S stock	**10 µl**
5. E_2	**1 µg/ml**
6. TCM 199 stock to	**10 ml**

Filter sterile and storage at 4°C for upto 1 week.

TCM199-Hepes (Stock)

1. TCM199 (Sigma, M-5017)	0.9990 g
2. Na pyruvate (Sigma, P-5280)	0.0056 g
3. Hepes (Sigma, H-4034)	0.5960 g
4. $NaHCO_3$ (Sigma, S-5761)	0.1250 g
5. Ultra pure water to	100 ml

Adjust pH to 7.25 and storage at 4°C for upto 1 week.

TCM199 + 10% FBS

1. TCM199 (Stock)	9 ml
2. FBS (Gibco-10270-098)	1 ml
3. P-S stock	10 µl

Filter sterile and storage at 4°C for upto 1 week.

TCM199-Hepes + 20% FBS

1. TCM199-Hepes (Stock)	8 ml
2. FBS (Gibco-10270-098)	2 ml
3. P-S stock	10 µl

Filter sterile and storage at 4°C for upto 1 week.

mSOFaa 10x stock

1. NaCl	6.294 g
2. KCl	0.534 g
3. KH_2PO_4	0.162 g
4. Phenol red	500 µl
5. $CaCl_2.2H_2O$	0.2514 g.
6. $MgCl_2.6H_2O$	0.0996 g.

Storage at 4°C for upto 1 month

mSOFaa Working solution

1. mSOF 10x stock	10 ml.
2. Essential AA (Sigma, B-6766)	2 ml
3. Non-essential AA (Sigma, M7145)	1 ml
4. $NaHCO_3$	0.2106 g
5. Na pyruvate	0.0036 g
6. L-glutamine	0.0146 g
7. Sodium lactate (60% syrup)	47 µl

Adjust volume to 100 ml and adjust pH to 7.40 and storage at 4°C for upto 1 week.

LAA Stock (100X)

1. mSOFaa working solution	**1.5 ml**
2. LAA (Sigma, L-8384)	**0.1500 g**

Storage at 4°C for upto 2 week

mSOFaa-LAA

1. mSOF working solution	**9.9 ml**
2. BSA	**0.0200 g**
3. LAA stock (100X)	**100 µl**

Filter sterile and storage at 4°C for upto 1 week.

α-MEM (Stock)

1. α-MEM (Sigma, M-0644)	**10.21 g**
2. $NaHCO_3$ (Sigma, S-5761)	2.2000 g
3. Ultra pure water to	1 l

Filter sterile and storage at 4°C for upto 3 months.

α-MEM + 10% FBS

1. α-MEM (Stock)	90 ml
2. FBS (Gibco, 10270-098)	10 ml
3. P-S (Stock)	100 µl

Storage at 4°C for upto 1 month.

PBS (-)

1. NaCl (Sigma, S-5886)	10.0000 g
2. KCl (Sigma, P-5405)	0.2500 g
3. Na_2HPO_4 (Sigma, S-5136)	1.4400 g
4. KH_2PO_4 (Sigma, P-5655)	0.2500 g
5. Ultra pure water to	1 l

Autoclave and storage at room temperature for upto 3 month.

mDPBS

1. NaCl (Sigma, S-5886)	4.0000 g
2. KCl (Sigma, P-5405)	0.1000 g
3. KH_2PO_4 (Sigma, P-5655)	0.1000 g
4. Na_2HPO_4 (Sigma, S--5136)	0.5750 g
5. Glucose (Sigma, G-7021)	0.5000 g
6. Na pyruvate (Sigma, P-5280)	0.0180 g
7. $CaCl_2 . 2H_2O$ (Sigma, C-7902)	0.0687 g
8. $MgCl_2 . 6H_2O$ (Sigma, M-2393)	0.0500 g
9. P-S (stock)	500 µl
10. Ultra pure water to	500 ml

Storage at 4°C for upto 3 months.

Trypsin/EDTA

1. Trypsin (Gibco, 2725-024)	0.2500 g
2. EDTA (BDH, 100935V)	0.0400 g
3. PBS (-)	100 ml

Filter sterile and storage at 4°C for upto 3 months.

0.2% Hyaluronidase

1. Hyaluronidase (Sigma, S-3506)	0.1000 g
2. PVP (Sigma, P-0930)	0.0500 g
3. mDPBS to	50 ml

Filter sterile and storage at -20°C for upto 3 months.

Hoechst 33342 (Stock)

1. Hoechst 33342 (Sigma, B-2261)	0.0020 g
2. DMSO (Sigma, D-1435)	1 ml

Aliquot into Eppendorf tube 5 µl/tube and storage at -20°C for upto 6 months.

Working Hoechst 33342

1. Hoechst (Stock)	5 µl
2. TCM199-Hepes + 10% FBS	1 ml

Do not storage after use.

Cytochalasin B (Stock)

1. Cytochalasin B (Sigma, C-6762)	1 mg
2. DMSO (Sigma, D-2650)	1 ml

Storage at -20°C for upto 6 months.

Working Cytochalasin B

1. Cytochalasin B (Stock)	5 µl
2. TCM 199-Hepes + 10% FBS	1 ml

Do not storage after use.

Zimmermann fusion medium (ZFM)

1. Sucrose (Sigma, S-1888)	5.4660 g
2. K_2HPO_2	0.0174 g
3. Glutathionine	0.0031 g
4. $Mg(C_2H_3O_2)_2.4H_2O$	0.0107 g
5. $Ca(C_2H_3O_2)_2$	0.0016 g
6. BSA no fatty acid (Sigma, A9647)	0.0010 g
7. Ultra pure water to	100 ml

Storage at 4°C for upto 3 months.

7% Ethanol

1. Emcare holding solution (ICP bio, ECHM-500)	930 µl
2. Absolute Ethanol (Carlo Erba Reagenti,414608)	70 µl

Do not storage after use.

Cytochalasin D (Stock)

1st stock

1. Cytochalasin D (Sigma, C-8273) 1 mg

2. DMSO (Sigma, D-1435) 1 ml

Aliquots into Eppendorf tube 100 µl/tube and storage at -20°C upto 6 months.

2nd stock

1. Cytochalasin D 1st stock 100 µl

2. TCM199 (steriled) 900 µl

Aliquots into Eppendorf tube 12.5 µl/tube and storage at -20°C upto 3 months.

Working Cytochalasin D

1. Cytochalasin D 2nd stock 12.5 µl

2. TCM 199 + 10% FBS 1 ml

Do not storage after use.

Cycloheximide (Stock)

1. Cycloheximide (Sigma, C-6798) 10 µl

2. TCM199 + 10% FCS 1 ml

Filter sterile, aliquot into Eppendorf tube 10µl/tube

and storage at -20°C for upto 3 months.

10 μg/ml Cycloheximide + 1.25 μg/ml Cytochalasin D

1. Cytochalasin D (2nd stock)	12.5 μl
2. Cycloheximide (stock)	10 μl
3. mSOF10	1 ml

Do not storage use immediately.

10% DMSO

1. α-MEM (Stock)	7 ml
2. FBS (Gibco, 10270-098)	2 ml
3. DMSO (Sigma, D-2650)	1 ml
3. P-S (Stock)	**10 μl**

Filter sterile and storage at at 4°C for upto 1 week.

Equilibration solution (10%EG + 10%DMSO in 199H-20)

1. 199H stock	6 ml
2. FBS	2 ml
3. P-S stock	10 μl
4. EG (Sigma, E-9129)	1 ml
5. DMSO (Sigma, D-1435)	1 ml

Filter sterile and storage at at 4°C for upto 2 weeks

Vitrification solution with 10%Ficoll

(20%EG + 20%DMSO + 10%Ficoll + 0.5M sucrose in 199H-20)

1.	Sucrose	1.7115 g
2.	Ficoll (Sigma, F-2878)	1 g
3.	199H stock upto	4 ml
4.	FBS	2 ml
5.	P-S stock	10 µl
6.	EG	2 ml
7.	DMSO	2 ml

Filter sterile and storage at at 4°C for upto 2 weeks

Vitrification solution without 10%Ficoll

(20%EG + 20%DMSO + 0.5M sucrose in 199H-20)

1.	Sucrose	1.7115 g
2.	199H stock upto	4 ml
3.	FBS	2 ml
4.	P-S stock	10 µl
5.	EG	2 ml
6.	DMSO	2 ml

Filter sterile and storage at at 4°C for upto 2 weeks

Warming solution (0.5M Sucrose solution)

1. Sucrose	1.7115	g
2. FBS	2	ml
3. P-S stock	10	µl
3. 199H stock upto	8	ml

Filter sterile and storage at at 4°C for upto 2 weeks

Materials

1. Inverted microscope (Karl Zeiss)
2. Micromanipulator (Narishige)
3. Fusion machine (SUT F-1, Suranaree University of Technology)
4. Hot air oven (Gallen Kamp)
5. CO_2 incubator (Shel Lab)
6. Tri-gases incubator (Thermo Forma)
7. Autoclave (Astell Scientific)
8. Refrigerator (Mitsubishi: j-elegance)
9. Deep freeze refrigerator (-70° C, Forma Scientific)
10. Suction pump (Millipore, 0295)
11. Analytical balance (Sartorius)
12. pH meter (Schott)
13. Stereo microscope (Olympus)
14. Laminar flow (Holten)
15. Ultrasonic cleaner (Crest)

16. Distill water machine (Fistreem Cyclone)

17. Ultra pure water machine (Millipore)

18. Liquid nitrogen tank (Cryodiffuse)

19. Centrifuge (Sigma, 2-15)

20. Portable incubator (Biotherm, Cryologic)

21. Desiccator

22. Auto pipette (Rainin)

23. Pipette pump (PiaAccu, Holten)

24. Magnetic stirrer (Heidolph MR2002)

25. Pasture pipette

26. Graduated pipette and pipette box

27. Tissue culture dish ∅ 60 mm (Nunc)

28. Tissue culture dish ∅ 35 mm (Nunc)

29. Bacteria dish ∅ 55 mm (Sterilin)

30. Tissue culture flask (Nunc)

31. Conical tube 50 ml (Corning)

32. Conical tube 15 ml (Falcon)

33. Cryovial (Nunc)

34. Cryo storage cane (Nunc)

35. Cryotop (Kitazato Supply Co., Tokyo)

36. Membrane filter pore size 0.2 μm (Pall Gelman)

37. Filter Holder ∅ 25 mm (Millipore)

38. Filter Holder ∅ 13 mm (Millipore)

39. Forcept

40. Syring (ERSTA)

41. Needle (Nipro)

42. Mouth piece

43. Rubber ball

44. Bottle (Duran)

45. Volumetric flask

46. Magnetic bar

47. Thermometer

48. Foam box

49. Goblet

BIBLIOGRAPHY

Chuti Laowtammathron was born in Khonkaen, Thailand on Tuesday July 19th, 1977. He finished high school from Khonkanewitayayon School in Khonkaen. In 2000, he received Bachelors Degree (B.Sc.) in Animal Production Technology from Institute of Agricultural Technology, Suranaree University of Technology. Then he continues studied Master degree in the field of Animal Biotechnology at School of Biotechnology, Institute of Agricultural Technology. His research topic was cloning of bovine embryos by using ear fibroblasts as donor cell : Comparison of survival rate after freezing in several media. This research has been presented as oral presentation in the Stem Cells and Somatic Cells Cloning Research : Present status and future trends on 3-4 December 2003 at Chulalongkorn University; the 42nd Kasetsart University Annual Conference 3-6 February 2004; Asian Reproductive Biotechnology on 12-14 April 2004 at Nong Lam University, Ho Chi Minh city, Vietnam. This work ever been presented as poster presentation in the Annual Conference of International Embryo Transfer Society (IETS) 2004 on 10-15 January 2004, Portland, Oregon, U.S.A.