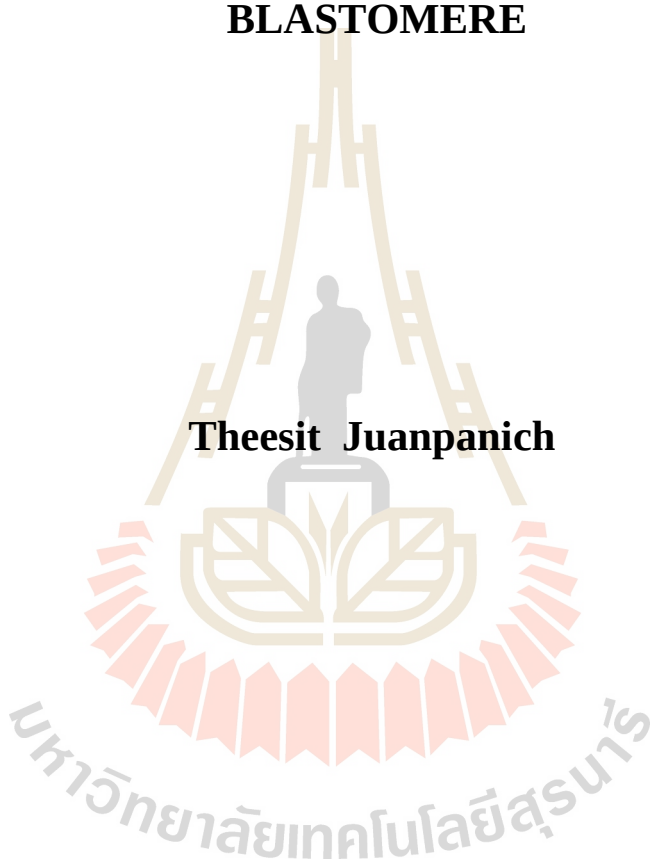
# EFFECT OF GEL EMBEDDED MATERIALS AND VITRIFICATION SOLUTIONS ON DEVELOPMENTAL COMPETENCE OF BOVINE SEPARATED BLASTOMERE

**Theesit Juanpanich**




**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology**

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# ผลของแท่งเจลและน้ำยาแช่แข็งที่แตกต่างกันต่ออัตราการเจริญเติบโตของตัวอ่อนโคแยกบลาสโตเมียร์

นายธีศิษฏ์ จ๋วนพานิช



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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**EFFECT OF GEL EMBEDDED MATERIALS AND VITRIFICATION SOLUTIONS ON DEVELOPMENTAL COMPETENCE OF BOVINE SEPARATED BLASTOMERE**

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ธีศิษฏ์ จ๋วนพานิช : ผลของแท่งเจลและน้ำยาแช่แข็งต่ออัตราการเจริญเติบโตของตัวอ่อนโคแยกบลาสโตเมียร์ (EFFECT OF GEL EMBEDDED MATERIALS AND VITRIFICATION SOLUTIONS ON DEVELOPMENTAL COMPETENCE OF BOVINE SEPARATED BLASTOMERE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. รังสรรค์ พาลพ่าย, 119 หน้า.

การแยกบลาสโตเมียร์จากตัวอ่อนโคระยะ 2 เซลล์ และ 8 เซลล์สามารถช่วยเพิ่มจำนวนตัวอ่อนระยะบลาสโตซิสต์เพื่อนำไปใช้ในการย้ายฝากตัวอ่อน อย่างไรก็ตาม การเลี้ยงตัวอ่อนแยกบลาสโตเมียร์ในน้ำยาเลี้ยงตัวอ่อนส่งผลให้การเจริญเติบโตของตัวอ่อนแยกบลาสโตเมียร์ลดลง การทดลองที่ 1 มีจุดประสงค์เพื่อพัฒนาระบบการเลี้ยงบลาสโตเมียร์ที่แยกจากตัวอ่อนโค การทดลองที่ 1.1 นำตัวอ่อนโคระยะไซโกตที่ผลิตโดยใช้ไข่จากโรงฆ่าสัตว์ปฏิสนธิในหลอดทดลอง จากนั้นนำไปเลี้ยงในแท่งเจล 1% agarose และแท่งเจล 1% calcium alginate และจานเลี้ยง Well of Well (WOW) จากการทดลองพบว่าตัวอ่อนระยะไซโกตที่นำไปฝังในแท่งเจล 1% agarose และ 1% calcium alginate สามารถเจริญเติบโตเป็นตัวอ่อนระยะบลาสโตซิสต์ (44% และ 49% ตามลำดับ) ซึ่งไม่แตกต่างกับตัวอ่อนที่เลี้ยงในหยดน้ำยาเลี้ยงปกติ (48%) การทดลองที่ 1.2 นำตัวอ่อนที่แยกและไม่แยกบลาสโตเมียร์จากตัวอ่อนที่ผลิตโดยใช้ไข่จากโรงฆ่าสัตว์ปฏิสนธิในหลอดทดลอง ระยะ 2 เซลล์ และ 8 เซลล์ ไปเลี้ยงในแท่งเจล 1% calcium alginate และจานเลี้ยง WOW สำหรับตัวอ่อนกลุ่มที่ไม่แยกบลาสโตเมียร์ ตัวอ่อนระยะ8 เซลล์ที่เลี้ยงในจานเลี้ยง WOW มีอัตราการเจริญเติบโตถึงระยะบลาสโตซิสต์สูงกว่า ตัวอ่อนระยะ 2 เซลล์ ที่เลี้ยงในแท่งเจล 1% calcium alginate อย่างมีนัยสำคัญทางสถิติ (68% และ 46% ตามลำดับ) แต่ไม่ต่างกับกลุ่มอื่นๆ สำหรับกลุ่มแยกบลาสโตเมียร์ จากตัวอ่อนระยะ 8 เซลล์ มีอัตราการเจริญเติบโตถึงระยะบลาสโตซิสต์ไม่แตกต่างกันทั้งกลุ่มที่เลี้ยงในจานเลี้ยง WOW และในแท่งเจล 1% calcium alginate (115% และ 111.7% ตามลำดับ) อย่างไรก็ตามบลาสโตเมียร์ที่แยกจากตัวอ่อนระยะ 2 เซลล์ กลุ่มที่เลี้ยงในจานเลี้ยง WOW มีอัตราเจริญเติบโตถึงระยะบลาสโตซิสต์สูงกว่าตัวอ่อนที่ฝังในแท่งเจล 1% calcium alginate (125.6% และ 88.3% ตามลำดับ) และมีอัตราการเจริญสูงกว่าบลาสโตเมียร์ที่แยกจากตัวอ่อนในกลุ่มอื่นๆ การทดลองที่ 1.3 นำตัวอ่อนที่แยกและไม่ได้แยกบลาสโตเมียร์จากตัวอ่อนระยะ 2 เซลล์ ที่ผลิตโดยใช้ไข่ที่เก็บด้วยวิธี OPU ปฏิสนธิในหลอดทดลอง นำไปเลี้ยงในจานเลี้ยง WOW พบว่าอัตราการเจริญเติบโตถึงระยะบลาสโตซีสต์ในกลุ่มบลาสโตเมียร์ที่แยกจากตัวอ่อนระยะ 2 เซลล์สูงกว่าตัวอ่อนที่ไม่ได้แยกบลาสโตเมียร์อย่างมีนัยสำคัญทางสถิติ (81.8% และ 24.4% ตามลำดับ) แต่พบว่าจำนวนเซลล์ของตัวอ่อนระยะบลาสโตซีสที่ได้จากการแยกบลาสโตเมียร์ มี

จำนวนน้อยกว่ากลุ่มของตัวอ่อนปกติ สรุปได้ว่าการเลี้ยงใน WOW มีความเหมาะสมสำหรับเลี้ยงบลาสโตเมียร์ที่แยกจากตัวอ่อน เนื่องจากความสามารถในการเจริญเติบโตถึงระยะบลาสโตซิสต์

การทดลองที่ 2 มีจุดประสงค์เพื่อศึกษาอัตรารอดและอัตราการเจริญเติบโตของตัวอ่อนและ
การทดลองที่ 2 มีจุดประสงค์เพื่อศึกษาอัตรารอดและอัตราการเจริญเติบโตของตัวอ่อนและ
บลาสโตเมียร์ที่แยกมาจากตัวอ่อนระยะ 2 เซลล์ และระยะ 8 เซลล์ ที่ผลิตโดยใช้ไข่จากโรงฆ่าสัตว์ปฏิสนธิในหลอดทดลองหลังผ่านการกระบวนการแช่แข็งด้วยวิธี Cryotop vitrification การทดลองที่ 2.1 เปรียบเทียบน้ำยาแช่แข็ง 2 สูตรในการแช่แข็งตัวอ่อนโครยะบลาสโตซีสต์ โดยน้ำยาแช่แข็งสูตรที่ 1 ประกอบด้วย Ethylene glycol (EG) + Propylene glycol (PG) และน้ำยาแช่แข็งสูตรที่ 2 ประกอบด้วย EG เพียงอย่างเดียว จากการทดลองพบว่าอัตรารอดหลังทำละลายของตัวอ่อนระยะบลาสโตซีสต์ในกลุ่ม EG+PG (88.4%) สูงกว่ากลุ่ม EG (76.9%) อย่างมีนัยสำคัญทางสถิติ การทดลองที่ 2.2 เปรียบเทียบน้ำยาแช่แข็ง 2 สูตรที่ใช้ในการทดลอง 2.1 ในการแช่แข็งตัวอ่อนโครยะ 2 เซลล์ และ 8 เซลล์ จากการทดลองพบว่ากลุ่มที่ใช้น้ำยา EG+PG หลังจากทำลายตัวอ่อนแช่แข็งระยะ 2 เซลล์ และ 8 เซลล์ ตัวอ่อนเจริญเติบโตต่อถึงระยะบลาสโตซีสต์ (0%, 0.5%) ซึ่งต่ำกว่าอย่างมีนัยสำคัญทางสถิติกับกลุ่มที่ใช้น้ำยา EG (34%, 42%) ตามลำดับ การทดลองที่ 2.3 ทำการแช่แข็งบลาสโตเมียร์ที่แยกมาจากตัวอ่อนระยะ 2 เซลล์ และระยะ 8 เซลล์โดยใช้น้ำยาสูตร EG พบว่าอัตราการเจริญต่อถึงระยะบลาสโตซีสต์ของบลาสโตเมียร์แช่แข็ง (21%, 18%) ต่ำกว่ากลุ่มบลาสโตเมียร์ที่ไม่ได้แช่แข็ง (44%, 50%) อย่างมีนัยสำคัญทางสถิติ

จากการทดลองทั้งหมดสรุปได้ว่าเทคนิคการแยกบลาสโตเมียร์สามารถเพิ่มจำนวนตัวอ่อนระยะบลาสโตซีสต์ได้ นอกจากนั้นบลาสโตเมียร์ที่แยกมาจากตัวอ่อนโครยะ 2 เซลล์ และระยะ 8 เซลล์ สามารถเจริญเติบโตในแท่งเจล 1% calcium alginate และสามารถนำไปแช่แข็งในน้ำยาแช่แข็งสูตร EG ได้



สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

THEESIT JUANPANICH : EFFECT OF GEL EMBEDDED MATERIALS AND VITRIFICATION SOLUTIONS ON DEVELOPMENTAL COMPETENCE OF BOVINE SEPARATED BLASTOMERE.
THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 119 PP.

BOVINE/SEPARATED BLASTOMERE/EMBEDDED EMBRYO/VITRIFICATION

Blastomere separation from 2-cell and 8-cell stages bovine embryos would be useful for increasing the number of embryos available for embryo transfer program. However, the small numbers of separated blastomere cultured in medium give rise to low developmental competence of embryo. The aim of experiment 1 was to develop culture system of blastomere separated from bovine embryos. In experiment 1.1, bovine zygotes derived from slaughterhouse oocytes fertilized *in vitro* were cultured in 1% agarose gel and 1% calcium alginate compared in Well of Well (WOW) culture system. The results showed that the zygotes embedded in 1% agarose and 1% calcium alginate gel could develop to blastocyst stage (44% and 49%) with no different from control (48%). In experiment 1.2, the intact and separated blastomere of slaughterhouse oocytes fertilized *in vitro* embryo at 2-cell and 8-cell stages were cultured 1% calcium alginate and WOW. In the case of intact embryo, the 8-cell stage embryo cultured in WOW showed significantly higher blastocyst rate (68%) than intact embryos at 2-cell stage embedded in 1% calcium alginate (46%) but was not different from the other groups. In separated blastomere from embryo at 8-cell stage, culturing the quadruple blastomere in 1% calcium alginate and WOW showed no different embryo development to blastocyst stage (111.7% and 115%). However, the blastocyst rate of blastomere separated from 2-cell cultured in WOW was higher than 1% calcium alginate group (125.6% and 88.3%) and was higher than the other separated blastomere groups. In experiment 1.3, intact and separated blastomere from

2-cell stage embryos derived from OPU-oocytes fertilized *in vitro* were cultured in WOW. The blastocyst rate of separated blastomere from 2-cell stage embryos culturing in WOW was significantly higher than intact 2-cell stage embryos (81.8% and 24.4%); however, the total numbers of cell in separated-derived blastocyst was lower than the intact-derived blastocyst.

The second experiment aimed to investigate the survival and *in vitro* developmental competence among intact and separated blastomere of 2 and 8-cell stages bovine embryos derived from slaughterhouse oocytes fertilized *in vitro* after freezing by using Cryotop vitrification method. In experiment 2.1, two different equilibration and vitrification solutions were used for cryopreservation of bovine expanded blastocysts. The first solution containing ethylene glycol (EG) + propylene glycol (PG) and the second solution containing only EG. The survival rate of vitrified-warmed expanded blastocysts using EG+PG (88.4%) was significantly higher than the EG group (76.9%). In experiment 2.2, the two vitrification solutions were used for cryopreservation of 2-cell and 8-cell stages bovine embryos. The results showed that in the EG+PG group, vitrified-warmed 2-cell and 8-cell embryos did not developed to blastocyst stage (0% and 0.5%, respectively) which were significantly lower than of the EG group (34% and 42%, respectively). In experiment 2.3, separated blastomeres from 2-cell and 8-cell stage embryos were vitrified-warmed by using EG-based solution. The blastocyst formation rates of vitrified-warmed separated blastomeres from 2-cell and 8-cell stage embryos (21% and 18%, respectively) were significantly lower than the fresh separated blastomeres (44% and 50%, respectively).

In conclusion, separated blastomere from 2-cell and 8-cell embryos embedded in 1% calcium alginate can develop to blastocyst stage. Furthermore, separated blastomere could be vitrified-warmed by using EG-based vitrification solution.

School of Biotechnology Student's Signature_____

Academic Year 2016 Advisor's Signature _____

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Theesit Juanpanich

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AI | = | artificial insemination |
| BM | = | based medium |
| BSA | = | bovine serum albumin |
| CL | = | corpus luteum |
| COCs | = | cumulus-oocyte-complexes |
| CPA | = | cryoprotective agent |
| CS | = | calf serum |
| DF | = | dominant follicle |
| DMSO | = | dimethyl sulfoxide |
| $E_2$ | = | estradiol |
| EG | = | ethylene glycol |
| ES | = | equilibration solution |
| FSH | = | follicular stimulating hormone |
| GnRH | = | gonadotrophin releasing hormone |
| Hpi | = | hours post insemination |
| ICM | = | inner cell mass |
| IOI | = | inter ovulatory interval |
| IVC | = | *in vitro* culture |
| IVF | = | *in vitro* fertilization |

# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| IVM | = | *in vitro* maturation |
| IVP | = | *in vitro* embryo production |
| LH | = | luteinizing hormone |
| $LN_2$ | = | liquid nitrogen |
| MHz | = | megahertz |
| ml | = | mililiter |
| mM | = | micromolar |
| MOET | = | multiple ovulation and embryo transfer |
| OPU | = | ovum pick-up |
| $P_4$ | = | progesterone |
| PBS | = | phosphate buffer saline |
| PF | = | preovulatory follicle |
| PG | = | propylene glycol |
| PGD | = | preimplantation genetic diagnosis |
| $PGF_{2\alpha}$ | = | prostaglandin F2-alpha |
| PI | = | propidium iodide |
| TE | = | trophectoderm |
| µl | = | microliter |
| VS | = | vitrification solution |
| WOW | = | wells of the well |
| ZP | = | zona pellucida |

# CHAPTER I

# INTRODUCTION

## 1.1 Introduction

Dairy cattle industry in Thailand has existed more than 50 years but still has few numbers of superior genetic dairy cattle with over 6,000 kg/cow/lactation of milk production found. Most farmers use conventional method to produce dairy cattle such as natural breeding and artificial insemination (AI). However, possibility of having male calf is 50:50 which cannot produce milk for milk industry. Dairy cattle breeding improvement by conventional breeding method is slowly progressing because bovine gestation period lasts 280 days. The United States of America has the highest milk production in the world which is about 9,500 kg/cow/year (French Ministry of Agriculture, Food and Forest, 2014) whereas Thailand can produce only 2,500-3,000 kg/cow/year (Thai Feed Mill Association, 2010.). In 2011, Thailand spends over 14 million baht on milk and milk product import from other countries due to inadequate of dairy cattle farm production.

Nowadays, *in vitro* embryo production (IVP) combined with ultrasound guided ovum pick-up (OPU) is an effective technique for producing progeny followed by embryo transfer in cattle. In 2014, 1,244,666 bovine embryos have been produced by *in vivo-derived* and *in vitro* procedure and 630,202 were IVP embryos (Perry, 2015). The number of OPU-IVP embryos has been increased because of OPU technology, which permits oocyte collection from high genetic and economic donor cattle. The

combination of frozen sexed semen and OPU-derived oocytes has enabled production of large number of high genetic merit, replacement heifers and herd expansion (Maxwell et al., 2004; Wheeler et al., 2006; Presicce et al., 2011). However, culture of low number of OPU-derived embryo gave rise to low embryo developmental rate and quality when compared with conventional group culture (Donnay et al., 1997; Gopichandran and Leese et al., 2006; Senatore et al., 2010). In previous study, gel embedded embryo such as calcium alginate gel (Kobayashi et al., 2006) and low melting point agarose gel (Senatore et al., 2010; Deb et al., 2011) can improve the developmental competence of small number of intact embryos by maintaining suitably embryo/volume ratio. Furthermore, early embryonic cells from zygote to morula stage of the embryos are classified as totipotent stem cell (Schramm et al., 2004), which have the potential to differentiate into any cell type in animal body and to generate new animal life. For this reason, the possibility to generate separated blastomere from cleaved embryos in term of improving number of blastocyst, to generate embryonic stem cell (ES cell) lines used in preimplantation genetic diagnosis (PGD) (Klimanskaya et al., 2006, Geens et al., 2009) and to study epigenetic reprogramming pattern (Chavez et al., 2014, Wu et al., 2014,) can be performed. Recently, a blastomere separation technique was developed for a simpler and more efficient procedure (Tagawa et al., 2008). The zona pellucida of embryo was removed by exposure with protease enzyme, followed by gentle pipetting with a tapered pipette. In cattle, twins (Takawa et al., 2008), triplets (Willadsen et al., 1981) and quadruplet monozygotic calves (Johnson et al., 1995) have been produced by blastomere separation technique. The stage of embryo which used for separation has been performed at two or eight cell stages because embryos are easy to manipulate. In

bovine progeny cryopreservation, many successful of oocytes and embryos vitrification which developed to blastocyst have been reported in immature oocytes (Spricigo et al., 2012; Liang et al., 2012; Sripunya et al., 2014), matured oocytes (Horvath and Seidel, 2006; Sripunya et al., 2010; Phongnimitr et al., 2013), zygote (Libermann et al., 2002; Hochi et al., 2004; Al-Hasani et al., 2007), early stages embryos (Campos-Chillon et al., 2009; Fathi et al., 2012), and blastocyst (Kuwayama et al., 1992; Park et al., 1999; Huang et al., 2007; Inaba et al., 2011) but there is no report in bovine separated embryos.

There are two objectives in this research. The first objective was to investigate the developmental rates and quality of OPU-derived embryos which are produced by blastomere separation and gel embedded techniques. The second objective was to investigate survival and *in vitro* developmental ability of bovine separated blastomere from 2-cell and 8-cell stages of embryos after vitrification using Cryotop method.

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# CHAPTER II

# LITERATURE REVIEW

## 2.1 The estrous cycle in cattle

The estrous cycle is defined as the time between estrus periods. The average length of the bovine estrous cycle are 21 days in cows and 20 days in heifers, with ranging from 18-24 days are considered in normal and can be divided in four phases as prestrus, estrus, metestrus and diestrus (Bearden and Fuquay, 1980). The various of different hormonal regulation that occur during the cycle, involving gonadotrophin releasing hormone (GnRH), follicular stimulating hormone (FSH), luteinizing hormone (LH), prolactin, inhibin, ovarian steroids/peptides hormone such as estradiol ($E_2$), progesterone ($P_4$) and prostaglandin F2-alpha ($PGF_{2\alpha}$) have been examined (Hansel and Convey, 1983; Peters, 1985). GnRH plays a crucial role in regulating ovarian activity during the cow's estrous cycle. This neuropeptide are synthesized and secreted in pulsatile pattern by hypothalamic secretory cell that stimulates the synthesis and secretion of FSH and LH by specific cell located in the anterior pituitary gland which promotes gonadal steroid synthesis (Figure 2.1) (Sealfon and Millar, 1995). The activity of ovarian estradiol is responsible for estrus phenomenon. The effect of estradiol appears to be "all or none" when an estradiol is higher than the threshold level, estrus induced intensity and duration of estradiol retained above the threshold during estrus period (Allrich, 1994). Synthesis of estradiol requires the coordinated activities of FSH and LH (Figure 2.2). Increasing of plasma estradiol

concentration derived from preovulatory dominant follicle (DF), after the decline of progesterone of the end of estrous cycle due to the regression of corpus luteum (CL) then induce a preovulatory of LH surge which secrete in low amplitude and high frequency pattern that occurs in the early of estrous before ovulation

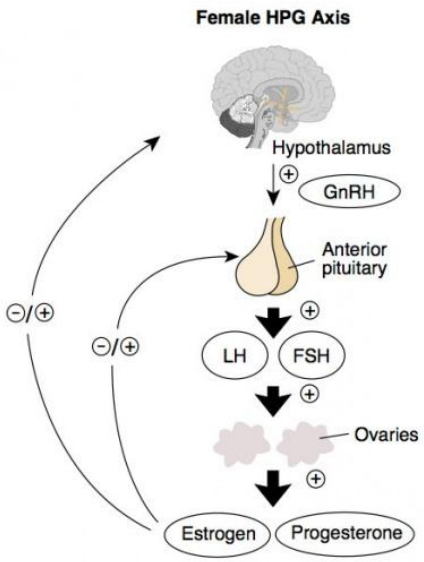


**Figure 2.1** Hypothalamus-Pituitary-Gonad endocrine axis. (Hiller-Sturmhöfel and Bartke, 1998).

(Roche, 1996). The various hormonal interactions and other events that present during the bovine estrus period as shown dramatically in Figure 2.3. Ovulation occurs 10-14 hours after estrous and followed by luteal phase of the estrous. Following expulsion of ovum, LH induces the transformation of ovulated follicular cell in to CL, a process known as luteinization, the protrusion of tissue and clots blood forms a structure known as the corpus hemorrhagicum. The consequences of LH surge including gene regulation, intracellular signaling and tissue remodeling within ruptured site of ovary

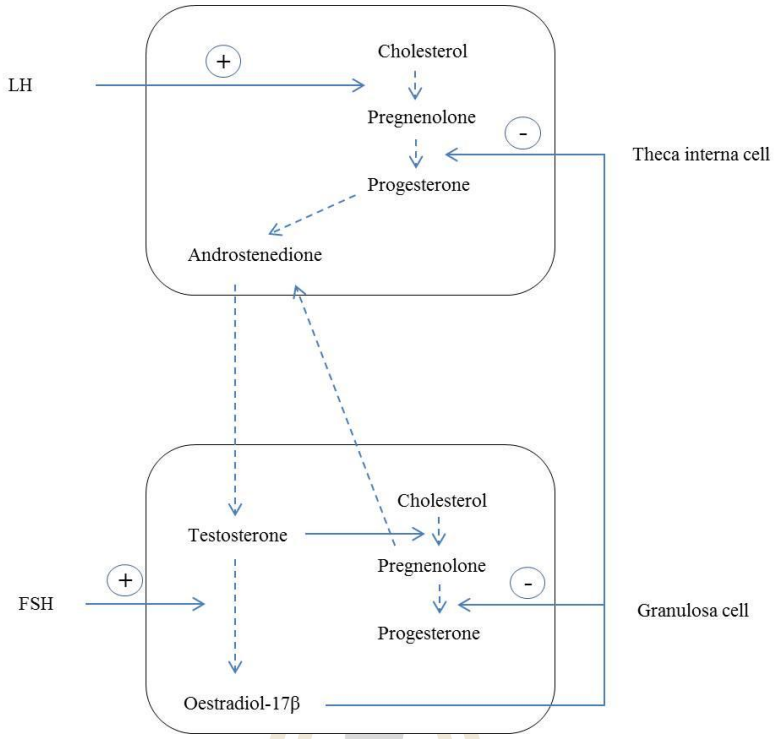


**Figure 2.2** Model of steroidogenesis in bovine preovulatory follicles. (Hansel and Convey, 1983).

(O'shea et al., 1978; Cran et al., 1979; Russell and Robker, 2007). After ovulation, the layer of granulosa cell is fallen into follicular antrum space. At the same period, the basement membrane that separated the granulosa cell layer from theca layer degrades. Then, theca interna and granulosa cells differentiate in to large and small luteal cells, respectively (Tomac et al., 2011). The large cells secrete progesterone and oxytocin whereas the small cells secrete progesterone and responsive to LH. During CL differentiation into cell which capable to producing progesterone is achieved by increased activity of enzyme necessary for conversion of cholesterol to progesterone (cytochrome P-450 complex and 3β-hydroxysteriod dehydrogenase/$\Delta^5$, $\Delta^4$ isomerase; 3β- HSD). Free cholesterol inside the cell is the precursor of progesterone production.

Inside of mitochrondria, the cytochrome P-450 enzyme cleaves the side chain from cholesterol to pregnenolone (Niswender et al., 2000). Pregnenolone is converted to progesterone by 3β- HSD enzyme (Figure 2.2). In luteal phase of estrus cycle, progesterone dominates the major of the estrous cycle; detectable amounts in the blood circulation 3-4 days after the day of CL formation. The circular progesterone rise for several days until the plateau is reached on day 8 of cycle.

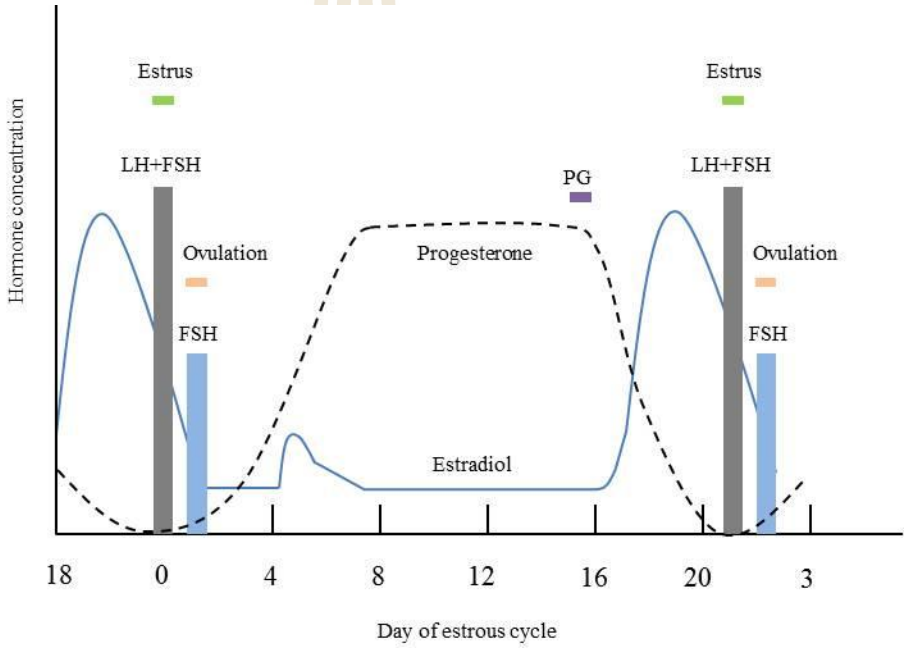




**Figure 2.3** Changing hormone concentrations during the bovine estrous cycle. (Peters, 1985).

In case of pregnancy, maintenance of CL is the result of maternal recognition between embryo and maternal uterus, as well as intrauterine autocrine and paracrine signals that modulate the endocrine function of luteal cells. In the absence of uterine and embryonic signals, rapid CL regression at the end of luteal phase (also known as Luteolysis), some 1-4 days prior to the onset of the new heat period, is a key of in the

bovine estrous cycle, resulting in progesterone circulation rapidly decrease (Zheng et al., 1994). Then, $PGF_{2\alpha}$ released from the endometrium cell resulting in CL regression and consequent dramatic fall in progesterone concentration that prepares the scene for new estrus cycle.

## 2.2 Follicular dynamics in cattle

The first follicular wave starts after ovulation, with 4-5 mm of follicles in diameter (Ginther and Hoffman, 2014). A follicular wave develops every 9 days in *Bos taurus*, resulting in 2-3 waves during the inter ovulatory interval (IOI) (Ginther et al., 2001) and the first wave dominant follicle (DF) undergoes atresia during 8-10 days of estrous cycle (Ginther et al., 1989a) (Figure 2.4). The interval seems similar between *Bos taurus* and *Bos indicus* species, but the average of largest of DF in *Bos taurus* is smaller than *Bos indicus* (Sartorelli et al., 2005). The growth of ovulated follicle is no regarded as a random process but is determined to be a part of well-organized and predicable events including recruitments of follicle, follicular selection and dominance of follicle.

### 2.2.1 Recruiments of follicle

Recruitment process is believed to be involved in the growth of the small follicles which the follicular size less than 3 mm are stimulated to grow (Webb et al., 1999). The concept of follicle recruitment is used for the entrance of follicles in the growing in wave-like growth pattern. The key hormone of recruitment process is FSH (Sunderland et al., 1994; Fricke et al., 1997). The two and three wave cattle had two and three FSH surge during the interovulatory interval (IOI), respectively (Adams et al., 1992). The peak of first FSH surge for wave 1 occurs near the day of ovulation or

when the largest follicle of wave is 4-5 mm (Adams et al., 1992). The second peak of FSH surge for wave 2 occur about 8 and 9 days after ovulation for three waves and two wave IOIs, respectively (Kulick et al., 2001).

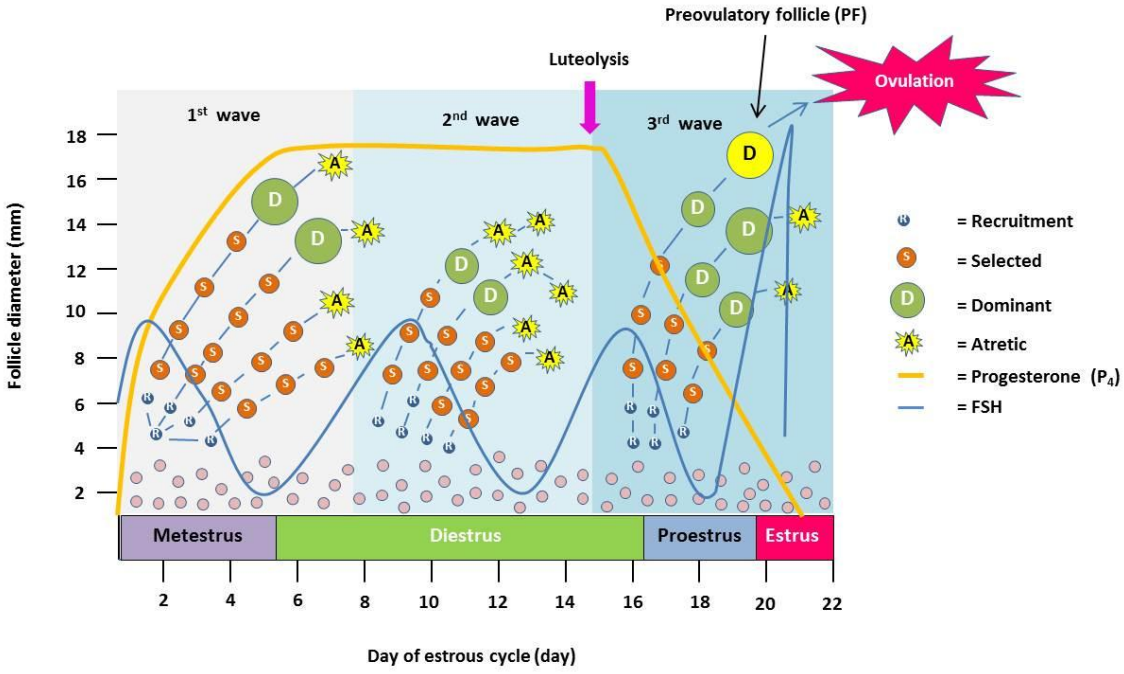


**Figure 2.4** The schematic pattern of follicular wave in cattle. (Crowe and Mullen, 2013).

The population of 2 mm in diameter follicles of wave 1 is higher in ovaries which presented of preovulatory follicle (PF) and extant regression of CL than the ovaries with both PF and CL or with CL alone. These differences in preovulatory patterns affect to the number of 2 mm follicle population in ovary (Ginther et al., 2015). In first follicular wave, it is not known that a group of 2 mm follicles begin to emerge at or before then beginning of FSH increase (Ginther et al., 2015). In follicular wave 2, the number of 1 to 3 mm follicles are maximum grow 1-2 days before the future

dominant follicle (DF) is 4 mm. The 1 to 3 mm follicles growth is induced by FSH levels (Jaiswal et al., 2004).

**2.2.2 Follicular selection**

Follicular selection is a process which determined one follicle becomes dominant and continues to ovulation process (Gordon, 1996). After recruitment, a small number of recruited follicles are continue growth until one follicle is selected to become a dominant while the remaining recruited follicles become static or apoptosis. The selection process takes 2-3 days after decreasing of FSH concentration (Austin et al., 2001). As follicle grew from 3-5 mm follicles, they gained the capacity to suppress FSH by using negative feedback effect of estradiol (Ginther et al., 2000). However, small follicle less than 3 mm had no detectable to suppress FSH (Gibbons et al., 1999). At the beginning of selection, more than 5 mm follicles contribute to reduce FSH concentrations (Gibbons et al., 1997; Ginther et al., 2000). At the same time, FSH levels are suppressed by peptide hormone such as inhibin (Ginther et al., 2001). In selection process, the dominant follicle contains high amount of higher molecular weight of inbibin while the subordinate follicles have increased amount of smaller molecular weight of inbibin (Austin et al., 2001). In 2003 Kanitz found that the largest follicles in selection process reach means of 8.5 and 7.7 mm in diameter at the end of selection period. Then, one of two largest follicle was subjected to be a dominant follicle by continue growing while the other was terminated. Moreover, Insulin like growth factor-1 (IGF-1) and its binding protein (IGFBPs) have been found to play a crucial role in follicular selection process. Because IGF-1 synergizes FSH by stimulating granulosa cell proliferation and steroidogenesis (Webb et al., 1999).

### 2.2.3 Dominance of follicle

The mechanisms and their factor leading to dominance of single follicle in cow when the follicle reaches 10 mm that suppressed the growth of the other follicles are not well clearly (Sartori et al., 2001). However, the endocrine environment during luteal phase cannot support growth and development of preovulatory dominance follicle, the dominance follicle undergoes atresia (Sirois and Fortune, 1990). Changes in progesterone levels appear to be particularly critical. In case of high progesterone concentrations, the dominant follicle becomes atretic due to negative feedback effect of the progesterone on LH secretion (Ireland et al., 2000). Moreover, it is believed that expose to low circulating levels of progesterone at the end of growth phase resulting in continued grow up to preovulatory follicle (Stock and Fortune, 1993).

## 2.3 Ovum-Pick Up (OPU)

Transvaginal ultrasound-guided follicle puncture, well known as OPU, is an efficient and repeatable method for obtaining competent oocytes from antral follicles in many species including humans (Gleichert et al., 1983; Dellenbach et al., 1984), cattle (Pieterse et al., 1984; Callesen et al., 1987), goats (Graff et al., 1995), horses (Meintjes et al., 1994), buffaloes (Pavasuthipaisit et al., 1995; Boni et al., 1996; Galli et al., 1998) and pigs (Bellow et al., 2001; Caamano et al., 2002). OPU techniques can also be applied in endangered wildlife animals such as rhinoceros (Hermes et al., 2009) and endangered gazelle (Berlinguer et al., 2008). Ultrasound-guided OPU is semi-invasive, highly repeatable, and can be applied to animals in various reproductive states (Bungartz et al., 1995 Ward et al., 2000; Merton et al., 2003; Chaubal et al., 2007). The technique can be performed without any side effects on the

donor's reproductive organ. Many physiological status of the donor is suitable for this technique with healthy oocyte recovery (Galli et al., 2000). Besides using OPU to treat infertile cows, it can be used on healthy donor of high genetic value, as a tool to shorten the generation interval and to increase the number of calves with excellent genetic potential (Kruip et al., 1994). The factors which associated with the OPU technique can be divided into technical and biological factors.

**2.3.1 The technical factor of OPU**

The technical factors that were investigated including correlation between size of aspiration needle and vacuum pressure (Bols et al., 1996; Fry et al., 1997), methodology for ovum pick-up (Santi et al., 1998) and type of ultrasound probe (Bols et al., 2003).

**2.3.1.1 The effects of aspiration vacuum and needle diameter**

The effects of aspiration vacuum and needle diameter on the morphology of the cumulus-oocyte-complexes (COCs) and developmental capacity of the oocyte after *in vitro* fertilization (IVF) were examined by Bols et al. OPU sessions operated by using 18-G needle with moderate aspiration vacuum (about 70 mmHg) is recommended for obtaining a maximum number of oocytes surrounded by a compact cumulus and high blastocyst rate after IVF (Bols et al., 1996).

**2.3.1.2 The ovum pick-up method**

To collect oocytes, Reichenbach et al., (1994) compared two OPU methods between laparoscopic technique (Figure 2.5) and ultrasound-guided oocyte aspiration (Figure 2.6). Both of OPU methods were efficient to retrieve oocytes from living animals and for *in vitro* production of bovine embryos. However, the

ultrasound-guided OPU procedure provides less traumatic to the vaginal especially the fornix and less peritoneal infection than laparoscopic-OPU.

**2.3.1.3 The type of ultrasound transducer**

A comparison was observed between two types of ultrasound transducer; a linear transducer and a mechanical multiple angle sector (MAP). Both of transducer types can be effectively used for picking-up the oocyte from living cattle. However, the MAP transducer system clearly demonstrated a superior abilty to visualize small follicles and greater number of oocytes recoverd per donor than the linear transducer (Bols et al., 2003).

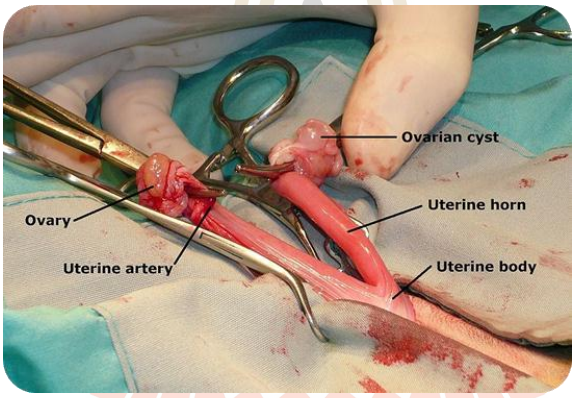


**Figure 2.5** Laparoscopic technique for oocyte collection (http://www.cannonvet.com/spay.htm).

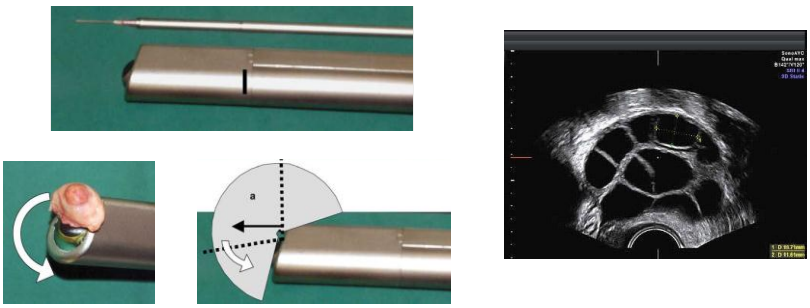

**Figure 2.6** Ultrasound-guided ovum-pick up (OPU) (Bols et al., 2003).

### 2.3.2 The biological factor of OPU

The biological factors that were investigated included hormonal stimulation (Roover et al., 2005; Presicce et al., 2010), timing and frequency of OPU sessions (Lopes et al., 2006; Roover et al., 2008), environmental status (Badinga et al., 1985; Al-Katanani et al., 2002; Takuma et al., 2010) and the donor animal herself for example; age (Majerus et al., 1999; Su et al., 2009 ), breed (Silva-Santos et al., 2011 ) and physiological status (Lopes et al., 2006 ).

#### 2.3.2.1 The hormonal stimulation and frequency of OPU sessions

Manipulation of follicular development with FSH starvation period following ovarian stimulation and administration of LH 6 hours before OPU produced highly developmental competent oocytes resulting in very high *in vitro* embryo production rates (Blondin et al., 2002). In fact, FSH administration as a single dose was adequate for ovarian stimulation; however, administering FSH in three intramuscular injection doses, 24 hours apart, significantly increased the follicular response and oocyte recovery and giving LH intravascular injection 6 hours prior to OPU resulting in significantly more morphologically superior oocytes, which produced significantly more blastocysts per OPU session (Chaubal et al., 2007). The species difference was found in responded to the same doses of FSH in terms of numbers and diameters of follicles and it was influenced the sizes but not the numbers of follicles (Roover et al., 2005).

The advantage of superstimulation prior to OPU increases a number of available follicles for puncture and give more oocytes retrieved, embryos, and produce less labor compared to non-superstimulated OPU. Moreover, embryo production in stimulated OPU session every 2 weeks (Roover et al., 2008) was higher

than non-stimulated OPU sessions (twice a week). The stimulated OPU session every 2 weeks reduced production costs per embryo because of reducing the procedure to collect oocytes, simplifying labor-consuming methods and increasing the number of oocyte batches per sessions. On the other hand, some studies concluded that non-superstimulated OPU twice a week provides a higher number and a better quality of oocytes (Galli et al., 2000; Merton et al., 2003). The reason explained by the lack of development of a dominant follicle (DF), which synthesizes high levels of estradiol and inhibin which is able to suppress FSH to basal levels inducing atresia in developing follicles (Fortune, 1994; Kruip et al., 1994; Mihm and Austin, 2002). In contrast, if OPU session was performed once weekly, the dominant follicle would grow, which affected number and quality of the recovered oocytes (Garcia and Salaheddine, 1998). However, Imai et al. (2002) examined the effect of frequency of OPU on the numbers of developing follicles in ovaries, under their particular condition, OPU depended on the individual skill of the operator and the aspiration at once a week was the most effective.

**2.3.2.2 Age of OPU donor**

A 7 to 12 month-old calves showed the possibility of producing blastocysts from oocytes collected by repeated OPU in non-stimulated calves were punctured at a high frequency (twice a week) and for a prolonged period (2 months). Cleavage rate resulting from 10 consecutive OPU sessions in the young cows were significantly higher than the middle-aged and old cows and no significantly difference between before and after puberty was observed for the mean numbers of follicles, oocytes and good quality oocytes. A prolonged OPU session did not affect the growth

rate or the reproductive physiology of the donors, growth remained linear during the 2-month period of puncture (Su et al., (2009).

**2.3.2.3 Breed of OPU donor**

For the follicular population, the number of oocytes recovered from *Bos indicus* subjected to ovum pick-up averaged 2-4 times higher when compared to *Bos taurus* cattle (Silva-Santos et al., 2011). Other reproductive characteristics (Figure 2.7), on average, *Bos indicus* cattle have more ovarian follicular waves (Figueiredo et al., 1997; Viana et al., 2000), more follicles per wave (Carvalho et al., 2008) , and a greater population of antral follicles < 5 mm in diameter than *Bos taurus* (Segerson et al., 1984). Therefore, oocytes retrieval from *Bos indicus* is higher than *Bos taurus* donors due to existing numerous small follicles. The average number of COCs per OPU session recovered from Nelore cattle (*Bos indicus*) was 18-25 COCs (Watanabe et al., 1999; Thibier et al., 2004; Rubin et al., 2005) and less than 8 COCs from Holstein cattle (*Bos taurus*) (Merton et al., 2009).

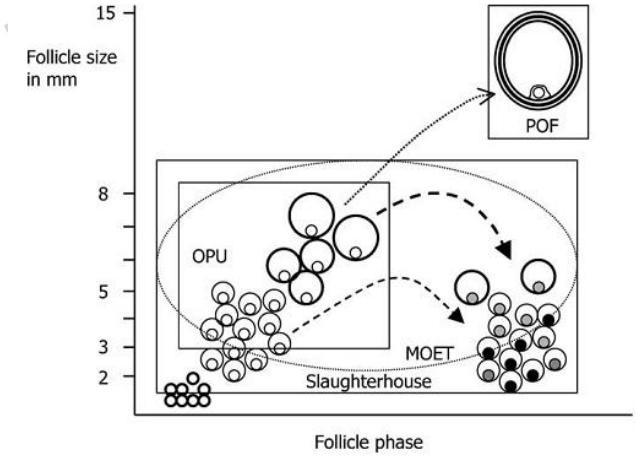


**Figure 2.7** The origin of the oocyte in the different embryo production systems: OPU; Ovum pick-up, MOET; multiple ovulation and embryo transfer and POF; preovulatory follicle (Merton et al., 2003).

### 2.3.3 The limitation of OPU

The limitation number of COCs from OPU was affected to embryo development because of the small number of zygote cultured in a medium droplet (Senatore et al., 2010). Actually, many studies reported a supportive effect on post-compacting embryonic development when group of embryos were cultured within the same droplet (Keefer et al., 1994; O'Doherty et al., 1997). An optimal number of presumed bovine zygotes must be cultured within a droplet in order to promote embryo developmental competence and quality by maintaining appropriate autocrine, paracrine signaling and embryotrophic factor (Brum et al., 2005; Gopichandran and Leese, 2006).

Blondin and Sirard (1995) compared the culture system of *in vitro* bovine embryos either single culture (one embryo /10 µl droplet) or in group culture (ten embryos /50 µl droplet) and they found significantly embryo development with group culture than individual culture. Senatore et al., 2010 showed the potential benefit of slaughterhouse helper-embryos which embedded in agarose gel can improve developmental competence of limited COCs number of OPU-derived oocytes fertilized with either conventional or sexed sperm. Moreover, agar-embedded slaughterhouse presumed zygotes may be cultured with small numbers of single cow OPU presumed zygotes as a group culture to improve OPU- derived embryo development and quality. In 2011, Deb et al. reported that slaughterhouse helper-embryos embedded in agarose gel improve OPU-derived embryo quality by increasing the gene expression of implantation and placental formation process.

### 2.3.4 Sex-sorted sperm

Reliable sex-sorted sperm through a fluorescence-activated flow cytometry has become popular in the dairy cattle industries to enhance and accelerate the diffusion of production traits. Over 90% accuracy of female IVF-derived blastocyst was produced from sexed sorted sperm (Presicce et al., 2010). In natural reproduction, the sex ratio is approximately 50:50. According to this natural limitation, overall profit margin of the dairy industry around the world is significantly reduced because only female animals are productive and most male calves are slaughtered with little financial return to the farmers.

In artificial insemination (AI), when one sexed semen straw is used, approximately 2 million x-sorted sperm are deposited into the female to fertilize one oocyte *in vivo*. On the other hand, *in vitro* fertilization (IVF), in which as few as 1,000 sperm used per oocyte (Yang et al., 1993). However, blastocyst yield with sexed-IVF has affected from bulls semens, fertilization techinique and culture method (Lu et al., 1999; Zhang et al., 2003; Lu and Seidel, 2004)

The application of OPU / *in vitro* production (IVP) and sex-sorted sperm can be combined together for producing high values of embryo by harvesting oocytes from preselected genetically superior living cows by ultrasound guided transvaginal aspiration, followed by *in vitro* maturation, fertilization with x-sorted sperm and culture until embryos reached the blastocyst stage and transferred non-surgically into recipients or for cryopreservation.

## 2.4 Blastomere separation

Early embryonic cells from zygote to morula stage of the embryo are classified as totipotent stem cell (Schramm et al., 2004), which have the potential to differentiate into any cell type in animal body and to generate new life of animal. In early embryonic stage, separated embryos have been established in cattle (Jonhson et al., 1995), sheep (Willadsen et al.,1980), goats (Tsunoda et al., 1984), pigs (Reichelt et al., 1994), horses (Allen et al., 1984), mouse (Illmensee et al., 2006), monkeys (Mitalipov et al., 2002) and humans (Hall et al., 1993). In cattle, this technique has been used to produce twins (Tagawa et al., 2008), triplets (Willadsen et al., 1981) and quadruplet monozygotic calves (Johnson et al., 1995). By this method, we can increase the number of embryos available for embryo transfer by using blastomere separation from cleavage-stage embryos. Blastomere separation procudure can divide in to 2 techniques; using surrogated zona pellucida technique and zona pellucida free technique.

### 2.4.1 Surrogated zona pellucida technique

In this technique, blastomere manipulation was performed by using manipulator connected with inverted microscope and tranfered into surrogated zona pellucida. Surrogate zona pellucida (ZP) has been prepared by previously removing all cellular blastomeres from donor embryos were selected and transferred into surrogate zona pellucida controlled by micromanipulator (Figure. 2.8). Even though, separated material from low quality oocytes or embryos. Single or half the number of blastomere by using micromanipulator was an efficacy method, this technique has many complications such as high cost of specialized equipment and technical skill of operator.

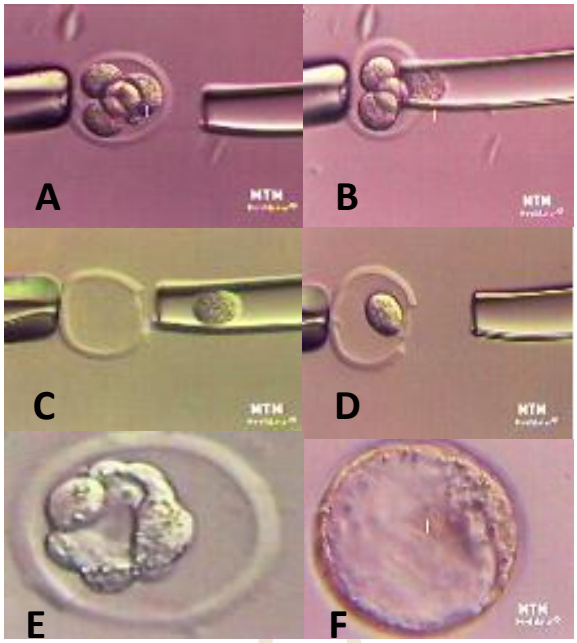


**Figure 2.8** Embryo splitting and *in vitro* culturing, the embryo is split into individual cells. (A and B). A single blastomere is shown in the transfer pipette, positioned for placement into a surrogate zona pellucida, and immobilized using a holding pipette (C and D). *In vitro* culturing of a split embryo to a blastocyst. (E and F) (Tang et al., 2012).

### 2.4.2 Zona pellucida free technique

The zona pellucida of embryo was removed by exposing with protease enzyme for 1–2 min, followed by gentle pipetting with a tapered pipette at room temperature condition. The blastomeres were isolated into two halves by gentle pipetting in culture medium with suitable tapered pipette. This technique was developed for a simpler and more efficient procedure (Tagawa et al., 2008). After separation, separated blastomere was transferred into two conical Wells of the Well culture system (WOW); microwells culture system. The amount of culture medium surrounding the embryo is very low (approximately 0.1 µl). In a quiet environment,

the dilution of certain factors in the WOW may be rather limited. In this way, the autocrine and paracrine factors may establish a more constant and suitable microenvironment for the embryo than in the large of the original well and the 20 µl drop (Vajta et al., 2000). Nagai et al. (2013) evaluated applicability of co-culture bovine separated blastomere with intact embryos (Figure. 2.9). They conclude that the morphological quality of blastocysts derived from separated blastomeres and the rate of blastocyst formation was slightly higher when the cells were co-cultured with intact embryos than the one cultured individually.

**Figure 2.9** The separated blastomere from zona pellucida free technique with WOW culture system.

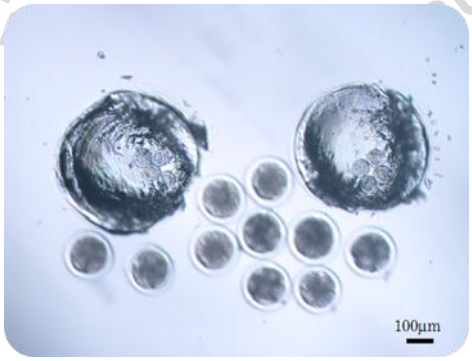


**Figure 2.10** The co-culture system of embryos derived from a separated blastomere with intact embryos (Nagai *et al.*, 2013).

The cost effective of separating the blastomeres from cleavage-stage embryos especially 2-cell and 8-cell stages of embryo in order to increase the number of embryos available for IVF and embryo transfer program must be evaluated. Multiplication of healthy offspring derived from embryo splitting especially, this technique combined with OPU/IVP and sex-sorted sperm has become and will continue to be an economic factor in dairy cattle industries.

## 2.5 Embryo cryopreservation

Embryo cryopreservation served as a tool for long term-storage and preserved of valuable genetic resource of livestock and endangered animals. Currently, there have two procedures used to cryopreserve mammalian embryos. The first to be developed was the slow freezing method (Whittingham, 1971; Wilmut, 1972). In general, slow freezing attempts to control biological properties of freezing with cryoprotective agent (CPA) to minimize intracellular ice crystal formation; embryo is gently immersed to low concentration of permeability CPA. These are usually glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) (Whittingham et al., 1972; Willadsen et al., 1976; Chen et al., 2005b; Luz et al., 2009); moreover, other non-permeability molecule also use including glucose, sucrose and trehalose (Diez et al., 2001; Barcelo-Fimbres and Seidel, 2007b; Somfai et al., 2014). The embryo loaded into the straw and equilibrated in -5 to -7$^{o}$C for several minutes. After equilibration, the solution is seeded to initiate extracellular freezing then cooled slowly about 0.3-0.5$^{o}$C/min between -30 to -65$^{o}$C. Then, the straw is plunged in to liquid nitrogen (-196$^{o}$C) for storage. By this method, seeding of the extracellular solution and slow cooling rate assure that freezing will take place only

outside of cell resulting in gradual dehydration until they reach the temperature which the intracellular matrix vitrifies (Mazur, 1963). However, they require specialized equipment to maintain the gradually cooling rate.

In contrast to vitrification method, embryos are frozen with rapid cooling rate (20,000°C/min), from room temperature to -196°C, by exposing to high concentration of CPA and plunged directly into liquid nitrogen that becomes an extremely viscous supercooled liquid without ice crystal formation (Rall, 1987). Since we know that small volume of CPA allow better heat transfer and higher cooling rate (Arav, 1992), many scientists have been developed techniques and devices to reduce volume of CPA including minimum drop size (Arav, 1992; Arav and Zeron, 1997; Park et al., 1999), solid surface (Dinnyes et al., 2000; Sripunya et al., 2010), close pulled straw (Chen et al., 2001), open pulled straw (Vajta et al., 1998; Gayer et al., 2008; Campos-Chillon et al., 2009), Hollow fiber (Matsunari et al., 2012; Uchikura et al., 2016), Cryoloop (Lane et al., 1999b) and Cryotop (Kuwayama and Kato, 2000; Ito et al., 2010; Inaba et al., 2011). Increasing cooling rate by decreasing the vitrified solution volume allows a moderate concentration of CPA resulting in reduced toxicity and osmotic hazardous effects (Yavin et al., 2009). However, vitrification using Cryotop method has been reported to be efficient for cryopreservation of bovine oocytes and embryos because of the small size of vitrification solution (less than 0.1µl), high cooling rate (22,800°C/min) and warming rate (42,100°C/min) (Kuwayama et al., 2005; De Rosa et al., 2007).

Many successful of oocytes and embryos vitrification have been reported in immature oocytes (Spicigo et al., 2012; Liang et al., 2012; Sripunya et al., 2014), matured oocytes (Sripunya et al., 2010; Horvath and Seidel, 2006; Phongnimitr et al.,

2013), zygote (Libermann et al., 2002; Hochi et al., 2004; Al-Hasani et al., 2007), early stages embryos (Campos-Chillon et al., 2009; Fathi et al., 2012), and blastocyst (Park et al., 1999; Inaba et al., 2011; Kuwayama et al., 1992; Huang et al., 2007) but no data reported in bovine separated blastomere. The differences between single cell such as oocyte or zygote and multiple cell development were discussed by Maneiro et al. (1991). They found that surface/volume ratio was increased in each division during embryo development due to uptake of nutrients necessary for growing. On the other hand, the lager embryo (low surface and high volume) might be expected to be more sensitive to freezing and thawing due to high lipid component in cytoplasm (Schmidt et al., 1987).

A successful of separated blastomere vitrification has been established in mouse (Kang et al., 1994; Kader et al., 2009) and human (Chung et al., 2008; Escriba et al., 2009). In mouse, Kang et al. found that the blastocyst rate of vitrified-thawed blastomere from 2-cell stage did not different from non-vitrified 2-cell embryo. Moreover, they also found that the rate of blastocyst was highest in 3M DMSO solution when compared with 1.5M and 4.5M DMSO. Form their experiment; they got four live singletons from after transfer into mouse recipients. Kader et al. (2009) also reported that non-apoptotic cell was found after vitrified single blastomere derived form 8-cell stages mouse embryo using a close loading device, although they did not show further development. Furthermore, Kobayashi et al. (1990) and Nagashima et al. (1991) reported that in mouse and rabbit blastomeres could be cryopreserved by the vitrification method without any protective treatment such as embedding in gel materials, suggesting that the vitrification method is suitable for freezing of the half embryos without a zona pellucida because of the method's

characteristic of avoiding ice crystal formation. In bovine, very few researches have been conducted on methods to cyopreserve bovine separated blastomere embryo. One reason is that blastocysts can be commonly produced by reliably for non-surgical transfer after *in-vitro* fertilization (IVF) and culture *in vitro*. Nowadays, we can increase the number of OPU-derived embryo by using blastomere separation technique, which simpler and efficiency, from early stages of embryo such as 2-cell and 8-cell stages. Cryopreservation of separated blastomere embryos would provide a valuable alternative in order to increase the number of OPU-derived embryo, allowing to study the developmental competence of separated embryo vitrified-thawed following cultured *in vitro*.

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# CHAPTER III

# EFFECT OF GEL CHIP ON DEVELOPMENTAL COMPETENCE OF SEPARATED BOVINE BLASTOMERE

## 3.1 Abstract

The objective of the present study was to examine the effect of gel material compared with WOW system on the developmental competence rates and quality of resultant blastocyst in bovine separated blastomere. In experiment 1, the intact zygotes were chosen to optimize the gel embedded method. The development rate was not difference among 1% agarose, 1% calcium alginate gel embedded and control; however, the 1% calcium alginate group was selected for the next experiment due to blastocyst recovery. In experiment 2, the blastocyst rate of separated embryo was higher than intact embryo group. Among separated blastomere (2- and 8-cell), these 2 culture systems (WOW and 1% calcium alginate) did not difference in blastocyst formation rate except in 2-cell alginate group; besides, the 2-cell WOW group showed the highest rate of blastocyst formation. In experiment 3, comparing WOW system, the rate of OPU derived-blastocyst from separated 2-cell embryo was significantly higher than intact 2-cell group; on the other hand, the total cell number in intact 2-cell was higher than separated 2-cell embryo. In conclusion, gel embedded can be used instead of WOW in bovine separated blastomere to provide the

blastocyst. However, the developmental competence of embedded-separated embryos was slightly lower than WOW-separated embryos.

## 3.2 Introduction

The combination between *in vitro* production (IVP) and ultrasound guided ovum pick-up (OPU) are effective techniques for producing more progeny than multiple ovulation and embryo transfer (MOET) in cattle (Pontes *et al*. 2009). In 2014, more than 1,244,666 bovine embryos have been produced by *in vivo*-derived and *in vitro* procedure, approximately 630,202 embryos were *in vitro* fertilized (IVF) embryos and increased dramatically in the future (Perry 2015). Commercial production of bovine embryos are produced from oocytes collected by OPU and slaughterhouse ovaries. OPU/IVF is provide more efficient way to produce embryos because it could be collected the oocytes from economic donor cattle with repeated collection from the same donor cows (Boni 2012). Moreover, this technique can be applied in farm animals with high genetic value. A combination with sexing semen and OPU-*in vitro* fertilization have enabled production a large number of high genetic merit, replacement heifers and herd expansion (Maxwell *et al*. 2004; Wheeler *et al*. 2006; Presicce *et al*. 2011). However, oocytes retrieval by OPU was 5 to 25 and less than 8 in Japanese black and Holstein cattle, respectively (Merton *et al*. 2009; Sugimura *et al*. 2013). Several studies reported that the embryos which were cultured individually or in small group, provided low blastocyst yield and quality of resultant blastocysts when compared with large group culture (Donnay *et al*. 1997; Gopichandran & Leese 2006; Senatore *et al*. 2010). Usually the basic group culture embryo is 20 to 25 bovine embryos in 100 µl of culture medium (Imai *et al*. 2006) or 40 to 50 embryos in 400 µl

of culture medium (Vajta *et al*. 1997a). These culture systems showed favorable results on blastocyst formation rate of nearly 50% (Vajta *et al*. 1997a; Holm *et al*. 1999). In previous studies showed that embedding embryos with gel materials such as calcium alginate gel (Kobayashi *et al*. 2006) and low melting point agarose gel (Senatore *et al*. 2010; Deb *et al*. 2011) could improve the developmental competence and quality of small number embryos and embedded embryos could develop to blastocyst stage.

Early embryonic cells from zygote to morula stage of the embryos are classified as totipotent stem cell (Schramm *et al*. 2004), which have the potential to differentiate into any cell type in animal body and to generate new life of animal. In bovine, separated embryos have been established to produce twins (Tagawa *et al*. 2008), triplets (Willadsen *et al*. 1981) and quadruplet monozygotic calves (Johnson *et al*. 1995). The approach including micromanipulation technique (Willadsen *et al*. 1981; Katayama *et al*. 2010) and exposing the embryo with proteolytic enzyme (Tagawa *et al*. 2008; Zhao *et al*. 2015) have been used for removing zona pellucida in blastomere separation; however, removing zona pellucida by micromanipulation technique has many complication such as high cost of specialized equipment and technical skill of operator. Thus, separating embryo using proteolytic enzyme is preferable to used more than micromanipulator technique. Tagawa *et al.* (2008) found that when culture the separated blastomere in 60 wells plate, the separated blastomere could not aggregated properly and grown like a bead structure and this problem may be due to zona pellucida removal. Vajta *et al.* (2000) developed a Well of Well (WOW) culture system which showed that in a microenvironment, culture medium surrounding the embryo is very low (approximately 0.1 µl); hence the autocrine and paracrine factors

can be established and supported more constant and suitable microenvironment for embryos than in original well and the 20 µl drop (Vajta *et al.* 2000). Several reports suggested separated blastomere should be culture in separated and small culture system such as WOW to solve this problem (Vajta *et al.* 2000, Tagawa *et al*. 2008, Tang *et al*. 2012). We hypothesized that embedded separated blastomere with gel material could be solve this problem as artificial zona pellucida.

To the best of our knowledge, there are no previous reports of separated blastomere embedded in gel material. The objective of present study was to investigate the developmental rates and quality of bovine separated embryos which are produced by blastomere separation and gel embedded technique. The possibility of separating the blastomeres from OPU-derived embryos in term of increasing the number of blastocyst for embryo transfer program should be investigated.

## 3.3 Materials and Methods

### 3.3.1 Animal management

Non-lactating Holstein cows were used (n=3; parity = 5.0±1.0; BCS at start of experiment = 2.4±0.1). All cows were housed in loose barn and fed basal on the Japanese Feeding Standard for Dairy Cattle (National Agricultural Research Organization 2006). The BCS was assessed on a 5-point scale (with 1 being extremely thin and 5 being extremely obese) with 0.25 increments (Ferguson *et al.* 1994).

### 3.3.2 Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were transported from slaughterhouse in 0.9% Sodium Chloride solution. Cumulus oocyte complexes (COCs) were aspirated from 2-6 mm follicle by using 5 ml syringe connected with 19-G needle. Both of slaughterhouse and OPU-derived COCs were washed 3 times in Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco-BRL, Grand Island , NY, USA) supplemented with 3% calf serum (CS, Gibco-BRL). Then, COCs were washed twice in IVM medium which consisted of TCM 199 (Gibco-BRL) supplemented with 5% CS and 0.02 armor units/ml follicular stimulating hormone (FSH, ANTORIN-R 10; Kyoritsu Seiyaku, Tokyo, Japan). A group of 20 COCs were cultured in 100 µl droplets of IVM medium covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) in 35-mm plastic dish at 38.5ºC under humidified atmosphere of 5%$CO_2$ in air for 20 h.\

### 3.3.3 *In vitro* fertilization (IVF) and *in vitro* culture (IVC)

Sperm preparation for IVF was previously described by Imai *et al*. (2006). Briefly, frozen semen was thawed at 37ºC, placed into a top layer of 4 ml 45-90% Percoll (GE Healthcare, Uppsala, Sweden) gradient solution and centrifuged at 670 × g for 20 min. The sperm pellets were re-suspended with 6 ml BO medium (Brackett and Oliphnat, 1975) solution supplemented with 10 mM Hypotaurine (Sigma Chemical, St. Louis, MO, USA) and 4 µl/ml Heparin (Novo-Heparin injection 1000; Aventis Pharma Ltd, Tokyo, Japan) then sediment using centrifugation at 543 × g for 5 min. The pellet was re-suspended in IVF medium which consisted of BO medium supplemented with 5 mM Hypotaurine, 4 U/ml Heparin and 10 mg/ml Bovine serum albumin (BSA) and adjusted the final concentration to $3\times10^6$ spermatozoa/ml. The

fertilization drop was prepared by making 100 µl droplets of sperm suspension in 35-mm plastic dish and covered with paraffin oil. Then, matured COCs were washed three times in IVF medium. A group of 20 COCs was placed into fertilization drop and cultured under a humidified atmosphere of 5% $CO_2$ in air at 38.5$^oC$ for 6 h. After IVF, the presumptive zygotes were denuded by gentle pipetting using fine glass pipette, and washed three times in CR1aa medium supplemented with 5% calf serum (Rosenkrans *et al*. 1993; Imai *et al*. 2002). A group of 20 presumptive zygotes was cultured in 100 µl droplets of CR1aa medium covered with paraffin oil under a humidified atmosphere of 5%$CO_2$, 5%$O_2$ and 90%$N_2$ at 38.5$^oC$ for 7-8 d.

### 3.3.4 Embedded bovine embryos

#### 3.3.4.1 1% Agarose gel solution

1% agarose gel was prepared according to Senatore *et al.* (2010) with some modifications. In brief, low gelling and melting point agarose powder (Takara: 5003) was prepared in phosphate buffer saline (PBS), sterilized by autoclaving and stored in room temperature (25-28$^oC$) as a solidified gel. For embedded embryo, solidified agarose gel was melted by heating to 600 watt microwave oven for 1 min 30 sec and waited until gel temperature was cooled down to 39$^oC$. A group of 5 presumptive zygotes were transferred to agarose solution by fine glass Pasture pipette, embedded as a sausage-like structure. Embryo gel chips were transferred to CR1aa medium and cultured in a humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5$^oC$ under paraffin oil for 7-8 days.

#### 3.3.4.2 1% Calcium alginate gel solution

The alginate encapsulation was performed as previous described by Kobayashi *et al*. (2006). First, the 1% sodium alginate solution was generated by

dissolving 1 g of alginate powder (Gibco-BRL) into 100 ml 0.9% NaCl solution and 0.1% $CaCl_2$ was prepared by dissolving 0.1g calcium chloride dihydrate (Wako Pure Chemical industries, Ltd., Japan) into 100 ml Lactate Ringer's solution as a crosslink solution used for gel formation. Embryo encapsulation was generated by putting 1% sodium alginate solution containing a group of 5 presumptive zygotes into 0.1% $CaCl_2$ solution. Alginate encapsulated embryos were cultured in CR1aa medium supplemented with 5% calf serum in humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5$^{o}$C under paraffin oil for 7-8 days.

### 3.3.5 Blastomere separation

Blastomere separation was previous described by Nagai *et al*. (2013). Briefly, the two-cell stage (31 hpi; hours post insemination) or eight-cell stage (51 hpi) of embryos were immersed into 0.25% protease solution (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan) for 2-3 min to removed zona pellucida, then gently separated into 2 single blastomeres (two-cell stage) or 2 quadruple blastomeres (eight-cell stage) by pipetting with a tapered Pasteur pipette at room temperature (25-28$^{o}$C). A pair of single blastomere (from 2-cell stage) or quadruple blastomere (from 8-cell stage) was cultured into Handmade WOW which produced by punching pin as a control group or embedding in gel material as a treatment group in CR1aa medium supplemented with 5% calf serum in humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5$^{o}$C under paraffin oil for 6 and 5 d, respectively.

### 3.3.6 Assessment of blastocyst quality

The quality of blastocyst was evaluated using the method previously described by Thouas *et al.* (2001). Briefly, the expanded blastocysts were washed

with 0.1 mg/ml Propidium Iodide (PI) and 0.2% (v/v) Triton X-100 dissolved in DPBS for 1 min. Then, stained with 25 µg/ml bisbenzimide (Hoechst 33342) dissolved in 99.5% ethanol for 5 min. The blastocysts were washed in glycerol, mounted on glass slide and flattened by cover slips to evaluate their quality. The numbers of trophectoderm (TE) and inner cell mass (ICM) were evaluated under UV light provided by a high-pressure mercury burner (Olympus, Tokyo, Japan) for fluorescence microscopy. The ICM and TE expressed in blue and red color, respectively.

### 3.3.7 Experimental design

*Experiment 1* investigated the effect of gel chip (1% agarose gel, 1% calcium alginate gel) on the developmental competence of intact embryos After insemination, the 5 presumptive zygotes were embedded using 1% agarose solution or 1% calcium alginate solution as a gel chip and cultured 4 gel chips in 100 µl drop of CR1aa supplemented with 5% CS in humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5$^{o}$C under paraffin oil for 7-8 days. A group of 20 presumptive zygotes were cultured in 100 µl in same condition and classified as a control. The rates of cleavage and blastocyst formation, total cell number and the rate of ICM were also recorded.

*Experiment 2* investigated the effect of gel chip on the developmental competence of separated blastomeres. After co-incubation, single blastomere (from two-cell stage; 31 hpi) or quadruple blastomere (from eight-cell stage; 51 hpi) were produced by blastomere separation method and divided into four groups which cultured in 2 conditions: embedded in 1% calcium alginate gel and WOW culture system. In each culture condition, they divided into single blastomere (from 2-cell)

and quadruple blastomere (from 8-cell). A pair of single blastomere (from two-cell) or quadruple blastomere (from eight-cell) were cultured in 50 µl droplet (5 embryos/drop) of CR1aa supplemented with 5% CS in humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5ºC under paraffin oil for 6 and 5 days, respectively. A group of 5 intact 2-cell and 8-cell stages of embryo were cultured in 50 µl droplet in WOW disc and classified as a control. The rates of blastocyst formation, total cell number and the rate of ICM were evaluated.

*Experiment 3* investigated the effect of culture type on the developmental competence of 2-cell OPU-derived embryos. The COCs were collected from non-stimulated Holstein cows using an ultrasound scanner (HS-2000, Honda Electronics Co. Ltd, Toyohashi, Japan) connected with 7.5 MHz convex vaginal transducer. The 2-6 mm follicles were aspirated with the aspiration needle (COVA needle; Misawa Medical, Tokyo, Japan). After 31 hpi, 2-cell embryos were divided into 2 groups; separated blastomere from 2-cell and intact 2-cell embryos. The zona pellucida of separated blastomeres from 2-cell embryos were removed by immersing into 0.25% protease (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan) for 2-3 min then gently separated into 2 single blastomeres by pipetting with a tapered Pasteur pipette at room temperature (25-28ºC). Then, separated blastomeres were transferred to Handmade-WOW and cultured in 50 µl droplet in CR1aa supplemented with 5% CS (5 embryos/drop) in humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5ºC under paraffin oil for 6 days. Intact 2-cell embryos were cultured in same condition classified as a control. The rates of blastocyst formation rate, total cell number and the rate of ICM were determined.

### 3.3.8 Statistical analysis

The rates of cleavage and blastocyst formation were showed in percentage. The total cell number was described in mean ± SEM and analyzed using one-way ANOVA. All statistical analyses were performed by using software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The Differences with P-value < 0.05 was defined as the significant level.

## 3.4 Results

### 3.4.1 Effect of gel chip (1% agarose gel, 1% calcium alginate gel) on the developmental competence of intact embryos

After insemination, presumptive zygotes were randomly allocated into control or embedded gel group (1% agarose gel and 1% calcium alginate gel). The rates of cleavage and blastocyst formation are shown in Table 3.1, which did not significantly difference among 1% agarose embedded gel, 1% calcium alginate embedded gel and control groups. Moreover, the total cell numbers also did not significantly difference between embedded and control groups (Table 3.2). Although the developmental competence and quality of resultant blastocyst were not different between gel embedded groups, approximately 30% of blastocysts derived from 1% agarose embedded were broken after removed from gel chip. Therefore, 1% calcium alginate gel was selected for following experiments.

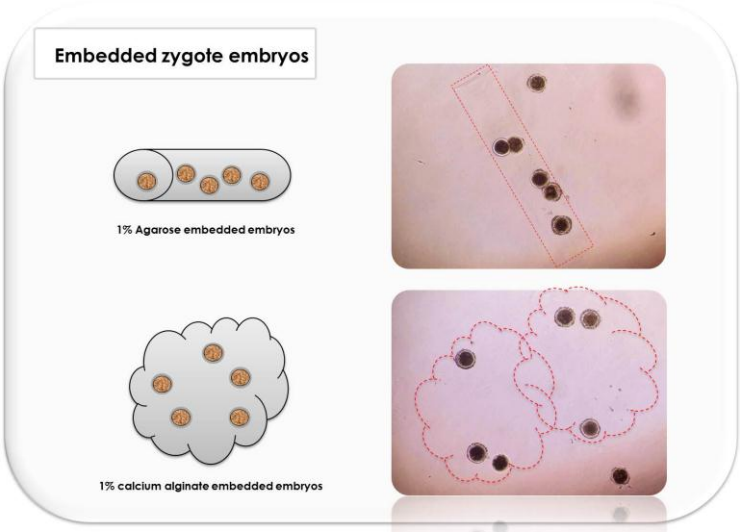


**Figure 3.1** Embeded zygote embryos with 1% low melting agarose gel and 1% calcium alginate gel.

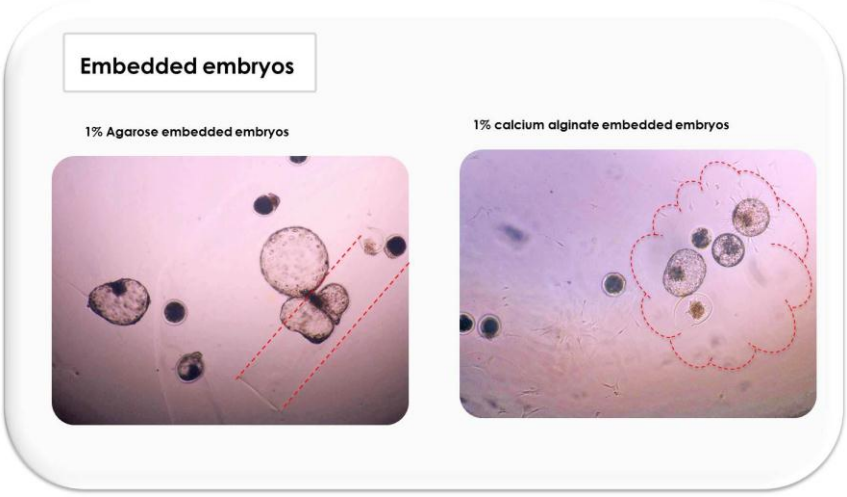


**Figure 3.2** Gel embedded derived-blastocysts on day 7-8.

### 3.4.2 Effect of gel chip on the developmental competence of separated blastomeres

After fertilization, intact 2 and 8-cell stages and separated 2 and 8-cell stages of embryos were designated to 1% calcium alginate embedded or individually cultured in Handmade-WOW disc. The rate of blastocyst formation (Table 3.3) of

intact 8-cell embryo cultured in WOW was significantly higher than intact 2-cell embryo embedded in 1% calcium alginate but did not different with the other group. In separation group, the quality of blastocysts from the 2-cell groups cultured in WOW was higher than 2-cell cultured in 1% calcium alginate but did not different from the other groups (Table 3.4, Figure 3.3). The total cell number of blastocyst derived from intact 8-cell embryo was higher than blastocyst derived from intact 2-cell embryo. The potential of blastocyst production rate from separated embryo was significantly higher than intact embryo group. The total cell number of blastocyst derived from intact embryo was higher than blastocyst derived from separated blastomere groups (Table 3.5, Figure 3.4-3.5). In separated blastomere embryo, the blastocyst formation rate of single blastomere (from 2-cell) embedded in 1% calcium alginate group was significantly lower than the other group but the group of single blastomere (from 2-cell) cultured in WOW showed the highest the percentage of blastocyst.


**Figure 3.3** Morphological quality of separated-derived blastocyst on day 7-8.

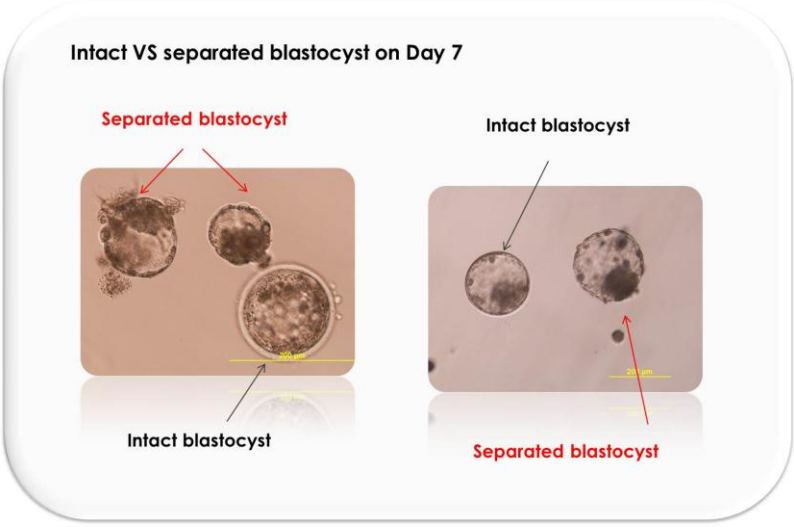


**Figure 3.4** Comparison between separated and intact-derived blastocyst on day 7-8.

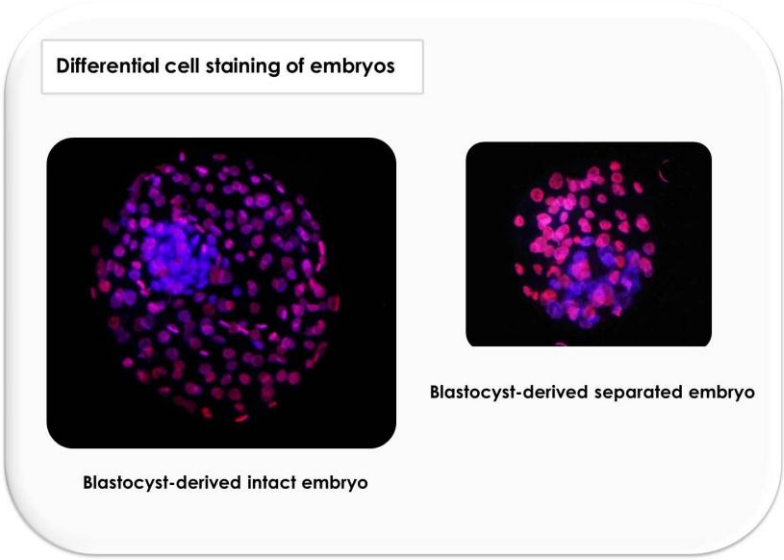


**Figure 3.5** Comparison between separated intact-derived blastocyst by using differential cell staining technique.

### 3.4.3 Effect of culture type on the developmental competence of 2-cell OPU-derived embryos.

A total 172 OPU-derived oocytes were collected from 3 non-stimulated Holstein cows and 145 oocytes were fertilized with frozen-thawed semen. At 31 hpi, a 46 from 91 two-cell embryos were separated and cultured in handmade-WOW system and 45 intact two-cell were cultured in same condition as a control. The potential of blastocyst formation from of separated 2-cell embryo was significantly higher than intact 2-cell group (Table 3.6). the numbers of cell in blastocyst derived from intact 2-cell was significantly higher than blastocysts derived from separated 2-cell embryo (Table 3.7).

**Table 3.1** Developmental competence of gel embedded IVF-derived intact bovine zygotes after culture *in vitro*.

| Group | No. oocytes inseminated | No. (% Mean ± SEM) cleaved embryos | No. (% Mean ± SEM) blastocyst on Day 7-8 |
|---|---|---|---|
| **Control** | 100 | 86 (86.0 ± 5.6) | 48 (48.0 ± 3.7) |
| **1% Agarose gel** | 100 | 72 (72.0 ± 5.8) | 44 (44.0 ± 5.8) |
| **1% Calcium alginate gel** | 100 | 85 (85.0 ± 1.6) | 49 (49.0 ± 5.8) |

Data in parentheses are presented as Mean ± SEM. No significant difference was detected among treatment groups at $P<0.05$ using one-way ANOVA.

**Table 3.2** Cell numbers in expanded blastocysts of IVF-derived intact bovine blastocysts after culture *in vitro*

| Group | No. blastocyst examined | No. nuclei ± SEM | | |
|---|---|---|---|---|
| | | ICM | TE | Total cell |
| **Control** | 48 | 35.7 ± 5.0 | 87.8 ± 4.9 | 123.5 ± 3.2 |
| **1% Agarose gel** | 44 | 38.2 ± 3.9 | 115.5 ± 15.3 | 153.7 ± 13.9 |
| **1% Calcium alginate gel** | 49 | 35.8 ± 4.9 | 92.6 ± 11.4 | 128.5 ± 15.2 |

Data in parentheses are presented as Mean ± SEM. No significant difference was detected among treatment groups at $P<0.05$ using one-way ANOVA.

**Table 3.3** Developmental competence of intact and separated 2-cell and 8-cell embryos when culturing in WOW and 1% calcium alginate embedded culture method.

| Stage of embryo | Type of embryo | Culture type | No. of embryos cultured | No. (% Mean ± SEM) blastocyst on Day 7-8 |
|---|---|---|---|---|
| 2-Cell | Intact embryo | WOW | 50 | 28(56.0 ± 2.4)[ab] |
| | | 1%alginate | 50 | 23 (46.0 ± 2.4)[a] |
| | 2 single blastomere | WOW | 50 | 61 (125.6 ± 4.2)[d] |
| | | 1%alginate | 50 | 41 (88.3 ± 9.6)[c] |
| 8-Cell | Intact embryo | WOW | 50 | 34 (68.0 ± 2.0)[b] |
| | | 1%alginate | 50 | 30 (60.0 ± 0.0)[ab] |
| | 2 quadruple blastomere | WOW | 50 | 56 (115.0 ± 10.6)[d] |
| | | 1%alginate | 50 | 55 (111.7 ± 5.6)[d] |

[a,b] Different superscripts in the same column indicate significant difference at $P<0.05$ using one-way ANOVA. Data in parentheses are presented as Mean ± SEM.

**Table 3.4** Quality of blastocysts developed from IVF-derived separated blastomere bovine embryos at 2-and 8-cell stages after cultured *in vitro*.

| Embryo satge | Groups | No. of blastocysts examined | Quality of blastocysts | | |
|---|---|---|---|---|---|
| | | | NO. (%) Grade 1 | NO. (%) Grade 2 | NO. (%) Grade 3 |
| 2-cell | WOW | 61 | 25 (49.2 ± 10.6)[a] | 25 (32.7 ± 8.8) | 11 (18.1 ± 6.5) |
| | 1% alginate | 41 | 7 (11.0 ± 7.8) [b] | 24 (50.1 ± 12.2) | 10 (38.9 ± 16.2) |
| 8-cell | WOW | 55 | 17 (36.0 ± 8.8) [ab] | 28 (48.6 ± 5.7) | 10 (15.4 ± 7.1) |
| | 1% alginate | 56 | 13 (23.8 ± 3.2) [ab] | 24 (43.4 ± 6.0) | 19 (32.8 ± 8.7) |

Data in parentheses are presented as mean±SEM. No significant difference was detected among treatment groups at P<0.05 using one-way ANOVA.

**Table 3.5** Cell numbers in expanded blastocysts of IVF-derived intact and separated bovine blastocysts after culture in WOW and 1%calcium alginate.

| Stage of embryo | Type of embryo | Culture type | No. blastocyst examined | No. nuclei ± SEM | | |
|---|---|---|---|---|---|---|
| | | | | ICM | TE | Total cell |
| 2-Cell | Intact embryo | WOW | 28 | 22.1 ± 1.6[b] | 80.1 ± 2.2[c] | 102.2 ± 1.3[c] |
| | | 1%alginate | 23 | 29.3 ± 2.0[c] | 106.7 ± 1.7[d] | 136.0 ± 1.0[d] |
| | 2 single blastomere | WOW | 34 | 16.0 ± 0.4[a] | 53.7 ± 0.9[b] | 69.7 ± 0.6[b] |
| | | 1%alginate | 13 | 17.7 ± 1.3[a] | 49.4 ± 2.0[ab] | 67.1 ± 1.0[b] |
| 8-Cell | Intact embryo | WOW | 34 | 33.0 ± 1.0[d] | 115.2 ± 1.2[e] | 148.2 ± 1.1[e] |
| | | 1%alginate | 30 | 35.5 ± 1.4[d] | 123.2 ± 2.5[f] | 158.7 ± 1.7[f] |
| | 2 quadruple blastomere | WOW | 27 | 16.0 ± 0.4[a] | 51.0 ± 1.3[b] | 67.1 ± 1.6[b] |
| | | 1%alginate | 20 | 15.5 ± 0.5[a] | 45.5 ± 0.5[a] | 61.0 ± 0.5[a] |

[a,b] Different superscripts in the same column indicate significant difference at $P<0.05$ using one-way ANOVA. Data in parentheses are presented as Mean ± SEM.

**Table 3.6** Developmental competence of OPU-derived 2-cell intact and separated bovine embryos after culture in WOW.

| Stage of embryo | Culture type | No. of embryos cultured | No. (% Mean ± SEM) blastocyst on Day 7-8 |
|---|---|---|---|
| 2-cell | Intact | 45 | 12 (24.4 ± 5.1)[a] |
| | 2 single blastomere | 46 | 38 (81.8 ± 16.5)[b] |

[a,b] Different superscripts in the same column indicate significant difference at P<0.05 using one-way ANOVA. Data in parentheses are presented as Mean ± SEM.

**Table 3.7** Cell numbers in expanded blastocysts of OPU-derived 2-cell intact and separated bovine embryos after culture in WOW.

| Stage of embryo | Culture method | No. blastocyst examined | No. nuclei ± SEM | | |
|---|---|---|---|---|---|
| | | | ICM | TE | Total cell |
| 2-Cell | Intact | 12 | 31.5 ± 2.0[a] | 88.5 ± 3.7[a] | 120.0 ± 4.9[a] |
| | 2 single blastomere | 36 | 14.5 ± 0.4[b] | 45.1 ± 0.5[b] | 59.6 ± 0.8[b] |

[a,b] Different superscripts in the same column indicate significant difference at $P<0.05$ using one-way ANOVA. Data in parentheses are presented as Mean ± SEM.

## 3.5 Discussion

Based on this study, 1% calcium alginate gel can support cell growth during blastomere aggregated process of separated bovine embryo form 2-cell and 8-cell stages. Traditionally, embryos had been cultured with in optimal embryo/volume ratio in order to maximize the rate of embryo development by maintaining appropriate autocrine and paracrine signaling (Gopichandran & Leese, 2006). In case of small number of embryos, the most commonly used culture method is to decrease the embryo/volume ratio of medium droplet. However, culturing embryos in small droplets may lead to technical problem and rapidly microenvironmental changes (Dai *et al*. 2012). Recently, Senatore *et al.* (2010) developed co-culture system which maintained the suitably embryo/volume ratio for small number of embryo by using gel embedded embryos. In the present study, the zygote embryos were embedded in 1% agarose and 1% calcium alginate gel. These embryos could develop to expanded blastocyst stage and develop to hatching stage of blastocyst by escaping from gel chip. There was no difference in developmental rate and total cell number between embedded embryo and non-embedded embryo. These results are in agreement with Kobayashi *et al*. (2006) who reported the encapsulated with 1% calcium alginate gel was not toxic to embryo development and Senatore *et al*. (2010) who reported that the developmental competence of embryos embedded in agarose chips was similar to normal intact embryo. In another aspect of the gel embedded embryos was helpful to separate individually embryo from each other which could be used as a gel embedded helper embryo co-culture systems in cultures of limited numbers of OPU/IVF oocytes (Senatore *et al*. 2010; Deb *et al*. 2011).

Together with these published reports that gel embedded embryos enhanced the developmental competence and embryo quality (Deb *et al*. 2011). There were no reports of further success in gel embedded embryos in bovine blastomere. As we known, the early stages of embryo are totipotent. Stem cells resembling totipotent blastomeres from early stages (zygote to morula) could develop to blastocyst stage (Tagawa *et al*. 2008; Nagai *et al*. 2013). Historically, blastomere separation for the purpose of producing multiple offspring in cattle (Jonhson *et al*. 1995), sheep (Willadsen *et al*. 1980), goats (Tsunoda *et al*. 1984), pigs (Reichelt *et al*. 1994), horses (Allen *et al*. 1984), mouse (Illmensee *et al*. 2006), monkeys (Mitalipov *et al*. 2002) and humans (Hall *et al*. 1993) have been published. Moreover, blastomere separation method provides the opportunities to study epigenetic reprogramming pattern (Chavez *et al*. 2014; Wu *et al*. 2014) and to generate embryonic stem cell (ES cell) lines used in preimplantation genetic diagnosis (PGD) (Klimanskaya *et al*. 2006; Geens *et al*. 2009). Blastomere separation method can be achieved by two different approaches; micromanipulation technique (Willadsen *et al*. 1981; Katayama *et al*. 2010) and exposing the embryo with proteolytic enzyme (Tagawa *et al*. 2008; Zhao *et al*. 2015). However, the protease enzyme was preferable for removing zona pellucida because it is simple and efficient procedure. In present study, the blastocysts-derived from blastomere separated embryos (from 2-cell and 8-cell stages) showed higher the proportion of blastocyst compared with intact embryos which was coincided with previous reports in mouse (Tang *et al*. 2012), monkey (Mitalipov *et al*. 2002) and cattle species (Tagawa *et al*. 2008; Zhao *et al*. 2015). We found that the percentage of blastocyst were not significantly different among 2-cell and 8-cell separated blastomere in both culture systems except the 2-cell blastomere separation in 1%

calcium alginate. Our result is in agreement with Tagawa *et al.* (2008) who reported the blastocyst rate was similar between 2-cell and 8-cell stages separated embryo in WOW culture system. Many studies indicated that even though blastomere separation could be used to multiply animal embryos, the total cell number of resultant blastocyst was decreased to approximately half of normal embryos (Tang *et al*. 2012; Nagai *et al*. 2013) which was in line with our results. Moreover, the total cell number of blastocyst derived from 1% calcium alginate embedded 8-cell stage was lower than other groups. The plausible cause was explained by a quadruple blastomere separated from 8-cell was disaggregated from each other when transferred to cross-link solution ($CaCl_2$; this phenomenon was not observed in 2-cell group). We suggested that the 1% calcium alginate was hereby authorized to be appropriated for the purposes of gel embedded blastomere separation and used an alternative to WOW culture system.

As mentioned previously for oocyte retrieval in OPU, factors such as donor and hormonal treatment influenced the total number of oocytes (Presicce *et al.* 2011). Many studies reported that the embryo producing by OPU/IVP method in Holstein cattle (*Bos taurus*) was lower than Nelore cattle because of small number of viable follicle for OPU (Segerson *et al*. 1984) and negative energy balance status (Esposito *et al*. 2014; O'Doherty *et al.* 2014). Due to the limited number of COCs retrieved per cattle in OPU, they resolved the problem by using WOW to provide suitable microenvironment for small group culture and the benefit of this culture system is likely provided autocrine and paracrine factors important for developmental competence of embryos (Vajta *et al.* 2000). Our results revealed that oocytes retrieval from different 3 donor cattle provided different number of oocytes (data not shown). This finding corroborated those by Su *et al*. (2009) who informed the donor has an

effect on number of oocytes collected by OPU. However, the rates of cleavage, 2-cell and blastocyst formation did not difference between 3 OPU donors (data not shown). In addition, the rate of OPU-derived blastocyst from separated 2-cell embryo was higher when compared with intact 2-cell embryo. Interestingly, the rate of inner cell mass did not difference between intact 2-cell and blastomere 2-cell groups; even though, the total cell number of separated 2-cell embryo was approximately two times lower than intact 2-cell embryos. This result is in concordance with previous reports by Tagawa *et al*. (2008), Nagai *et al*. (2013) and Zhao *et al*. (2015) who demonstrated that the total cell number of blastocyst produced from blastomere separation was only 50% of blastocyst produced from intact embryos. As we known, the total cell number is very important predictor of embryo quality. Many researches have been proved that the embryo derived from blastomere separation could be able to develop into normal offspring after embryo transfer. In spite of low total cell number of resultant blastocyst, Costa *et al*. (2011) found after transferred on day 7, demi-embryo which produced from blastocyst bisection on day 7 presented in a similar size with normal embryo at day 42 of pregnancy. It was also reported the normal offspring produced from blastomere separation by Willadsen *et al*. (1981), Johnson *et al*. (1995), and Tagawa *et al*. (2008). These findings confirmed that blastomere separation blastocysts by their ability to produce offspring did not depend on the total cell number of blastocyst.

## 3.6 Conclusions

In conclusion, both of 1% calcium alginate gel and 1% agarose had no adverse effect on developmental competence of bovine zygote embryo; however, some of expanded blastocysts were broken after blastocyst recovery from 1% agarose gel. Our

study further demonstrates that 1% calcium alginate gel could support cell dividing during blastomere aggregated process of separated bovine embryos. For gel embedding, the manipulation of single blastomere is easier than quadruple blastomere. However, the developmental competence of embedded-separated embryos was slightly lower than WOW-separated embryos.

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# CHAPTER IV

# EFFECT OF VITRIFICATION ON SURVIVAL AND DEVELOPMENTAL COMPETENCE OF SEPARATED BOVINE BLASTOMERE

## 4.1 Abstract

Generating techniques to enhance the success of blastomere separation is important for bovine economy due to increases the yield of transferable embryos. This study aimed to compare the survival and *in vitro* developmental ability of intact and separated embryos at the 2- cell and 8-cell stages after Cryotop vitrification. In experiment 1, expanded blastocysts were subjected to 2 different vitrification solutions: 1) ethylene glycol (EG) + propylene glycol (PG) and 2) EG groups. The survival rate of blastocysts in the EG + PG group was higher than the EG group. In experiment 2, intact 2-cell and 8-cell stage embryos were subjected to vitrification solutions as same as expanded blastocyst. We found that the EG + PG group produced more dead embryos than the EG groups ($P < 0.05$). In EG group, the blastocyst formation rate was not difference among vitrified 2-cell, vitrified 8-cell and non-vitrified 2-cell groups. In experiment 3, there was no difference in the rate of blastocyst formation and total number of cells between 2 vitrified blastomere groups. In summary, this study confirmed that EG-based vitrification solution could be used

for the vitrification of intact and separated blastomere 2- cell and 8-cell stages bovine embryos by Cryotop vitrification.

## 4.2 Introduction

Vitrification is frequently referred to as a novel technology of cryopreservation in embryology that reduces the time commitment and equipment expense required for conventional slow freezing. Successful vitrification could be achieved through the combination of the following factors including high concentrations of CPA to avoid ice crystallization, high cooling rates (20,000 °C/min) and minimizing the volume of CPA solution. Various types of vitrification devices have been developed to reduce the volume of CPA, including minimum drop size (Arav 1992; Arav & Zeron 1997; Park *et al.* 1999), Cryotop (Kuwayama & Kato 2000; Ito *et al.* 2010; Inaba *et al.* 2011), Solid surface (Dinnyes *et al.* 2000; Sripunya *et al.* 2010), Open pull straw (Vajta *et al.* 1998; Gayer *et al.* 2008; Campos-Chillon *et al.* 2009), and Hollow fiber (Matsunari *et al.* 2012; Uchikura *et al.* 2016). The Cryotop vitrification method has been reported to be efficient for the cryopreservation of bovine oocytes and embryos because of the small size of the vitrification drop (less than 0.1 µl), high cooling rate (22,800 °C/min), and high warming rate (42,100 °C/min) (Kuwayama *et al.* 2005; ; De Rosa *et al.* 2007).

Successful vitrification has been reported in many studies including immature oocytes (Spicigo *et al.* 2012; Liang *et al.* 2012; Sripunya *et al.* 2014), matured oocytes (Horvath & Seidel 2006; Sripunya *et al.* 2010; Phongnimitr *et al.* 2013), zygote (Liebermann *et al.* 2002; Hochi *et al.* 2004; Al-Hasani *et al.* 2007), early stages embryos (Campos-Chillon *et al.* 2009; Fathi *et al.* 2012), and blastocysts (Kuwayama *et al.* 1992; Park *et al.* 1999; Huang *et al.* 2007; Inaba *et al.* 2011). The blastomeres

which were isolated from cleavage stage embryos could be vitrification and further develop to blastocyst stage (Kang *et al.* 1994; Escriba *et al.* 2009; Kader *et al.* 2009); however, they have been no report for bovine separated blastomeres.

During the early embryonic stage, embryonic cells from the zygote to the morula stage are totipotent stem cells (Schramm *et al.* 2004) which have the potential to differentiate into any cell type in the body and to generate new life in animals. They applied this property in blastomere separation in order to increase the number of embryos for *in vitro* fertilization and embryo transfer (IVF-ET) program and also in high genetic merit embryos (Willadsen & Polge 1981; Johnson *et al.* 1995). The methods which used for blastomere separation have 2 techniques including micromanipulation technique (Willadsen & Polge 1981, Katayama *et al.* 2010) and exposing the embryo with proteolytic enzyme (Tagawa *et al.* 2008); however, the technique for blastomere separation by using protease enzyme is frequently used more than micromanipulation technique due to simple and efficient procedure. Another benefit of blastomere separation was reported by Klimanskaya *et al.* (2006) who applied in regenerative medicine for testing preimplantation genetic diagnosis (PGD) from vitrified isolated blastomere in human (Chung *et al.* 2008; Escriba *et al.* 2009; Kader *et al.* 2009).

To our knowledge, research has not been conducted to verify whether bovine separated embryos could be vitrified by the Cryotop method. In the present study, we investigated the survival and *in vitro* developmental ability of bovine blastomeres separated from the 2-cell and 8-cell stages of embryos after vitrification using the Cryotop method.

## 4.3 Materials and methods

### 4.3.1 Chemicals and media

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise stated.

### 4.3.2 Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were transported from a slaughterhouse in 0.9% Sodium Chloride solution. Cumulus oocyte complexes (COCs) were aspirated from follicles of 2–6 mm in diameter by using a 5 ml syringe connected with a 19-G needle. COCs were washed 3 times in *Dulbecco's phosphate buffered saline* (D-PBS, Gibco-BRL, Grand Island, NY, USA) and supplemented with 3% calf serum (CS, Gibco-BRL). Then, COCs were washed twice in IVM medium, which consisted of TCM-199 (Gibco-BRL) supplemented with 5% calf serum and 0.02 armor units/ml follicular stimulating hormone (FSH, ANTORIN-R 10; Kyoritsu Seiyaku, Tokyo, Japan). A group of 20 COCs was cultured in 100 µl droplets of IVM medium covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) in a 35-mm plastic dish at 38.5 °C under humidified atmosphere of 5% $CO_2$ in air for 20 h.



### 4.3.3 *In vitro* fertilization (IVF) and *in vitro* culture (IVC)

Sperm preparation for IVF was performed as previously described (Imai *et al.* 2006). In brief, frozen semen from a Holstein bull was thawed in a water bath set to 37°C, and then placed into the top layer of 4 ml 45–90% Percoll (GE Healthcare, Uppsala, Sweden) gradient solution and centrifuged at 670 × g for 20 min. The sperm pellets were re-suspended in 6 ml BO medium (Brackett & Oliphant 1975) solution supplemented with 10 mM hypotaurine (Sigma Chemical, St. Louis, MO, USA) and 4

µl/ml heparin (Novo-Heparin injection 1000; Aventis Pharma Ltd, Tokyo, Japan), and were centrifuged at 543 × g for 5 min. The pellet was re-suspended in IVF medium, which consisted of BO medium supplemented with 5 mM hypotaurine, 4 U/ml heparin, and 10 mg/ml bovine serum albumin (BSA), and was adjusted to a final concentration of $3\times10^6$ spermatozoa/ml. The fertilization drop was prepared by making 100 µl droplets of sperm suspension in a 35-mm plastic dish that was covered with paraffin oil.

Matured COCs were then washed 3 times in IVF medium. A group of 20 COCs was placed into the fertilization drop and cultured under a humidified atmosphere of 5% $CO_2$ in air at 38.5 °C for 6 h. After IVF, the presumptive zygotes were denuded by gentle pipetting using a fine glass pipette, and were washed 3 times in CR1aa medium supplemented with 5% calf serum (Rosenkrans *et al.* 1993; Imai *et al.* 2002). A group of 20 presumptive zygotes was cultured in 100 µl droplets of CR1aa medium covered with paraffin oil under a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 °C for 7–8 d.

### 4.3.4 Blastomere separation

Blastomere separation was performed as previously described (Nagai *et al.* 2013). In brief, embryos at the 2-cell stage (31 hpi; hours post insemination) or 8-cell stage (51 hpi) were immersed in 0.25% protease (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan) for 2–3 min to remove zona pellucida (ZP). The embryos were then gently separated into 2 single blastomeres (2-cell stage) or 2 quadruple blastomeres (8-cell stage) using a tapered Pasteur pipette at room temperature (25–28 °C). After separating the blastomere embryos, they were allocated to vitrified and non-vitrified groups in experiment 2.

### 4.3.5 Vitrification of intact embryos

The intact embryos at the 2-cell, 8-cell, and expanded blastocyst stages were vitrified and warmed by 2 different protocols. In the first treatment (EG + PG group), embryos were treated as previously described (Somfai *et al.* 2013), with some modifications. Specifically, the embryos were washed 3 times in base medium (BM) that consisted of 25 mM HEPES-buffered TCM-199 (Gibco-BRL) supplemented with 20% CS. The embryos were then placed into equilibration solution that was composed of BM supplemented with 7.5% ethylene glycol (EG, Wako Japan) and 7.5% propylene glycol (PG, Wako Japan) for 10 min at room temperature (25–28 °C). Then, the embryos were transferred to vitrification solution that was composed of BM supplemented with 20% EG, 20% PG, and 0.5 M trehalose (Hayashibara Biochemical Laboratories Inc., Japan) for 30 sec at room temperature. A group of 4 intact embryos was placed onto the surface of the Cryotop device (Kitazato BioPharma, Shizuoka, Japan) and immediately immersed in liquid nitrogen ($LN_2$). The vitrified embryos in the EG + PG group were warmed by placing the Cryotop into BM supplemented with 0.5 M and 0 M trehalose for 5 min in each step at 38 °C.

In the second treatment (EG group), the vitrification procedure was performed as previously described (El-Danasouri & Selman 2001), with some modification**s.** Embryos were washed in BM, and were then placed in equilibration solution that was composed of BM supplemented with 1.5 M (8.4%) EG solution for 5 min at room temperature. Then, the embryos were transferred to vitrification solution that was composed of BM supplemented with 7 M (39%) EG and 0.6 M trehalose for 30 sec at room temperature. A group of 4 embryos were placed onto the surface of the Cryotop and immediately immersed in $LN_2$. The vitrified embryos in the EG group

were warmed by placing the Cryotop into BM supplemented with 1, 0.5, 0.25, and 0 M trehalose for 3 min in each step at 38 °C.

The vitrified-warmed blastocysts from both groups were washed 3 times in culture medium, which contained TCM-199 supplemented with 20% CS and 0.1 mM beta-mercaptoethanol (Gibco-BRL). Then, a group of 10 blastocysts was cultured in 50 µl droplets of culture medium covered with paraffin oil in a 35-mm plastic dish under a humidified atmosphere of 5% $CO_2$, in air at 38.5 °C for an additional 2 d. Vitrified-warmed 2-cell and 8-cell stage embryos were washed 3 times in culture medium containing CR1aa medium supplemented with 5% CS. A group of embryos was culture in 50 µl droplets of culture medium covered with Paraffin oil in a 35-mm plastic dish under a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 °C for 6 and 5 d, respectively.

### 4.3.6 Blastomere vitrification

Pairs of single blastomeres (from the 2-cell stage) or quadruple blastomeres (from the 8-cell stage) were vitrified and warmed using the second treatment (EG group) described in the previous experiment. The vitrified-warmed single blastomere and quadruple blastomere were washed 3 times in CR1aa medium supplemented with 5% CS, and were cultured in a handmade well of well (WOW, 5 embryos/50 µl) covered with paraffin oil in a 35-mm culture dish under a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 °C for 6 and 5 d, respectively.

### 4.3.7 Assessment of blastomere quality

The quality of the blastocysts was evaluated using a previously described method (Thouas *et al.* 2001). In brief, the hatched blastocysts were treated with 0.1 mg/ml propidium iodide (PI) and 0.2% (v/v) triton x-100 in DPBS for 1 min. Then,

the blastocysts were stained with 25 µg/ml bisbenzimide (Hoechst 33342) dissolved in 99.5% ethanol for 5 min. The blastocysts were washed in glycerol, mounted on glass slide, and flattened by cover slips to evaluate their quality. The number of trophectoderms (TE) and the inner cell mass (ICM) were evaluated under ultra violet (UV) light provided by a high-pressure mercury burner (Olympus, Tokyo, Japan) for fluorescence microscopy. ICM and TE were expressed as blue and red color, respectively.

### 4.3.8 Experimental design

*Experiment 1* investigated the effect of the 2 different vitrification methods on the survival and hatching ability of intact IVF-derived bovine expanded blastocyst stages. After warming, the expanded blastocyst stages were cultured in culture medium in a 35-mm culture dish for an additional 2 d. The non-vitrified embryos were cultured under the same conditions and classified as controls. The survival and hatched rates, ICM, TE, and total cell numbers were examined.

*Experiment 2* investigated the effect of the 2 different vitrification methods on the developmental competence of intact IVF-derived bovine embryos at the 2-cell, 8-cell stages. After warming, the 2-cell and 8-cell stages were cultured in culture medium in a 35-mm culture dish for an additional 6 and 5, respectively. The non-vitrified intact embryos were cultured under the same conditions and served as controls. The blastocyst formation rate, ICM, TE, and total cell numbers were examined.

*Experiment 3* investigated the developmental competence after vitrification and warming blastomeres separated from IVF-derived embryos at the 2- and 8- cell stages of the embryo. After separation, a pair of single blastomeres (from

the 2-cell stage) and quadruple blastomeres (from the 8-cell stage) was subjected to vitrification. After warming, the blastomeres were cultured in microenvironmental condition of handmade-Well of Well dish (WOW) under a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 °C under paraffin oil for 6 and 5 d, respectively. The non-vitrified blastomeres were cultured under the same condition and served as controls. The survival and blastocyst formation rate, ICM, TE, and total cell numbers were examined.

#### 4.3.9 Statistical analysis

Data were showed as mean ± SEM. The experiments were replicated 5 times. The percentage data were subjected to arcsine transformation. The transformed data were analyzed using one-way ANOVA. Between-treatment differences were determined using post-hoc Fisher's protected least significantly difference test. All statistical analyses were performed by using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was set as the significance level.

## 4.4 Results

#### 4.4.1 The effect of two different vitrification methods on the survival and and hatching ability of intact IVF-derived bovine expanded blastocyst stages

Grade 1 and 2 of expanded blastocysts on Day 7-8 were selected for Cryotop vitrification using 2 different equilibration and vitrification solution methods. The survival rate of vitrified-warmed blastocysts in the EG + PG group was significantly higher than that of the EG group but both of these groups were

lower than that of the non-vitrified group (Table 4.1). The total cell number of vitrified-warmed blastocysts in the EG group was also lower than that in the EG + PG and control groups (Table 4.2).

### 4.4.2 The effect of two different vitrification methods on the developmental competence of intact IVF-derived bovine embryos at the 2-cell, 8-cell stages

There was no significant difference in the rate of blastocyst formation between the intact 2-cell and 8-cell stages after vitrified-warmed with EG-based treatment (Table 4.3). However, the rate of blastocyst formation in the fresh 8-cell group was significantly higher than the other groups. The number of cells per blastocyst and the number of trophectoderm cells in the vitrified 2-cell group were significantly lower than those recorded in the other groups (Table 4.4).

### 4.4.3 The effect of vitrification on survival and developmental competence of separated IVF derived bovine embryos at 2-cell, 8-cell stages

The 2-cell and 8-cell stages of the embryo were separated into 2 single blastomeres and 2 quadruple blastomeres. Then, the separated blastomeres were vitrified using the EG-based method. The rate of blastocyst formation from the non-vitrified (single blastomere and quadruple blastomere) groups was significantly higher than those from the vitrified groups (Table 4.5). The quality of blastocysts from the non-vitrified groups was similar to those of the vitrified groups (Table 4.6). The rate of monozygotic paired blastocyst formation of fresh quadruple blastomeres derived from the 8-cell stage was higher than that of the vitrified group, but did not differ to

the other groups. The total number of cells per blastocyst was significantly higher in the fresh groups compared to the vitrified groups (Table 4.7).

**Table 4.1** Survival and hatched rates of vitrified-warmed IVF-derived intact bovine expanded blastocyst after culture *in vitro*.

| Groups | No. of blastocysts cultured | No. (%) survived at 24 h | No. (%) hatched blastocysts at 48 h |
|---|---|---|---|
| **Fresh control** | 50 | 50 (100 ± 0.0 )[a] | 42 (84.1 ± 2.2 ) |
| **EG + PG** | 50 | 43 (88.4 ± 3.7)[b] | 36 (78.0 ± 7.1) |
| **EG** | 50 | 39 (76.9 ± 4.7)[c] | 36 (67.0 ± 13.4) |

Data in parentheses are presented as mean±SEM. [a,b] Different superscripts in the same column indicate significant difference at P<0.05 using one-way ANOVA.

EG+PG: Embryos were vitrified by placing in 7.5%EG+7.5%PG for 10 min and 20%EG+20%PG+ 0.5M trehalose for 30 sec.

EG: Embryos were vitrified by placing in 1.5M (8.4%) EG for 5 min and in 7M (39%) EG + 0.6M trehalose for 30 sec

**Table 4.2** Cell numbers in hatched blastocysts of vitrified IVF-derived intact bovine expanded blastocysts after culture *in vitro*.

| Groups | No. of blastocysts examined | No. of nuclei ±SEM | | |
|---|---|---|---|---|
| | | ICM | TE | Total cells |
| **Fresh control** | 42 | 54.5 ± 3.7[a] | 172.5 ± 3.2[a] | 227.0 ± 4.6[a] |
| **EG + PG** | 36 | 48.7 ± 2.0[ab] | 174.1 ± 10.4[ab] | 219.3 ± 9.7[a] |
| **EG** | 36 | 40.9 ± 3.4[b] | 127.8 ± 6.5[b] | 168.6 ± 8.7[b] |

Data in parentheses are presented as mean±SEM. [a,b] Different superscripts in the same column indicate significant difference at P<0.05 using one-way ANOVA.

EG+PG: Embryos were vitrified by placing in 7.5%EG+7.5%PG for 10 min and 20%EG+20%PG+ 0.5M trehalose for 30 sec.

EG: Embryos were vitrified by placing in 1.5M (8.4%) EG for 5 min and in 7M (39%) EG + 0.6M trehalose for 30 sec

**Table 4.3** Developmental competence of vitrified-warmed IVF-derived intact bovine embryos at 2- and 8-cell stage after culture *in vitro*.

| Stages of embryo | Group | No. of embryo | Blastocyst (%) |
|---|---|---|---|
| **2-cell** | Fresh control | 50 | 22 (44.0 ± 4.0)[b] |
| | EG + PG | 50 | 0 (0.0 ± 0.0)[a] |
| | EG | 50 | 17 (34.0 ± 6.8)[b] |
| **8-cell** | Fresh control | 50 | 34 (64.0 ± 6.8)[c] |
| | EG + PG | 50 | 21 (0.5 ± 1.0)[a] |
| | EG | 50 | 1 (42.0 ± 8.6)[b] |

Data in parentheses are presented as mean±SEM. [a,b] Different superscripts in the same column indicate significant difference at P<0.05 using one-way ANOVA.

EG+PG: Embryos were vitrified by placing in 7.5%EG+7.5%PG for 10 min and 20%EG+20%PG+ 0.5M trehalose for 30 sec.

EG: Embryos were vitrified by placing in 1.5M (8.4%) EG for 5 min and in 7M (39%) EG + 0.6M trehalose for 30 sec

**Table 4.4** Cell numbers in blastocysts of IVF-derived intact bovine embryos at 2-and 8-cell stages vitrified with 1.5M EG and 7M EG +0.6 trehalose and after warming they were culture *in vitro*.

| Stages of embryo | Group | No. of blastocysts examined | No. of nuclei ±SEM | | |
|---|---|---|---|---|---|
| | | | ICM | TE | Total cells |
| 2-cell | Fresh control | 22 | 27.0 ± 0.8 | 82.5 ± 3.1[a] | 109.5 ± 3.1[a] |
| | Vitrified | 17 | 23.4 ± 3.0 | 68.4 ± 3.4[b] | 91.8 ± 3.7[b] |
| 8-cell | Fresh control | 32 | 26.0 ± 0.3 | 91.2 ± 2.7[a] | 117.3 ± 2.6[a] |
| | Vitrified | 21 | 28.3 ± 2.1 | 82.2 ± 7.00[a] | 110.5 ± 8.3[a] |

Data in parentheses are presented as mean±SEM. [a,b] Different superscripts in the same column indicate significant difference at $P<0.05$ using one-way ANOVA.

**Table 4.5** Developmental competence of vitrified-warmed IVF-derived separated blastomere bovine embryos at 2-and 8-cell after culture *in vitro*.

| Stage of embryo | Groups | No. of embryo cultured | No. (%) blastocyst | No. (%) monozygotic paired blastocysts |
|---|---|---|---|---|
| 2 single blastomere from 2-cell stages | Fresh control | 100 | 44 (44.0 ± 7.0)[a] | 14 (46.5 ± 13.6)[ab] |
| | Vitrified | 100 | 21 (21.0 ± 5.5)[b] | 5 (18.3 ± 13.0)[ab] |
| 2 quadruple blastomere from 8-cell stages | Fresh control | 100 | 50 (50.0 ± 3.5)[a] | 17 (59.0 ± 14.9)[a] |
| | Vitrified | 100 | 18 (18.0 ± 4.7)[b] | 3 (8.3 ± 5.3)[b] |

Data in parentheses are presented as mean±SEM. [a,b] Different superscripts in the same column indicate significant difference at $P<0.05$ using one-way ANOVA.

**Table 4.6** Quality of blastocysts developed from IVF-derived separated blastomere bovine embryos at 2-and 8-cell stages vitrified with 1.5M EG and 7M EG +0.6M trehalose after warming they were cultured *in vitro*.

| Stages of embryo | Groups | No. of blastocysts examined | Quality of blastocysts | | |
|---|---|---|---|---|---|
| | | | NO. (%) Grade 1 | NO. (%) Grade 2 | NO. (%) Grade 3 |
| 2 single blastomere from 2-cell stages | Fresh control | 44 | 15 (31.3 ± 7.1) | 21 (46.9 ± 10.2) | 8 (21.8 ± 9.7) |
| | Vitrified | 21 | 10 (40.8 ± 17.7) | 7 (32.5 ± 13.5) | 4 (16.7 ± 9.5) |
| 2 quadruple blastomere from 8-cell stages | Fresh control | 50 | 21 (43.7 ± 8.0) | 22 (44.7 ± 9.6) | 7 (11.7 ± 7.3) |
| | Vitrified | 18 | 6 (30.0 ± 9.4) | 6 (21.7 ± 11.0) | 6 (28.3 ± 12.7) |

Data in parentheses are presented as mean±SEM. No significant difference was detected among treatment groups at P<0.05 using one-way ANOVA.

**Table 4.7** Numbers of cells in blastocysts derived from separated and vitrified bovine blastomere at the 2-cell and 8-cell stages.

| Stages of embryo | Groups | No. of blastocysts examined | No. of nuclei ±SEM | | |
|---|---|---|---|---|---|
| | | | ICM | TE | Total cells |
| 2 single blastomere from 2-cell stages | Fresh control | 36 | 16.0 ± 0.4[a] | 47.4 ± 1.2[ab] | 63.4 ± 1.3[a] |
| | Vitrified | 18 | 15.6 ± 0.8[a] | 42.2 ± 1.5[a] | 57.7 ± 1.2[b] |
| 2 quadruple blastomere from 8-cell stages | Fresh control | 33 | 15.6 ± 0.6[a] | 51.0 ± 0.8[b] | 66.6 ± 0.9[a] |
| | Vitrified | 14 | 11.9 ± 1.2[b] | 43.4 ± 2.8[a] | 55.4 ± 2.0[b] |

[a,b] Different superscripts in the same column indicate significant difference at P<0.05 using one-way ANOVA.
Data in parentheses are presented as mean±SEM.

## 4.5 Discussion

Our results confirm that early stage of bovine separated blastomeres could be successfully cryopreserved by the Cryotop vitrification method, and had the ability to develop to the blastocyst stage. The present experiment showed that although the survival rate of the EG-vitrified blastocyst group was significantly lower than that of the vitrified combination of EG + PG, the hatched ability did not significantly difference between 2 groups which was agree with Villmil *et al*. (2011). Moreover, the total cell number of blastocysts vitrified with EG + PG was significantly higher than that of EG alone. In blastocyst vitrification, most of the CPA that is commonly used for vitrification is a combination of EG with other substances or EG alone. This phenomenon might maintain the ability of blastocysts to hatch after the vitrified-thawing process (Takahashi *et al.* 2005; Liebermann & Tucker 2006; Liebermann 2009, 2011). In the current study, although the EG + PG and EG alone exhibited high potential to vitrify bovine blastocysts, few blastocysts were produced after vitrify 2-cell and 8-cell stages of the embryo. In comparison, the use of EG-based CPA solution in early cell stage of embryos might help generate blastocysts after vitrification and thawing process. The toxicity of CPA may be a key limiting factor for cryopreservation, the CPA toxicity could be achieved at non-toxic level by using combination of CPA substances (Ali & Shelton 1993). EG is more commonly used for the early stages of embryo vitrification procedures, and also used in combination with other chemicals, such as DMSO (Ghorbani *et al.* 2012) and disaccharide molecules, which help draw out more water from the cells (Szell *et al.* 1989; Mukaida *et al.* 1998), and reduced the exposure time of CPA to toxins (Elnahas *et al.* 2013). This result was in agreed with that obtained by Tamas *et al*. (2013), who reported that the

rate of blastocyst formation of EG + PG was reduced compared with non-vitrified control and those vitrified in EG-based CPA. Due to their high lipid content, the bovine oocytes tend to be sensitive to chilling injury (Sripunya *et al.* 2014). Chilling injury is a major limiting factor for the developmental competence of oocytes (Ghetler *et al.* 2005). Several studies reported that PG permeates into the oocyte, 1-cell, and 2-cell stages faster than EG results in large volumetric change and damage to embryos in morphology, function and cytoskeletal organization (Arakawa *et al*. 1990; Mazur & Schneider 1986; Oda *et al*. 1992; Pedro *et al*. 2005; Elnahas *et al.* 2013). There shrinkage and swelling occur immediately causing damage to the oocyte and embryo. In addition, the surface area per volume ratio of the early stage of embryos is lower than the blastocyst stage (Maneiro *et al.* 1991). Furthermore, EG has better heat transfer efficiency and low viscosity compared with PG (The Dow chemical company, 2008ab). At room temperature, a little different in viscosity between EG and PG has no effect to heat transfer activity. However, if the temperature below $0^{o}$F, the heat transfer is much better with EG (The Dow chemical company, 1998). The reason could be explained why EG + PG did not work well in early stage of embryo. Moreover, high concentrations of PG have been reported a negative effect on the developmental competence of oocytes and embryo due to the high toxicity (Kasai *et al.* 1990).

Blastomere separation which is the way to increase the number of transferable embryo has been successfully achieved for several livestock species (Willadsen 1980; Tsunoda *et al.* 1984; Allen & Pashen 1984; Reichelt & Nieman 1994; Illmensee *et al.* 2006). Our results showed that EG alone is suitable CPA for cryopreserving the 2-cell and 8-cell stages of embryos. Base on this experiment, EG-based CPA solution was

selected for vitrified separated blastomeres from the 2-cell and 8-cell stages. A blastocyst formation rate derived from separated bovine blastomere vitrification was lower than that of the non-vitrified group. This result is contrast with mice and human which showed similar rate of blastocyst production between vitrified and non-vitrified group (Kang *et al.* 1994; Escriba *et al.* 2009). To date, limited data have been available on the application of vitrification in the bovine blastomere. During blastomere separation, the ZP was removed by immersing it in protease, and then the blastomere was removed by gently using a Pasture pipette. ZP is a glycoprotein layer that surrounds the plasma membrane of oocytes, and has many functions, including promoting communication between oocytes and the follicle during follicular development and regulating interactions between oocytes and spermatozoa during the fertilization process. ZP has been reported to have an important role in the cryopreservation of oocytes during vitrification, because it protects oocytes by resisting mechanical stress (Gupta *et al.* 2012). Choi *et al*. (2015) showed that the microencapsulation of mammalian cells without ZP in ZP-like hydrogel represents an effective strategy to improve their survival rate after vitrification. The rate of blastocyst formation after vitrification might be reduced due to an increased the cytoplasmic flow rate of CPA in the absence of the ZP. Alternatively, differences in the osmolality between the inside and outside of cells might be reduced in ZP-free embryos (Wassarman *et al.* 1999; Gupta *et al.* 2012). The total number of cells in separated blastomeres was significantly lower than that of non-separated blastomeres. This result is consistent with that obtained by Katayama *et al*. (2010), who detected a decline in the total number of cells in the separated blastomere of mouse embryos

compared with control. However, the ratio of TE/ICM cell did not difference between the blastocyst derived from separated and non-separated embryos (data not shown).

## 4.6 Conclusion

In conclusion, we successfully completed the cryopreservation of single and quadruple blastomere separated bovine embryos. Moreover, we confirmed that EG-based vitrification solution could be used for the vitrification of the intact and blastomere separated bovine embryos at the 2- and 8-cell stages by using the Cryotop vitrification method.

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# CHAPTER V

# OVERALL CONCLUSION

Ultrasound guide ovum-pick up (OPU), *in vitro* embryo production and blastomere separation are becoming important tool for producing high genetic merit offsprings. However, low numbers of OPU-derived oocytes could be adverse affected to developmental abilty of embryo by decreasing the embryo/volumn ratio. Using gel embedded embryo can be applied for maintaining the suitably embryo/volume ratio for small number of embryo. Our study demonstrated that 1% low melting point agarose gel and 1% calcium alginate gel had no adverse effect on slaughterhouse-derived zygote and separated blastomere from 2- and 8-cell embryos. However, some of expanded blastocysts were broken after recovering from 1% low melting point agarose gel chips. In case of separated embryo, the rate of blastocyst formation did not different between quadruple blastomere (from 8-cell) cultured in WOW and 1% calcium alginate gel whreas the blastocyst rate of single blastomere (from 2-cell) in WOW was higher than 1 % calcium alginate gel. In practically, culturing of separated embryos in WOW is simpler and less technical problem than embedded in gel materials.

In second experiment, our study compared between 2 different equilibration vitrification solutions; base medium containing ethylene glycol (EG) + propylene glycol (PG) and base medium with EG for embryo cryopreservation. The survival rate of vitrified-warmed expanded blastocyst did not different between EG + PG and EG

based only. However, the quality of blastocyst in term of total nubers of cell in EG + PG group was higher than EG based only. Interestingly, most of 2- and 8-cell were dead after vitrified with EG + PG based solution, only 1 low quality of blastocyst obtained from vitrified-warmed 8-cell embryo. On the other hand, EG-based vitrification solution can be used for vitrification of the intact and blastomere separated bovine embryo from 2 and 8-cell stages by using Cryotop vitrification method.

# BIOGRAPHY

Mr. Theesit Juanpanich was born in Bangkok on 12 April 1985, Thailand. He studied high school at Yothinburana School. After that, he received his Doctor of Veterinary Medicine degree (DVM) from Facultly of Veterinary Medicie, Kasetsart University, Thailand. After graduation, he worked as an exotic and small animal practitioner at Kwuncum Animal Hospital for 2 years. In 2011, he received the scholarship from the Royal Golden Jubilee Ph.D program and studied Ph.D. course in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. During Ph.D cource, he performed his experiment about Ovum pick-up (OPU), *in vitro* embryo production and blastomere manipulation with Assoc. Prof. Dr. Rangsun Parnpai laboratory and Prof. Dr. Kei Imai's laboratory at Rakuno Gakuen University, Hokkaido, Japan.