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Effect of Trehalose and Natural Honeybee as Cryoprotectant on the Viability of Vitrified Thawed Matured Bovine Oocytes

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours

Faculty of Agro Based Industry

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Student

Name:

Date:

I certify that the report of this final year project entitled "Effect of Trehalose and Natural Honeybee as Cryoprotectant on the Viability of Vitrified-Thawed Matured Bovine Oocytes" by Tai Cheah Jie, matric number F15A0230 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

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Supervisor

Name:

Date:

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LIST OF ABBREVIATION

BSA	Bovine Serum Albumin
BW	Body Weight
COVA	Cow Ova Vacuum Needle
COC	Cumulus Cells Complexes
CPA	Cryoprotectant
DMSO	Dimethyl Sulfoxide
E_2	Estradiol
EG	Ethylene Glycol
FSH	Follicle Stimulating Hormone
HB	Honeybee
IVF	*In vitro* Fertilization
IVM	*In vitro* Maturation
IVP	*In vitro* Embryo Production
LH	Luteinizing Hormone
NBCS	New Born Calf Serum
OPU	Ovum Pick Up
PVP	Polyvinylpyrrolidone
FBS	Fetal Bovine Serum
FDA	Fluorescence Diacetate
GnRH	Gonadotropin-Releasing Hormone
GV	Germinal Vesicle
LN_2	Liquid Nitrogen

mDPBS	Modified Dulbecco's Phosphate Buffered Saline
MII	Metaphase II
OPS	Open Pull Straw
p-FSH	Polyvinylpyrrolidone dissolved in FSH
P-S	Penicillin-Streptomycin
PNU	Princess of Naradhiwas University
RS	Rising Solution
SSV	Solid Surface Vitrification
T1	Treatment 1
T2	Treatment 2
TCM-199	Tissue Culture Medium - 199
VS 1	Vitrification Solution 1
VS 2	Vitrification Solution 2
WS 1	Warming Solution 1
WS 2	Warming Solution 2

LIST OF SYMBOLS

%	Percentage
=	Equal Sign
≥	Greater or equal to
®	Registration Mark
°C	Degree Celsius
cc/mL	Cubic Centimetre/Millilitre
G	Gauge
kg	Kilogram
M	Molarity
MHz	Mega Hertz
mg/kg	Milligram/Kilogram
mm	Millimetre
mL	Millilitre
mL/min	Millilitre/Minute
mg	Milligram
mmHg	Millimetre of Mercury (Pressure)
µg/mL	Microgram/Millilitre
nm	Nanometre
w/v	Weight/Volume

Effect of Trehalose and Natural Honeybee as Cryoprotectant on the Viability of Vitrified-Thawed Mature Bovine Oocytes

ABSTRACT

Vitrification has been extensively used in cryobiology studies especially in oocytes studies. Each vitrification medium produces different vitrification capabilities depending on the vitrification medium components. Until today there is still a possibility of finding better vitrification medium by altering the sugar component in vitrification medium to find new superior combination. The objectives of this study are (1) To determine the effectiveness between Trehalose and Honeybee as cryoprotectants on viability of vitrified-thawed bovine oocytes (2) To determine the effectiveness of superstimulation protocol for ovarian follicular growth before Ovum Pick Up (OPU). Two cattle were subjected superstimulation protocol, per session conducted for total of five days where three days both cattle were administrated with 200 mg follicle stimulation hormone (FSH) within 24 h once and two days of "resting period" (FSH starvation) totalling for two session. The "coasting period" (FSH starvation) between first and second session were three days (72 h). OPU occurred at the fifth day (120 h) for oocytes collection. The ovarian growth was observed by ultrasonographic examination right before OPU. Prior vitrifying oocytes by solid surface vitrification (SSV) with treatment Trehalose media (T1) and Honeybee media (T2) followed by warming protocol, oocytes subjected to *in vitro* maturation (IVM). The oocytes viability were evaluated by fluorescein diacetate (FDA) staining. The follicular growth from first session were large size follicles for both cattle but not the second session. Number of oocytes obtained from first session were (n=60) and second session (n=0). Oocytes viability for treatment Honeybee media (90.9%) was significantly higher Trehalose media (70.4%) at student *t*-test ($p \geq 0.05$). The presence study suggests the follicular growth are affected by FSH administration. The presence study also suggests the used of honeybee might be a superior alternative as sugar component in vitrification medium for vitrifying bovine oocytes.

Keyword: Follicle Stimulating Hormone (FSH), OPU, Cattle, Oocytes, Honeybee, Trehalose

Kesan daripada Trehalose dan Madu Lebah Sebagai Krioprotektan pada Daya Kehidupan Bovin Oosit yang telah Divitrifikasi dan Pematangankan

ABSTRAK

Vitrifikasi telah digunakan secara meluas dalam kajian kriobiologi terutamanya dalam kajian oosit. Setiap media vitrifikasi menghasilkan keupayaan vitrifikasi yang berbeza bergantung kepada komponen media vitrifikasi. Sehingga hari ini masih terdapat kemungkinan untuk mencari medium vitrifikasi yang lebih baik dengan mengubah komponen gula dalam medium vitrifikasi untuk mencari kombinasi unggul baru. Objektif kajian ini adalah: (1) Untuk menentukan keberkesanan and Trehalose dan Madu sebagai krioprotektan pada daya kehidupan bovin oosit yang telah divitrifikasikan. (2) Untuk menentukan keberkesanan superstimulasi untuk pertumbuhan folikel ovari sebelum *Ovum Pick Up* (OPU). Due lembu terlibat untuk protokol superstimulasi, setiap sesi dijalankan selama lima hari yang tiga hari kedua-dua lembu diberi dengan 200 mg hormon stimulasi folikel (FSH) dalam tempoh 24 jam sekali dan dua hari lagi "tempoh berehat" (kekurangan FSH) berjumlah dua sesi. "Tempoh pemancaran" (kekurangan FSH) antara sesi pertama dan kedua adalah tiga hari (72 jam). OPU berlaku pada hari kelima (120 jam) untuk pengumpulan oosit. Pertumbuhan ovari diperhatikan oleh peperiksaan ultrasonografi tepat sebelum OPU. Sebelum oosit divitrifikasikan dengan cara permukaan pepejal vitrifikasi (SSV) menggunakan rawatan media Trehalose (T1) dan Madu (T2) diikuti dengan protokol pemanasan, oosit tertumpukan pada *in vitro* pematangan (IVM) dahulu. Daya kehidupan oosit dinilaikan dengan pewarnaan fluoresenin diasetat (FDA). Jumlah oosit yang diperoleh dari sesi pertama adalah (n = 60) dan sesi kedua (n=0). Pertumbuhan folikel dari sesi pertama adalah folikel bersaiz besar untuk kedua-dua lembu tetapi bukan sesi kedua. Daya kehidupan melalui rawatan media vitrifikasi Madu (90.9%) lebih tinggi berbanding Trehalose (70.4%) dalam Ujian T ($p \geq 0.05$). Kajian ini menunjukkan pertumbuhan folikel dipengaruhi oleh administrasi FSH dan penggunaan Madu berpotensi dijadikan sebagai alternatif yang lebih efetik sebagai komponen gula dalam medium untuk vitrifikasi bovin oosit.

Kata kunci: Hormon Stimulasi Folikel (FSH), OPU, Lembu, Oosit, Madu, Trehalose

CHAPTER 1

INTRODUCTION

1.1 Research Background

The first successful cryopreservation was done by Whittingham, Leibo, and Mazur (1972). Polge, Smith, and Parkes (1949) whom were the first individuals reported on successful cryopreserved mammalian oocytes and embryos while Wilmut (1972) also successfully cryopreserved mouse embryo with slow freezing method. More successful cryopreserved studies emerged even since. Luyet and Hodapp (1938) successfully cryopreserved frog spermatozoa with vitrification method was an important breakthrough in the fields of cryopreservation studies for instances, the occurrence of two successful pregnancies of cryopreserved human embryo (Zeilmaker, Alberda, van Gent, Rijkmans, & Drogendijk, 1984). Based on a study by Noyes, Porcu, and Borini (2009) indicated that currently about 900 born infants that been successfully delivered with the help from cryopreservation technologies. Research by Noyes et al. (2009) also indicated that successfully delivered infants with the aid from cryopreservation technologies shows no any apparent congenital anomalies. The successfully cryopreserved cells further popularise cryopreservation studies that also able to assist in the fields of reproductive technologies.

There had been many studies that attempted to devise better protocols for cryopreservation that able to achieve high survivability of embryos and cells that subsequently reduces labour that worked effortlessly for both freezing and thawing procedures (Mochida & Ogura, 2010). To date, the current cryopreservation protocol still causes low survival and farrowing rates with slow freezing method (Gábor Vajta, 2013) which this study is attempting at finding a cryopreservation protocol that can achieve high viability cells. Despite the lack of prominent protocol, two cryopreservation protocols that successfully cryopreserved under many studies which is known as slow freezing or 'controlled slow freezing' and vitrification (Almiñana & Cuello, 2015). In the past, a study indicated by Sherman and Lin (1958) show vitrification protocols causes damaged to the intracellular and easy formation of crystallization due to the requirement of high concentration of cryoprotectants (CPAs).

Sugar incorporation to the vitrification solution able to reduce the damage and formation of ice crystal (Leibo, 1984). Various sugars have been tested as cryoprotectant agents (CPA) in vitrification solution, namely lactose (Rayos, Takahashi, Hishinuma, & Kanagawa, 1994) ; trehalose (Saha, Rajamahendran, Boediono, Sumantri, & Suzuki, 1996) ; sucrose (Saito, Imai, Tomizawa, & Livestock, 1994). Honey also to provide positive nutritional effect to the cells and bacterial pathogen (Fuselli, García De La Rosa, Eguaras, & Fritz, 2008). Honey bee will be used in this study as it viewed as an alternative for expanding the vitrification solution.

1.2 Problem Statement

Slow freezing and vitrification techniques in cryopreservation field are methods that still currently used widely in preserving embryos and oocytes. Numerous of studies with successful cryopreserved embryos/oocytes with vitrification techniques which expanded upon into the fields of cryopreservation of human embryo, mammalian embryos and oocytes. However, there always have been an ongoing dispute regarding which are the best embryonic stages for vitrification (Zhou et al., 2005). The ongoing dispute shows studies that related to this preformed in low uniformity and have exceeding expectation (Sunde et al., 2016).

Vitrification can also be performed on oocytes. Oocytes can be vitrified with or without the presences of Cumulus Cells Complexes (COCs). There always been a discourse and debate about whether vitrification on oocytes should be done with or without COCs. Numerous successfully vitrified oocytes with the presences of COCs such as: (1) An increase of oocytes survival and higher maturation rate oocytes (Bogliolo et al., 2007) ; (2) Presented with higher rate of cleavage of oocytes development compared to denuded oocytes (Shirazi et al., 2012). Despite many investigations are successful with vitrified COCs oocytes, author Zhang, Nedambale, Yang, and Li (2009) indicated that vitrification does not give any deletions effects at non-COCs on survival, cleavage and development of blastocyst rates. Studies by Mo et al. (2014) shows similar results for survival, cleavage and blastocyst formation of matured ovine oocytes whether with or without cumulus cells after warming procedures.

There are also numerous studies that surround vitrification solution that uses sugar as cryoprotectant. The used of sugar as cryoprotectant successfully aided embryo vitrification (Kasai et al., 1992; Yoshino, Kojima, Shimizu, & Tomizuka, 1993). Successfully vitrified embryo and oocytes subsequently further improved *in vitro* embryo production (IVP) program (Caamaño et al., 2015; G. Vajta, Holm, Greve, & Callesen, 1996) indicating vitrification protocol have great potential. Sugar as cryoprotectant in vitrification media in previous studies compared with different sugars to evaluate the ability to cryopreserve oocytes and embryos (Kuleshova, MacFarlane, Trounson, & Shaw, 1999; Lestari et al., 2018). However, the similarity of those studies were all oocytes from local slaughterhouse and usually it transported elsewhere for further evaluation. Thus, this study explores the possibility of obtaining the oocytes samples through transvaginal ultrasound guided follicular aspiration (OPU) in hopes of retrieving high quality oocytes. Despite there are several studies integrated vitrification protocol and ovum pick up (OPU) and presented with high number of oocytes yield and quality (Chasombat, Nagai, Parnpai, & Vongpralub, 2013; Denpong, Thevin, Suporn, & Saksiri, 2012), most of the studies only involved common sugars such as glucose, fructose, sorbitol, sucrose, raffinose, and Trehalose (Kuleshova et al., 1999). Therefore, it is evident that there are still different sources of sugars that yet to explore which this study utilises Honeybee (HB) as its cryoprotectant component in vitrification solution. It is also evident that little to none researches that have been done on such combination which are those recovered oocytes obtained via OPU that utilises sugar such as Honeybee (HB) as cryoprotectant in vitrification solution.

1.3 Hypothesis

The study comprised of two hypotheses:

H_O: The effectiveness for Trehalose are less great as sugar for cryoprotectant in vitrification solution compared to Honeybee.

H_1: The effectiveness for Trehalose are greater as sugar for cryoprotectant in vitrification solution compared to Honeybee.

H_O: The effectiveness of superstimulation of ovarian follicular growth before ovum pick up (OPU) show high responsiveness.

H_1: The effectiveness of superstimulation of ovarian follicular growth before ovum pick up (OPU) show low responsiveness.

1.4 Objectives

The objectives of this study are:

1. To determine the effectiveness of Trehalose and Honeybee as sugar for cryoprotectant in vitrification medium on the viability of vitrified-thawed matured bovine oocytes.
2. To determine the effectiveness of superstimulation for ovarian follicular growth before ovum pick up (OPU).

1.5 Scope of study

This study directed its focus towards 2 different objectives, in which this study determines to prove and justify its presented hypotheses to be true. The first objective is to determine the effectiveness of Trehalose and Honeybee (HB) as sugar component for cryoprotectant in vitrification solution on the viability of vitrified-thawed matured bovine oocytes. The second objective is to determine the effectiveness of superstimulation protocol of ovarian growth before ovum pick up (OPU). The study begins from the superstimulating the cattle for follicular growth before OPU. The superstimulation protocol took place three days consecutively by administrating follicle stimulating hormone (FSH) and rest for two day before performing OPU for oocytes collection. The follicular growth were then observed with a portable ultrasound. Thereafter, oocytes pick up (OPU) via transvaginal ultrasound-guided follicular aspiration. Collected oocytes were then subjected to *in vitro* maturation (IVM) and followed by vitrifying it using solid surface vitrification (SSV) method which thereafter freezes it with liquid nitrogen (LN_2). Matured oocytes were separated into two different vitrification solution group: Trehalose treatment (T1) ; Honeybee (HB) treatment (T2). The separation of treatment groups allows evaluation and comparison of effectiveness between Trehalose and HB as cryoprotectant in vitrification solution on oocytes derived from follicular aspiration. Oocytes from both groups were further subjected to fluorescence diacetate (FDA) staining and observed with a fluorescence microscope to evaluate the oocytes viability.

1.6 Significance of Study

In the cryopreservation field, much of the studies are interested in further improving the standards of vitrification protocols. However, vitrification protocols only applicable according to the species of animal. Thus, this proposed study aims to further modify the vitrification protocol using Honeybee (HB) instead of common sugars in the vitrification medium to give the protocol more versatility in hopes that the new vitrification solution protocol will translate well to any animals species. There are numerous studies with satisfactory results presented around different protocol that utilities ovum pick up (OPU): (1) Obtained highly competent oocytes development that derived from Indian breed cattle up (Manik, Singla, & Palta, 2003) ; (2) Buffalo that subjected to bovine somatotropin (bST) treatment improves follicular population prior to OPU program (Sá Filho et al., 2009) ; (3) Genetic variation and improvement on cost efficiency for *in vitro* embryo production (IVP) (Merton et al., 2009) ; (4) Integration of progesterone prior to OPU did not alter oocytes yield but further encourages *in vitro* embryo production (IVP) (Gimenes et al., 2015). However, throughout the years there are still an absence of Honeybee (HB) as cryoprotectant in vitrification solution protocol that vitrify and thaw matured bovine oocytes which derived from transvaginal ultrasound-guided follicular aspiration (OPU). Time limitation hinders this research and was only able to evaluate the viability of oocytes but unable to proceed to *in vitro* fertilisation (IVF).

1.7 Limitation of study

There are some limitation exists in this particular study. Time constraint prevents this study to proceed further next to *in vitro* fertilization (IVF) of recovered oocytes to further verify the oocytes survivability. The recovered oocytes are also highly dependent on the experimental animal performance and the skills in performing the oocytes collections. The sample that obtained through Ovum Pick Up (OPU) method are external factors that directly affects the results of the experiment.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Cryopreservation

The existence of cryopreservation started about 200 years ago, where Gey Lussac founded the every first method to cryopreserve by observing clouds that is not frozen even though with subzero temperatures while ascending in a hot air balloon (Arav, 2014). Cryopreservation, however, able to integrate itself well in the medical field. This is evident when the two pregnancies were successfully transferred of cryopreserved human embryo (Zeilmaker et al., 1984). Exploration of cryopreservation started with human embryo, that subsequently instilling interest of devising better protocols that can minimise the loss, better survival and higher fertilization rate when those human embryos being assessed (Kansal Kalra et al., 2011). The first frog's spermatozoa that successfully vitrified from the old protocol procedures (Luyet & Hodapp, 1938) subsequently leads to the expansion of cryobiology studies to the animals. The preservation of frozen material made possible with cryopreservation which can act as a back-up storage when catastrophic does happen which ensure the preservation of biodiversity continues to strive (Critser & Russell, 2000).

2.1.1 Cryopreservation Method

All of the cryopreservation involved about the same principle which includes reduction of temperature, dehydration of cellular, thawing and freezing. The cryopreservation methods causes cells to form ice crystal, but the used of cryoprotectant prevents formation of ice crystallization (Mara, Casu, Carta, & Dattena, 2013). Whittingham et al. (1972) and Wilmut (1972) were two different authors that successfully cryopreserved a mouse embryo with a technique called slow-freezing. However, vitrification techniques success did not widely celebrated in the past as it overshadowed by the success slow freezing techniques because of the used of high concentration cryoprotectants (Arav, 2014). The studies on cryopreservation field became more popular when investigation on alteration of the original techniques and protocols able to achieve better cells viability. Author Willadsen, Polge, and Rowson (1978) modified the original protocol that still widely used today. However, there is still the absences of universal protocol despite its potential in this field of study. According to Kolibianakis, Venetis, and Tarlatzis (2009) there is still lack of evidences that able to prove suitable techniques or protocols that can produce desirable results.

2.1.2 Slow Freezing Method

The first successfully cryopreserved mammalian oocytes and embryos with the mistakenly used glycerol as cryoprotectant was performed by Polge et al. (1949) in the efforts to duplicate the results obtained from Luyet and Hodapp (1938). These leads to the discovery of slow freezing as a method for cryopreservation (Arav, 2014). Two

different authors that successfully freeze embryos including Whittingham et al. (1972) and Wilmut (1972) that lay the foundation for slow freezing method studies.

The idea or principle behind 'slow freezing' generally means with the administration of cryoprotectants that allows the biophysical properties such as cooling and warming to be possible along with the minimization of cellular injury and also intracellular ice crystal formation (Wallach, Friedler, Giudice, & Lamb, 1988). However, the implication comes when different exposure time of those oocytes to cryoprotectant, whereby the cooling rate should be slow enough for dehydration of oocytes while fast enough to prevent oocytes from obtaining toxicity because of prolonged exposure times (Tao & Del Valle, 2008). Method such as slow freezing and thawing integrated into procedure as it able to minimize intracellular ice formation and the reduction of structural damage (Fabbri et al., 1998).

2.1.3 Ultrafast Freezing Method (Vitrification)

The very first studies that expanded the field of vitrification was started with feasibility of mouse embryo. The mouse embryo has successfully cryopreserved by vitrifying at -196^{0}C (Rall & Fahy, 1985). Vitrification was gaining more traction in which a human cleavage-stage embryo successfully vitrified resulting a live birth (Gordts, Roziers, Campo, & Noto, 1990). Author Kono, Suzuki, and Tsunoda (1988) was the first whom successfully vitrified rat blastocysts that further perpetuate vitrification popularity. Pervious literatures pointed that vitrification were better alternatives to slow freezing cryopreservation (Lilia L. Kuleshova & Lopata, 2002) including better survival rate

(Abdelhafez, Desai, Abou-Setta, Falcone, & Goldfarb, 2010), inexpensive and various mammalian embryos that are possible to be vitrified (Gábor Vajta & Nagy, 2006).

Vitrification is meant by a process that solidify the living cells, and the avoidance of ice crystal formation during cooling happens with the used of high concentration of cryoprotectant solution along with rapid cooling and thawing rates (Lilia L. Kuleshova & Lopata, 2002; Pereira & Marques, 2008; Gábor Vajta & Nagy, 2006). Any forms of cryoinjuries occurs more prominently at vitrification. Thus, solution to cryoinjuries is to have high cooling rates and concentration of cryoprotectants (Gábor Vajta & Nagy, 2006). However, most of the vitrification methods uses are standard straw that holds the embryos that limits the high cooling rate (Matsumoto, Jiang, Tanaka, Sasada, & Sato, 2001) before the discovery of other alternatives. The use of electron microscope grids (Martino, Songsasen, & Leibo, 1996), Open Pulled Straws (OPS) (G. Vajta et al., 1998) that allows only small amount of vitrification solution (VS) which leads to the increased of post thaw viability (Hochi, Akiyama, Minagawa, Kimura, & Hanada, 2001) and cryoloops (Lane, Schoolcraft, Gardner, & Phil, 1999).

2.1.4 Thawing/Warming

The significant differences in its protocol between the words slow freezing and vitrification, while thawing and warming protocols are very similar. Both thawing and warming similar in terms high heat rates usage, with the low concentration of CPA for the avoidance of ice crystal formation. Despite the similarity of its procedures, the term ‘freezing’ is mainly used for slow freezing while ‘thawing’ is used for vitrification (Almiñana & Cuello, 2015). Studies done by Leibo (1984) shows the frozen embryo can

be directly transfer without the removal of CPAs. Another study done by Seki and Mazur (2009) suggest that warming rate are significant towards the survival rate of embryos in comparison to the cooling rate. Further supporting this narrative, that rapid warming is essential for minimizing the formation of ice crystal and the revised protocols was able to yield oocytes with increased survival and high developmental potential (Seki & Mazur, 2008). However, some research suggest that low warming rates can be lethal to the cells that leads to minor ice formation (AbdelHafez, Xu, Goldberg, & Desai, 2011).

2.2 Ovum Pick Up (OPU)

In vivo oocytes collection initially performed on human with the possibility of retrieving female gametes. Performing oocytes collection on humans pave such methodology which can be done on animals (Raffaele Boni, 2012). Many new developments of better protocols which better suited *in vivo* oocytes collection in animals: (1) Oocytes collection that uses follicular aspiration and observed by endoscopy via right paralumbar fossa in 50 heifers (Lambert et al., 1983) ; (2) The oocytes recovery utilised ultrasonically guided aspiration of bovine follicles after superovulated the bovines (Callensen, Greve, & Christensen, 1987). After the discoveries of better and efficient protocols that allows retrieval of repeated oocytes using transvaginal ultrasound-guided follicle puncture as initially described by Pieterse, Kappen, Kruip, and Taverne (1988). It leads to the continuation discovery of new protocol which is combination of OPU with *in vitro* fertilisation (IVF) turns into an ideal method to allows large producing of embryos (Manik et al., 2003) which subsequently further improving *in vitro* embryo production (IVP) that well adapted with both researches and commercial production programs

(Merton et al., 2009). Despite the continuation of OPU integrated with IVP that resulting improvement in genetic information in buffalo and oocytes quality (Sá Filho et al., 2009), many other operations shown otherwise. The recovered cumulus cell complex (COC) significantly reduced per OPU procedure (Neglia et al., 2003), and the decreased of cleavage and blastocyst formation rate (Gimenes et al., 2015) are some operations that show inefficiency of OPU incorporated with IVP.

The inefficiency of OPU protocols subsequently leads to the search for better protocols that better optimising OPU to further improve IVP operations. The main objectives of improving OPU protocols are through obtaining high efficiency, number and quality of recovered oocytes. Needle with aspiration pump that able to aspirate high number and quality oocytes (Raffaele Boni, 2012). This needle with aspiration protocol for oocytes recovery that leads to the replacement of original shorter steel needle which are easier to change between donor cattle, thus able to reduce cross contamination between cattle (Bols, Vandenheede, Van Soom, & de Kruif, 1995). It also indicated by Bols et al. (1995) that those needles which were cheap, sterile and readily to be dispose further improves OPU protocol as a result. However, needle with aspiration protocol shown decrease in oocytes quality in these particular scenarios: (1) Probability of mechanical damage to the recovered oocytes associated with needle size and length (Raffaele Boni, 2012) ; (2) Aspiration pump that utilised high aspiration pressure recovered decrease compact cumulus in oocytes (Ward, Lonergan, Enright, & Boland, 2000) ; (3) The sharpness and tip bevel of the needle (Bols, Ysebaert, Van Soom, & de Kruif, 1997).

The collection of oocytes is also possible with utilisation of laparoscopic (L-OPU) via transvaginal guided aspiration which was developed by Reichenbach, Wiebke, Besenfelder, Mödl, and Brem (1993). L-OPU is efficient for routine use with high number

of animals involved, and successful aspiration within shorter time intervals (Reichenbach et al., 1993). L-OPU presented with several positive results which were decreased of ovaries injuries and the ability of clear observation to the ovaries and the aspiration process taken place inside the bovine when compared to transvaginal ultrasound-guided ovum pick up (U-OPU) (Santl et al., 1998). However, some studies indicates L-OPU would require the animal to be anaesthetised and positioned in dorsal recumbency that the animal can be traumatised from such situation (Holland et al., 1981). Thus, U-OPU as indicated by Pieterse et al. (1991) is a less traumatic alternative then L-OPU as it does not required surgery procedures.

2.3 Hormonal Stimulation In OPU

Before hormonal stimulation incorporated with ovum pick up (OPU) procedures, hormonal stimulation is a method that vastly contributed to *in vitro* embryo production (IVP) program. The used of bovine somatotropin (bST) significantly affects the number of follicle and improved quality of cumulus cells (Sá Filho et al., 2009). Thus, many operations with hormonal stimulation prior OPU significantly improved IVP programs: (1) Utilisation of porcine FSH (p-FSH) for IVP program in bovine *Bos taurus* resulting higher embryo yields per OPU session (Sendag, Cetin, Alan, Hadeler, & Niemann, 2008) ; (2) The administration of Gonadotropin-releasing hormone (GnRH) to promote high production follicular wave at predictable time (Hamid, Herménégilde, D, J, & LA, 1998) ; (3) Heifers that FSH-stimulated prior to OPU recovered significantly higher number of oocytes initially and decreases on subsequent OPU session (Reis, Staines, Watt, Dolman, & McEvoy, 2002). However, FSH yield the best results with high number of follicles

aspirated and recovered oocytes within the duration of two to four days (Merton et al., 2003).

IVP significantly improved efficiency with hormonal superstimulation required "coasting" period (the resting duration between last hormonal administration and OPU) before OPU (Blondin, Bousquet, Twagiramungu, Barnes, & Sirard, 2002). It also indicated by Blondin et al. (2002) that costing for 48 hours produced more follicles. A study by the authors Nivet et al. (2012) proves that accurate coasting period produces the best yielding result with given FSH to bovine. Coasting for 48 hours between administration of p-FSH and OPU increases oocytes competence because of suitable follicular environment that permits completion of oocytes maturation (van Wagtendonk-de Leeuw & de Ruigh, 1999).

Despite improvements made to OPU with hormonal stimulations, there is still some presence of shortcomings. Hormonal stimulation requires reintroduction of prestimulation (van Wagtendonk-de Leeuw, 2006). Hormonal stimulation prior to OPU may also result in higher number of embryo but deceases per OPU sessions and hormonal stimulation are limited to once a week only (Merton et al., 2003). However, a study conducted by Chaubal et al. (2006) suggested FSH administration prior to OPU for once a week provided the best result. Thus, there are also OPU performed without hormonal administration that showed positive results as well. An OPU session with no hormonal administration can be extended to certain period of time without much detrimental effect towards the cattle performance (Chastant-Maillard et al., 2003). OPU that performed up to three times within a week produces high number of embryo and also can be cost effective since it performed without any hormonal injection (Merton et al., 2003).

2.4 Cryoprotectants (CPAs)

Cryoprotectants (CPA) are agents that prevents the formation of intracellular ice crystals in cells that exposed to liquid nitrogen for the purpose of preservation (Hainaut & Vaught, 2017). CPA can also maintain cells quality after thawing cells tissue fragments (Unni et al., 2012). Wide range of CPA protocols have been used on embryos, which most of the protocols can be divided into three groups: Low molecular weight permeating CPAs; Low molecular weight non-permeating CPAs ; High molecular weight non-permeating CPAs (Palasz & Mapletoft, 1996). Glycerol, ethylene glycol (EG) and dimethyl sulfoxide (DMSO) are examples of low molecular weight permeating CPAs. Permeating CPA agents forms hydrogen bond with the cells intracellular water molecules and prevents the formation of crystallization (Pereira & Marques, 2008). High molecular weight non-permeating CPAs, however, able to reduce the required cryoprotectants in vitrification protocols, subsequently reduces toxicity in CPAs (Pereira & Marques, 2008). High molecular weight non-permeating CPAs are the combination of more than one cryoprotectants that minimizes the toxicity effects in CPAs (Orief & Schultze-Mosgau, 2005).

Despite the attempts of developing wide arrays of protocols, cryoinjuries to cells structure are unavoidable (Buarpung, Tharasanit, Comizzoli, & Techakumphu, 2013). Limited amount of CPAs used may leads to osmotic injuries (Almiñana & Cuello, 2015) due to presences of toxicity (Fahy, 1986). There are also studies suggested that other cryoinjuries that were associated exposure of CPAs: (1) Depolymerization of microtubules and microfilaments with the traditional vitrification protocol (Almiñana & Cuello, 2015) ; (2) Changes in polyploidy of mouse oocytes (Eroglu, Toth, & Toner,

1998) ; (3) Hardens of zona pellucida structure of mouse oocytes (Carroll, Depypere, & Matthews, 1990).

Better suppression of toxicity from CPAs is one solution for further optimising cryopreservation protocol (Fahy, 1986). These leads to various studies that cryopreserve with the used of CPAs. Some studies proven that minor alteration of protocols with usage of CPAs permits favourable results. The combination of two permeable CPAs agent reduces the toxicity effect during vitrification (Ishimori, Takahashi, & Kanagawa, 1992; Vicente & García-Ximénez, 1994). Some studies also indicates that combination of two CPA would bring less toxicity and yield better results than single CPA studies (Best, 2015). Vitrification with addition of non-permeating CPAs significantly reduces toxicity of CPAs (Liebermann et al., 2002). The widely accepted CPAs agent were ethylene glycol (EG) as the toxicity is low and high permeability (Emiliani, Van Den Bergh, Vannin, Biramane, & Englert, 2000) and an effective CPA agent for vitrification (Orief & Schultze-Mosgau, 2005).

Minimizing the cryoinjuries would be possible with the alteration of standard protocol with different CPAs agent (Best, 2015; W.-X. Liu et al., 2009). However, high cooling rate decreases CPA concentration and leads to the decease of toxicity level in the solution (Liu, Phy, & Yeomans, 2012). There are some studies that proves that dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glycerol as combined CPAs can lead to successful vitrification of mouse blastocyst (Gautam, Verma, Palta, Chauhan, & Manik, 2008).

2.4.1 Sugar Incorporated As CPAs

Since the existence of cryopreservation that started about 200 years ago (Arav, 2014) that leads to the expansion of cryopreservation studies that begins with the first successfully cryopreserved mammalian cells (Polge et al., 1949). The success of cryopreservation because of discovery that is effective and efficient strategies that still used widely until today which are slow freezing (Whittingham et al., 1972) and vitrification or fast freezing (Rall & Fahy, 1985). However, the first study that subjected embryos cryopreservation that uses sugar as CPA (Leibo, 1986). The successful result for such integration between sugar as cryopreservation subsequently leads various studies that performed on embryos. The addition of sugar as CPA allows the embryo to withstand equilibration in numerous vitrification solution, reduces the toxicity effects acting on embryo and assisted in dehydration of embryos (Yoshino et al., 1993). Large amount of sugar as CPA permits high survivability of extreme dehydration condition (Wright, Eroglu, Toner, & Toth, 2004). Thus, a study by the author Hotamisligil, Toner, and Powers (1996) speculated that sugar able to protect the membrane integrity and preserving the structural shape of the embryo.

Sugar classified according to number of sugars that bonded through glycosidic bonding. The linkage resulting into different structural and molecular properties that leads to different protection during freezing (Tsai, Chong, Meng, & Lin, 2017). Such molecular properties of sugar means the ability of protection to the cells are highly dependent on the sugar during cryoprotection (Yildiz, Kaya, Aksoy, & Tekeli, 2000). Disaccharide are commonly used in vitrifying oocytes and embryo in numerous animals specie (L. L. Kuleshova et al., 1999). The addition of disaccharides such as sucrose and Trehalose for vitrification solution also significantly reduces the toxicity effect by shorting the exposure

time (Orief & Schultze-Mosgau, 2005). However, an study investigated also by author L. L. Kuleshova et al. (1999) suggested the combination of sugar from different saccharides can increase the cells survival rate. Thus, the differences in molecular properties of each sugar provide different level of protection to the cells which indicates the level of protection depends on the sugar during cryopreservation (Yildiz et al., 2000).

2.4.2 Trehalose and Honeybee in Cryopreservation Studies

Trehalose is a non-permeable disaccharides sugar that have larger presences in sperm cryopreservation. Various species spermatozoa have subjected to cryopreservation with the used of Trehalose including bull (Iqbal, Naz, Ahmed, & Andrabi, 2018), buck (Karunakaran et al., 2018), boar (Athurupana, Takahashi, Ioki, & Funahashi, 2015) and ram (Tonieto et al., 2010). Trehalose also used as a component in vitrification procedures which vitrify embryo (Saha et al., 1996). The estimated 2.5 times larger in hydration radius than other sugar (Sola-Penna & Meyer-Fernandes, 1998) might have gave Trehalose the flexibility as CPA during cryopreservation. The large hydration radius subsequently led to dehydration suppression of shell protein subsequently improving the protein stability of cells (Lin & Timasheff, 1996). An investigation by the authors Crowe, Crowe, Rudolph, Womersley, and Appel (1985) mentioned that trehalose can further stabilised with the interactions between membrane of cells and groups of phospholipid.

The chemical properties in honey are natural mixture of 25 sugars which comprises mostly fructose and glucose with the estimation of 95%-97% as dry matter (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). The combination of such chemicals properties have elements of enzymes, vitamins and minerals that grants honey with the

ability to provide pharmacological effects in living cells (Christy E., Anna.M, & Roland.N, 2011). Sperm cryopreservation using honey is more prominent than oocytes and embryo. A study shows that spermatozoa that subjected to honey during cryopreservation obtained energy from honey that are essential to spermatozoa metabolic processes (El-Sheshtawy, El-Badry, El-Sisy, El-Nattat, & Abo Almaaty, 2016). However, an investigation by author Alfoteisy (2012) mentioned honey can used as vitrification media to oocytes because of its suitable concentration. Thus, it can observed that the natural combination chemical properties of honey resulting a more superior antioxidant effect compared to single antioxidant element (Christy E. et al., 2011).

2.4.3 Cryoinjury

Cells that subjected to cryopreservation experiences wide array of stress that can leads to cells death (Mazur, Leibo, & Chu, 1972). However, the extent of injury largely depends on the cells shape, permeability of membrane, and quality of the oocytes (Prentice & Anzar, 2011). The principle of vitrification is the total elimination of ice formation when cells suspending in the vitrification medium (Gábor Vajta & Nagy, 2006). High concentrated CPAs may cause toxicity and osmotic injury, which method used by Stachecki (2004) able to elevate the problem with the used of lowest concentration CPAs to achieve highest possible cooling and warming rates. Despite the changes that made in the protocol of cryopreservation, major adverse consequences such as toxicity from CPAs, ice crystal formation, chilling that leads to unavoidable changes in intracellular cytoskeleton and organelles (Gábor Vajta, 2000). A study according to Ebrahimi, Valojerdi, Eftekhari-Yazdi, and Baharvand (2012) demonstrated that there will be

irreversible deleterious effects on morphology, ultrastructure, meiotic spindle and chromosomal of oocytes after vitrification. The main cause of low fertilization rate due to harden of zona pellucida after vitrification/warming protocols (Asgari et al., 2012).

2.5 Bovine Oocytes

Initial observation of bovine oocytes was done by Edwards (1965). However, bovine oocytes cultivated *in vitro* by various different studies produces ranges of success rate (Edwards, 1965; Sreenan, 1970). Recent advancement in the studies of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes (Martino, Mogas, Palomo, & Paramio, 1995). Oocytes are the largest cells with low surface to volume ratio in which surrounded by zona pellucida (Prentice & Anzar, 2011). Despite its large in size, oocytes are still prone to chilling and low permeability towards cryoprotectants (Woods, Benson, Agca, & Critser, 2004). Plasma membrane, cortical granules and spindle formation are some of the distinct differences in both oocytes and embryos (Chen et al., 2003). Ovaries derived slaughterhouses are significantly cheaper and also available in large numbers of oocytes (Martino et al., 1995). Oocytes that received from the slaughterhouse derived ovaries are usually at germinal vesicle (GV) stages (Prentice & Anzar, 2011).

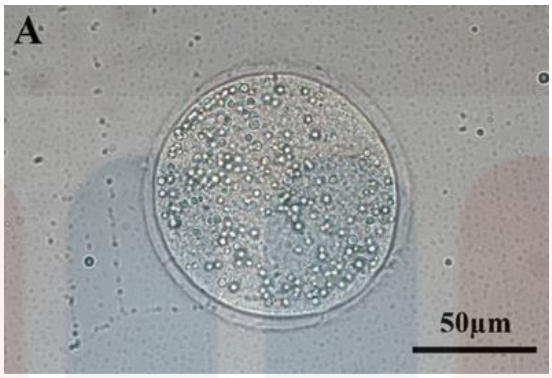

Figure 2.1: Oocytes visualization in bright field at 50 µm.
Sources: Silva et al. (2014).

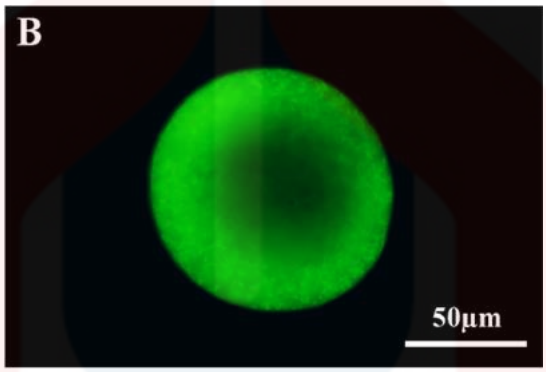

Figure 2.2: Characterization of viable oocytes after staining with calcein-AM at 50 µm.
Sources: Silva et al. (2014).

2.5.1 Age

There are several species of animals shows that ages are associated with decline of female reproductive capabilities (Ottolenghi et al., 2004). There are several different types of decline capabilities which includes changes occurs in hypothalamic-pituitary-ovarian axis (Klein et al., 1996), subsequently causes decreased of oocytes quality (Eichenlaub-Ritter, Vogt, Yin, & Gosden, 2004). Study performed by Hamatani et al.

(2004) further suggested that oocytes quality can affected by maternal age whenever comparing the mRNA expression profile of MII of mice from different age groups. Thus, age and the quality oocytes are highly correlated. There are findings also suggests that oocytes that derived from older mice presented with more sensitive extracellular stress in comparison to younger mice (Yan et al., 2010). A study by Goto et al. (2011) pointed by human embryos found with the decease of clinical pregnancy rate, viable pregnancy rate and delivery rate highly associated with the increased of age. Number of studies also shows the support of deleterious effects of increased age: (1) Lowered survival and cleavage of oocytes after vitrification (Saragusty & Arav, 2011) ; (2) Deceased in number of oocytes (Yan et al., 2010).

2.5.2 Species

The limiting number of cryopreservation preformed with embryo transfer usually highly associated with types of animal species. Lack of studies on domestic animal species in which factors including difficulty and high cost of studies (Almiñana & Cuello, 2015). A study by Jin et al. (2011) shows that different species of oocytes/embryo have its distinctive tolerance towards CPAs due to the differences of higher membrane permeability. Thus, numerous of studies demonstrated various modification towards cryopreservation protocols for better optimization in various species animals.

2.5.3 Cumulus Oocytes Complexes (COCs)

The presence of cumulus cells are important as it can ensure development of cells (Imoedemhe & Sigue, 1992). Thus, as indicated from a previous study by Vincent, Pickering, and Johnson (1990) that cumulus cells can affect the fertilisation rate when zona pellucida hardens but improves upon warming/thawing procedures. Cumulus cells plays a vital role in the maturation process by establishing mutual network of complex interaction between function and physical (Bogliolo et al., 2007).

Number of studies involved around cumulus cells expansion. A study by Moawad et al. (2012) mentioned that vitrified oocytes have retarded cumulus cells expansion. Further supporting that narative, vitrifying oocytes with DMSO as CPA significantly reduces in cumulus cells expansion, subsequently damages and interrupted the expansion of cumulus cells as a result (Somfai et al., 2010). Vitrified oocytes causes damaged to cumulus cells and distort the actin filaments with the presences of CPAs during vitrification procedures (Bogliolo et al., 2007). The high sensitivity of oocytes to cryoprotectant exposure (Chaves, Souza-fabjan, & Mermillod, 2014) also leads to the occurrences of depolymerisation tubulin and microtubule distortion in vitrified oocytes (Succu et al., 2007). There are some protocols that can elevate the problems with the use of Cytochalasin B (CCB) that can reduce cryodamage in cumulus cells: (1) Improved the stability of microtubules (Rho et al., 2002) ; (2) Preservation of gap junction between oocytes and cumulus cells (Vieira et al., 2002).

2.5.4 Matured Oocytes (Metaphase ll, Mll)

Vitrification can be performed in 2 different meiotic stages which were germinal stage (GV, immature) and MII (metaphase II, matured) oocytes (Fernández-Reyez et al., 2012). Oocytes that undergoes vitrification experiences membrane stability during chilling resulting chromosomal aberrations and an increased in polyploidy which gives implication towards fertilization (Tharasanit, Colenbrander, & Stout, 2006). The used of immature oocytes have distinct differences. Vitrification of immature oocytes has the potential to change spindle and chromatin configuration (Bogliolo et al., 2007). Vitrification also affects the ability of the oocytes to survive during and after vitrification (Mo et al., 2014). Despite low survival rate of vitrified matured oocytes, higher resistance to vitrification with higher cleavage rate still presented when comparing to immature oocytes (Shirazi et al., 2012). The effects of cryopreservation towards nuclear stages is not fully understood, however GC stage oocytes were still shows higher resistance towards to cryodamage due to smaller size of the cell (Purohit, Meena, & Solanki, 2012). Vitrification matured oocytes able to present normal structure and higher cleavage rate that vitrified GV oocytes (Quan et al., 2014). Matured oocytes are more suitable for vitrification as those cells have higher tolerance indicated by Quan et al. (2014). However, the constant debate on whether the used of mature or immature oocytes (Isachenko, Soler, Isachenko, Perez-Sanchez, & Grishchenko, 1998) are able to produce more favourable results during and after vitrification. It is undeniable that further studies is a necessity to further improve the vitrification method (Andrade et al., 2014) which suitable for both mature and immature oocytes.

2.6 *In vitro* Maturation (IVM)

Immature oocytes IVM plays a prominent roles for elevating the problems of infertility in domestic animals (Quero, Villamandos, Millan, & Valenzuela, 1995). There are many infants that delivered through IVM (Jurema & Nogueira, 2006). Thus, many attempted to further improve the IVM rate for both oocytes and embryos (Kim et al., 2011). Hence, current IVM technology main intention have shift from culturing oocytes to retrieving immature oocytes (Cha et al., 1991).

The usage of TCM-199 for maturation medium have been done in numerous studies. A study by Andrade et al. (2014) shows that TCM-199 able to induce activation probably with the presence of several nutrients such as inorganic salts, antioxidants and amino acids. However, Andrade et al. (2014) also noted that modified TCM-199 does not gives any effects towards the growth of oocytes. Amino acid from TCM-199 speculated that able to improve the cells viability (Gardner & Lane, 2003) and lowered oxidative stress (Liu & Foote, 1995). An independent research by Bing, Nagai, and Rodriguez-Martinez (2001) shows that TCM-199 with constant volume of Follicle-stimulating hormone (FSH) causes oocytes to maturation with addition of cysteamine. More application have been done with TCM-199 by Beker, Colenbrander, and Bevers (2002) which shows TCM-199 that supplemented with 17β-estradiol (E_2) could significantly decrease the total amount of MII oocytes, while increases the percentage of nuclear aberration. However, high incidences revolving around the affects from 17β-estradiol (E_2) which leads to unequal segregation of chromosomes of cells (Ochi, 1999).

2.7 Assessment Of Vitrified Oocytes

There have been various method that publish for the cryopreservation of embryos (Rall, 1992). Vitrification is the most widely used method which can be an alternative for slow freezing method (Lilia L. Kuleshova & Lopata, 2002) in the cryopreservation field. Vitrification techniques exposed the selected oocytes/embryos to high concentration cryoprotectants for a short duration that will suppress the cells to form ice crystal and followed by direct contact of cells with liquid nitrogen (LN_2) (Shin et al., 2011). Utilization of high concentration of cryoprotectant induces various cryoinjuries and stresses that exert on the cells such as formation of ice crystal (Leibo, McGrath, & Cravalho, 1978). Thus, there should be a method to observe the viability of the cells at any stages after cryopreservation. A study by Mohr and Trounson (1980) able to devised a technique with the used of Fluorescein diacetate (FDA) to stain the cells. Viable cells will be stained with FDA while non-viable cells are not. Thus, limitation of FDA staining is the inability to observed the development stages of the cells (Yang et al., 2003).

CHAPTER 3

MATERIALS AND METHOD

All the chemicals and media used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and all the apparatus were brought from Thermo Fisher Scientific Company Ltd. (Bangkok, Thailand) unless stated otherwise.

3.1 Experimental Design

This study begins with selecting two suitable cattle by evaluating with a portable ultrasound and suitable cattle bodyweight. Superstimulation protocol subjected both cattle with per session conducted for total of five days (120 hours) in which three days (72 hours) both cattle were administrated with follicle stimulating hormone (FSH) once in every 24 hours and two days (24 hours) of "resting period" (FSH starvation) totalling for two sessions. The "coasting period" (FSH starvation) between first and second sessions were three days (72 hours). Ovum pick up (OPU) for oocytes collection occurs at fifth day (120 hours) for both sessions. The same OPU operator was conducted by same person, Dr. Jakkhaphan. Prior to OPU, the ovarian growth was monitored by portable ultrasound. The collected oocytes subjected to *in vitro* maturation (IVM), followed by exposure to cryoprotectant agent (CPA) of vitrification solution. Vitrified oocytes

immediately freeze with method solid surface vitrification (SSV) and stored for ten days followed by warming/thawing procedure. Vitrification-thawed method and procedures were carried out with two different treatments which comprises Trehalose and Honeybee (HB) as the sugar for cryoprotectant agent (CPA) with the aimed of comparing the effectiveness of both of those sugar as CPA that OPU derived oocytes. Treated oocytes divided into two different groups. Oocytes with Trehalose treatment assigned as Treatment 1 (T1), while oocytes with HB treatment assigned as Treatment 2 (T2). Oocytes from both groups were then subjected to fluorescence diacetate (FDA) staining and observed with a fluorescence microscope to evaluate the oocytes viability.

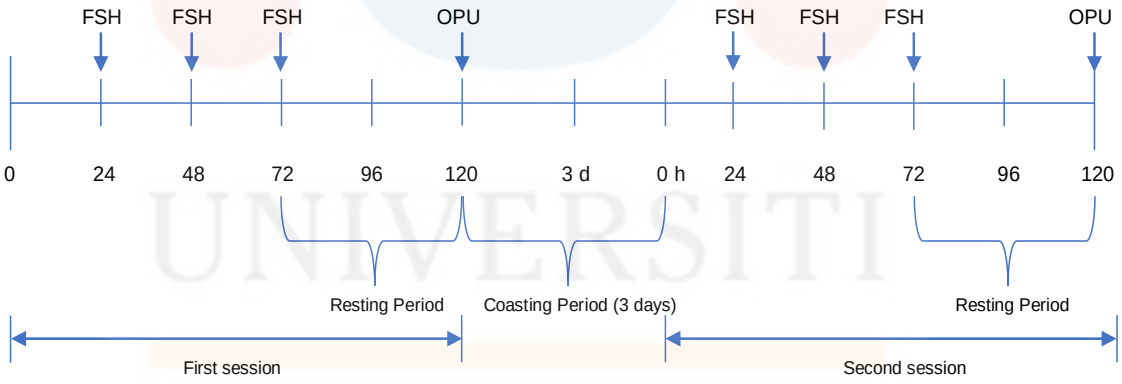

Figure 3.1: The superstimulation protocol for both sessions. Resting and coasting period were subjecting both cattle to FSH starvation or ceasing FSH support. Both "Resting period" and "Coasting period" serves the same function but termed differently for easy differentiation sessions.

3.2 Experimental Animal

This study was conducted at cattle farm located at Faculty of Agriculture, Princess of Naradhiwas University (PNU), Narathiwat province, Thailand (Latitude 6.4557° N, Longitude 101.7898°E). The experimental animals used were Brahman cattle with normal reproductive cycle and were free from any illness or any reproductive abnormalities (n=2). Both cattle were raised at the cattle farm of PNU which diet were kept with pasture and feed supplementation. Prior to cattle selection, cattle were inspected with ultrasonographic examination and estimation bodyweight of 200 kg. The research protocol and management in current study was approved by Animal Ethics Committee of PNU.

3.3 Superstimulation Protocol Using Follicle Stimulating Hormone (FSH)

This experiment adopted its superstimulation protocol that previously devised from Chasombat et al. (2013) with some minor modification that suited for this experiment. Each selected cattle were given total of 10 mL (200 mg) of FSH (Folltropin®, Vétoquinol USA, Inc., Texas, USA) within three days with the first two days were administrated with 2 mL while the third day administrated with 6 mL of FSH totalling 10 mL (200 mg). Each FSH administration performed once within 24 hours. The FSH was administered via 2.6 mL via 18G x 1.5” needle and 10 cc/mL NIPRO Hypodermic Syringe (NIPRO, Boulevard Bridgewater, New Jersey) totalling to 10 mL (200 mL). The dosage were given three days consecutively (72 hours) and given rest for two days before follicular aspiration sessions starts (Figure 3.1) . The follicular growth were examined

with portable ultrasound device that comes with intravaginal 7.5-MHz transducer (Honda®HS-2100, Honda Electronics Co. Ltd, Japan) with total two superstimulation and OPU sessions. Thus, the cattle were classified in Cattle 1 and Cattle 2 for easy identification to obtained results.

3.4 Ovum Pick Up (OPU) For Oocytes Collection Via Follicular Aspiration

The aspiration protocols in this experiment were accordance with some minor modification to the protocol from Chasombat et al. (2013) where was adopted from original protocol by the author Bols et al. (1997) and thus, this aspiration procedure was performed by Dr. Jakkhaphan Pitchayapipatkul from Faculty of Agriculture, Princess of Naradhiwas University. Some preparation was prepared before proceeding to follicular aspiration. Preparation of aspirated follicular solution, modified Dulbecco's phosphate buffered saline (mDPBS) was prepared for 1000 mL which served as stock solution. The mDPBS was further supplemented with 1% fetal bovine serum (FBS) and 2% Heparin sodium salt from porcine intestinal mucosa. The supplemented mDPBS was prepared for 250 mL which served as oocytes washing. The cattle were properly restrained before follicle aspiration can begin. Cattle were properly trapped with stanchion to limits its movement and immediate administration of 0.1 mg/kg body weight (BW) Xylazine (X-lazine, L.B.S. Laboratory Ltd., Part, Bangkok, Thailand) which waited 10 minutes for the cattle become less aggressive, followed by 5 mL of 2% Lidocaine Hydrochloride (Locana, L.B.S. Laboratory Ltd., Part, Bangkok, Thailand) for anaesthetised and complete immobilisation of the cattle. Thus, the follicles were aspirated with a portable ultrasound device that comes with intravaginal 7.5-MHz transducer (Honda®HS-2100, Honda

Electronics Co. Ltd, Japan) and 17 G x 490 mm cow ova vacuum needle (COVA Needle; Misawa Medical, Tokyo, Japan). An aspiration pump (Welch Model No.2515C-75, Gardner Denver Welch Vacuum Technology, Inc. Sheboygan, Wisconsin, USA) aspirated the follicle by punctured the vaginal wall with the COVA needle with ultrasound guided probe. The vacuum was set to 120 mmHg and 22 mL/min aspiration rate via the COVA needle. The aspirated follicles were collected to a 50 mL tube containing small volume of mDPBS follicular fluid which act as a saline and washing solution for aspirated follicles. The classification of aspirated oocytes or follicles were based accordance to the oocytes morphology which as previously described by Chaubal et al. (2006). Prior to *in vitro* maturation (IVM), the recovered cumulus cells were classified as: (1) Grade A, > four layers of cumulus cells ; (2) Grade B, three or four layers of cumulus cells ; (3) Grade C, one or two layers of cumulus cells ; (4) Grade D, denuded oocytes ; (5) Grade E, oocytes that are expanded with the cumulus cells. Thus, only oocytes from Grade D and E were not used and continue to maturation, whilst the rest proceeded to *in vitro* maturation (IVM).

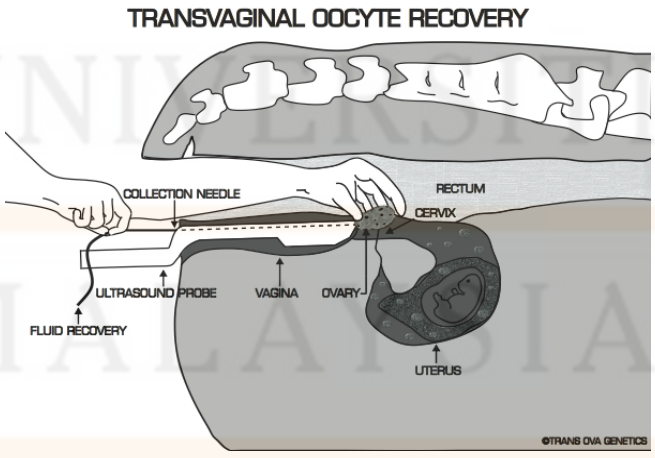

Figure 3.2: Ovum Pick Up (OPU) for oocytes collection via transvaginal oocytes aspiration using ultrasound probe

Sources: Trans Ova Genetics

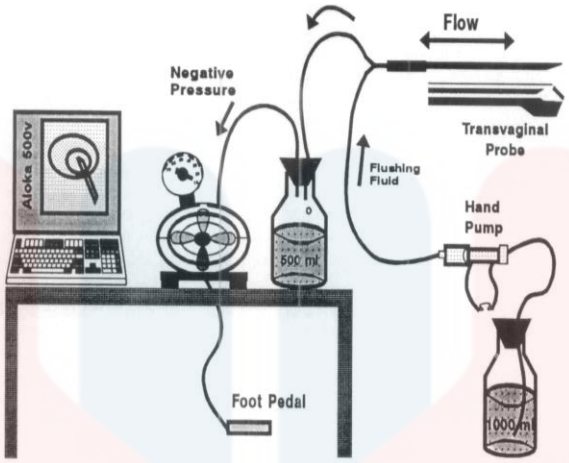

Figure 3.3: The OPU method preparation before oocytes collection with some minor modification that simply the procedures.

Source : Meintjes et al. (1995)

3.5 *In vitro* Maturation (IVM) Of Bovine Oocytes

Recovered oocytes from all cattle from aspiration protocol proceeds to *in vitro* maturation (IVM) procedure with some minor modification that been described before by Chasombat et al. (2013) which were adopted from pervious study mentioned by Ratto, Peralta, Mogollon, Strobel, and Correa (2011). Prior IVM, oocytes were washed several times with drops of 100 µL in 100 mm dish, with four times in TCM-199 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin stock (P-S stock) followed by three times in TCM-199 with 10% FBS and 1% P-S stock further supplemented with follicular stimulating hormone (FSH ; 1 µg/mL) + luteinizing hormone (LH ;1 µg/mL) + estradiol hormone (E_2 ; 1 µg/mL). The oocytes were washed for the last time with 35 mm dish containing mDPBS supplemented with FBS + P-S stock + 5% polyvinylpyrrolidone (PVP). The oocytes were then transferred to 35 mm dish containing four droplets of 100 µL (Figure 3.4) with immediate separation accordance to oocytes grade. Droplets

containing oocytes were covered with paraffin oil (Figure 3.4) and dishes were place into the CO_2 incubator at 38.5°C, 5% CO_2 in air at maximum humidity for 24 hours.

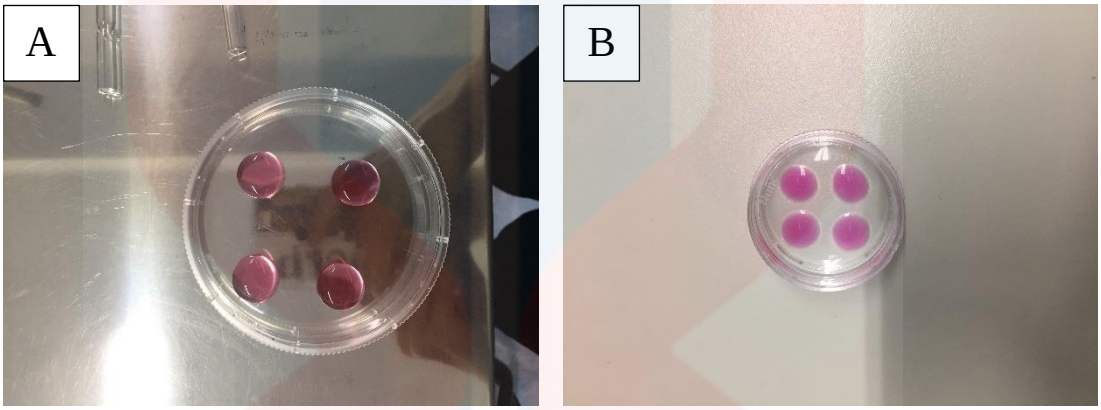

Figure 3.4: Preparation for oocytes before IVM, where (A) The oocytes were transferred into 100 mm dish for washing (B) Droplets containing oocytes with paraffin oil in 35 mm dish

3.6 Vitrification Of Matured Bovine Oocytes

The vitrification protocols were adopted with some alteration from pervious study Jakkhaphan Chasombat, Nagai, Parnpai, and Vongpralub (2015) which the protocol originated from the author Dinnyés et al. (2014). Prior to the vitrification procedures, the oocytes were separated into two different groups: T1 (27 oocytes) and T2 (33 oocytes). The vitrification procedure started with T1 followed by T2. The cumulus cells of oocytes from both groups were removed gently with pipetting in rising solution (RS) and thus, all the washing procedure were done in a 4 well dish. Oocytes from T1 were washed once with RS containing TCM-199 supplemented 20% newborn calf serum (NBCS) + 0.1% P-S stock and then continue washed three times in an vitrification solution 1 (VS 1) consisting TCM-199 + 20% NBCS + 1% P-S stock supplemented with 2% ethylene

glycol (EG) + 2% dimethyl sulfoxide (DMSO) and suspended for 14 minutes before exposing oocytes to vitrification solution 2 (VS 2) (17.5% EG + 17.5% DMSO + 50 mg/mL PVP + 0.3 M Trehalose in TCM-199 with 20% NBCS + 1% P-S stock) for washing three times within one minute totalling 15 minutes in duration for vitrification procedure. They were then dropped immediately as droplets for 1-2 µL containing 5 oocytes per drop (Solid surface vitrification method-SSV). The droplets were directly dropped onto the surface with folded aluminium foil that cooled around -150 to -180ºC by float on the surface of liquid nitrogen (LN_2). Prior to the vitrified droplets moved into 15 mL tube, the tubes were immersed into LN_2 to reduce the temperature. Utilising nitrogen-cooled forceps to move vitrified droplets into cooled 15 mL tube. Thereafter, tubes containing vitrified droplets were moved and stored in LN_2 tanks for ten days. The procedure above were also used on T2 with information of Honeybee (HB) based from Alfoteisy (2012) except T2 oocytes were exposed to different VS 2 (17.5% EG + 17.5% DMSO + 50 mg/mL PVP + 1 M HB (21.74% w/v) in TCM-199 containing 20% NBCS + 1% P-S stock) which the rest of the protocol remain the same.

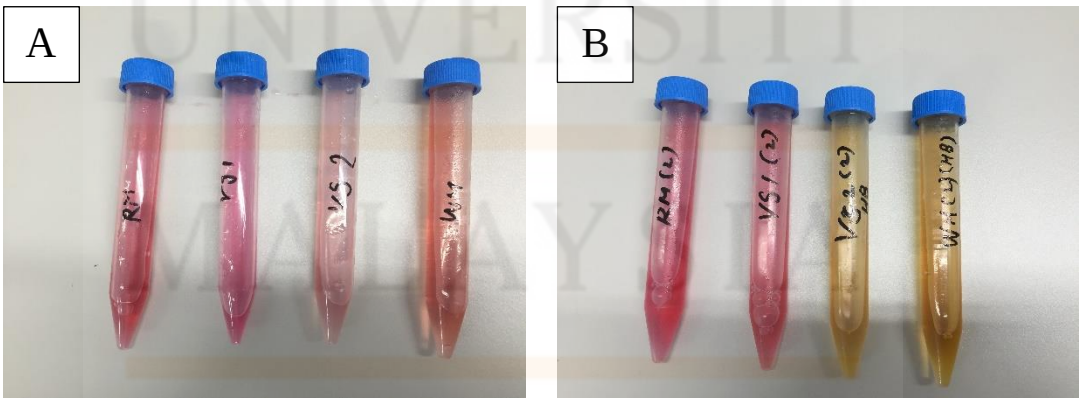

Figure 3.5: Vitrification media preparation for oocytes vitrfication, where from left to right RM, VS1, VS 2, WS which (A: T1 = Trehalose Treatment) and (B: T2 = Honeybee Treatment)

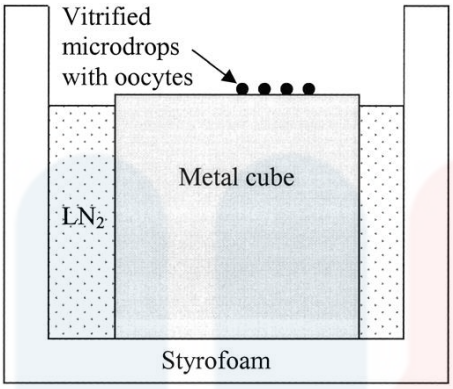

Figure 3.6: The solid surface vitrification (SSV) method setup. Instead the used of metal cube, aluminium foils were used and floated on liquid nitrogen (LN_2). The droplet oocytes were instantaneously vitrified upper surface of cooled aluminium foil.

Sources: Dinnyés et al. (2014)

3.7 Thawing Of Matured Vitrified Bovine Oocytes

The thawing procedure were also adopted from pervious study Jakkhaphan Chasombat et al. (2015) which were originated from author Dinnyés et al. (2014). Prior to thawing of oocytes, warming solutions (WS) were prepared, where T1 uses WS 1 (0.3 M Trehalose + 20% NBCS + 0.1% P-S stock in TCM-199), while T2 uses WS 2 (1 M HB (21.74% w/v) + 20% NBCS + 0.1% P-S stock) respectively. The vitrified droplets containing oocytes in the 15 mL tubes were move from the LN_2 tank using nitrogen-cooled forceps to a 4 well dish containing TCM-199 further supplemented with 0.15 M, 0.075 M and 0.0375 M Trehalose of WS 1 for one minute each. Thereafter, the oocytes were washed for the last time with TCM-199 until the following procedures. The procedures were also similar for T2, except vitrified droplets containing oocytes were transferred to a 4 well dish consisting of TCM-199 and supplemented with 0.5 M and 0.125 M HB of WS 2 for one minute each.

3.8 Viability Assessment

The viability assessment method was based with some modification from the author Jakkhaphan Chasombat et al. (2015) that was originally adopted from Mohr and Trounson (1980). Both group from T1 and T2 undergoes the same assessment method for verifying oocytes condition. The vitrified-thawed oocytes were stained with 2.5 µg/mL fluorescence diacetate (FDA) containing in mDPBS that further supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5°C for 2 minutes in a dark room without any emitting light sources. The stained oocytes were then washed three times with mDPBS in 5 mg/mL of BSA. The viability of those oocytes were observed under a fluorescence microscope (Eclipse TS 100; Nikon, Tokyo, Japan) with wavelength excitation of 460-495 nm and emission at 510 nm been used for better observation. Stained oocytes that projected with bright green fluorescence light regarded as live while oocytes that showed otherwise regarded as dead.

3.9 Statistical Analysis

The raw data for the oocytes viability vitrified-thawed with Trehalose and Honeybee (HB) were express through statistical analysis. Statistical Analysis System software (SAS 9.1) were used for assessing the raw data of oocytes viability. Student's T-test were used to allow proper analysis of raw data addresses the relationship between viability of oocytes and treatment of Trehalose and Honeybee (HB). The limitation of raw data in current study led to the used of student *t*-test for data analysis.

CHAPTER 4

RESULTS AND DISSCUSSION

4.1 The Ovarian Follicular Growth After FSH Superstimulation Protocol

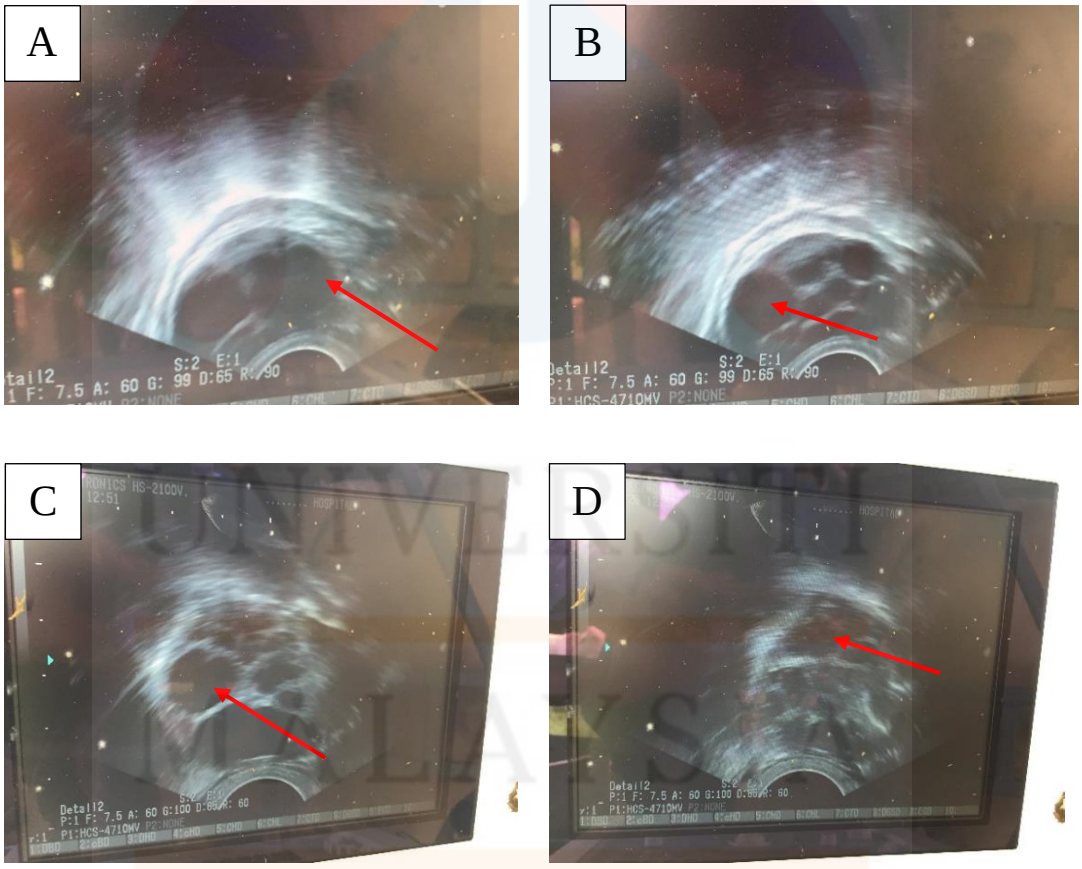

Figure 4.1: The ovarian follicular growth for Cattle 1 (A) left ovary and (B) right ovary. Similar responsiveness for Cattle 2 (C) left ovary and (D) right ovary. All of these follicular responses were from pre-OPU superstimulation of first session. All the superstimulation were responsive. Arrow indicates follicles that contains oocytes.

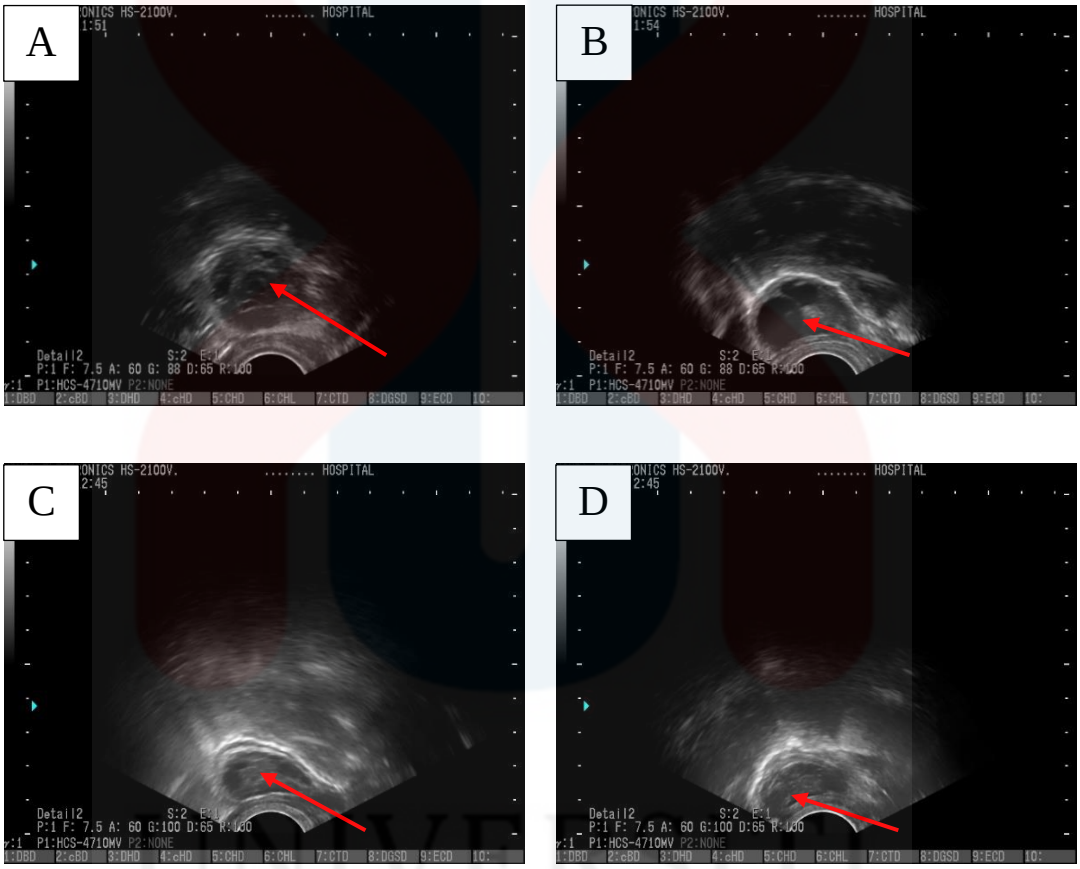

Figure 4.2: The ovarian follicular growth for Cattle 1 (A) left ovary and (B) right ovary. Similar responsiveness for Cattle 2 (C) left ovary and (D) right ovary. All of these follicular responses were from pre-OPU superstimulation of second session. All the superstimulation were poor. Arrow indicates follicles that contains oocytes.

Both two cattle were selected before superstimulation programme. Per session. each cattle were given 200 mg follicle stimulating hormone (FSH) for three days consecutively and rested for two days (FSH starvation) before follicular aspiration (OPU) with total for two sessions. The ovarian follicular growths were observed with a portable ultrasound. The first session both cattle shows high responsiveness of the FSH hormone injection. The size of the follicles (Figure 4.1) were large for both the Cattle 1 and Cattle 2. Both the left and right ovaries for each of the cattle showed positive responsiveness for the administration of FSH. However, the second session both Cattle 1 and Cattle 2 showed low responsiveness despite following the same hormonal administration protocol. Both left and right ovaries follicle sizes is small for both cattle in the second session (Figure 4.2).

Interesting finding in this study was unusual ovarian follicular growth in second session (Figure 4.2) rather than first session (Figure 4.1) after proceeded with current superstimulation protocol in this study. The first session has large ovarian follicular size and oocytes recovery after the superstimulation protocol. The result from this study agrees with a pervious study by Chaubal et al. (2007) that administration of FSH over three days able to increase the follicular response and oocytes recovery. A study by Goodhand, Staines, Hutchinson, and Broadbent (2000) showed similar trend, where multiple injections of FSH produces the highest number of oocytes compared to single FSH injection. Thus, emergence of new follicles are directly relation to the increased to the FSH (R. Boni, Roelofsen, Pieterse, Kogut, & Kruip, 1997; Sakhong, Sirisathien, Thammawung, Phasuk, & Yongpraub, 2014). Contradictory on the second session FSH injection in comparison which presented with small ovarian follicular size despite both first and second sessions follows same hormonal administrations protocols which FSH administration supposed significantly increased medium and large size follicles

(Goodhand et al., 2000). Such unusual follicular growth in this present study, might cause by the long "coasting period" (FSH starvation) between first and second session which lasted for three days (72 hours). The coasting period lasted 48 hours with additional 15 hours does not inhibit follicular growth and reduction of smaller follicular growth (Jeyakumar, 2004) which current study suggesting that extended coasting period might resulted in poor and inhibition of follicular growth. This study also in agreement that coasting for 48 hours improve oocytes competence because the follicular environment from exogenous FSH support permits oocytes maturation despite the used of p-FSH (van Wagtendonk-de Leeuw & de Ruigh, 1999) than only common FSH in current study. Coasting period that lasted for 92 hours, decreased oocytes competence containing large growing ovarian follicle (Nivet et al., 2012).

Such unexplainable phenomenon could also cause by the reduced amount of FSH administration in the second session due to lack of FSH supply. Exogenous FSH treatment influences the follicular growth numbers and size of follicle (Chasombat et al., 2013) which might able to explain the reduced amount of follicle when FSH administration were limited. Some previous studies, synchronises the cattle cycle in estrus and remove dominant follicles to initiate of new follicular wave followed by FSH superstimulation (Chasombat et al., 2013; Viana et al., 2010) which did not perform both the sessions in this current study which might explains the greater follicular growth in the first session rather than the second session.

OPU protocol in this present study involves ultrasound guided transvaginal follicular aspiration that might be responsible for adverse effect towards the cattle and oocytes. Thus, the OPU protocol could provide explanations for low or none oocytes recovery in the second session. The first session in this study presented with high number of follicles population which it would require more number of punctures from the needle

to aspirate the follicles that could led to ovarian lesion (Sá Filho et al., 2009) but dissimilar with author Chastant Maillard et al. (2003) that pointed OPU protocol does not have adverse impact towards the cattle. Successive repeated follicular punctures during follicular aspiration might result in increase of connective tissues in albuginea which subsequently led to the hardening of ovarian consistency (Petyim, Bage, Forsberg, Rodríguez-Martínez, & Larsson, 2001). Follicular aspiration can cause injuries and alteration to the ovarian stroma (Espey, 1994) when attempting to aspirate small follicles (Backer, Kanitz, Nürnberg, Kurth, & Spitschak, 1996). Author Fry, Niall, Simpson, Squires, and Reynolds (1997) noted that various needle sizes with different vacuum pressure used can impact the oocytes quality and caused various oocytes recovery rate which 18 gauge needle size with 120 mmHg vacuum pressure utilized in this study led to inconsistently results. Both the sessions were conducted by the same operator in this study which the operator has years of experience performing OPU. Thus, operator might not be the contribution to the significant influence to the low oocytes recovery rate as pointed by author Fry et al. (1997). The OPU protocol in current study only performed once a week for both sessions which might led to the follicular dynamics not fasten enough to cause rapid emergence of follicular population (Raffaele Boni, Campanile, & Zicarelli, 1995). Thus, it might able to explain the poor follicular response for the second session in the present study despite several previous studies have shown the follicular puncture does not affect the ovary functionality (Petyim et al., 2001).

The frequency of follicular aspiration also be discussed here. A study by Sá Filho et al. (2009) also indicated that relatively short inter-aspiration interval could have approximately reduction of 27% in the number of follicles due to the short duration time for the ovaries to heal from the previous aspiration session. Those mention effects could be the possible explanation to the poor follicular performance in the second session

despite the mentioned effects were from short inter-aspiration interval (twice a week) while the present study was longer in interval for aspiration (once a week). The used of multiple different cattle with similar characteristics but subjected to same OPU protocols which accordance to the individual studies which are 80 cattle (Chasombat et al., 2013), 10 cattle (Denpong et al., 2012) and 27 cattle (Bols et al., 1995). In contrast with current study, only two same cattle were involved for both session that might have prevent dominant follicle negatively influences the smaller follicles to produce corpus luteum and estrogen subsequently disrupt the cycle (van Wagtendonk-de Leeuw, 2006) indicating short interval of follicular aspiration can result in poor follicular response in second session.

4.2 Oocytes Collection From Ovum Pick Up (OPU)

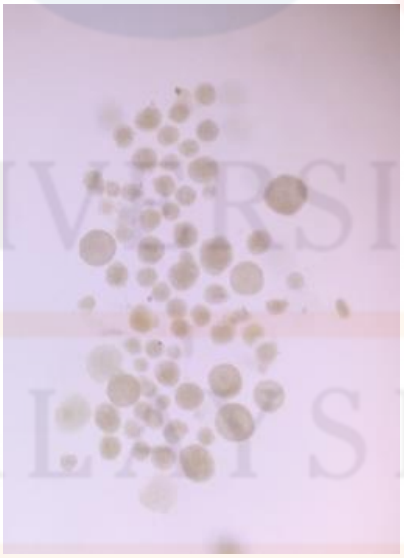

Figure 4.3: Recovered oocytes from first OPU session before *in vitro* maturation (IVM).

Table 4.1: Recovered oocytes classification prior to *in vitro* maturation (IVM)

Oocytes classification	**First session**	
	n	**%**
Grade A	35	58.3
Grade B	12	20.0
Grade C	10	16.7
Grade D	3	5.0
Grade E	0	0
Total	**60**	

Oocytes classification based from pervious author by Chaubal et al. (2006) (Refer page 33)

Interestingly, the superstimulation protocol in this study had led to the high number of Grade A (n=35) and B (n=12) oocytes which this study in agreement with author Goodhand et al. (2000) that oocytes quality improved quality with the existence of increased follicular size when there were exogenous FSH administration. Thus, this study also suggesting that following the superstimulation protocol in this study could result in high number of Grade A and Grade B oocytes while lower number for Grade C, D and E but further improve the protocol by reducing the coasting period between sessions.

4.3 Matured Vitrified Oocytes before FDA staining

Table 4.2: Viability of post-warmed oocytes from each treatment after *in vitro* maturation (IVM) and before FDA staining.

Treatments	Vitrified Oocytes (*n*)	Retrieved Oocytes (*n*)	Degenerated Oocytes (*n*)	Viability* (%)
Trehalose (0.3 M)	27	27	0	100
Honeybee (1.0 M)	33	33	0	100

***** Percentage of viability bovine oocytes were before FDA staining.

Table 4.2 summarised the numbers of vitrified, retrieved, degenerated bovine oocytes and viability percentage of bovine oocytes. All the data presented were oocytes from the first session after *in vitro* maturation (IVM) but before FDA staining. The vitrified oocytes from trehalose treatment (T1) were 27, while vitrified oocytes honeybee treatment (T2) were 33. The uneven separation of oocytes due to the difficulty of oocytes handling when transferring the oocytes. The number of retrieved oocytes remain same throughout the process. However, the bovine oocytes viability from both group which based morphological damage which observed directly from the microscope. Thus, the bovine oocytes viability were 100% before FDA staining.

4.4 Matured Vitrified oocytes after FDA staining

Table 4.3: The percentage of viability and number of vitrified oocytes according to treatment (Trehalose and Honeybee) after FDA staining.

Treatment	Number of vitrified oocytes*	Viability (*n*) (%)
Trehalose (0.3 M)	27	19/27 (70.4)[a]
Honeybee (1.0 M)	33	30/33 (90.9)[b]

Different superscript letter within the same column are significantly differed according to student *t*-test ($p \geq 0.05$).

* Oocytes were the combination from both Cattle 1 and Cattle 2 in the first session.

Table 4.3 shows a total of 60 oocytes from both cattle were separated into two different groups. The group with Trehalose treatment (T1) were assigned with 27 oocytes, while Honeybee (HB) treatment (T2) were assigned with 33 oocytes. The uneven number of oocytes separations were due to the difficulty of oocytes handling when transferring the oocytes. Thus, all these oocytes were subjected to different vitrification medium. Table 4.3 also shows, the percentage of oocytes viability from T2 (90.9%) were significantly higher compared to oocytes from T1 (70.4%) with total of vitrified 60 oocytes. It shows that the HB more effective as cryoprotectant in vitrification medium compared to Trehalose in 0.3 M. Due to the different total number of vitrified oocytes, Student's *t*-test (SAS 9.1) were used to properly determine the relationship between the treatment (Trehalose and Honeybee) and the oocytes viability due to the limitation of raw data obtained. Thus, T1 (90.9%) were significantly different compared to T2 (70.4%) despite the close presented percentage.

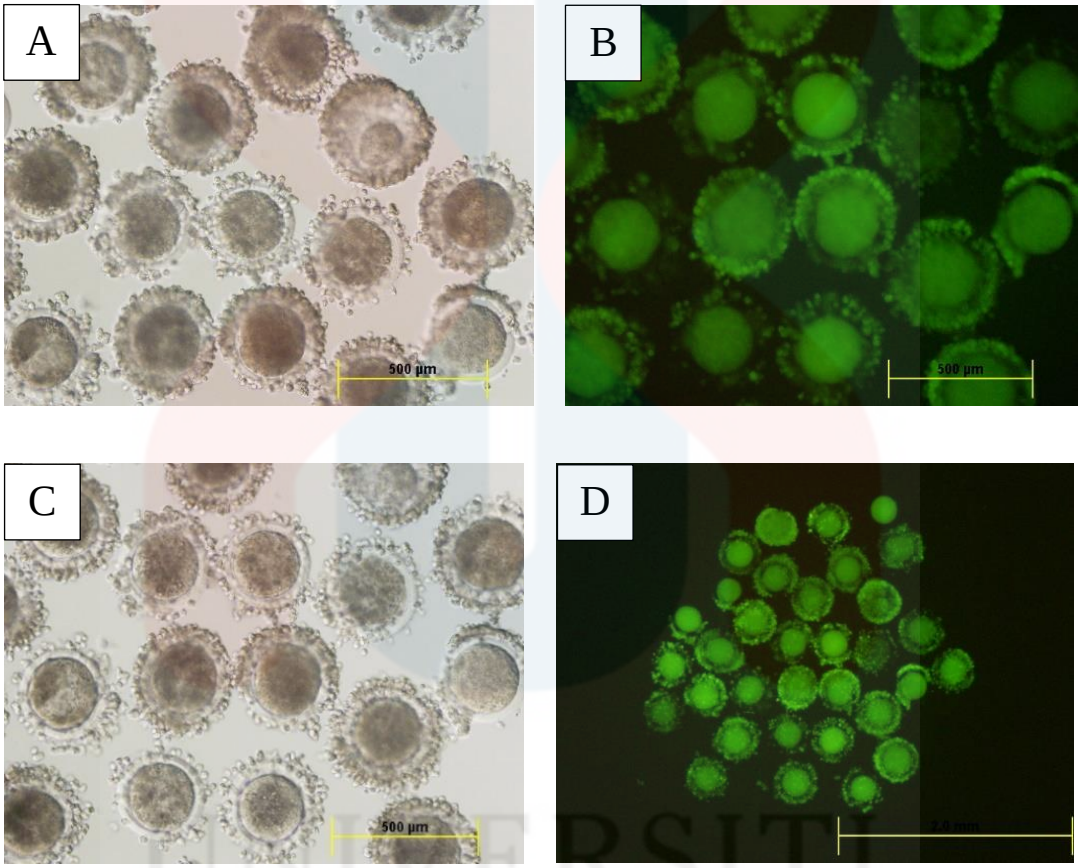

Figure 4.4: Fluorescein diacetate (FDA) staining of matured vitrified-thawed bovine oocytes in treatment 1 (T1) Trehalose (1.0 M) (A: Bright field and B: FDA) and treatment (T2) Honeybee (HB) (C: Bright field and D: FDA).

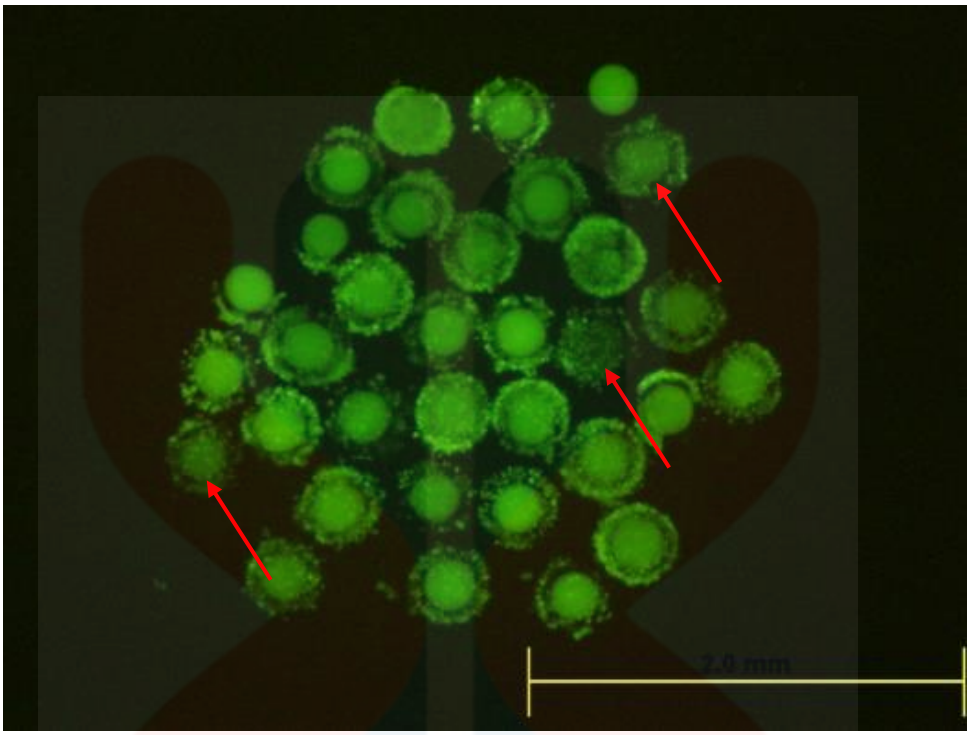

Figure 4.5: Oocytes viability are visible under FDA staining. Arrow indicates oocyte was no longer viable, while oocytes without arrow with bright green fluorescence light indicates viable. All the oocytes from treatment 2 (T2) Honeybee (HB).

The novelty finding of this current study would be high viability achieved from bovine oocytes after vitrifying with HB as the cryoprotectant integration into vitrification protocol. Such performance of HB in this study might due to similar dehydration and volumetric alteration status of bovine oocytes compared to sucrose (Alfoteisy, 2012). The superior dehydration of HB might have enhances cell osmotic gradient across the cell membrane (Alfoteisy, 2012) which subsequently prevents formation of intracellular ice crystals in cells when oocytes were expose to LN_2 (Hainaut & Vaught, 2017). Such preventive intracellular ice crystal formation might due the chemical properties in HB which naturally existed as a mixture of 25 sugars that were mostly glucose and fructose with the estimation 95%-97% of dry matter content (Bogdanov et al., 2008) that lead to high oocytes viability in this study. HB seen more success in spermatozoa cryopreservation by utilizing HB as extenders (El-Sheshtawy et al., 2016; Fakhrildin &

Alsaadi, 2014) which probably able to provide some explanation of higher bovine oocytes viability than Trehalose in current study. The combination of sugar such as monosaccharides, disaccharides, oligosaccharides and polysaccharides from HB (Bogdanov et al., 2008) allows spermatozoa to use those sugar as source of energy that lead to the increased of survival and motility during spermatozoa exposed to cryopreservation protocol (Yimer et al., 2015). The natural combination of HB composition that present in HB also provides a more superior antioxidant capabilities compared to single element antioxidant (Christy E. et al., 2011) which explains HB has been known for protecting cell organelle from oxidative damage or oxidative stress (Bogdanov et al., 2008) which further suggesting the superiority of HB than Trehalose in this current study. Vitrifying oocytes with HB as cryoprotectant resulted in greater oocytes viability and embryonic development than sucrose (Alfoteisy, 2012). The greater post-warming of blastocyst formation rate as speculated by Alfoteisy (2012) might due to the various bioactive composition that existed in HB. Amino acid from HB also might be responsible for the speculation of some amino acid that able to stabilize phospholipid cells (Anchordoguy, Carpenter, Loomis, & Crowe, 1988).

Another interesting highlight in current study was the current cryoprotectant (CPA) media with the integration of Honeybee (HB) as the sugar component for vitrification subsequently achieved high oocytes viability. CPA limits or inhibits the crystallization of intracellular in cells when exposed to LN_2 (Hainaut & Vaught, 2017). High concentration from the CPA subsequently led to toxicity and chilling effect that can cause cytoskeleton and organelles changes (Gábor Vajta, 2000). In the present study, combination of both ethylene glycol (EG) and dimethyl sulfoxide (DMSO) as CPA for vitrification might had led to better survivability of bovine oocyte which current study in agreement with Vicente and García-Ximénez (1994) that combination of both EG and DMSO permits better

survivability compared only EG *per se*. The superiority combination of both EG and DMSO was higher comparing to only DMSO alone for bovine oocytes vitrification (Cetin & Bastan, 2006). The addition of sugar as CPA media able to inhibit injuries from dehydration (Wright et al., 2004). This present study showed that higher bovine oocytes viability ($P \geq 0.05$) was achieved with HB medium (n=33) compared to Trehalose medium (n=27). Superior antioxidant and anti-inflammatory effect from HB natural composition (Bogdanov et al., 2008) might had led HB to be the superior as CPA media than Trehalose in this study. Despite Trehalose was less superior compared to HB based only from oocytes viability in this study, Trehalose was superior than other sugars in various aspect such as maintaining membrane stabilization and preserving the biologic materials in cells (J. H. Crowe, Carpenter, & Crowe, 1998).

HB consist mixture of 25 sugars but were mainly glucose and fructose (Bogdanov et al., 2008) which may suggest glucose and fructose that presence in HB might have contributed to higher viability bovine oocytes than Trehalose in this study. Author Fernández-Reyez et al. (2012) suggested by that monosaccharides sugar such as fructose and glucose are suitable for supplementation for spermatozoa freezing extenders. Pervious literatures showed that monosaccharides (glucose and fructose) are more effective compared to disaccharides (Trehalose) in spermatozoa cryopreservation as extenders. Molinia, Evans, and Maxwell (1994) pointed that monosaccharides are more effective than disaccharides when cryopreserving ram spermatozoa resulting and lead to a higher motility. Study by Garcia and Graham (1989) mentioned that monosaccharides and disaccharides are more effective that trisaccharide for bovine spermatozoa. Trehalose are also capable as extenders in spermatozoa cryopreservation with achieving good levels of motility, viability and acrosome integrity for boar spermatozoa (Athurupana et al., 2015). However, author Fernández-Santos et al. (2007) pointed that different

spermatozoa species can result in various extender performance status which this study speculating the types of animal species might contributed to the lower viability bovine oocytes. To date, there are little to no studies that directly compare the performance of both HB and Trehalose as sugar for bovine oocytes cryopreservation which this current study attempting to answer despite all the data refers solely only from bovine oocyte viability.

The other interesting finding in this current study was high viability of oocytes by solid surface vitrification (SSV) with Honeybee (HB) as integrated sugar component into the vitrification solution. To date, there was poor documentation of utilizing SSV with HB integrated into the sugar component in vitrification solution. Despite vitrification solution with HB treatment as CPA in current study did not subjected to toxicity test, this novel vitrification protocol still able to achieve high oocytes viability. Vitrification of cell with high CPA concentration can led to toxicity effect to cell (Kuwayama, Vajta, Kato, & Leibo, 2005) which high concentration being utilized in this study. Thus, this study was suggesting that the used high concentration of CPA with HB treatment was possible with SSV method. The natural combination of HB composition allows HB to have superior antioxidant and anti-inflammatory effect (Bogdanov et al., 2008) which might permits the utilization of higher CPA concentration. SSV method also might had contributed to such high oocytes viability. SSV utilized microdrops for direct contact with LN_2 to achieve high cooling rate and heat exchanged for cells (Dinnyés et al., 2000). SSV vitrification method could yield high surviving rate of bovine oocytes that subsequently proceeded oocytes with IVF (Sripunya et al., 2010). However, SSV requires extensive practice in controlling oocytes containing droplet and which oocytes tend to loss in the glass capillary (Sripunya et al., 2010) which in this study SSV was performed by an experience person which gives the explanation that the number of retrieved oocytes

remain same throughout the process (Table 4.2). The competence of SSV can be clearly reflected in fertilized oocytes which porcine oocytes vitrified by SSV can develop in blastocysts stage *in vitro* (Gupta, Uhm, & Lee, 2007) and even potential nuclear transfer (Tamás Somfai et al., 2006).

The intrinsic and extrinsic factors that responsible were beyond the control of this study that might led to the unexplainable phenomenon that occurred in second session with small follicular growth in this study. Both cattle in present study were subjected to short-term hormonal treatment during hot weather that might cause impaired follicles and poor oocytes qualities (Roth et al., 2001). A study by Roth, Arav, Braw-Tai, Bor, and Wolfenson (2002) also indicates a more effective hormonal treatments were necessary for improving the oocytes quality during autumn because poor performance presented in the rate of blastocyst formation despite bovine were administrated with bovine somatotrophin (bST) and FSH. The superstimulation protocol (3 days, 2 rests) that been utilized in current study might not be suitable for Brahman breed cattle as different breed response differently to follicular growth from different superstimulation protocol in Holstein cows (De Roover, Genicot, Leonard, Bols, & Dessy, 2005; Sendag et al., 2008), Angus cross cow (Chaubal et al., 2006), and Thai native cattle (Chasombat et al., 2013). Superovulation of some cattle donors were fairly consistent in yielding of large number of oocytes while otherwise for some donor cattle (Hasler et al., 1995) indicating both cattle in this study have fairly inconsistent superovulation cycle. The dietary intake can influence the reproductive function including oocytes quality and changes in ovaries function (Boland, Lonergan, & O'Callaghan, 2001) that the dietary intake could had influence both cattle in this study. The dietary feed given to both cattle not present due to limitation of resources in this study.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

High viability can be achieved by vitrifying bovine oocytes using cryoprotectant (CPA) with the integration of Honeybee (HB) with the media. The natural composition of HB could had resulted in the superior antioxidant effect that inhibits the crystallization of intercellular of cells than Trehalose. Vitrifying with SSV using HB vitrification media can also permits high viability bovine oocytes.

Superstimulation protocol prior to OPU are effective for ovarian growth. The first session with FSH support presented with positive follicular growth. The prolonged coasting period might have interfered with follicular growth dynamics which causes poor follicular growth in the second session.

Limitation of resources and time constraint were the major limitation in this study. Various minor limitation also existed in this study. Only two session of superstimulation performed in this study were the direct result from limitation of resources and time. Conventional superstimulation protocol usually proceeded multiple of times rather only two sessions which gives the poor follicular growth in the second session. The limitation of resources also led to the prolonged coasting period (FSH starvation) as well as

exogenous FSH support given to cattle were limited by the last FSH administration in the second session. Two cattle rather than multiple different cattle which performed by usual superstimulation protocol were direct result from limited resources. Data that details about follicular size and growth were insufficient to evaluate the effectiveness of current superstimulation protocol. Other minor limitation that affects the results would be different cattle breed respond to superstimulation protocol differently which varies the overall results. Pasture with supplementation given to cattle in this study were not properly evaluated and presumed suitable to be given to cattle. The hot weather in Southern Thailand could also detriment the cattle in follicular growth performance.

5.2 Recommendation

Further studies should subject those vitrified bovine oocytes with HB vitrification media to *in vitro* fertilization (IVF) for better gauging the performance of this novel HB integrated media. Oocytes that subjected to LN_2 during vitrification experienced hardening of zona pellucida (Ambrosini et al., 2006) that subsequently preventing spermatozoa penetration into the oocytes (Denpong et al., 2012). Successful penetration of spermatozoa to vitrified oocytes indicating HB media has the capabilities similar to other sugar that permits the integration sugar component in vitrification media. The used of HB in oocytes cryopreservation led the foundation to more possibilities of HB potential as sugar component to be integrated into CPA that can led to various oocytes cryopreservation performance.

Further studies can also be done by varying the vitrification method. Despite high oocytes viability achieved by SSV in this study, SSV requires extensive practice in

controlling droplets containing oocytes which losses some oocytes in glass capillaries during transfering were common (Sripunya et al., 2010). Vitrifying oocytes with SSV were not easily accessible by masses which utilizing conventional straw, open pulled straw (OPS), cryotop and cryoloop as open carrier could result in similar oocytes viability that achieved in this study. Vitrification with open carriers could improves warming rates that led to *in vitro* development of cells (Chaves et al., 2014). Utilization of novel HB vitrification media with different vitrification method permits better evaluation on the flexibility of this novel vitrification media.

The poor follicular response from the second session might resulted from the extended coasting period in the superstimulation using FSH administration. Previous studies noted that coasting period yielded greater oocytes competence at 48 hours (Jeyakumar, 2004; van Wagtendonk-de Leeuw & de Ruigh, 1999) indicating negative impacts on extended coasting period. Reduce coasting period could led to more responsive and greater ovarian follicular growth. Time constrain and limitation of resources led to only two session, but cattle subjected to multiple sessions as other conventional superstimulation protocol can be used for modification to current protocol for future work. Two session inhibits the clear information for ovarian follicular growth dynamics and thus does not project the superstimulation protocol effectiveness. Synchronization of cattle cyclic would allow better prediction on FSH support influencing the follicular dynamics of the cattle.

Detailing the follicular size in the further study can be taken into consideration as follicle size influences oocytes competence (Blondin, Vigneault, Nivet, & Sirard, 2012). Exogenous FSH support to cattle with alteration to current superstimulation protocol could result yield larger follicular size. Another important modification to future work is the used of multiple different cattle but similar characteristic rather than the used of same

cattle in this study (n=2). Extensive follicular punctures may occur only on two cattle than multiple different cattle which should be the alteration to current experimental design for greater follicular growth and oocytes emergence.

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APPENDIX

mDPBS stock (1000 mL)

Sodium Chloride (NaCl) = 8.0 g

Potassium Chloride (KCl) = 0.2 g

Monopotassium Phosphate (KH_2PO_4) = 0.2 g

Disodium Hydrogen Phosphate (Na_2HPO_4) = 1.15 g

Glucose ($C_6H_{12}O_6$) = 1.0 g

Pyruvic Acid ($C_3H_4O_3$) = 0.036 g

Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) = 0.1374 g

Magnesium Chloride Hexahydrate ($MgCl_2$-$6H_2O$) = 0.1 g

P-S stock = 1.0 mL

mDPBS stock for OPU (250 mL)

mDPBS stock = 242.5 mL

FBS = 2.5 mL

Heparin = 5 mL

mDPBS stock for oocytes washing (30 mL)

mDPBS stock = 28.1 mL

5% PVP = 1.5 g (1.5 mL)

FBS = 0.2 mL

P-S stock = 0.2 mL

Other stocks used in this study;

FSH stock = 50 mg/mL

LH stock = 10 mg/mL

E_2 stock = 1 mg/mL

P-S stock = 1 mg/mL