DEVELOPMENT OF CLONED SWAMP BUFFALO EMBRYOS RECONSTRUCTED WITH VITRIFIED OOCYTES

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การเจริญของตัวอ่อนกระบือปลักโคลนนิ่งจากโอโอไซท์แช่แข็งด้วยวิธี Vitrification

นางสาวสุจิตรา หมื่นไธสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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จุดประสงค์ของงานวิจัยคือ การศึกษาความสามารถในการเจริญของตัวอ่อนกระบือปลักจากโอโอไซท์แช่แข็งด้วยวิธี Vitrification โดยแบ่งการแช่แข็งโอโอไซท์เป็น 2 กลุ่ม คือ แช่แข็งโอโอไซท์ระยะเมตาเฟส ทู (กลุ่ม MII) และแช่แข็งโอโอไซท์หลังจากการ enucleation (กลุ่ม Enu) การแช่แข็งทำโดยนำโอโอไซท์ไป equilibrate ในน้ำยา 7.5% ethylene glycol (EG) + 7.5% dimethylsulfoxide (DMSO) ที่ละลายใน TCM199-Hepes (TCM199H) + 20% fetal bovine serum (FBS) นาน 4, 7 หรือ 10 นาที จากนั้นนำไป vitrify ในน้ำยา 15% EG + 15% DMSO ที่ละลายใน TCM199H + 20% FBS นาน 1 นาที จัดกลุ่มโอโอไซท์ครั้งละ 5 ใบ วางที่ปลายของ Cryotop แล้วจุ่มลงไนโตรเจนเหลวทันที การละลายโอโอไซท์ ทำโดยนำ Cryotop ออกจากไนโตรเจนเหลว แล้วจุ่มลง 0.5M ซูโครส ที่อุณหภูมิระหว่าง 22-24 °C นาน 5 นาที แล้วย้ายสู่น้ำยา TCM199H + 20%FBS จากนั้นคัดเลือกโอโอไซท์ที่มีความปกติมาใช้เป็นไซโตพลาสผู้รับในการทดลอง Parthenogenetic activation (PA) และการย้ายฝากนิวเคลียสด้วยเซลล์ร่างกาย (scNT) จากผลการทดลองหลังละลายโอโอไซท์แช่แข็งพบว่าประมาณ 71% มีชีวิตรอดหลังการละลายและประมาณ 83% ของการทดลองที่ไม่รวมกลุ่ม Enu พบว่ามี First polar body โดยหลังการทำ scNT พบว่าตัวอ่อนจากทั้งวิธี PA และ scNT ที่ได้จากโอโอไซท์แช่แข็งมีความสามารถในการพัฒนาของตัวอ่อนไปสู่ระยะบลาสโตซีสได้ ถึงแม้ว่าอัตราการแบ่งตัวและอัตราการเจริญสู่ระยะบลาสโตซีสน้อยกว่ากลุ่มโอโอไซท์สด ทั้งนี้ได้นำตัวอ่อนระยะบลาสโตซีสวันที่ 6.5 ของการเลี้ยงมาย้อมสีตัวอ่อนเพื่อนับจำนวนเซลล์ระหว่าง inner cell mass cell และ trophectoderm cell พบว่าจำนวนเซลล์เฉลี่ยของตัวอ่อนที่ได้จากโอโอไซท์แช่แข็งเมื่อเทียบกับโอโอไซท์สดมีจำนวนเซลล์น้อยกว่า การทดลองนี้ได้แสดงว่าเวลาในการ equilibrate โอโอไซท์ในขั้นตอนการแช่แข็งมีผลต่ออัตราการอยู่รอดของตัวอ่อน โดยที่โอโอไซท์กระบือปลักมีความสามารถทนต่อความเป็นพิษของน้ำยาแช่แข็งได้นาน 5- 11 นาที ดังนั้นโอโอไซท์กระบือปลักสามารถนำมาใช้แช่แข็งด้วยวิธี Vitrification เพื่อใช้สำหรับ Cryopreservation ได้

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2548

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SWAMP BUFFALO/OOCYTE/VITRIFICATION/EMBRYOS/CLONING

The objective of this study was carry out in order to evaluate the developmental potential of cloned swamp buffalo embyos recontructed with cryopreserved matured oocytes by vitrification technique. The *in vitro* matured oocytes were separated into two groups, vitrified at metaphase II stage (MII) and vitrified after enucleated MII oocyte (Enu). The oocytes were equilibrated with 7.5% ethylene glycol (EG) + 7.5% dimethylsulfoxide (DMSO) in TCM199-Hepes (TCM199H) + 20% fetal bovine serum (FBS) for 4, 7 or 10 min and vitrified in 15% EG + 15% DMSO in TCM199H + 20% FBS for 1 min. Five of each group of oocytes were placed on a sheet of cryotop device (Kitazato Supply Co., Tokyo) and immediately submerged in liquid nitrogen. One hour before starting the experiment, the vitrified oocytes were warmed by directly place the tip of cryotop in 0.5M sucrose in TCM199H + 20% FBS at 22-24^{o}C for 5 min then moved to TCM199H + 20% FBS. The normal morphology oocyte were used for toxicity test by parthenogenetic activation (PA) and also used for recipient cytoplasts in somatic cell nuclear transfer (scNT) experiment. After warming, appproximately 71% of the vitrified oocytes survived and found that 83% of these contain first polar body. The scNT embryos derived from vitrified oocytes can develop to blastocyst stage as fresh oocytes. However, cleavage and blastocyst development rates of vitrified oocytes

However, cleavage and blastocyst development rates of vitrified oocytes following PA and scNT were lower than the fresh oocytes. At day 6.5, embryos at blastocyst stage were differentially stained for the inner cell mass cell and trophectoderm cell. The mean cell number in the blastocysts derived from vitrified oocytes was lower than the fresh oocytes. In conclusion, the equilibration time of vitrified buffalo oocytes effect the survival rate of the embryos. The matured swamp buffalo oocytes could tolerate the toxicity of cryoprotectants for 5-11 minutes. The vitrified buffalo oocytes could develop to blastocysts. Therefore, swamp buffalo oocytes can be cryopreserved by using vitrification technique.

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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

1.1 The introduction of buffalo

1.1.1 Classification of swamp buffalo (*Bubalus bubalis*)

There are two types of buffalo in Thailand, river buffalo (Figure 1) and swamp buffalo (Figure 2). Both of them have the same scientific name, *Bubalus bubalis*. However, the chromosome number are different, the river buffalo has 50 chromosomes but the swamp buffalo has 48 chromosomes. Most of the buffalo in Thailand are swamp buffalo.

1.1.2 Taxonomy of swamp buffalo

Kingdom *Animalia*

Phylum *Chordata*

Class *Mammalia*

Order *Artiodactyla*

Family *Bovidae*

Subfamily *Bovinae*

Genus *Bubalus*

Species *Bubalus bubalis*

1.1.3 Characteristics of swamp buffalo

Most of the swamp buffalo in Thailand are completely black in color. Only a few of them are white. The white buffalo are not albino but the color is due to uncertain genetic effects. The dark color makes buffalo heat intolerant, which is why it must stay near the swamps. On average, mature male buffalo weigh 450 – 600 kg, the mature females weigh 350 – 450 kg. They generally contain v-shape like hairs on the neck. Puberty is reached at about 18 months, the gestation period are about 300 – 340 days, and weaning occurs after 6 – 9 months. The swamp buffalo is chiefly a grazer, feeding in the morning and evenings and lying up in dense cover or submerging in wallows during mid-day. During the mid-day heat, the swamp buffalo frequently wallows in water or muddy pools, sometimes almost completely submerged, with only its nostrils showing. In addition to keeping it cool, wallowing helps to remove skin parasites, biting flies, and other pests.

Figure 1. Murrah type; river buffalo in Thailand.

Figure 2. Swamp buffalo in Thailand.

1.1.4 Buffalo situation in Thailand

The buffalo populations are mainly in Asia. According to the report of Food and Agricultural Organization of the United Nations (FAO) (1996), Thailand had the fourth largest buffalo population after India, China and Pakistan. Although, the world's buffalo numbers are increasing about 1.3% annually. However, the numbers of buffalo in Thailand are dramatically decreasing. According to the report of Information and Statistics Group, Information Technology Center, Department of Livestock Development of Thailand, there were about 5 million buffaloes in 1993 and decreased to 1.4 million in 2004. If situation allowed to continue like this, buffalo could be extinct from Thailand in 20 years. There are several reason for the reduction in the number of Thai swamp buffalo, which mainly affected by agricultural modernization. The industry invasion into Thai rural areas. The reproductive system of buffaloes are also not as good as that of cattle. The buffalo has long been considered a poor breeder such as slow to mature and slow to re-breed after calving. Poor management and nutrition has contributed much to this reputation. Their gestation period is about a month longer than that of cattle. Buffalo estrus is difficult

to detect and mating occur mainly at night so that the farmer or producer is likely to encounter more problems breeding buffaloes than cattle. Therefore, many scientists and organizations try to develop several techniques to increase the number of buffalo. Assisted reproductive technology (ART) is one of the techniques that can preserve the buffalo by artificial insemination (AI), *in vitro* fertilization (IVF) (Pavasuthipaisit et al, 1992; Wani et al., 2004) or nuclear transfer technology (Parnpai et al., 1999; 2002).

Recently, somatic cell nuclear transfer has been proven as one of the techniques that can conserve and multiply valuable genetic individual. As previous reports demonstrated that cloned buffalo embryos could be derived from fetal fibroblasts, adult ear fibroblasts and granulosa cells (Parnpai et al., 1999, 2000a, 2002, 2003; Kittiyanant et al., 2001). The buffalo oocytes are highly required to use as recipient cytoplasm for cloning experiment, however, the number of buffalo oocytes are not enough to run the experiment from day to day. From this problem, the topic of “How to store the oocyte for further used?” has been raised several years ago. Moreover, many research have to consider the efficiency of cryopreservation techniques to be used which lead to obtain the high survival rate of oocyte.

1.2 The overview of cloning technology

1.2.1 The history of animal cloning

Clone, also spelled Clon, derived the term stems from the ancient Greek “klon”, which means twig, and refers to the fact that a twig could give rise to an identical tree. Clone is a population of genetically identical cells or organisms that are derived originally from a single original cell or organism by asexual methods.

Cloning is fundamental to most living things, since the body cells of plants and animals are clones ultimately derived from the mitosis of a single fertilized egg. In approaching cloning or nuclear transfer, it is dividing into two groups. One is cloning by blastomere separation (embryo splitting) and cloning by nuclear transfer (NT) technology.

The first cloning experiment were performed in 1902 by Spemann which used a strand of hair as a noose, successfully split apart the cells of a two-celled salamander embryo and obtained a normal salamander from each individual cell. Subsequently, Spemann performed the first nuclear transfer in 1928. He transferred the nucleus of 16-cell embryo to a single salamander embryo cell with no nucleus. The cell took up the nucleus and developed into a normal salamander. The fact that a nucleus from a matured embryo cell could direct the development of a complete organism proved that genetic information did not diminish as a cell developed. From these results, Spemann, in 1938, proposed the “fantastical experiment” of cloning an organism from differentiated or even adult cells using the nuclear transfer method.

However, cloning as Spemann proposed was not completed until the success of Robert Briggs and Thomas King (1952, 1955). They cloned northern leopard frogs (*Rana pipients*) using a method of nuclear transfer. However, gastrula nuclei from mesoderm or endoderm injected into eggs were unable to develop to mature frog (Briggs and King, 1960). Until 1962, Gurdon reported the using of nuclei from intestine of tadpoles to produce fertile adult frogs.

In mammals, the first report of nuclear transfer into enucleated eggs was published by Karl Illmensee and Peter Hoppe (1981). They transfer of ICM nuclei into an enucleated zygote resulted in three live-born mice whereas the transfer of

trophectodermal nuclei failed to support development. However, these results, and the techniques that were described, could not be reproduced by others and have not been to this day (Tsunoda and Kato, 1998; Wakayama et al., 2000; Zhou et al., 2000).

Therefore, throughout the 1980s, research teams sought new techniques. Because mammalian eggs are so much smaller than amphibian eggs, and blastomere nuclei are so much larger than somatic cell nuclei, the techniques of nuclear transplantation into mammalian eggs had to be perfected. To avoid puncturing the plasma membrane of the mammalian egg, Davor Solter and colleagues took advantage of the property of some proteins, most notably those expressed on the surface of Sendai virus, to cause two membrane to fuse together. In 1983, McGrath and Solter demonstrated that nuclei from cleaving blastomeres could also be fused to enucleated zygotes by inactivated Sendai virus (strain HVJ, the hemagglutinating virus of Japan). With this technique, the offsprings were born after embryo transfer.

Subsequently, Steen Willadsen (1986) published a description of the first farm animal that resulted from the transfer of the nuclei of 8- or 16-cell stage sheep embryos into an enucleated ovulated egg. He used an oocyte that had been arrested at the metaphase stage of second meiotic division and from which the spindle and metaphase plate had been removed as the recipient cytoplast. Similar results were reported for cattle from Prather and colleauges (1987).

In 1989, Smith and Wilmut reported the birth of lambs developed from embryos reconstituted from either 16-cell blastomeres or ICM cells fused with unfertilized sheep eggs. Enucleated oocytes receiving ICM cells of cattle (Keefer et al., 1994; Collas and Barnes, 1994), and mice (Tsunoda and Kato, 1998) also developed into live offspring. Development of embryonic stem (ES) cells to term has

been limited. Live calves were produced after transfer of short-term cultured ICM cells of blastocyst into enucleated oocytes (Sims and First, 1993).

Since then, a number of cattle (Prather et al., 1987; Bondioli et al., 1990), pig (Prather et al., 1989), mouse (Tsunoda et al., 1987) rabbit (Stice and Robl, 1989; Yang et al., 1990; Collas and Robl, 1990; Yang, 1991), rhesus monkey (Meng et al., 1997) have been produced after transfer of blastomeres of two-, four-, and eight-cell and morula-stage embryos to enucleated oocytes at the second metaphase.

With the successful of the world's first clone from adult somatic cells (Wilmut et al., 1997), Dolly, a viable lamb resulting from a cultured cell derived from somatic mammary gland cells harvested from a 6-year-old ewe. From these results several laboratories have used various modifications of the nuclear transfer technique to produce cloned animals.

A large number of cloned female and male sheep (Wilmut et al., 1997; Schnieke et al., 1997), mice (Wakayama et al., 1998; Kato et al., 1999; Wakayama and Yanagimachi, 1999; Ogura et al., 2000; Ono et al., 2001), cattle (Kato et al., 1998, 2000; Cibelli et al., 1998; Vignon et al., 1998; Zakhartchenko et al., 1999b; Wells et al., 1999; Renard et al., 1999; Shiga et al., 1999; Kubota et al., 2000; Lanza et al., 2000; Parnpai et al., 2000b), goats (Baguisi et al., 1999), and pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000) have been produced after nuclear transfer of somatic cell cultured from various tissues of fetuses, newborns, and adults.

Cloned animals have been produced after nuclear transfer of somatic cells from organs 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), fetal genital ridge cells (Betthauser et al., 2000), fetal skin fibroblasts

(Onishi et al., 2000), adult skin fibroblasts (Parnpai et al., 2000b), granulosa cells (Polejaeva et al., 2000), gonad (Wakayama and Yanagimachi, 2001) and others. Table 1 shows the successful of live offspring produced from somatic cell cloning.

Table 1. The successful of live offspring produced from somatic cell cloning *

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. 1999b, 54: 264-272.
	Fetal germ cells	Died within 24h	Zakharchenko et al., Mol.Reprod.Dev. 1999c, 52: 421-426.
	Adult fibroblasts	Alive	Kubota et al., Proc. Natl. Acad. Sci. 2000, 97: 990-995.
	Senescent fibroblasts	Alive	Lanza et al., Science 2000, 288: 665-669.

Table 1. The successful of live offspring produced from somatic cell cloning (continue)*

Species	Donor cell	Offspring	Reference
Cattle (cont.)	Transfected fetal fibroblasts	Alive	Zakharchenko et al., Mol.Reprod.Dev. 2001, 60: 362-369.
	Ear fibroblasts	Alive	Parnpai et al., Theriogenology 2002, 57: 443.
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.
	Fetal fibroblasts	Alive	Ono et al., Biol. Reprod. 2001, 64: 44-50.
Cat	Cumulus cells	Alive	Shin et al., Nature 2002, 415: 859.

Table 1. The successful of live offspring produced from somatic cell cloning (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.
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
Dog	Skin fibroblasts	Alive	Lee et al., Nature 2005, 436: 641.

*modified from Brem and Kuhholzer (2002) and Solter (2000).

1.2.2. Cloning techniques

1.2.2.1 Enucleation

Cloning or nuclear transfer is a technically demanding process. There have been attempting to eliminate micromanipulation for some of the most complicate procedures, such as enucleation. Enucleation is the method for remove the nucleus out of the oocyte. Alternatives have included aspiration of polar body and adjacent cytoplasm (Prather et al., 1987; Stice and Robl, 1989; Smith and Wilmut, 1989), rejection of the half with evidence of polar body or chromatin (Willadsen, 1986), gradient centrifugal enucleation (Tatham et al., 1995), chemical enucleation (Fulka and Moor, 1993), functional enucleation (Wagoner et al., 1996), telophase enucleation (Bordingnon and Smith, 1998), and nuclear transfer without micromanipulators (Peura et al., 1998; Vajta et al., 2001).

1.2.2.2 Fusion

In cloning procedures, fusion may be accomplished by electrical stimulation (Wilmut et al., 1997; Kato et al., 1998; Polejaeva et al., 2000), polyethylene glycol (PEG), or Sendai virus (McGrath and Solter, 1983), with electrical pulse being by far the most popular method. Efficient fusion by electrical pulse depends on healthy cell membranes, physical contact of both oocyte and blastomere membranes to be fused and direction of the fusion current primarily through the point of membrane contact (Zimmerman and Vienken, 1982). In some case, 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).

1.2.2.3. Activation

Oocyte activation and embryo culture are the next steps in the cloning process. Because of cloning process lack of sperm-induced fertilization steps, an oocyte must be activated for completion of the second meiotic division (MII) and progress through subsequent stages of embryo development. There are many choices for the method of artificial activation and many theories as to the optimal timing between fusion and activation as well as for embryo culture development. However, somatic cell cloning needs high kinase levels which is necessary for reprogramming. Aging of the oocyte or repeated pulsing might represent a limitation because the somatic nucleus will be expose to decreased levels of MPF (maturation promoting factor). At present, the most common protocols are based on physical or chemecal stimuli. These protocol performed to induce calcium oscillations to degrade cell cycle proteins repeatedly and or block new protein synthesis.

For example, ethanol (Kishikawa et al., 1999), calcium ionophore (Koo et al., 2001), ionomycin (Rho et al., 1998) or calcium ionophore followed by 6-DMAP (dimethylaminopurine) (Susko-Parrish et al., 1994) and strontium (Whittingham and Siracusa, 1987; Wakayama and Yanagimachi, 1999), including electric pulses (Prather et al., 1987; Stice and Robl, 1989; Ware et al., 1989; Bondioli et al., 1990; Smith and Wilmut, 1990; Mayes et al., 1995; Nagashima et al., 1997) had also been used for activation.

After activation, the culture requirements of cloned embryos should be the same as normally fertilized embryos. In the initial experiments, embryos culture were performed *in vivo* into the sheep oviduct after agar embedding (Willadsen, 1986). Recently, the embryos culture were performed *in vitro* with the refinement of

in vitro protocols. *In vitro* cultured appeared to be approximately half (13%) that obtained from *in vivo* cultured in sheep oviducts (23-35%) (Bondioli et al., 1990).

1.2.2.4 Culturation

The culture of embryos requires an appropiate environment especially *in vitro* cultures so that the early embryo can undergo several cleavage develop to reach the blastocyst stage. Embryo culture can seperate to *in vivo* and *in vitro* system. For *in vivo* culture system, their have used a variety of animal systems, including mice, rabbits, sheeps, cattle and even chicken eggs for culture of early bovine embryos. The use of oviduct as an *in vivo* culture system helped to focus attention on the unique properties of this part of the female reproductive systems. All the evidence suggests that the fallopian tube plays a crucial role in the life of the gametes and in the early embryo in the days after fertilization.

For *in vitro* culture system, the cells of the oviduct were used in the first successful coculture systems in sheep and cattle. The use of oviductal monolayer culture system in sheep marked a particularly important (Gandolfi et al., 1986; Gandolfi and Moor, 1987). Subsequenly, it became apparent that not only oviductal cells but a wide variety of other somatic cells are capable of providing an environment in several animal embryos can develop. However in the optimal embryo culture system, it is essential to think in terms of chemically defined media that are based on a knowledge of embryo metabolism and embryo preferences for energy substrates and other essential nutrients. In recent years, embryo culture media specialized for optimum development of embryos from individual species have been developed such as bovine, synthetic oviductal fluid (SOF) (Tervit et al., 1972); mice,

Chatot/Ziomek/Bavister (CZB) medium (Chatot et al., 1989); and pigs, North Carolina State University-23 (NCSU-23) medium (Petters and Wells, 1993).

1.2.2.5 Pregnancy and Efficiency of nuclear transfer

Pregnancy rates after transfer of cloned embryos are lower than with normally fertilized embryos in all species. The lower pregnancy rates are probably due to incomplete nuclear reprogramming leading to a fuilure of the placental membranes to develop and function normally. However, the success of cloning an entire animal from differentiated adult cell demontrated that genes inactivated during tissue differentiation can be re-activated by a process called nuclear reprogramming. This process that means the reversion of a differentiated nucleus back to a totipotent status. Somatic cell cloning may be used to generate multiple copies of desirable genetic characteristics, to produce transgenic animals for pharmaceutical protein or xeno-transplantation, or to preserve endangered species. In addition, cloning has become an essential tool for studying gene function, genomic imprinting, genomic re-programming, regulation of development, genetic disease, and gene therapy, as well as other topics.

1.3 The principle of cryopreservation technology

Cryobiology is the study of the effects of extremely low temperatures on biological systems, such as cells or organisms. Cryopreservation is an applied aspect of cryobiology, which has resulted in methods that permit low temperature maintenance of a diversity of cells. The objective of cryopreservation is to minimize damage to biological materials, including tissues, mammalian cells, bacteria, fungi, plant cells and viruses, during low temperature freezing and storage. Cryopreservation

provides a continuous source of tissues and genetically stable living cells for a variety of purpose, including research and biomedical processes.

The basic principle of cryopreservation is the extent of freezing damage depends on the amount of free water in the system and the ability of water to crystallize during freeznig. Water is the major component of all living cells and must be present for chemical reactions to occur within a cell. During freezing, most of the water changes to ice, and cellular metabolism decreased.

Ice formation initiates in the extracellular environment, resulting in increased salt concentrations as water is removed to form ice. This ice formation results in an osmotic imbalance. Then water leaves the cells by osmosis cause cellular dehydration. Excessive dehydration can be damage to cell recovery.

Potentially damaging effects of dehydration and ice can be minimized by the following methods 1) controlling the cooling rate by using an appropiate controlled rate freezer, 2) using cryoprotective agents in appropiate solutions, 3) maintaining appropiate storage temperatures and 4) controlling the rewarming rate. All of these events interact to influence the outcome of cryopreservation.

The cryopreservation procedures have already been defined in several mammalian species to maintain variety of cells such as sperms, oocytes and embryos in frozen state. In 1776, Spallazani and colleagues reported that the sperm and egg silk could be preserved in snow frozen at low temperature. Subsequently, cryoprotectant is widely used for cryopreservation in many species.

The first successful studies in the freeze-thawing of mammalian embyros were those reported by Whittingham (1972) in the mouse embryos at 8-cell stage. Then, Wilmut and Rowson (1973) were the first to show cattle embryos would survive after

freezing. Subsequently, Friedler and colleagues (1988) reported the frozen of matured oocytes. Following early experiments, the procedures were used in several mammalian species and several different protocol which were applied around the world.

1.4 Types of cryopreservation technology

1.4.1 Slow freezing or conventional freezing method

Slow freezing method of cryopreservation has been developed to accommodate the consequences of ice formation. Matured mouse oocytes were first successfully cryopreserved in a solution of 1.5 M dimethylsulfoxide (DMSO) and a slow controlled cooling rate (Whittingham, 1977). Conventional slow freezing procedures have the following distinctive steps (Figure 3): 1) oocytes exposed at room temperature to molar concentrations of low molecular weight permeating cryoprotectants, such as ethylene glycol (EG), glycerol, DMSO or propylene glycol until equilibrium is reached between cryoprotectants solution; 2) induction of ice crystals (seeding) at -5º to -7ºC and holding at this temperature for 5-10 min; 3) controlled, slow cooling around 0.2 to 2.0 ºC/min were used to decrease temperature; 4) plunging at -30º to -70ºC by use cooliing rate 0.3ºC/min and storage in liquid nitrogen (-196ºC); 5) controlled thawing at around 250ºc/min (e.g. 25ºC water bath without stirring); 6) removal of cryoprotectant at room temperature and prior to culture or transfer. In order to survive cryopreservation, cells must remain undamaged and physiologically functional during the entire procedure. Therefore, appropiate supplement of each step is absolutely critical.

Conventional slow freezing procedures for mammalian oocytes are remarkably similar to the protective mechanism developed by different freeze-tolerant animals against freezing environment (Storey and Storey, 1990). The keys factors in the survival of freezing is the production of large amounts of low molecular weight cryoprotectants such as glycerol, DMSO and variety of other alcohols and sugars (Baust, 1981; Bowler and Fuller, 1987; Salt, 1957; Somme, 1982; Zachariassen, 1980). These permeating compounds apparently protect cell structure by preventing intracellular ice crystal formation during freezing and thawing (Bowler and Fuller, 1987; Schneider, 1986).

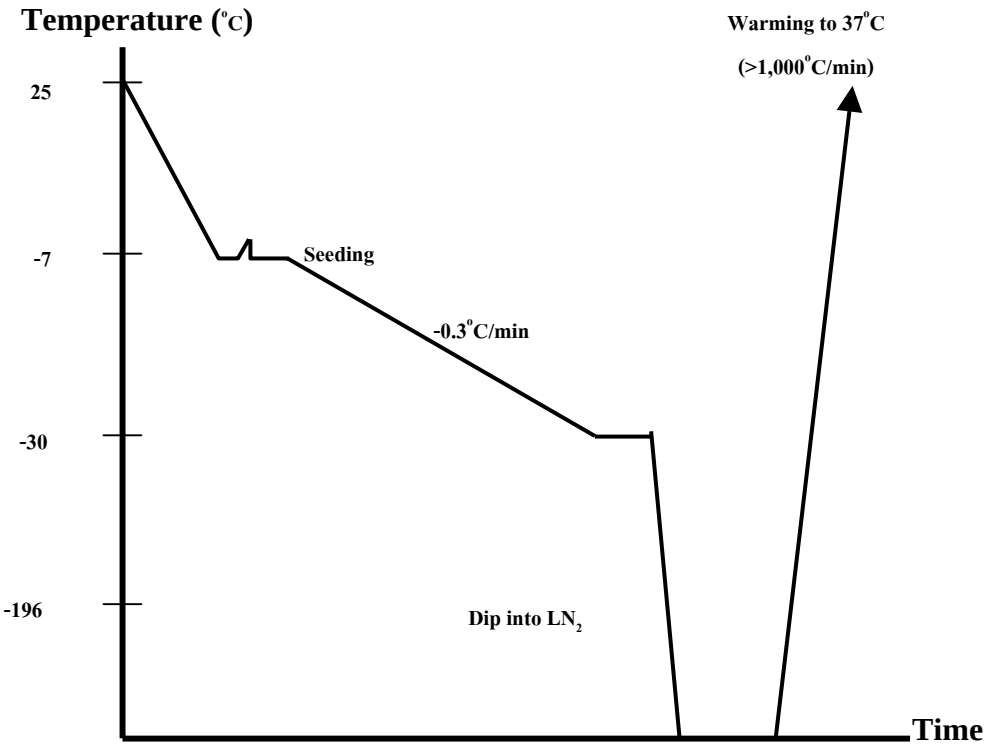

Figure 3. The cryopreservation procedure by slow freezing.

During the time that intracellular fluids become equilibrated with high concentration of a nontoxic, permeating cryoprotectant and the temperature drops

below zero, ice crystal growth is induced in freeze-tolerance animals by specific ice-nucleating proteins (Zachariassen et al., 1980). Induction of ice crystals, also known as seeding, prevents supercooling and the detrimental effects of rapid freezing which occurs when spontaneous ice nucleation occurs (Duman, 1982). Once ice crystals are induced, there is a tendency of smaller crystals to grow into larger crystals, a process which is lethal to the cells and which must be prevented (Knight and Duman, 1986). In the laboratory, the ice crystal formation is controlled by utilizing slow cooling rates (less than 2ºC/min) which allow dehydration of cells during freezing (Schneider, 1986).

Slow freezing procedures can be highly damaging to oocytes of chill-sensitive species. They involve prolonged exposure to the temperature at most damage occurs. This may explain the protocols which minimize exposure to the temperature at most damage occurs, e.g., by using high equilibration temperature combined with rapid or very rapid cooling rates can get good results for oocytes of chill sensitive species (Martino et al, 1996; Isachenko, 1997; Le Gall and Massip, 1999; Vajta et al., 1998).

This technique has been widely used in several animal species such as cattle (Ohkoshi et al., 1997; Kubota et al., 1998; Booth et al., 1999; Peura et al., 1999), mouse (Whittingham, 1977; Carroll et al., 1993), rabbit (Bank and Maurer, 1974), human (Fugger et al., 1988; Abdalla and Leonard, 1988), sheep (Willadsen et al., 1976), goat (Bilton and Moore, 1976), cat (Dresser et al., 1988; Luvoni and Pellizzari, 2000), horse (Yamamoto et al., 1982), swamp buffalo (Kasiraj et al., 1993) and hamster (Lane et al., 1999a). Since the first report of live-born young from cryopreserved mouse embryos, similar success has been reported in other mammalian species (Table 2).

Table 2. The lives offspring of mammalian animals after cryopreserved and transferred embryos to recipients.

Species	Years	References
Mouse	1972	Whittingham et al.
Cattle	1973	Wilmut et al.
Rabbit	1974	Bank et al.
Sheep	1974	Willadsen et al.
Rat	1975	Whittingham et al.
Goat	1976	Bilton et al.
Horse	1982	Yamamoto et al.
Human	1984	Zeilmaker et al.
Baboon	1984	Pope et al.
Mamoset	1986	Summers et al.
Cynomolgus macaque	1987	Balmaceda et al.
Cat	1988	Dresser et al.
Rhesus macaque	1989	Wolf et al.
Pig	1989	Dixon et al.
Wapidi	1991	Wenkoff et al.
Hybrid macaque	1992	Cranfield et al.
Swamp buffalo	1993	Kasiraj et al.
Fallow deer	1994	Morrow et al.
Mongolian gerbil	1999	Mochida et al.
Hamster	1999	Lane et al.

Modified from Rall, W.F., 2001.

1.4.2 Ultra-rapid freezing or vitrification method

Vitrification is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. In 1985, Rall and Fahy showed for the first time, successful cryopreserved murine embryos by vitrification. Vitrification does not create ice crystal by the use of extreme elevation in viscosity during cooling (Fahy et al., 1984). Vitrification as an ultra-rapid cooling technique is based on direct contact between the vitrification solution containing the cryoprotectant agents (CPAs), and the liquid nitrogen (LN_2).

In contrast to slow freezing protocols, during vitrification the entire solution remains unchanged and the water does not precipitate, so no ice crystals are formed (Fahy, 1986). At low temperature, the physical definition of vitrification is the solidification of a solution which water is rapidly cooled and formed into a glassy, and not by ice crystallization, but by extreme elevation in viscosity during cooling (Fahy et al., 1984). However, vitrification is a result of high cooling rates associated with high concentration of CPAs. Vitrification as a cryopreservation method has many advantages such as no ice crystal formation. Vitrification is relatively simple, requires no expensive programmable freezing equipment (Figure 4), and relies on the placement of the cell in very small volume of vitrification medium. The comparison between the advantages and disadvantages of conventional freezing and vitrification are shown in Table 3.

Figure 4. Programmable freezing machine.

1.5 Variables in vitrification

1.5.1 Cooling and warming rates

There are two parameters for the success of cryopreservation: the speed of freezing (cooling rate), and the effects of the dissolved substances, i.e. the concentration of the CPAs. The optimal cooling rate is the rate that permists most water to move out of the cells and freeze/vitrify extracellularly. Therefore, a primary stategy of any vitrification protocol must be to pass rapidly through the critical temperature zone of 15 to -5ºC to decrease chilling injuries.

Regarding warming, in the most successful experiments, high warming rates have been used, which means warming of cells by directly plunging them into the warming solution. Isachenko and colleagues (2001) obtained hightly successful results with the vitrification of bovine-GV-oocytes in open pulled straw (OPS) using quick warming (Isachenko et al., 2001).

1.5.2 Concentration of the CPAs

To achive high cooling rates requires the use of high concentration of the cryoprotectant solution, which depresses ice crystal formation. There is critical concentration required for vitrification. The minimal concentration can lead to either osmotic or chemical toxicity. Minimizing the toxicity of the CPAs resulting from the high cryoprotective concentration as well as reducing the cooling rate can be achieved by substituting an amino group for the hydroxyl (OH-) group of an alcohol, and increasing the hydrostatic pressure of the solution. Kanno and colleagues (1975) were able to demonstrate that the temperature at which crystallization begins can be reduced through an increase in the hydrostatic pressure (Kanno et al., 1975). Finally, reducing the cooling rate can be achieved by reduction of minimal concentration through the additional use of polymers which are non-permeable and therefore remain in the extracellular area (Fahy et al., 1984). In addition, minimizing the toxicity of the CPAs can also be achieved by using a combination of two CPAs, and a stepwise exposure of cells to pre-cooled concentrated solutions. Some studiess mixed other permeating agents, such as DMSO, to reduce the concentration of single CPAs (Ishimori et al., 1993; Vajta et al., 1998; Yokota et al., 2000).

1.5.3 Sample size and carrier systems

The container used and the total volume of the sample to be frozen determines the size of the contact area with the liquid nitrogen, and the cooling rate. In vitrification techniques, the smallest possible volumes of vitrification solutions are used in specialized carrier or support devices, giving cooling rates approaching 10,000 ºC/min to 20,000 ºC/min. To improve chances that the sample is surrounded

with liquid and not vapor. The sample size should be minimized and the duration of any vapor coat is reduced. Including increase of the cooling rate. Furthermore, to facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution. To minimize the volume of the vitrification solution, special carriers are used during the vitrification process.

Martino and colleagues (1996) demonstrated the first successful cryopreserved *in vitro*-matured bovine oocytes by vitrification. Vajta and colleagues (1998) reported a novel way of vitrification using open pulled straws (OPS) for bovine embryos as well as oocytes. Later on the improved OPS methods using glass capillary (Kong et al., 2000; Hochi, et al., 2000; 2001) and gel-loading tip (Tominaga et al., 2001) or the flexipet-denuding pipette (FDP) (Liebermann et al., 2002), micro drops (Papis et al., 2000; Atabay et al., 2003), electron microscopic (EM) grids (Martino et al., 1996; Arav and Zeron, 1997; Hong et al., 1999; Park et al., 1999, 2000; Chung et al., 2000), hemi-straw system (Kuwayama and Kato, 2000; Vandervorst et al., 2001; Liebermann and Tucker, 2002), nylon mesh (Matsumoto et al., 2001), cryoloop (Oberstein et al., 2001; Liebermann and Tucker, 2002; Lane et al., 1999a, 1999b; Yeoman et al., 2001) and cryotop (Kuwayama and Kato, 2000) has been performed.

1.5.4 Buffering solutions

Vitrification solutions are aqueous cyoprotectant solutions, which do not freeze or change pH when cooled at high cooling rates to very low temperature. Therefore, the buffered medium base used for vitrification is either phosphate-buffered saline (PBS), or HEPES-buffered culture medium.

1.5.5 Cryoprotectant agents (CPAs)

The discovery of glycerol as a cryoprotectant was first done with bull spermatozoa by Polge and colleauges (1949). The CPAs used in early experiments are glycerol (Wilmut and Rowson, 1973; Willadsen, 1977). Glycerol and DMSO (dimethylsulfoxide) are the most commonly used CPAs. Fetal bovine serum (FBS) is often used in mammalian cryopreservation solutions, but it is not a cryoprotective agents. Salts have been reported to be CPAs such as magnesium chloride ($MgCl_2$) (Karow and Carrier, 1969). CPAs protect slowly frozen cells by one or more of the mechanisms such as suppressing high salt concentrations, reducing cell shrinkage at a given temperature, reducing the fraction of the solution frozen at a given temperature and minimizing intracellular ice formation. Ice formation can be eliminated entirely, both within the cells and the extracellular matrix, when CPAs are used in extremely high concentrations (i.e., at least 50% volume/volume) (Fahy, 1988). It is known that there are permeability differences between species. The cells undergo rapid contraction and expansion as a result of marked changes in the osmolarity of cryoprotectant medium in which they are held. Cell damage may result from exposure to rapid changes in osmotic conditions therefore, CPAs have usually been added or diluted out in a series of steps.

1.5.6 Disaccharides and macromolecules

Osmotic shock is an aspect of cell cryopreservation that is likely to be responsible for damage to the cells. An alternative approach is to place the cells in a medium containing a high concentration of a non-penetrating molecule such as sucrose. This enables the cells to reach equilibrium, in terms of its isotonic volume.

The use of sucrose as an osmotic buffer was first described by Leibo and Mazur (1978). In this way, the cells is transferred directly from concentrated CPAs solution into sucrose solution that is isotonic with the cryoprotectant. The non-permeating sucrose prevents swelling and the absence of cryoprotectants in the extracellular solution permits the rapid efflux of the intracellular solute. Sucrose concentration between 0.25M to 1.0M have been used by various researchers. Addition of large molecular weights such as disaccharides like sucrose or trehalose do not penetrate the cell membrane, but can significantly reduce the amount of CPAs.

Naturally, cells contain high concentration of protein, which are helpful in vitrification. Higher concentrations of CPAs are needed for extracellular vitrification than for intracellular vitrification. The addition of a polymer with a high molecular weight such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG) or Ficoll is sufficient in order to vitrify extracellularly with the same cryoprotective concentration used intracellularly. Furthermore, the polymers may be able to build a viscous matrix to encapsulate the oocytes/embryos, and also prevent crystallization during cooling and warming (Kasai et al., 1990; Kuleshova et al., 2001). O'Neill and colleagues (1997) observed that addition of PEG resulted in greatly improved viability of oocytes following cryopreservation, and vastly reduced the variablility seen with vitrification solution alone. The other investigators added Ficoll in the vitrification solution which was thought to stabilize the glass formation and form a protective coating around embryos (Kasai et al., 1990; Park et al., 2000).

1.5.7 Toxicity of vitrification solution

The toxicity of vitrification solution has been tested for oocytes and embryos cryopreservation (Rall, 1987; Szell et al., 1989; Kasai et al., 1990; Huang et al., 1992; Mahmoudzadeh et al., 1993; Rall and Wood, 1994; Songsasen et al., 1995; Suzuki et al., 1995; Martino et al., 1996). The early studies of cryopreservation were more toxic than present day (Rall, 1985; Ishimori et al., 1993; Rall and Wood, 1994) because of the high cryoprotectant concentration. It was neccessary to formulate a nontoxic and efficient solution for vitrification procedures.

Several studies have compared the toxicity of cryoprotectants. Ethylene glycol (EG), with the characteristics of low toxicity and rapid permeation of the cell, it has became an important component of vitrification solution. EG is also widely used when compare to propanediol or glycerol (Kasai, et al., 1990; Miyamoto et al., 1978; Songsasen et al., 1995; Szell et al., 1989). Many researches used the combination of cryoprotectant such as sugars and polymers in the vitrification solution for reducing the concentration of cryoprotectant (Ali and Shelton, 1993; Mukaida et al., 1998; Kuleshova et al., 1999; 2001).

Large-molecular weight polymers can reduce the incidence of damage to the zona pellucida (Dumoulin et al., 1994; Gutierrez et al., 1993) from their osmotic pressure. They can fully or partially act as a serum substitute for mouse oocytes (Carrol et al., 1993) and embryos (Dumoulin et al., 1994). Some of these non-permeating cryoprotective agents have direct protective effects on the cell membrane. However, the primary mechanism of action appears to be the induction of vitrification (extracellular grass formation).

1.6 The oocyte

1.6.1 The origin of oocyte

Oocytes present in the adult ovary develop from a definite number of primodial germ cells (PGCs) that are formed in the yolk sac epitheliam of the embryo. Once PGCs have reached the developing ovary the cells begin to differentiate into oogonia. The population of oogonia proliferates until shortly before birth at which time the oogonia enter meiosis and are termed primary oocytes (Gosden and Bownes, 1995). The oogonia are the stem cells that give rise to all the oocytes in the ovary (Rüsse, 1983). The population of oogonia goes through a predetermine, species-specific, number of mitotic cycles until the cells enter the prophase of meiosis and become oocytes (Figure 5). The prophase of meiosis is traditionally separated into five sequential stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Primary oocytes progress only through part of meiosis and arrest at the pachytene or dictyate stage of the first meiotic prophase (Byskov and Hoyer, 1994; Picton, 2001). The nucleus of diplotene oocytes is known as germinal vesicle (GV) (Figure 6A). The oocyte at the GV stage is a diploid cell (2n), which has twice the normal amount of DNA. The mammalian ovary has only a finite supply for oocytes. Oocytes remain in meiotic arrest for many months or even years. The process of meiosis will halves the number of chromosomes resulting in the creation of haploid oocytes that are ready for fertilization by sperm carrying the other half of the genome.

The oocyte is a unique and large cell with high volume-surface ratio, surrounded by a zona pellucida (ZP) and several layers of granulosa cells forming the cumulus oocyte complex (COC). The cumulus cells are the subpopulation of ovarian granulosa cells that surrounds the oocyte. The cumulus cells in close contact with the

oocyte are known as corona radiata. They are in close contact with the oocyte through cytoplasmic extensions which penetrate the ZP and terminate in bulbous swelling closely associated with the oocyte membrane (De Loos et al., 1991). The corona radiata communicates the oocyte with other cumulus cells through the gap junctions which play an important role in the metabolic cooperation between oocyte and cumulus cells during the growth phase of the oocyte (Van Soom et al., 2002).

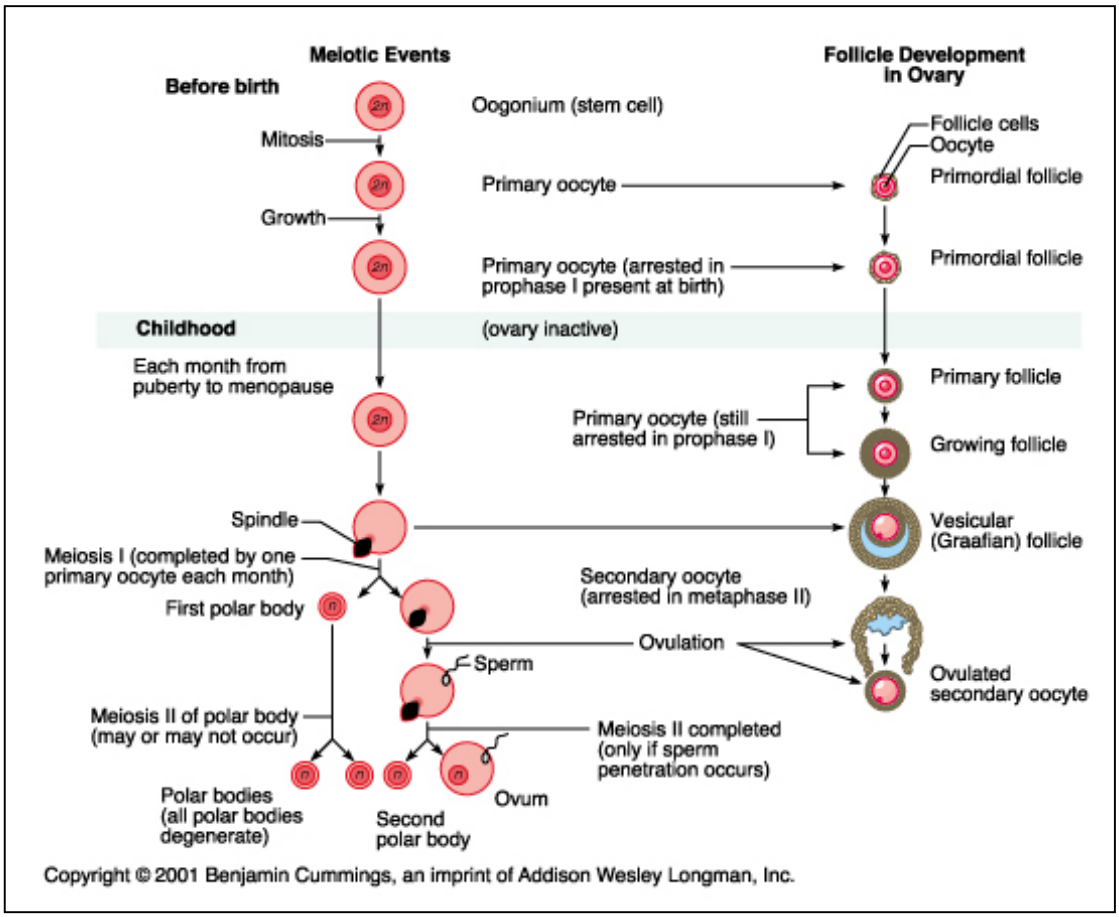

Figure 5. The meiotic event, follicle development in ovary and fertilization by sperm in mammalian species.

1.6.2 Stage of maturation

Oocyte maturation is a complex phenomenon during which the oocyte progresses from the diplotene to the metaphase II (MII) stage (Figure 6B). The transition from the diplotene stage to metaphase is called diakinesis. The oocyte resumes meiosis in response to the ovulatory LH surge (Callesen et al., 1986; Channing et al., 1978; Dieleman et al., 1983; Ireland and Roche, 1982; Masui and Clarke, 1979; Peng et al., 1991;) or removal from the follicle (Pincus and Enzmenn, 1935), allows the oocyte to reach the MII stage. During diakinesis, the nuclear membrane starts to fold, the nuclear pores disappear and then the nuclear membrane fragments before rapidly disappearing to leave only small sacs with double walls (Kubelka et al., 1988; Szollosi et al., 1972). These events are known as germinal vesicle breakdown (GVBD), which is the first visible sign of meiotic resumption. The second meiotic division without chromosome replication takes place immediately and the oocyte reaches the MII. The oocyte remains arrested at the MII stage until fertilization takes place and the oocyte completes meiosis and forms the pronucleus. In cloning, the lack of sperm-induced fertilization steps necessitates the application of an artificial activation in order to trigger further development.

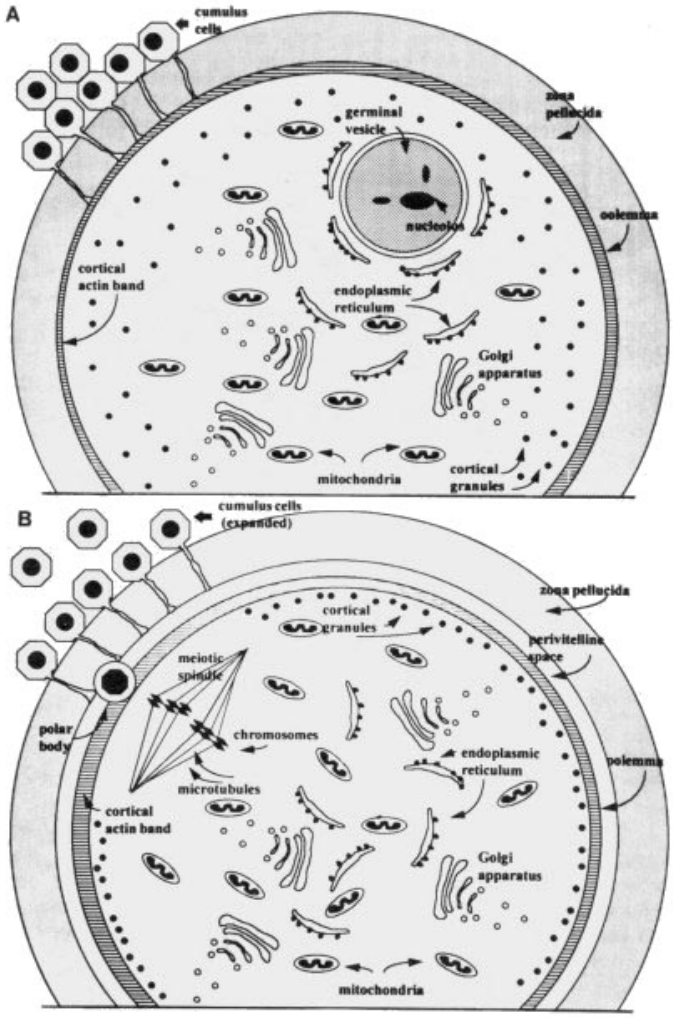

Figure 6. Schematic representation of the cytological features of Germinal vesicle (A) and Metaphase II (B) mammalian oocytes. From Parks, J.E. (1997). Hypothermia and mammalian gametes. In: Karow A.M., Critser, J.K. eds. Reproductive Tissue Banking Scientific Principles. San Diego: Academic Press. P 229-261.

1.7 Cryopreservation of oocytes

Two main techniques have been used for cryopreservation of oocytes: slow freezing and vitrification. These two systems have been used for cryopreservation of

mammalian oocytes with high variation in experimental detail. Experiments on cloning need a lot of oocytes. The main source of oocyte is from slaughter house. When oocytes are aspirated from follicles on ovaries derived from a slaughter house, they are immature at the GV stage of maturation. Until *in vitro* culture in maturation medium, they are mature at the MII stage of maturation.

Although Sherman and Lin (1958) investigated the survival of unfertilized mouse oocytes during cooling and warming, it was not until 1977 (Whittingham, 1977) that live births from frozen-thawed MII mouse oocytes were obtained. Mouse oocytes have been more broadly study compared with other mammalian species oocytes. Van Blerkom (1989) first attempted to cryopreserve mouse GV oocytes, and the first live birth was obtained by Candy and colleauges (1994) using a slow freezing method in the presence of 1.5M DMSO. Subsequently, the research of oocyte cryopreservation has concentrated on mature oocytes of cattle and human. Recently, the improvements of the conditions may significantly increase the efficiency of oocyte cryopreservation for bovine and other species (Pollard et al., 1994; Martino et al., 1996; Vajta et al., 1997, 1998). However, there are very few report in success rate of cryopreserved buffalo oocytes.

Many specific problems have been described for oocytes at different meiotic stages of maturation, including those associated with the cryopreservation of ovulated oocytes like spindle disorganization (Mandelbaum et al., 2004), loss or clumping of microtubules resulting in some scattering of chromosomes (Sathananthan et al., 1998), increased polyploidy after fertilization (Al-Hasani et al., 1987; Carroll et al., 1989; Glenister et al., 1987), and a decrease in fertilization (Glenister et al., 1987; Wood et al., 1992; Mandelbaum et al., 2004).

An important characteristic of the GV stage oocyte is that the genetic material contained at early prophase. Since no spindle is present, this type of oocyte is assumed to be less prone to microtubular and chromosomal damage. Freezing of immature oocytes at the GV stage might circumvent these problems because genetic material is contained within the contours of a nuclear envelope. Nevertheless, very low survival rates have been reported for cryopreserved immature oocyte of bovine (Lim et al., 1992; Otoi, et al., 1995; Suzuki et al., 1996), buffalo (Das et al., 1999; Dhali et al., 2000; Wani et al., 2004), or mouse (Van Blerkom, 1989; George et al., 1994; Cooper et al., 1998).

The other stage that is traditionally cryopreserved is the mature oocyte at the MII stage of maturation. A series of cytoskeletal elements play an active role during the maturation process of the oocyte. For example, microfilaments of actin are involved in cell shape modifications and movements and microtubules form the spindle apparatus (Parks and Ruffing, 1992). Exposeing oocytes to CPAs and/or cooling them from about 37ºC to 20 ºC or below causes various cytoskeletal and chromosomal alterations. Nevertheless, offspring have been obtained from mature oocytes that have been frozen slowly (Fuku et al., 1992; Otoi et al., 1993, 1995; Candy et al., 1994; Kubota et al., 1998) as well as from vitrified ones (Hamano et al., 1992; Hamano and Kuwayama, 1992; Vajta et al., 1998; Hong et al., 1999; Papis et al., 2000; Le Gal et al., 2000; Choi et al., 2000; Chung et al., 2000; Hochi et al., 2004).

In the majority of investigations studying the effect of cooling on mammalian oocytes, a negative cryoinfluence is explained in terms of the effect on cytoskeleton elements. Many reports of cooling of mouse oocytes causes depolymerization of

cytoskeleton protein structures to take place (Magistrini and Szollosi, 1980; Maro et al., 1985; Pickering and Johnson, 1987). Most mouse oocytes cooled to 25ºC for to 10 min have abnormal cytoskeleton (Pickering and Johnson, 1987). After exposure to 4ºC for 20 min, completely disassembled spindles were observed. This process of depolymerization is reversible. Spindles of mouse oocytes returned to normal appearance after warming at 37ºC for 60 min. Spindles of about half of human oocytes exposed to room temperature for 10 min returned to normal after 4 hours of culture at 37ºC (Pickering and Johnson, 1987). Bovine oocytes are also sensitive to decrease in temperature (Aman and Parks, 1994). Parks and Ruffin (1992) have shown that 56% of oocytes exposed to 25ºC and 90% of oocytes exposed to 4ºC for 1 min had abnormal spindles. Martino and colleagues (1995) reported that the developmental rate of GV-bovine oocytes is also impaired after exposure to 10ºC for 30 min, or 0ºC for only 30 sec. Data on the sensitivity of porcine and buffalo oocytes to low temperature are limited. Didion and colleagues (1990) examined the viability of pig GV-oocytes following cooling or freezing by conventional methods that the cumulus-intact GV-porcine oocytes did not survive cooling to temperature at or below 15ºC.

However, many report shows that the GV stage oocyte is more sensitive to cryopreservation than any other nuclear stages (Parks and Ruffing, 1992; Otoi et al., 1995; Parks et al., 1997). One of the major concerns caused by oocyte freezing is possible effects on the cytoskeletal structures. The meiotic spindle is a particularly sensitive structure and showing only partial ability to restore its original organization following cooling and thawing (Pickering et al., 1990). The meiotic stage also seems

to influence the survival of mammalian oocytes after cryopreservation (Rall and Fahy, 1985; Miyake et al., 1993).

It is known that MII oocytes are more resistant to freeze damage than GV-stage oocytes. This may be due to differences in the properties of cytoskeleton elements. One important differences is that the configuration of microtubules and microfilaments is different at these two stages of oocytes maturation. Cytoskeleton elements in GV-oocytes appear straight and rigid, while the appearance of microfilaments and microtubules in MII stage oocyte is undulating and flexible (Allworth and Albertini, 1993). The interaction between the lipid phase of cells and the elements of the cytoskeleton is complex. Hardening of these lipids might cause deformation and disruption of the cytoskeleton. In the case of the rigid GV-oocyte cytoskeleton this apparently results in permanent damage, while in the more flexible MII-oocyte cytoskeleton, permanent damage is absent (Men et al., 2003).

The cryopreservation of mammalian oocytes could assist the application of several reproductive biotechnologies, such as *in vitro* production of embryos (Hasler, 1992), treatment of infertility (Tucker et al., 1998.), nuclear transfer (Kubota et al., 1998; Wolf et al., 1999) and cell banking (Wildt et al., 1997). Because immature oocytes at the germinal vesicle (GV) stage are often difficult to mature *in vitro* and most applications require mature oocytes, therefore, oocyte cryopreservation attempts have focused on the metaphase II (MII) stage. Vitrification is a promising method for oocyte cryopreservation because the chilling injury (spindle disorganization) and ice formation can be avoid.

1.8 Vitrification of oocytes

To survive cryopreservation, the oocyte must tolerate a sequence of volumetric contractons and expansions. Unlike all stages of preimplantation embryos, oocytes are more vulnerable to the cryopreservation procedures involving ice crystallization. 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 7. This can be explained by the decrease in permeability of the cytoplasmic membranes of oocytes (Ruffing et al., 1993). It is well known that the sensitivity of oocytes to osmotic swelling is very high, which can occur during the removal of cryoprotectant from cryopreserved cells.

Cryopreservation of mouse oocytes has been well documented and has resultd in greater success than studies with other mammalian species. However, vitrification has been used successfully for the cryopreservation of oocytes in mice (Kasai et al., 1990; Miyake et al., 1993; Wood et al., 1993; Chen et al., 2000a), bovine (Hamano et al., 1992; Saha et al., 1996; Vajta et al., 1998; Booth et al., 1999; Hurtt et al., 2000; Papis et al., 2000), equine (Hurtt et al., 2000; Oberstein et al., 2001) and human (Hunter et al., 1995; Chen et al., 2000b; Chung et al., 2000; Yokota et al., 2000; Park et al., 2000) oocytes can survive cryopreservation by vitrification. There are reports on the maturation of oocytes after their vitrification in case of buffalo as well (Nag and Maurya, 1997; Saxena and Maurya, 1999; Dhali et al., 2000). The results from recent studies highlight that the high cooling rate is an important factor to improve the effectiveness of oocyte vitrification (Leibermann et al., 2002; Leibermann and Tucker, 2002).

Otoi and colleagues (1998) vitrified bovine oocytes that were very sensitive to chilling with EG-based CPAs in conventional straws. They achieved results better than those observed with the slow freezing method. Vajta and colleagues (1998) developed OPS to hold bovine oocytes with a very small amount of solution for vitrification. They reported that the developmental capacity and the pregnancy potential of oocyte vitrified using OPS were improved when compared with those vitrified using conventional straws.

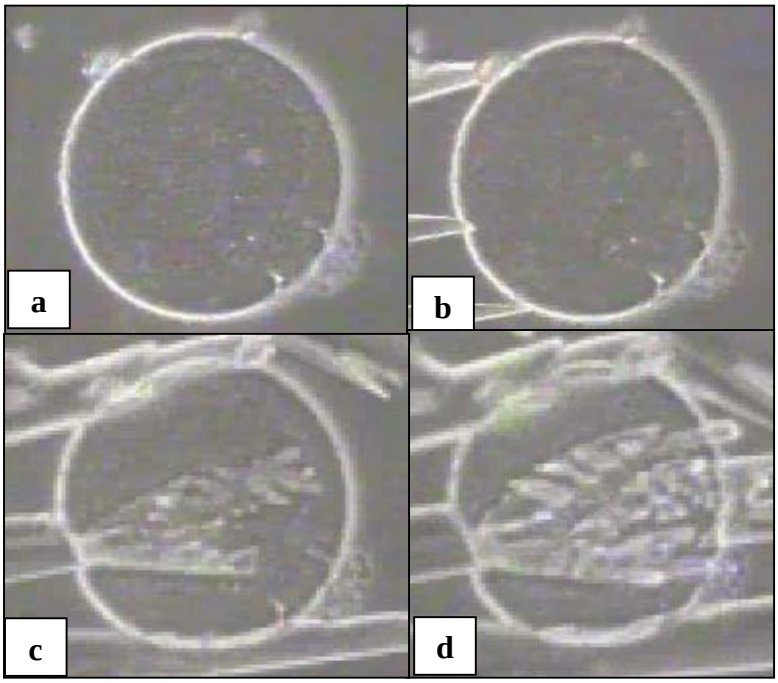

Figure 7. Ice crystal growing through the oocyte, it is a major cause to destroy the oocyte during cooling method.

Table 3. 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).

1.9 Differential staining

1.9.1 Blastocyst evaluation by means of Differential staining

The blastocyst stage embryos can be evaluate for the normal development by differential staining method. It is clear that an exact estimation of embryo quality at the blastocyst stage is very important for both the clinicians and the researchers. A proper evaluation of blastocyst quality remains an important challenge for researchers

to estimate and select the best embryos for transfer. Blastocyst quality can be evaluated by different approaches: if the selected embryo has to be transferred, the technique to estimate embryo quality must be noninvasive. However, more accurate data can be acquired from invasive techniques (Table 4). Attention is focused on the physiology of differentiation of the blastocyst into inner cell mass (ICM) cells and trophectoderm cells (TE), which can be visualized by means of differential staining.

1.9.2. Invasive technique

Invasive assessment of embryo quality mostly involves a kind of fixation or staining of the embryo. Total cell number and allocation of cells to ICM and TE after differential staining of these cells (Van Soom et al., 1996) give more detalis on embryo quality. Mitochondria status can be assessed either by fluorescent staining and confocal imaging (invasive) (Barnett et al., 1997). Techniques to stain ICM cells and TE cell differentially in embryos was first performed by studying reconstructions of serially sectioned embryos such in the mouse (Barlow et al., 1972) and in the horse embryo (Bruyas et al., 1993). However, these microscopic slides are tedious to evaluate and problems in interpretation of cell position can arise. Also micromanipulation was used to separate the ICM from TE cells (Gardner et al., 1973), but this was rather impractical when large groups of embryos have to be compared. More recently, a quick procedure to differentiate between ICM and TE cells was developed starting from an immunological approach.

1.9.3 Zona removal

Outer cells of the blastocyst form a seal of zunular tight junctions. The consequence of this is that outer cells can selectively be lysed by complement after

being labelled with antibodies. However, the inner cells are protected by the seal of the outer cells from antibody-mediated complement lysis. The procedure to lyse the outer TE cells selectively is called immunosurgery (Solter and Knowles, 1975). However, in recent years other techniques have become available for selective labelling of outer and inner cells such as species-specific antiserum complement lysis (Handyside and Hunter, 1984), chemical labelling nonspecific antiserum complement lysis (Hardy et al., 1989), chemical lysis of TE cells (De la Fuente and King, 1997) and artificial labelling of TE cells with lectin (De la Fuente and King, 1998).

In each of the techniques, it is essential that the embryos are completely zona-free before starting the staining procedure. This means that the zona should be removed by means of chemical (acid Tyrode solution) (Handyside and Hunter, 1984; Rivera et al., 1996; Hardy et al., 1989) or enzymatic (pronase) (Handyside et al., 1987; Iwasaki et al., 1990; Van Soom et al., 1996; Pampfer et al., 1990; Narula et al., 1996) treatment or by both (Giles and Foote, 1995; Du et al., 1995). An alternative is tearing the zona by means of needles or micromanipulation (Papaioannou and Ebert, 1988). Techniques for zona removal have been applied in different species. It is also possible to use hatched blastocysts.

1.9.4 Species-specific antiserum complement lysis method

Differential staining after lysis induced by species-specific antiserum followed by complement which is the fast techniques to stain the embryos. The first approach was describes by Handyside and Hunter in 1984 for murine embryos. They used a heat-inactivated antiserum generated in rabbits against mouse spleen cells, followed by complement (Handyside and Hunter, 1984). Outer cells were sensitive to lysis by

complement which had been exposed to antiserum. Propidium iodide (PI) which had been added to the complement solution stained the nuclei of the lysing cells. This staining could be assessed by means of an inverted microscope equipped with fluorescence.

Cell inside the embryo (ICM cells) were protected against the complement action by the seal formed by the TE cells, as antibodies will not go through this single cell layer. Therefore, ICM cells will not be lysed and will not be stained by PI but can be stained by bisbenzimide or Hoechst 33342 or 33258. To prepare the antiserum, spleen cells from the target species must be homoginized and injected in rabbits, with subsequent booster injections. Before collecting the blood of the rabbits, the antibody titre and the specificity of the antiserum have to be tested. In this way, the right concentration to induce cell lysis by complement treatment can be determined. Since the amount of antiserum is limited and generated only against one species, its use is restricted to the target species. Only in mouse and rat embryos the binding of antibodies is nonspecies-specific (Spielman et al., 1980). To applied correctly the techniques for selective labelling of outer and inner cells, differential staining can be used to obtain a better estimation of embryo quality and embryo differentiaton.

Table 4. Different noninvasive and invasive techniques to estimate embryo quality.

Noninvasive methods	Invasive methods
Embryo morphology	Cytogenetic analysis
Timing of development	Cell number
Metabolic tests	Differential staining
Blastocyst formation and hatching	TUNEL staining
Vital staining	Cell biological components
Freezing resistance	Electron microscopy

Modified from Van Soom et al., 2001.

1.10 Objectives

1. To examined the developmental potential of cloned swamp buffalo embryos derived from fresh and vitrified matured oocytes.
2. To investigate the appropriate conditions of the period of time in equilibration solution to vitrified buffalo oocytes in order to maintain high survival after warming.

CHAPTER II

MATERIALS AND METHODS

2.1 Preparation of donor cell

The small pieces of buffalo ear skin (5x5 mm) was cut and kept at 4ºC in modified Dulbecco's phosphate buffer saline (mDPBS) during transported to laboratory. The buffalo skin tissues were removed from cartilage and cut into small pieces (about 1x1 mm) before being placed in 60 mm culture dishes and the tissues were covered with glass slide. Five milliliters of alpha modification minimal essential medium Eagle (αMEM) + 10% fetal bovine serum (FBS) was added into the dishes and culture under humidified atmosphere of 5% CO_2 in air at 37^{o}C for 8-10 days. The fibroblasts outgrowth from ear skin tissues were harvested using 2.5 µg/ml Trypsin and 0.4 µg/ml EDTA in phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+} (Trypsin/EDTA) and cultured on 25 cm^2 culture flask (Nunc) in αMEM + 10% FBS. The ear fibroblasts were frozen with 10% dimethylsulfoxide (DMSO) in αMEM + 20% FBS at the third cell culture passages and stored in liquid nitrogen. Before nuclear transfer, frozen fibroblasts were thawed and cultured in α-MEM + 10% FBS, these cells were used for nuclear transfer until passages 8. Sub-confluence fibroblasts were harvested by standard trypsinization. Then cells were pelleted and dissociated to be single cells suspension in Emcare holding medium (ICP bio)**.**

2.2 Preparation of recipient cytoplast

The buffalo ovaries were transported from a slaughterhouse to the laboratory in saline solution (0.9% NaCl) at room temperature (Figure 8). The oocytes were harvested from the ovaries by manually aspirating from follicles (2-6 mm in diameter) using 10 ml syringe attached with 21G needle. Only cumulus oocyte complexed (COCs) with at least three layers (Figure 9) were washed five times in mDPBS supplemented with 0.1% polyvinylpyrrolidone (PVP) and three time in maturation medium. For *in vitro* maturation (IVM) culture, groups of 20 oocytes were placed in 100 µl droplets of maturation medium covered with mineral oil (Sigma) under humidified atmosphere of 5% CO_2 in air at 38.5ºC for 21 h. The culture medium for IVM was TCM199 (Sigma) supplemented with 10% FBS, 0.02 AU/ml follicle stimulating hormone (FSH, Antrin®, Denka Phamaceutical), 50 iu/ml human chorionic gonadotropin (hCG, Chorulon®, Intervet) and 1 µg/ml 17β estradiol (E_2, Sigma).

Figure 8. Buffalo oocytes from slaughter house in saline solution.

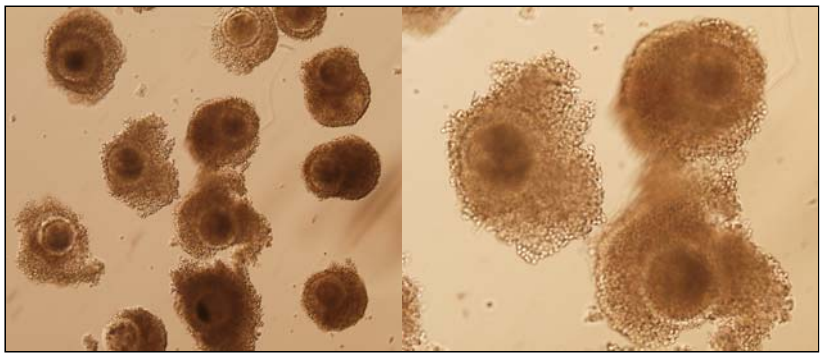

Figure 9. Buffalo oocytes with cumulus cells after manually aspirating (10x, 40x).

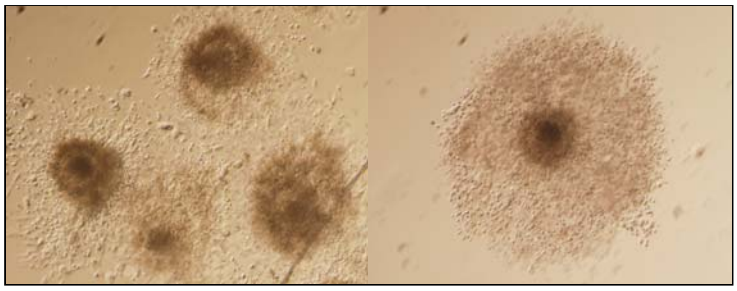

Figure 10. Buffalo oocytes with expanded cumulus cells after IVM (10x).

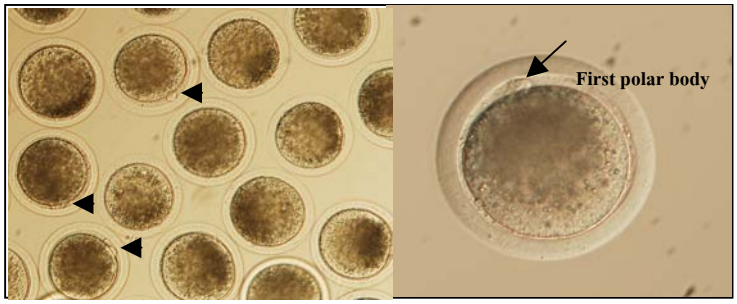

Figure 11. Matured buffalo oocytes at metaphase II with first polar body (arrow) after cumulus cells removal (10x, 100x).

2.3 Enucleation of oocytes

At 21h after IVM (Figure 10), the cumulus cells were mechanically removed by repeated pipetting using 0.2% hyarulonidase and subsequently washed 5 times in

Emcare holding medium. Oocytes at metaphase II (MII) were selected by the presence of the first polar body (1st PB, Figure 11). Enucleation was performed by micromanipulator under inverted microscope. Oocytes were placed in Emcare holding medium containing 5 μg/ml cytochalasin B (CB) for 15 min. The zona pellucida above the first polar body was cut with a sharp glass needle and about 10% of cytoplasm under the first polar body was squeezed out of the zona pellucida (Figure 12). Successful enucleation was confirmed by Hoechst 33342 (Bis-benzimide, Sigma) fluorescent staining of the squeezed-out karyoplasts (Figure 13). After enucleation, the enucleated oocytes were washed 5 times in Emcare holding medium and kept in this medium until vitrification or nuclear transfer.

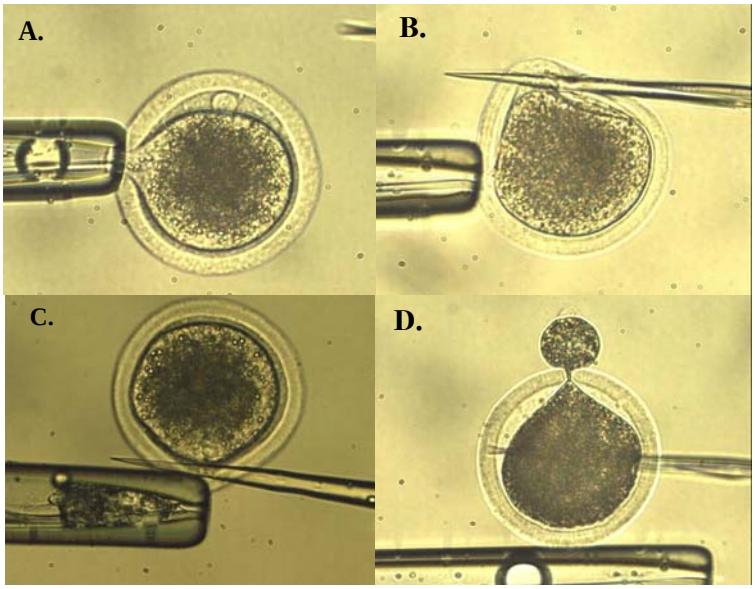

Figure 12. Manipulated the mature oocyte by enucleation (100x).

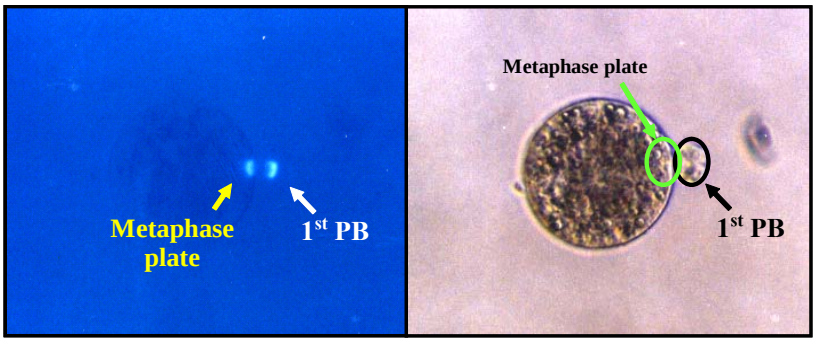

Figure 13. Successful enucleation was confirmed by Hoechst 33342 staining of the squeezed-out karyoplasts (100x).

2.4 Toxicity test of oocytes

The MII oocytes at 21h after IVM were selected to test toxicity of cryoprotectants. Before toxicity test, the MII oocytes were washed four times in TCM199 + 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) + 20% FBS (199H+20). The MII oocytes were then exposed to equilibration solution containing 7.5% ethylene glycol (EG) + 7.5% DMSO in 199H+20 for 4, 7 or 10 min. Then, placed in vitrification solution containing 15% EG + 15% DMSO + 0.5M sucrose in 199H+20 for 1 min. There are termed of 4+1, 7+1 and 10+1, respectively. Subsequently, The oocytes were directly placed in 3 ml of 0.5M sucrose in 199H+20 for 5 min, then transferred into 199H+20 for 5 min. The normal morphologies of tested oocytes were identified as the presence of 1st PB and the intact of zona pellucida and ooplasm membrane. The tested oocytes were kept in 199H+20 under humidified atmosphere of 5% CO_2 in air at 38.5ºC until 26 h after IVM, those tested oocytes were used for parthenogenetic activation. Oocytes with normal morphologically were counted and cultured in groups of 10-20 oocytes in 100 µl of *in vitro* embryos culture medium.

2.5 Vitrification of oocytes

The MII oocytes were separated into two groups. The first group, MII oocytes were immediately vitrified before enucleation (MII) and the second group, MII oocytes were vitrified after enucleation (Enu). The MII group were vitrified at 22 h after IVM whereas Enu group were vitrified at 23 h after IVM under 22 – 24^{o}C. For vitrification, the oocytes were washed 4 times in 199H+20. After that, the oocytes were placed in equilibration solution containing 7.5% EG + 7.5% DMSO in 199H+20 for 4, 7 or 10 min. Then, placed in vitrification solution containing 15% EG + 15% DMSO + 0.5M sucrose in 199H+20 for 1 min. There were termed of 4+1, 7+1 and 10+1, respectively. Five of each oocytes were loaded onto the end tip of cryotop (Kitazato Supply Co., Tokyo) (Figure 14, 15) and immediately submerged into liquid nitrogen.

2.6 Warming of oocytes

Both MII and Enu groups of vitrified oocytes were warmed using one-step dilution method. The cryotop containing oocytes were removed from liquid nitrogen tank and directly dip the end tip of cryotop into 3 ml of 0.5M sucrose in 199H+20 for 5 min, then transferred to 199H+20 for 5 min. The morphology of cytoplasm and polar body were examined under stereomicroscope. The normal morphologies of vitrified oocytes were identified as the presence of 1st PB and the intact zona pellucida and ooplasm membrane (Figure 16). The vitrified oocytes were kept in 199H+20 under humidified atmosphere of 5% CO_2 in air at 38.5ºC before used in nuclear transfer and parthenogenetic activation.

Figure 14. Cryotop, vitrification device which contained oocyte at the end tip of cryotop sheet (circle).

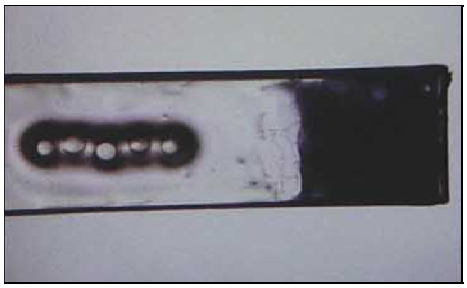

Figure 15. Oocyte location at the end tip of the cryotop sheet.

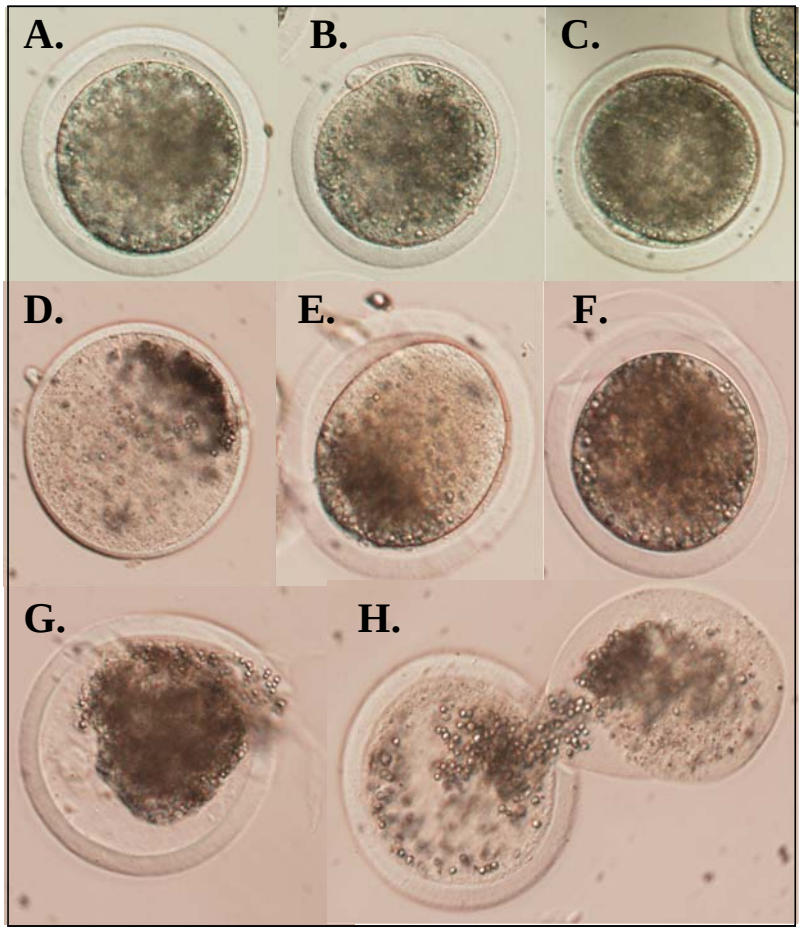

Figure 16. Morphologies of vitrified oocytes consists of normal: 1st PB still present, zona pellucida and ooplasm membrane intact (A-C), and abnormal: cytoplast lysis (D, G-H), zona fracture (F-H) and cytoplast abnormal shape (E).

2.7 Nuclear transfer

One hour after warming, the vitrified MII oocytes were enucleated and confirmed by Hoechst 33342 staining. Individual ear fibroblast (Ø14-16 μm) was injected into the perivitelline space of each successfully enucleated oocyte (Figure 17). Then somatic cell cytoplast complexes (SCCCs) were transferred into

Zimmerman fusion medium (ZFM) (Zimmermann and Vienken, 1982) and individual SCCC was placed at both tips of fusion electrodes (Figure 18) to electrostimulate with 2 DC pulses of 26V/SCCC each pulse was 17 μsec apart and was delivered by fusion machine (SUT F-1, Suranaree University of Technology). The reconstructed embryos were activated by placed in 7% ethanol for 5 min and incubate in 10 μg/ml cycloheximide (CHX) and 1.25 μg/ml cytochalasin D (CD) under humidified atmosphere of 5% CO_2 in air at 38.5°C for 5h.

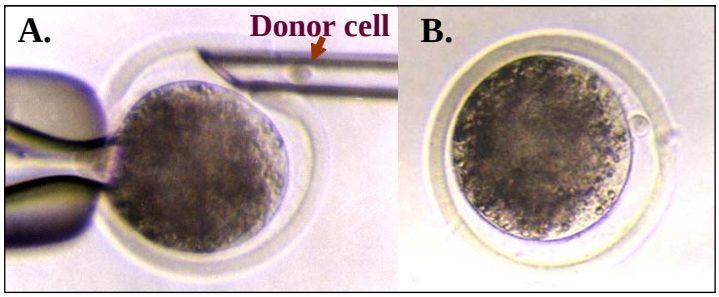

Figure 17. Fibroblast donor cell was injected into perivitelline space of enucleated oocyte.

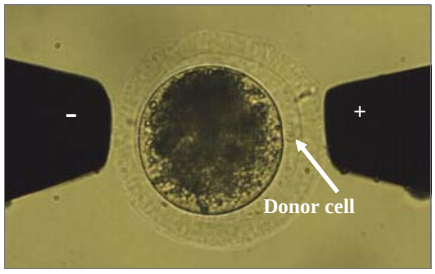

Figure 18. SCCCs was fused by electrode probes.

2.8 Parthenogenetic activation of oocytes

The oocytes from fresh MII, toxicity test and vitrified MII group after warming were subjected to parthenogenetic activation. All oocytes were activated at

26h after IVM by placed in 7% ethanol for 5 min and cultured in 10 µg/ml CHX + 1.25 µg/ml CD for 5 h under humidified atmosphere of 5% CO_2 in air at 38.5ºC. Then activated oocytes were *in vitro* cultured for further development.

2.9 In vitro embryos culture

The parthenogenetic activated fresh MII, toxicity test, vitrified MII oocytes and activated reconstructed embryos were cultured in modified synthetic oviductal fluid (mSOFaa) medium (Gardner et al., 1994) supplemented with 3 mg/ml fatty acid free BSA (A-6003, Sigma) in the ratio of 20 oocytes per 100 µl under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5^{o}C for 2 days. After that, the 8-cell stage embryos was selected and and co-cultured with bovine oviductal epithelium cells (BOEC) under humidified atmosphere of 5% CO_2 in air at 38.5^{o}C for 5 days. Half of the medium were changed daily and the development of embryos was daily observed.

2.10 Differential staining

The zona pellucidae of blastocyst stage embryos at day 6.5 were removed by 0.5% protease and washed in mSOFaa medium. The zona-free embryos were incubated in 100 µl of 10% rabbit anti-buffalo spleenocyte for 45 minutes and then further incubated in 100 µl of 10% guinea pig complement (S-1639, Sigma) + 75 µg/ml propidium iodide + 100 µg/ml Hoechst 33258 for 45 min. The embryos were mounted on glass slide and covered with glycerol. Then, the mounted embryos were counted to determine the number of trophectoderm (TE) and inner cell mass (ICM) cells under fluorescense microscope. From these, the TE showed blue color of

Hoechst 33258 and ICM showed red color of propidium iodide. The ratio between ICM and TE cells can be use for evaluated the normal embryo development from fresh and vitrified oocyte.

2.11 Statistical analysis

Each treatment was replicated five times. The significant differences were determined by ANOVA using SAS program ($P<0.05$).

CHAPTER III

RESULTS

3.1 Morphological survival and 1st PB

The exposure of MII oocytes to the varies equilibration time of the cryoprotectants used for the vitrification of oocytes, followed by the removal of cryoprotectants in warming solution did not affect ($P > 0.05$) the normal morphology of vitrified buffalo MII oocyte immediately after warming and still present of 1st PB of the oocytes. From the Table 5 showed that a total of 3,223 vitrified-warmed buffalo oocytes were used to observed the morphology of the oocytes, of which 2,296 (71.2%) were normal. And 1,131 of 1,356 of all group except the enu group (83.4%) show 1st PB. However, the number of normal morphology and the present of 1st PB in toxicity test (TT) group were significant different higher ($P<0.05$) than vitrified groups in both of PA and scNT. However, among the vitrified groups, the higher normal morphology was obtained in PA groups than scNT groups but were not significantly different. There was no different in the percentage of 1st PB presented of PA and scNT groups, but were lower ($P<0.05$) than toxicity groups. The normal morphology of equilibration time for 4 (72.8%; 748/1027) and 7 (71.4%; 713/999) min were significantly higher than equilibration time of 10 (69.8%; 835/1197) min (Table 6).

Table 5. The normal morphology of vitrified buffalo MII oocyte and the present of 1st PB immediately in warming solution.

Groups	No. oocyte warmed	Normal morphology (%)	1st PB (%)
TT. MII 4+1	120	105 (87.5)[a]	95 (90.5)[a]
TT. MII 7+1	121	99 (81.8)[a]	91 (91.9)[a]
TT. MII 10+1	124	109 (87.9)[a]	99 (90.8)[a]
Total	**365**	**313 (85.8)**	**285 (91.1)**
PA.Vitri.MII 4+1	165	119 (72.1)[b]	98 (82.4)[b]
PA.Vitri.MII 7+1	169	124 (73.4)[b]	97 (78.2)[b]
PA.Vitri.MII 10+1	197	141 (71.6)[b]	109 (77.3)[b]
scNT.Vitri.MII 4+1	313	220 (70.3)[b]	173 (86.5)[b]
scNT.Vitri.MII 7+1	298	203 (68.1)[b]	171 (84.2)[b]
scNT.Vitri.MII 10+1	371	236 (63.6)[b]	198 (83.9)[b]
Total	**1513**	**1043 (68.9)**	**846 (81.1)**
scNT.Vitri.Enu 4+1	429	304 (70.8)[b]	-
scNT.Vitri.Enu 7+1	411	287 (69.8)[b]	-
scNT.Vitri.Enu 10+1	505	349 (69.1)[b]	-
Total	**1345**	**940 (69.9)**	**-**

Values with different superscripts (a, b) within a column are significantly different (P<0.05).

Table 6. The normal morphology of vitrified buffalo MII oocyte immediately in warming solution.

Groups	No. oocyte warmed	Normal morphology (%)
TT. MII 4+1	120	105 [a] (87.5)
PA.Vitri.MII 4+1	165	119 [a] (72.1)
scNT.Vitri.MII 4+1	313	220 [a] (70.3)
scNT.Vitri.Enu 4+1	429	304 [a] (70.8)
Total	**1027**	**748 (72.8)**
TT. MII 7+1	121	99 [a] (81.8)
PA.Vitri.MII 7+1	169	124 [a] (73.4)
scNT.Vitri.MII 7+1	298	203 [a] (68.1)
scNT.Vitri.Enu 7+1	411	287 [a] (69.8)
Total	**999**	**713 (71.4)**
TT. MII 10+1	124	109 [b] (87.9)
PA.Vitri.MII 10+1	197	141 [b] (71.6)
scNT.Vitri.MII 10+1	371	236 [b] (63.6)
scNT.Vitri.Enu 10+1	505	349 [b] (69.1)
Total	**1197**	**835 (69.8)**

Values with different superscripts (a, b) within a column are significantly different ($P<0.05$).

3.2 *In vitro* development of swamp buffalo embryos following toxicity test

As shown in Table 7, there was no significant differences in the rates of cleavage and development to the morula stage at day4 between the fresh and TT.MII group. However, the development to 8-cell stage and blastocyst stage at day7 of fresh (70.3%; 83/118, 26.5%; 27/102) and TT.MII 4+1 (70.5%; 74/105, 25.3%; 23/91) were higher (P<0.05) than those TT.MII 7+1 (64.6%; 64/99, 18.1%; 15/83) and 10+1 (59.6%; 65/109, 16.7%; 15/90), respectively. There was significant differences in development to blastocyst stage at day6 of fresh (21.6%; 22/102) oocytes which were higher (P<0.05) than TT.MII 10+1 (12.2%; 11/90).

Table 7. *In vitro* development of swamp buffalo embryos by toxicity testing.

Group	Cultured	Cleaved (%)	8-cell (%)	Mor. at d4 (%)	Blast. at d6 (%)	Blast. at d7 (%)
Fresh	118	102/118 (86.4)	83/118 [a] (70.3)	60/102 (58.8)	22/102 [a] (21.6)	27/102 [a] (26.5)
TT. MII 4+1	105	91/105 (86.7)	74/105 [a] (70.5)	53/91 (58.2)	17/91 [a,b] (18.7)	23/91 [a] (25.3)
TT. MII 7+1	99	83/99 (83.8)	64/99 [b] (64.6)	48/83 (57.8)	13/83 [b,c] (15.7)	15/83 [b] (18.1)
TT. MII 10+1	109	90/109 (82.6)	65/109 [b] (59.6)	52/90 (57.8)	11/90 [c] (12.2)	15/90 [b] (16.7)

Values with different superscripts (a, b, c) within a column are significantly different (P<0.05).

3.3 *In vitro* development of swamp buffalo embryos following parthenogenetic activation

The results summarized in Table 8. show that the cleavage rate of fresh (78.4%; 80/102) and Vitri.MII 4+1 (76.5%; 91/119) oocytes were higher ($P<0.05$) than those PA.Vitri.MII 7+1 (63.7%; 79/124) and PA.Vitri.MII 10+1 (62.4%; 88/141), respectively. The development to 8-cell stage of fresh (63.7%; 65/102) oocytes was higher ($P<0.05$) than those PA.Vitri.MII 7+1 (45.0%; 57/124) and 10+1 (44.7%; 63/141), respectively. There was no significantly differences in the development to morula stage at day4 between fresh and vitrified oocytes. However, the development to blastocyst stage at day6 and day7 of fresh (23.8%; 19/80, 30.0%; 24/80) oocyte were significant differences higher ($P<0.0.5$) than those PA.Vitri.MII 4+1 (15.4%; 14/91, 17.6%; 16/91) and 7+1 (15.2%; 12/79, 16.5%; 13/79) and 10+1 (14.8%; 13/88, 15.9%; 14/88), respectively.

Table 8. *In vitro* development of swamp buffalo embryos by parthenogenetic activation.

Group	Cultured	Cleaved (%)	8-cell (%)	Mor. at d4 (%)	Blast. at d6 (%)	Blast. at d7 (%)
Fresh	102	80/102 [a] (78.4)	65/102 [a] (63.7)	46/80 (57.5)	19/80 [a] (23.8)	24/80 [a] (30.0)
PA.Vitri. MII 4+1	119	91/119 [a] (76.5)	65/119 [a,b] (54.6)	49/91 (53.8)	14/91 [b] (15.4)	16/91 [b] (17.6)
PA.Vitri. MII 7+1	124	79/124 [b] (63.7)	57/124 [b] (45.0)	42/79 (53.2)	12/79 [b] (15.2)	13/79 [b] (16.5)
PA.Vitri. MII 10+1	141	88/141 [b] (62.4)	63/141 [b] (44.7)	46/88 (52.3)	13/88 [b] (14.8)	14/88 [b] (15.9)

Values with different superscripts (a, b) within a column are significantly different (P<0.05).

3.4 *In vitro* development of cloned swamp buffalo embryos following somatic cell nuclear tranfer

In vitro development of cloned swamp buffalo embryos from scNT in Table 9 shows the rates of fusion, cleavage and development to 8-cell stage of fresh (84.0%; 110/131, 78.0%; 85/109, 60.6; 66/109, respectively) oocytes were significantly higher (P<0.05) than scNT.Vitri. Enu 10+1 (76.6%; 131/171, 70.2; 92/131, 45.0%; 59/131, respectively). However, there was no significant differences in development to morula stage at day4 between fresh and vitrified oocytes. The development to

blastocyst stage at day6 and day7 of fresh (15.3%; 13/85, 18.8%; 16/85) oocytes were significant differences higher ($P<0.05$) than those scNT.Vitri. MII 10+1 (8.0%; 7/88, 9.8%; 9/88) and scNT.Vitri. Enu 10+1 (5.4%; 5/92, 9.8%; 9/92), respectively.

Table 9. *In vitro* development of cloned swamp buffalo embryos from somatic cell nuclear transfer.

Group	Fused (%)	Cultured	Cleaved (%)	8-cell (%)	Mor. at d4 (%)	Blast. at d6 (%)	Blast. at d7 (%)
Fresh	110/131 [a] (84.0)	109	85/109 [a] (78.0)	66/109 [a] (60.6)	51/85 (60.0)	13/85 [a] (15.3)	16/85 [a] (18.8)
scNT.Vitri.MII 4+1	124/159 [a,b] (78.0)	113	81/113 [a,b] (71.7)	60/113 [a,b] (53.1)	47/81 (58.0)	9/81 [a,b,c] (11.1)	11/81 [a,b] (13.6)
scNT.Vitri. Enu 4+1	120/146 [a,b] (82.2)	102	70/102 [a,b] (68.6)	50/102 [a,b] (49.0)	38/70 (54.3)	8/70 [a,b] (11.4)	9/70 [a,b] (12.9)
scNT.Vitri.MII 7+1	105/139 [a,b] (75.5)	100	71/100 [a,b] (71.0)	53/100 [a,b] (53.0)	32/71 (45.1)	8/71 [a,b,c] (11.3)	9/71 [a,b] (12.7)
scNT.Vitri. Enu 7+1	97/131 [a,b] (74.0)	97	61/97 [a,b] (62.9)	40/97 [a,b] (41.2)	28/61 (45.9)	6/61 [a,b,c] (9.8)	7/61 [a,b] (11.5)
scNT.Vitri.MII 10+1	119/154 [a,b] (77.3)	119	88/119 [a,b] (73.9)	56/119 [a,b] (47.1)	40/88 (45.5)	7/88 [b,c] (8.0)	9/88 [b] (9.8)
scNT.Vitri. Enu 10+1	131/171 [b] (76.6)	131	92/131 [b] (70.2)	59/131 [b] (45.0)	42/92 (45.7)	5/92 [c] (5.4)	9/92 [b] (9.8)

Values with different superscripts (a, b, c) within a column are significantly different (P<0.05).

3.5 Blastocyst differential staining

Average of TE, ICM, total cell number and ratios of ICM:TE for *in vitro* cultured swamp buffalo blastocyst at day6.5 are summarized in Table 10. The total cell number and average of mean of TE, ICM were 103 ± 21.2, 80 ± 16.1 and 24 ± 6.1, respectively. The ratios of ICM:TE was similar in groups of TT, PA, scNT.fresh and scNT.Vitri. 4+1 which were around 1:3.2 to 1:3.5. In addition, there was a increasing trend of the ratios of ICM:TE in groups of scNT.Vitri. MII 7+1, scNT.Vitri. Enu 7+1, scNT.Vitri. MII 10+1 and scNT.Vitri. Enu 10+1 which were 1:4.0, 1:3.8, 1:3.9 and 1:4.0, respectively.

In Figure 19 - 21 shows dark-field fluorescent photomicrographs of differentially stained swamp buffalo blastocyst at day6.5 of development. The intense blue colour represents the chromatin in nuclei of TE cells and red colour represents the ICM nuclei. In the case of hatching or hatched swamp buffalo blastocysts with larger diameters and greater cell numbers, ICM nuclei were more densely packed and overlapped, which sometimes made numbers difficult to resolve.

Table 10. Cell number of swamp buffalo embryos from blastocyst stage of day6.5

Groups	Means of TE ± S.D.	Means of ICM ± S.D.	Means of total cell no. ± S.D.	Means of ICM:TE
TT. Fresh	99 ± 11.5	27 ± 4.5	126 ± 17.0	1:3.3
TT. MII 4+1	81 ± 12.6	24 ± 3.6	106 ± 16.1	1:3.3
TT. MII 7+1	70 ± 11.8	20 ± 2.7	90 ± 10.3	1:3.5
TT. MII 10+1	78 ± 19.2	25 ± 9.3	104 ± 27.6	1:3.3
PA. Fresh	95 ± 25.3	31 ± 7.7	127 ± 32.7	1:3.2
PA. MII 4+1	86 ± 29.0	26 ± 9.3	112 ± 37.8	1:3.3
PA. MII 7+1	91 ± 24.5	26 ± 7.9	117 ± 32.2	1:3.3
PA. MII 10+1	101 ± 12.6	31 ± 7.3	132 ± 18.0	1:3.4
scNT. Fresh	103 ± 20.8	32 ± 8.0	135 ± 28.6	1:3.3
scNT-Vitri. MII 4+1	62 ± 19.5	18 ± 6.4	80 ± 24.9	1:3.5
scNT-Vitri. Enu 4+1	62 ± 18.9	20 ± 6.1	82 ± 24.4	1:3.2
scNT-Vitri. MII 7+1	68 ± 5.5	18 ± 2.3	86 ± 6.5	1:4.0
scNT-Vitri. Enu 7+1	79 ± 7.2	22 ± 5.7	101 ± 12.6	1:3.8
scNT-Vitri. MII 10+1	72 ± 15.4	19 ± 4.1	90 ± 19.4	1:3.9
scNT-Vitri. Enu 10+1	68 ± 13.6	17 ± 3.6	85 ± 17.0	1:4.0
Average	**80 ± 16.1**	**24 ± 6.1**	**103 ± 21.2**	**1:3.5**

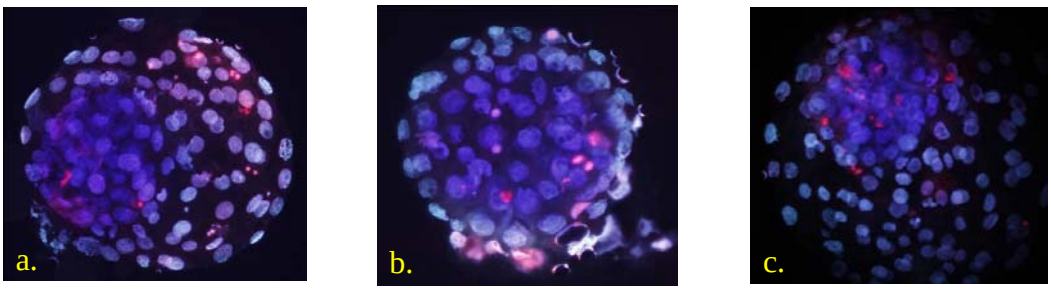

Figure 19. Differential staining of swamp buffalo blastocysts: (a) TT.MII 4+1, (b) TT.MII 7+1, (c) TT.MII 10+1 (100x).

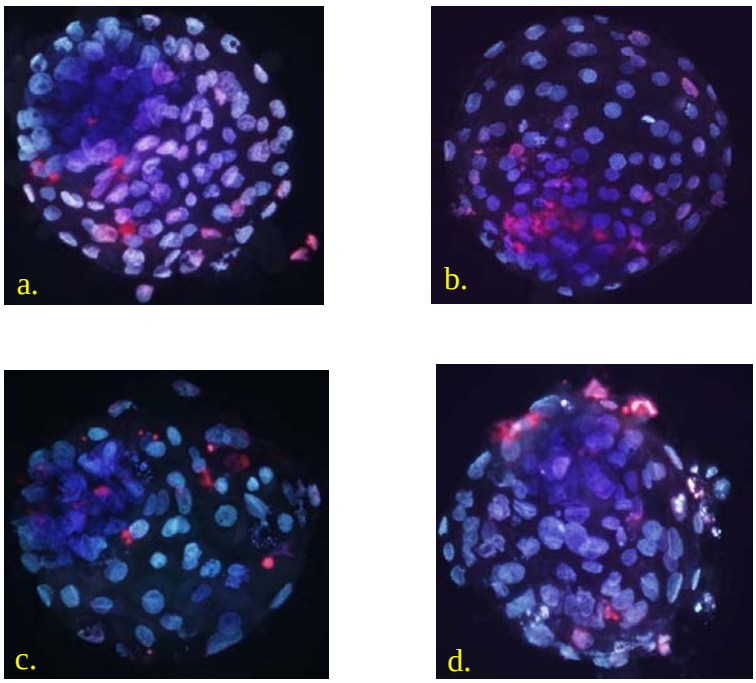

Figure 20. Differential staining of swamp buffalo blastocysts: (a) PA. Fresh, (b) PA. MII 4+1, (c) PA. MII 7+1, (d) PA. MII 10+1 (100x).

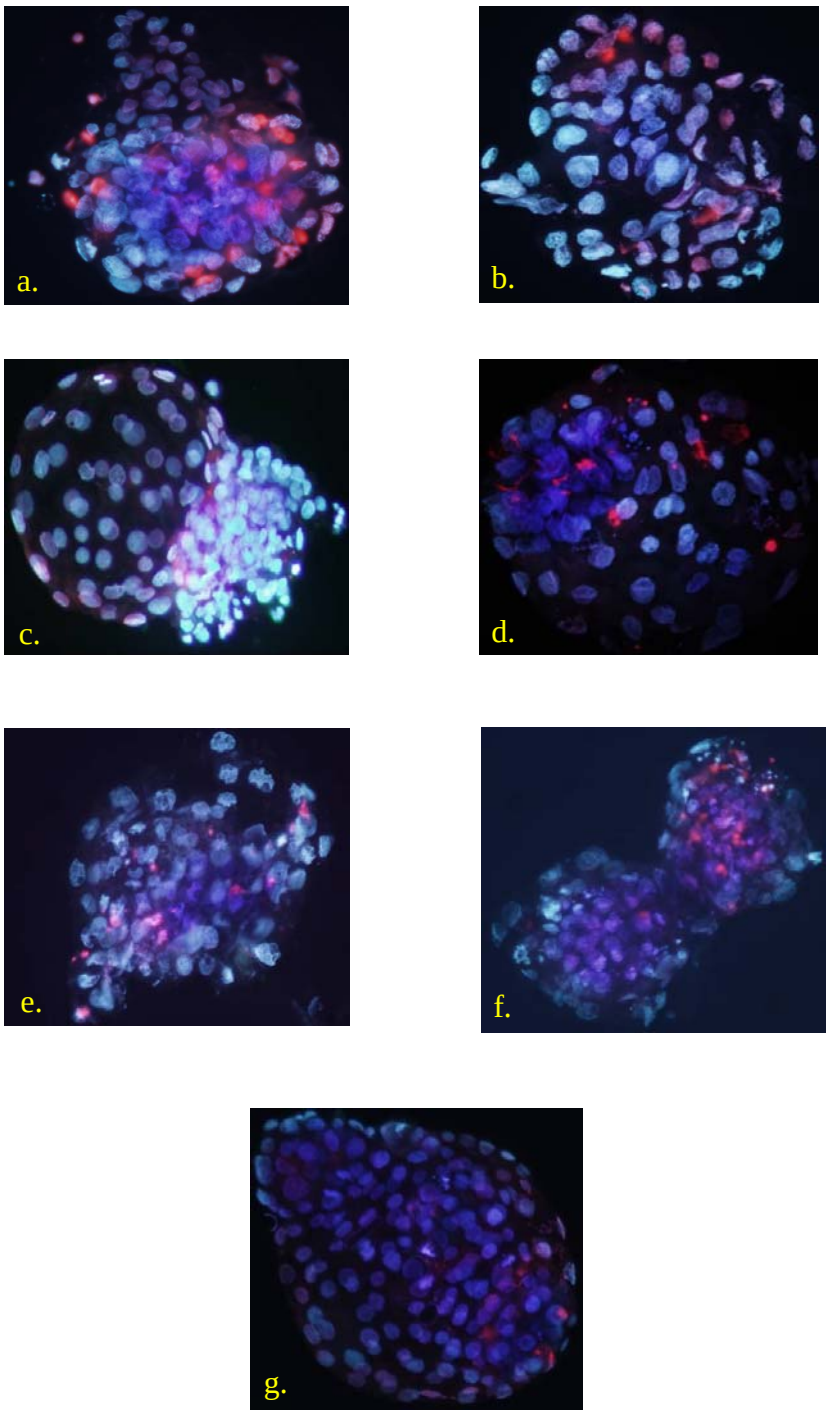

Figure 21. Differential staining of swamp buffalo blastocysts: (a) scNT. Fresh, (b) scNT.Vitri. MII 4+1, (c) scNT.Vitri. Enu 4+1, (d) scNT-Vitri. MII 7+1, (e) scNT.Vitri. Enu 7+1, (f) scNT.Vitri. MII 10+1, (g) scNT.Vitri. Enu 10+1, respectively (100x).

CHAPTER IV

DISCUSSION AND CONCLUSION

The developmental potential of swamp buffalo embryos derived from fresh and vitrified matured oocytes were studied. Approximately 71% of the vitrified buffalo oocytes observed survive after warming. This rate was slightly lower than the open pulled straw vitrification techniques (Das et al., 1999; Dhali et al., 2000). Cryotop was used as a container in this study. Cryotop offers many advantages over other methods for cryopreservation of oocytes. Most important is the higher cooling and warming rates when compared to slow freezing. In addition, the cryotop sheet is thin which resulting in the increase of cooling rate during vitrification. This container requires very small volumes of an oocyte suspension (less than 1 (l). This method does not effect the volume change during cooling which cause zona pellucida damage.

Men and colleagues (2003a) demontrated that greater than 90% of oocytes are viable immediately after warming as assessed by morphological evaluation and confirmed by membrane permeability test. Nevertheless, the detrimental effect of cryopreservation was expressed gradually during in vitro culture. In other studies reported that high cooling rate could prevent the sensitivity of cells from chilling injury and allow no time for spindle depolymerization (Pollard and Leibo, 1994; Martino et al., 1996; Vajta et al., 1998).

Slow freezing gives acceptable results for the oocytes that are not sensitive to chilling due to low lipid content like mouse, cat, rabbit and humans, whereas poor

results have been reported for other species. Particularly in those that are sensitive to cooling (e.g. pig, cattle) due to more lipid content. Especially in buffalo oocytes which have more lipid content and are more sensitive to cooling (Ledda et al., 2001). During vitrification, no ice is formed but the osmotic changes are often more severe than during freezing (Kuwayama et al., 1994).

This study is the first reports on vitrified matured swamp buffalo oocytes for somatic cell nuclear transfer using cryotop device. Recently, Wani and colleagues (2004) evaluate the different cryoprotectants with varying concentration to use in the vitrification of immature buffalo oocytes. They suggested that DMSO, EG and PROH can be used to vitrify immature buffalo oocytes for subsequent utilisation in IVM/IVF and embryo transfer. However, there was no report on vitrified mature buffalo oocytes for somatic cell nuclear transfer.

The results of the toxicity test in this study indicated that a short exposure (4 min) in equilibration solution has no effect to the in vitro development of swamp bufalo embryos compared to fresh groups. However, in PA and scNT groups, the results show effected to development to blastocyst compared to fresh groups. This may be due to the oocytes from toxicity testing which were not kept in liquid nitrogen. The greater lipid content present in the buffalo oocytes may also be one of the factors responsible for embryo development. As it has been reported that more lipid content present in the oocytes makes them more sensitive to chilling injury (Ledda et al., 2001). Martino and colleagues (1996) reported that the osmotic stress produced by the cryoprotectants has been deleterious effects on survival of mature bovine oocytes.

There were reported on development ability of oocytes frozen at GV stage to be much lower than that of in vivo or in vitro matured oocyte in mice (Schroeder et al., 1990) and cattle (Lim et al., 1992). Dhali and colleagues (2000) presented the cryopreserve buffalo immature oocytes by vitrification using a combination of 3.5 M EG and 3.4 M DMSO with an exposure time of 3 min. Wani and colleagues (2004) reported the successful production of buffalo blastocysts from immature oocyte cryopreserved by vitrification. Their study reported the percentage of morula and blastocyst formation from the cleaved embryos did not vary in fresh and vitrified oocytes.

Men and colleagues (2002) demonstrated that MII stage oocytes survive cryopreservation better than GVBD oocytes, which may due to cytoskeleton in the first meiotic division particularly susceptible to damage. MII oocyte exhibit a more flexible cytoskeleton, probably less sensitive to cryodamage (Alworth and Albertini, 1993). Study on chilling sensitivity conducted on MII stage oocytes found that the main damage occured were due to meiotic spindle disorganization followed by microtubule depolymerization (Rall, 1987; Sathananthan et al., 1988; Shaw et al., 2000; Men et al., 2002). Asada and colleagues (2002) reported the exposure to cryoprotectants contained in a vitrification solutuion affects mitochondria, vesicles, and/or microvilli and reduces embryonic development of MII oocyte, probably as a consequence of osmotic shock. Interestingly, the cytoplasmic strucrture of the vitrified bovine oocyte was not altered, as evidenced by location and morphology of cellular membrane, organelled, corticol granule, lipid droplets, mitochondria and cytoplasmic vesicles, resembling structures in fresh oocyte (Diez et al., 2005). After vitrification procedures of MII bovine oocytes, chromosomal aberrations have been

observed may due to apoptosis (Fuku et al., 1995a; Fuku et al., 1995b; Men et al., 2003; Shaw et al., 2000) and disorganization of MII spindle (Aman and Parks, 1997; Saunders and Parks, 1999; Hyttel et al., 2000). Diez and colleagues (2005) show that combination of meiotic arrest and vitrification under their conditions is responsible for serious damages at the cellular level, making the oocytes unable to undergo IVF and subsequent embryonic development. Although their vitrification procedure by straw yields low blastocysts rates, they suggested the matured oocyte as an appropiate stage to progress in research in oocyte vitrification. However, most results were from bovine oocytes. Most study on buffalo vitrified oocytes are needed for the advancement of assisted reproductive technology and cryopreservation.

The temperature of solution during vitrification also affected the penetration rate of cryoprotectant and its toxicity. The permeation rate of cryoprotectant into the cell will increased if the temperature increased (Martino et al, 1996; Isachenko, 1997; Le Gall and Massip, 1999; Vajta et al., 1998). So during vitrification and warming in this study, the temperature was controlled at 22-24(C to reduce the toxicity of cryoprotectant.

In this study, it appears that zona pellucida fracture and cytoplast lysis after vitrification and warming can happen. It may be due to the 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 fracture 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).

A tool study in initiation of embryos develoment mechanisms is parthenogenetic activation which used to optimized the activation protocols of oocytes or cytoplasts to improve the efficiency of nuclear transfer technology. This preliminary study parthenogenetic activation of vitrified buffalo oocytes with fresh oocytes in vary of time to equilibrated oocytes at 26h after IVM. It demonstrated that the possibility of chemically activating buffalo vitrified oocytes because all treatments in this studies were able to sustain vitrified embryos development up to the blastocyst stage. In this study, the activation of reconstructed swamp buffalo embryos from fresh and vitrified oocytes with 7% ethanol followed by culture in the combination of CHX and CD had high development rates as previously reported by Dinnyes et al., 2000, Gassparrini et al., 2004, and Saikhun et al., 2004. Miranda and colleagues (2002) reported a better response of buffalo oocytes to ethanol activation which evaluated by pronuclear formation. It was observed at 30h versus 24h post-maturation. It appears that the time for successful oocyte activation is very narrow in several species. An optimal period is between 21 and 24 h whereas further aging seen to negatively affect after parthenogenetic development of buffalo oocytes (Gasparrini et al., 2004).

Differences in the efficiency of parthenogenetic activation of buffalo blastocyst development after used vitrified oocytes with different equilibration time have been observed between fresh and vitrified oocytes. As in this results, the development to buffalo blastocyst of fresh (30.0%) oocytes were higher than vitrified 4+1 (17.6%), 7+1 (16.5%), 10+1 (15.9%). It has been demonstrated in vitrified buffalo oocytes that the embryo development dependent on the time of oocytes equilibration. It was also demonstrated that shorter exposure (4 min) in equilibrated

swamp buffalo oocytes could develop to blastocyst stage higher than those longer exposure time (7 and 10 min).

Although high recovery rate of morphologically normal oocytes in all groups of vitrified oocytes in this study were obtained the developmental rate of blastocyst embryo was lower in vitrified-thawed oocytes compared to that of fresh oocytes. Higher developmental rate was observed in oocytes vitrified for 4 min in equilibration solution.

From the results of in vitro development of scNT swamp buffalo embryos show that the vitrified buffalo oocytes contained the developmental potential to blastocyst stage which was similar to the fresh oocytes. However, the vitrified MII oocytes before enucleation and after enucleation of equilibration time for 4 and 7 min had no effected to development into blastocyst but differ from 10 min. The development rates of scNT embryos from vitrified oocytes was lower after enucleation and prolong exposure in equilibration solution. This may due to vitrification effect. After cryopreservation, the oocyte presents compromised developmental competence. This can be primarily explained by nuclear damage (Men et al., 2003a; 2003b), including spindle disorganization and loss of clumping of microtubules (Schroeder et al., 1990; Men et al., 2003a; Rodriguez and Farin, 2004). As well as other ultrastructural alterations seen in vitrified oocytes (Blondin et al., 1997).

Interestingly, several studies reported that the buffalo embryos develop 24-36h earlier than domestic cattle embryos collected on the corresponding days (Drost et al., 1986; Karaivanov et al., 1987; Misra et al., 1990). The buffalo embryos reach the early- and mid-blastocyst stages at 141 and 156-176h after estrus, respectively

(Anwar and Ullah, 1998). Atabay and colleagues (2004) demonstrated the intergeneric NT embryos reached the early blastocyst stage at 128h after fusion. Similarly, Parnpai and colleagues (1999) showed that buffalo scNT embryos developed half-day faster to blastocyst compared to bovine scNT embryos. Their study demonstrated that buffalo fetal fibroblasts could be reprogrammed after transferring into recipient cytoplasts for producing the cloned embryos up to blastocyst stage. We also found the same results in this study.

Differential staining of ICM after isolation by micromanipulation (Gardner et al., 1973) or immunosurgery using complement mediated lysis (Solter and Knowles, 1975) has been previously described and has since been modified by the introduction of staining with fluorochrome (Handyside and Hunter, 1984; Papaioannou and Ebert, 1998). This method had reported the problems associated with efficiency of staining which is highly dependent on the state of chemical stability of the antibodies and maintenance of the conditions at which these antibodies are incubated.

Theoretically, differential staining performed using PI and BIS dye which ICM nuclei labelled with BIS appeared blue and TE cell (labelled with both BIS and PI) appeared red when examined by fluorescence microscopy. On the contrary, this study converted the results as previous reported (Hardy et al., 1989; Van Soom et al., 1996; Kaidi et al., 2001). The ICM cells appeared red and TE cells appeared blue in colour. This may affected from the concentration of PI and BIS used in this study which were much higher concentration than the previous studies.

This study, the swamp buffalo blastocysts after day6.5 of embryo development were differential stained. The mean cell number in the blastocysts derived from vitrified oocytes was lower than that in the blastocysts derived from fresh oocytes. In

this study, the mean cell number in the blastocysts derived from scNT-fresh oocytes was higher than that of the previous study (Parnpai et al., 1999; Atabay et al., 2004). On the other hand, The mean cell number in the blastocysts derived from vitrified oocytes was similar to previous report (Parnpai et al., 1999; Saikhun et al., 2004; Atabay et al., 2004). Kochhar and colleague (2002) reported that cattle and buffalo embryos which were produced *in vitro* under the same culture condition, total cell number of buffalo blastocysts was reported to be smaller than that of cattle blastocysts.

There were same reports of damage to embryos after vitrification includes a decrease in numbers of microvilli in TE cells, loss of plasma membrane integrity, mitochodria changes and swelling of the rough endoplasmic reticulum, formation of small vesicles, and distinct intramembrane particle aggregations in the plasma membranes (Vajta et al., 1997; Ohboshi et al., 1998; Kaidi et al., 1999). Some of these changes are apparently reversible (Vajta et al., 1997; Ohboshi et al., 1998; Kaidi et al., 1999), however, cell death also occurs as suggested by the decrease in blastocyst cell number (Kaidi et al., 1999). In case of cells were dendely packed and more overlapped which made numbers difficult to resolve. This uneven compression or non-specific staining of the mount, contributed to inefficient staining in approximately 20% of buffalo blastocysts. However, this method have efficacy to assess by comparison of ICM and TE ratios for day6.5 buffalo blastocyst. Thouas and colleague (2001) reported the blastocyst differential staining patterns indicate that this technique represents a simple and reliable alternative to current bichromatic blastocyst staining techniques for the differential assessment of cell numbers and may

be useful for the assessment of blastocyst derived from *in vitro* maturation, culture systems and advanced reproductive technologies such as cloning.

To date, no reports on cryopreservation of scNT buffalo embryos derived from vitrified matured oocytes. In addition, oocyte cryopreservation has only limited success in buffalo species. It is necessary to study for improvement the vitrification method (e.g. optimization of vitrification solution contents and/or warming method, container device) to facilitate the application of assisted reproductive procedures which may improve development to the blastocyst stage. From the results of this experiment vitrified oocytes supported embryonic development as same as fresh oocytes. It can concluded that equilibration time to vitrified buffalo oocytes were related in order to maintain high survival rates of cloned swamp buffalo embryos after vitrification and warming. Therefore, it is possible to cryopreserve swamp buffalo oocytes using cryotop as a novel easily handled container by vitrification.

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APPENDIX

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 (15%EG + 15%DMSO in 199H-20)

1. 199H stock	32.5 ml
2. FBS	10 ml
3. P-S stock	50 μl
4. EG (Sigma, E-9129)	7.5 ml
5. DMSO (Sigma, D-1435)	7.5 ml

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

Vitrification solution (15%EG + 15%DMSO in 199H-20)

1. Sucrose 8.5575 g
2. 199H stock upto 25 ml
3. FBS 10 ml
4. P-S stock 50 µl
5. EG 7.5 ml
6. DMSO 7.5 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. Analytical balance (Sartorius)
2. Auto pipette (Rainin)
3. Autoclave (Astell Scientific)
4. Bacteria dish ∅ 55 mm (Sterilin)
5. Bottle (Duran)
6. Centrifuge (Sigma, 2-15)
7. CO_2 incubator (Shel Lab)
8. Conical tube 15 ml (Falcon)
9. Conical tube 50 ml (Corning)
10. Cryo storage cane (Nunc)
11. Cryotop (Kitazato Supply Co., Tokyo)
12. Cryovial (Nunc)
13. Deep freeze refrigerator (-70° C, Forma Scientific)
14. Desiccator
15. Distill water machine (Fistreem Cyclone)
16. Filter Holder ∅ 13 mm (Millipore)
17. Filter Holder ∅ 25 mm (Millipore)
18. Foam box
19. Forcept
20. Fusion machine (SUT F-1, Suranaree University of Technology)
21. Goblet
22. Graduated pipette and pipette box

23. Haematocytometer

24. Hot air oven (Gallen Kamp)

25. Inverted microscope (Karl Zeiss)

26. Laminar flow (Holten)

27. Liquid nitrogen tank (Cryodiffuse)

28. Magnetic bar

29. Magnetic stirrer (Heidolph MR2002)

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

31. Micromanipulator (Narishige)

32. Mouth piece

33. Needle (Nipro)

34. Pasture pipette

35. pH meter (Schott)

36. Pipette pump (PiaAccu, Holten)

37. Portable incubator (Biotherm, Cryologic)

38. Refrigerator (Mitsubishi: j-elegance)

39. Rubber ball

40. Stereo microscope (Olympus)

41. Suction pump (Millipore, 0295)

42. Syring (ERSTA)

43. Thermometer

44. Tip

45. Tissue culture dish ∅ 35 mm (Nunc)

46. Tissue culture dish ∅ 60 mm (Nunc)

47. Tissue culture flask (Nunc)

48. Tissue homoginize

49. Tissue paper

50. Tri-gases incubator (Thermo Forma)

51. Ultra pure water machine (Millipore)

52. Ultrasonic cleaner (Crest)

53. Volumetric flask

54. Water bath

BIBLIOGRAPHY

Suchitra Muenthaisong was born on Sept 1st, 1979 in Bangkok, Thailand. She studied high school at Ratchasimavittayalai School. She graduated with a Bachelor of Science degree from Department of Biology, Faculty of Science, Khonkaen University, in 2002. After graduation, she worked as a research assistant for 1 year in the animal cloning laboratory at Suranaree University of Technology with Dr. Rangsun Parnpai. Then, she decided to further her study in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. During her master degree enrollment, she was also an assistant researcher with Dr. Rangsun Parnpai. She conducted the research in the topic of Development of cloned swamp buffalo embryos reconstructed with vitrified oocyte as thesis work. The results of this project have been presented as oral presentation at the Asian Reproductive Biotechnology Symposium April 12-14, 2004 at Nong Lam University, Ho Chi Minh city, Vietnam. The 43rd Kasetsart University Annual Conference in Bangkok, Thailand February 1-4, 2005. Research Network Development of Higher Education Alliance in Nakhon Ratchasima, Thailand on June 24th, 2005; The 12th International Congress on Biotechnology in Animal Reproduction in Ching Mai, Thailand on August 4-6, 2005.