# Effect of Transportation in Liquid Nitrogen on Viability Of Bovine Oocytes

By

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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:

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I certify that the report of this final year project entitled “Effect of Transportation in Liquid Nitrogen on Viability of Bovine Oocytes” by Ong Fei Fun, matric number F14A0323 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, University Malaysia Kelantan.

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_____

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**Effect of Transportation in Liquid Nitrogen on Viability of Bovine Oocytes**


## ABSTRACT

Oocyte cryopreservation has significantly increased as freezing technology improves. Excess oocytes obtained during assisted reproduction technology therapies can be stored safely and indefinitely with cryopreservation. This technology promises improvement in the establishment of oocytes banks, permitting female genetic material to be stored unfertilized until the desired male germplasm is selected. However, there is concern regarding the transportation effect as the movement to and from the central laboratory may have adverse effects on vitrified oocytes. Currently, studies on the effect of transportation towards the viability of oocytes are still scarce. Studies showed that vibrations that occur during transportation will disrupt the cells cytoskeleton. In contrast, it has also been suggested that the low frequency of vibration facilitates the pregnancy rate of embryo. The objectives of this study were 1) to investigate the effect of transportation on oocytes and 2) to determine the viability of oocytes after transportation. In this study, bovine ovaries that were collected from slaughterhouses were sliced to retrieve oocytes for *in vitro* maturation (IVM). After 24 hours of IVM, the oocytes were vitrified using open-pulled straw and plunged directly into liquid nitrogen. The vitrified oocytes were divided into three groups whereby one was the control group and other groups were exposed to vibrations of 180 and 300 rpm for one hour (to simulate transportation via airplanes and trucks), respectively. The viability of oocytes was examined using fluorescein diacetate stain. Result showed that there was no significant difference between control and experimental groups ($P>0.05$). The viability of control, truck and airplane groups recorded were 95%, 100% and 100%, respectively. The mechanical vibration is suggested to have no harmful effect but serves as a stimulation to induce intracellular communication which is essential in cell differentiation. In conclusion, the vibration had no adverse effect on the viability of vitrified bovine oocytes.



Keywords: Transportation, vibration, liquid nitrogen, oocytes, viability

**KESAN PENGANGKUTAN DALAM NITROGEN CECAIR TERHADAP KADAR PENGHIDUPAN OSIT BOVIN**


## ABSTRAK

Kriopreservasi oosit untuk pemeliharaan kesuburan telah meningkat dengan ketara disebabkan teknologi pembekuan yang bertambah canggih. Oosit yang berlebihan semasa terapi teknologi pembiakan dibantu boleh disimpan dengan menggunakan teknologi vitrifikasi. Teknologi ini menyumbang kepada penubuhan bank oosit, membolehkan oosit disimpan dengan keadaan baik sehingga sperma yang sesuai dipilih. Walau bagaimanapun, terdapat kebimbangan mengenai kesan pengangkutan terhadap oosit dibeku kerana pergerakan ke dan dari makmal pusat mungkin mempunyai beberapa kesan buruk terhadap oosit yang telah dibeku. Pada masa kini, maklumat mengenai kesan pengangkutan terhadap viabiliti oosit masih tidak mencukupi. Beberapa kajian menunjukkan getaran yang berlaku semasa pengangkutan akan mengganggu sitoskeleton sel. Manakala beberapa kajian menyimpulkan bahawa frekuensi rendah getaran memudahkan kadar kehamilan embrio. Oleh itu, kajian ini dijalankan dengan objektif untuk menyiasat kesan pengangkutan terhadap oosit dan menentukan viabiliti oosit selepas pengangkutan. Dalam kajian ini, ovari lembu telah dikumpul dari rumah sembelih. Oosit dikeluarkan dan dipilih untuk pematangan *in vitro*. Selepas dikultur dalam media pematangan selama 24 jam, oosit dikeluarkan dan dibeku dengan menggunakan *open pulled straw* yang diubahsuai. Oosit yang telah dibeku telah dibahagikan kepada tiga kumpulan, salah satu kumpulan dijadikan kumpulan kawalan, kumpulan lain telah terdedah kepada getaran di frekuensi 180 dan 300 rpm selama satu jam untuk mensimulasikan pengangkutan kapal terbang dan trak. Penilaian viabiliti terhadap oosit kumpulan kawalan dan rawatan telah dijalankan dengan menggunakan *fluorescein diacetate* selepas pencairan. Keputusan menunjukkan perbezaan antara viabiliti kumpulan kawalan dengan eksperimen adalah tidak signifikan (P> 0.05). Viabiliti kumpulan trak dan kapal terbang adalah sangat tinggi, iaitu masing-masing 95%, 100% dan 100%. Viabiliti yang tinggi menunjukkan bahawa getaran tidak mempunyai kesan buruk terhadap viabiliti oosit.



Kata kunci: Pengangkutan, getaran, nitrogen cecair, oosit, viabiliti

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## LIST OF ABBREVIATION

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ART | Assisted reproductive technology |
| $CO_2$ | Carbon dioxide |
| cm | Centimetre |
| COC | Cumulus-oocyte complex |
| DMSO | Dimethyl sulfoxide |
| EG | Ethylene glycol |
| FCS | Fetal calf serum |
| FDA | Fluorescein diacetate |
| FSH | Follicle-stimulating hormone |
| g | Gram |
| Hz | Hertz |
| IVM | *In vitro* maturation |
| LN2 | Liquid nitrogen |
| L | Litre |
| LH | Luteinizing hormone |
| mRNA | Messenger ribonucleic acid |
| µg | Microgram |
| mg | Milligram |
| ml | Millilitre |
| M | Molar |
| OPS | Open-pulled straw |
| OPU | Ovum picked-up |

| | |
|---|---|
| PB1 | Phosphate buffer 1 |
| PBS | Phosphate buffer solution |
| rpm | Revolutions per minute |
| TCM-199 | Tissue culture medium-199 |

## LIST OF SYMBOLS

| | |
|---|---|
| % | Percentage |
| °C | Degree celcius |
| > | More than |
| < | Less than |
| ± | Plus-minus |

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

Cryopreservation is commonly used to preserve intact living cells and tissues at a very low temperature. Whittingham et al. (1972) reported the first successful cryopreservation of mouse embryos and later the first birth after cryopreserving mouse oocytes in liquid nitrogen was recorded in 1977. Fuku, Kojima, Shioya, Marcus, & Downey (1992) also reported the first success in obtaining calves after freezing and thawing in-vitro mature bovine oocytes. Until now, there are two basic cryopreservation methods that have been introduced, slow freezing which is the first technique to be developed and vitrification. Slow freezing is known as equilibration freezing whereby extracellular water slowly crystallizes under controlled-cooling rate conditions and the resulting osmotic gradient draws water from the intracellular compartment.

In contrast, vitrification is a non-equilibrium method where both intra and extracellular components are vitrified after dehydration (Saragusty J. & Arav A., 2011). This provides a higher survival rate and minimizes the deleterious effects on post-warming oocyte and embryo morphology (Rezazadeh Valojerdi, Eftekhari-Yazdi, Karimian, Hassani, & Movaghar, 2009). However, it requires extremely high cooling rate and higher concentration of cryoprotectants to achieve rapid freezing with minimum

freezing injury (Gábor Vajta & Nagy, 2006). Rall & Fahy (1985) were the first to report a high proportion of mouse embryos survived after vitrification at -196°C. Five years later, another attempt for human cleavage-stage embryo was done with successful delivery (Gordts, Roziers, Campo, & Noto, 1990). There are several reports that showed that vitrified oocytes gave higher post-thaw survival, fertilization, implantation and pregnancy rates compared to slow freezing method (Cobo & Diaz, 2011; Herrero, Martínez, & Garcia-Velasco, 2011; Zhang, Liu, Xing, Zhou, & Cao, 2011). Today, vitrification has been widely used to cryopreserve reproductive cells in assisted reproductive technology (ART).

Oocyte cryopreservation has significantly increased in fertility preservation as the freezing technology has improved. The excess oocytes during ART therapies could be stored safely and improve the establishment of oocyte banks, permitting female genetic material to be stored unfertilized until the desired male germplasm is selected. However, there is concern regarding the transportation of vitrified oocytes as the movement to and from the central laboratory may have several adverse effects on vitrified oocytes (Gandhi, Allahbadia, Kagalwala, & Madne, 2015). McDonald et al. (2011) had investigated the effect of transporting cryopreserved oocytes and found that the survival rate 3 hours after thawing of vitrified and shipped oocytes is significantly lower than that of non-shipped vitrified oocytes. The effect of transportation raises the concern in researchers to improve the survival rate, viability and the ability to be fertilized of vitrified, shipped oocytes.

The objective of this study was to determine the transportation effect with different vibration frequencies on the viability of oocytes. In this experiment, the ovaries were collected from slaughterhouse and transported to laboratory within 2 hours after

slaughtering. The collected ovaries were washed using phosphate buffer solution (PBS) with antibiotic, and then followed by oocyte recovery. Oocytes recovered were allowed to mature *in vitro* to standardize the development stages before vitrification. The vitrified oocytes were divided into three groups, whereby one is control, and the remaining two groups were exposed to 300 and 180 rpm of vibration load to mimic the transportation environment of a truck and an airplane, respectively.

## 1.2 Problem Statement

There are many livestock breeds experiencing a gradual diminishment of genetic diversity. Artificial selection pressure increased rapidly due to the wide use of artificial reproductive techniques such as artificial insemination, embryo transfer and the use of sexed semen (Powell, Norman, & Sanders, 2003; Sørensen, Voergaard, Pedersen, Berg, & Sørensen, 2011). The high intensity of artificial selection makes the recovery of underestimated traits quickly impossible. Therefore, overall genetic resources must be conserved by the international community in order to conserve the potential to face future challenges such as climate changes, emerging diseases, pressure on land and water and shifting market demands.

Oocyte cryopreservation is important to conserve maternal genome for establishment and maintenance of genetic banks (Vieira et al., 2002). The oocytes are safely stored in a frozen state until an appropriate male germplasm is selected. However, the vitrified oocytes have to be transported from central laboratory to other areas for research or breeding purposes. The journey of transportation varies depending on the destination, as well as the travel time. Generally, researchers would use land transport to reach their destination within one day but international collaboration requires air freight

transportation to minimize the travel time. During transportation, vibration is the main concern as a large vibration could give adverse effect to the oocytes. The viability and the quality of oocytes might be reduced after the transportation and resulting large loss of biological sample, time and money. In order to minimize the damage towards oocytes, the effect of transportation should be studied to maintain the viability of oocytes after transporting from one place to another.

## 1.3 Objectives

1. To investigate the effect of transportation with different vibration frequencies on oocytes using liquid nitrogen.
2. To determine the viability of oocyte after transportation.

## 1.4 Scope of Study

The study was carried out using bovine oocytes collected from slaughterhouse. Recovered oocytes with one or more layer of cumulus cells and evenly granulated oocytes were selected for *in vitro* maturation (IVM). After 24 hours of IVM, the mature oocytes were exposed to ethylene glycol (EG) and dimethyl sulfoxide (DMSO) for equilibration purposes. Then, the oocytes were immediately transferred to a vitrification solution that contained a higher concentration of EG and DMSO with the addition of sucrose for 45 seconds, followed by loading of oocytes into open-pulled straw (OPS). Vitrified oocytes were divided into three groups, one served as a control group which remained in the liquid nitrogen tank and not shipped, while the other two were in experimental groups that given vibration loads of 180 rpm and 300 rpm to mimic transportation for 1 hour. All groups of oocytes were warmed and examined after the

transportation. The viability of control groups and experimental groups were compared and examined using fluorescein diacetate (FDA) stain.

## 1.5 Significance of Study

The transportation of oocyte is crucial as the journey of the transportation might cause damage to oocytes, reduced viability and cause irreversible fertility loss. These detrimental effects should be avoided as these poor quality oocytes are not able to be utilized for research or breeding purposes. This results the wastage of oocytes from potential breed stock as well as financial loss. Thus, this study will provide information on the effect of transportation on the viability of oocytes. If any detrimental effects caused by transportation are seen, further research will be needed to facilitate ways to minimize it.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Ovaries

Ovary is the primary female reproductive organ that located in the abdominal cavity of cattle. The paired ovaries are oval to bean-shaped, 1 – 1.5 inches long and pale pink in colour. It serves as a site of gamete production where germ cells form follicles, develop and mature. The germ cells produced are named oocytes and carrying the maternal genome. Besides that, ovaries are also playing an important role as endocrine glands. They are producing steroid hormones such as oestrogen and progesterone that are necessary for reproduction (Stefansdottir, Fowler, Powles-Glover, Anderson, & Spears, 2014). Oestrogen is crucial in the development of female sex characteristics whereas progesterone is responsible for the uterus preparation before pregnancy, and preparing mammary gland during lactation too.

Histologically, there are three developmental stages of ovarian follicles, primordial, growing and mature follicles as known as Graafian follicles. Primordial follicles are the earliest stages of mammalian oocytes development during gestation (Baillet & Mandon-Pepin, 2012). Each follicle contains an oocyte that is not growing and surrounded by a layer of flattened granulosa cells. When the follicle enters growing stage, the oocyte will starts enlarging and the granulosa cells proliferate to become cuboidal (Fair, Hulshof, Hyttel, Greve, & Boland, 1997). In between the oocyte and proliferated

granulosa cells, the thick coat called zona pellucida is formed. At this stage, the follicle is identified as primary follicle.

As the oocytes grow, the layers of granulosa cells will continue proliferate and form the fluid-filled antral cavity called antrum. The formation of antrum results a dramatic increase in follicle size and become secondary follicles. Once the secondary follicle matures, it is identified as Graafian follicle which will expel its oocyte during ovulation. The granulosa cells will then differentiate to corpus luteum and responsible for progesterone production (Parker & Mathis, 2014; Stefansdottir, Fowler, Powles-Glover, Anderson, & Spears, 2014).

### 2.1.1 Ovaries Collection

Slaughterhouse is the most economical source of oocytes compared to other recovery methods such as ovum-picked up (OPU) technique. This allows large production of embryo with minimum cost. From slaughterhouse, pairs of fresh ovaries could be collected from slaughtered cattle, and transport to laboratory for processing. Studies reported that the time in between animal being slaughtered and oocytes recovery has a direct effect on the oocyte quality. Saleh (2017) demonstrated that longer period of time after slaughter results lower yield of oocytes with bad quality. The highest yield and quality was recorded in the first two hours after slaughter where the lowest yield was after 24 hours. This result was supported by Lv et al. (2010) and Lonergan & Fair (2016) that suggested the time interval between ovaries collection and oocyte recovery is the dominant factor to yield fertility impaired oocytes.

Other than the time elapsed after slaughter, temperature is one of the factors affecting oocyte quality. During transportation to laboratory, the fluctuation of temperature is extremely important to be avoided. In general, ovaries are transported in saline solution at the temperature between 30°C and 37°C to maintain the oocytes quality. A thermo-protective container or cooler insulated container with a bottle of phosphate buffer solution could be used to provide a consistent environment for ovaries (Hatzel & Carnevale, 2016).

### 2.1.2 Oocytes recovery

Currently, there are number of methods to recover oocyte for *in vitro* embryo production. *In vivo* matured oocytes could be recovered from live animals using laparoscopic or surgical methods such as OPU that first established by the Dutch team (Pieterse et al., 1991). This technique enables researchers to retrieve oocytes from the particular donor without killing. However, laparoscopic or surgical methods are expensive and the yield is very small (Pawshe, Totey, & Jain, 1994). In contrast, collecting ovaries from slaughterhouse is a cheaper alternative with abundant source of oocytes.

There are several methods to recover oocytes from collected ovaries, but the most common are slicing and aspiration method. Slicing method can be done easily by scoring the surface of ovary using a sterile surgical blade. After scoring, the scored surface need to be rinse and tap on the collection medium immediately to maximize the oocytes recovered. Previous reports reported that slicing method yield more oocytes than aspiration especially in cattle and goat (Martino, Palomo, Mogas, & Paramio, 1994; Saleh, 2017) This statement was supported with the fact that slicing method could release the oocytes from surface follicles as well as follicles from the deeper cortical

stroma, while aspiration could release oocytes from surface follicles only (Pawshe et al., 1994; Das, Jain, Solanki, & Tripathi, 1996).

## 2.2 *In vitro* Maturation (IVM)

Oocytes recovered from the slaughterhouse are usually from various stages, some are immature oocytes which is in germinal vesicle stage or mature oocytes (metaphase II). In this study, the oocytes recovered will undergoes IVM before exposing to vitrification solution. This is to standardize the developmental stage of oocytes for vitrification. (Fasano, Demeestere, & Englert, 2012) concluded that IVM procedure is more efficient when it is performed before oocyte vitrification as mature oocyte has higher membrane permeability than immature oocytes (Fuku et al., 1992). This makes the access of cryoprotectant into the oocytes much easier and results in a higher survival rate after freezing and thawing. Besides that, mature oocytes possess stable ultrastructure that able to protect them from freezing damage (Thoa, Huong, Ly, Anh, & Huong, n.d.).

### 2.2.1 Maturation medium

During *in vitro* maturation process, the immature oocytes will undergo a series of cytoplasmic changes and results variable competence of the embryos (Moor, Mattioli, Ding, & Nagai, 1990). The synthesis and storage of mRNA and protein during IVM and early stage of embryonic development are influenced by the composition of maturation medium. Hence, the role of each composition in the media has to be identified to make sure the oocytes are matured in a good condition (Motlík & Fulka, 1986; Sagirkaya et al., 2007).

In bovine and equine, tissue culture medium-199 (TCM-199) is commonly used to handle and culture the oocytes with the presence of fetal calf serum (FCS). This formulation of medium has significantly enhanced the maturation of oocytes and subsequent embryonic development compared to synthetic oviductal fluid supplemented with serum. However, the effect of FCS as a protein supplement was dependent on the maturation medium used in IVM of bovine oocytes. (Lonergan, Carolan, & Mermillod, 1994; Sagirkaya et al., 2007; Gómez et al., 2008)

Despite adding FCS into TCM-199, the maturation medium used also containing steroids such as oestradiol to improve the completion of maturation changes. In bovine, the addition of oestradiol during IVM was reported to increase the maturation rate in some studies (Fukui, Fukushima, Terawaki, & Ono, 1982; Younis, Brackett, & Fayrer-Hosken, 1989). Moreover, the addition of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and oestradiol to a medium can improve the ability of IVM bovine oocytes to be fertilized (Fukushima & Fukui, 1985). However, the addition of 5 μg/ml LH in TCM-199 failed to enhance the rate of maturation, but FSH and oestradiol did so significantly.

The formulation of Earle's salt in TCM-199 is commonly used for incubation in 5% carbon dioxide ($CO_2$). The bicarbonate buffer solution helps in maintaining the pH at 5% $CO_2$ environment and provide a consistent environment for IVM (Foss, Ortis, & Hinrichs, 2013). Before incubating, mineral oil was added onto the maturation medium to prevent evaporation during incubation (Tokoro et al., 2015). In this study, the oocytes were incubated in maturation medium for 24 hours. The 24 hours IVM protocol is widely used in experiment as the developmental competence of oocytes yield is similar with *in vivo*

(Heinzmann et al., 2015). Prolonged maturation should be avoided as the blastocyst rate and polyploidy will be decreased in cattle (Demyda-Peyrás et al., 2013).

## 2.3 Cryopreservation

Cryopreservation is essential for widespread application of assisted reproductive technologies (ART). It can facilitate the storage of germplasms such as oocytes, sperm and embryos for a long duration yet no or little genetic alteration occur (Rodrigo Marques dos et al., 2006). The frozen or vitrified human embryo is proved to be able to store up to 20 years with no effect on any of the parameters evaluated including post-thaw survival, rates of implantation, clinical pregnancy, miscarriage, and live birth (Riggs et al., 2010).

Recently there is a survey said that more than 50 % of transferred bovine embryos had been previously frozen (Thibier, 2003), this shows the freezing process is critical in maintaining the quality of transferred embryos. Although Parks & Ruffing (1992) and Aman & Parks (1994) had concluded that bovine oocytes are more difficult to freeze compared to embryos in cleavage stage, there are still many researches and study on the improvement on oocyte cryopreservation. This is because the cryopreservation of oocytes had efficiently decreased the dependence on the fresh oocytes. In addition, oocyte cryopreservation would provide a source of genetic materials with greater flexibility for the application of other technologies such as somatic cell nucleus transfer (Atabay et al., 2004).

### 2.3.1 Slow Freezing

In 1972, Whittingham et al. introduced slow freezing method and published the first successful cryopreservation of mouse embryos. In 1977, Whittingham reported on the first birth after cryopreservation of mouse oocytes in liquid nitrogen at -196ºC. In 1983, Trounson & Mohr reported the first human pregnancy from embryo that was cryopreserved using slow freezing. Slow freezing is also known as equilibrium freezing because equilibrium will be achieved when the fluid is exchanging between extracellular and intracellular spaces of living cells. The speed of freezing is about 0.3 to 2°C per minute, which is slow enough for the water to be removed from the living cells through osmotic gradient created by cryoprotectant. This results in a safe freezing without any serious osmotic and deformation effects due to intracellular crystallization (Mazur, 1990).

Furthermore, this technique had been accepted as a safe procedure due to the use of low concentration of cryoprotectant that would not lead to serious toxic and osmotic damage. Hence, most previous study using slow freezing to cryopreserved ovarian tissues. However, the low concentration of cryoprotectants used might insufficient for preventing the crystallization to occur. This might result detrimental effects on living cells and reduces in quality. In addition, this technique is time consuming due to its low freezing rate and requires an expensive programmable freezing machine. This high cost and time consuming technique causing embryologists to find another cryopreservation protocol which is vitrification (Rezazadeh Valojerdi et al., 2009).

### 2.3.2 Vitrification

In 1985, vitrification was first reported by Rall & Fahy with a high proportion of mouse embryos survived after vitrification at -196°C. It was then further developed in animal reproduction to improve the assisted reproductive technologies. In 1999 and 2000, the first successful pregnancies and deliveries after vitrification of human oocytes were reported (Gordts et al., 1990). Until now, vitrification has been widely used to cryopreserve human and animal oocytes in different developmental stages (Yoon et al., 2003; Kuwayama, Vajta, Kato, & Leibo, 2005). Vitrified oocytes gave a high survival rate of 80 to 85% and near 100% for blastocysts after warming (Nawroth et al., 2005). The terms freezing and thawing are commonly used for slow freezing while vitrifying and warming are used for vitrification procedures. (Rezazadeh Valojerdi et al., 2009)

According to Nawroth et al. (2005), the physical definition of vitrification is the solidification of a solution at ultralow temperature by extreme elevation in viscosity during cooling, such that the living cells are exposed to the higher concentration of cryoprotectant and plunged directly into liquid nitrogen. In short, vitrification is an ultra-rapid cooling technique that requires a higher concentration of cryoprotectant to solidify the solution. During the vitrification process, the water will be largely replaced by the cryoprotectant and transform from the liquid phase to a glassy state. This production of glassy state will make the water behave like a solid, but without any crystallization or ice formation.

Crystallization or ice formation is one of the factors that can potentially decrease the viability of cryopreserved living cells. In order to avoid this, a higher concentration of cryoprotectant will be used to cryopreserve the bovine oocytes. Besides that, a rapid

cooling rate (15.000 to 30.000 °C/min) is also achieved by directly plunging the oocytes into liquid nitrogen (Babaei, Derakhshanfar, & Kheradmand, 2007). In a previous study, Parks & Ruffing, (1992) concluded that rapid cooling rates can efficiently reduce the toxicity of the cryoprotectant and also diminish the length of time oocytes are exposed to temperature which they are sensitive.

### 2.3.3 Oocyte Cryopreservation

The development of oocyte cryopreservation is playing important role in the field of reproductive biology especially in conserving maternal genome for establishment and maintenance of genetic banks (Vieira et al., 2002). According to the Food and Agriculture Organization, about 20% of the livestock breeds in the world are currently at the high risk of extinction. Farm animals especially cattle are experiencing rapid artificial selection pressure due to the widely used of artificial reproductive techniques such as artificial insemination and embryo transfer (Powell et al., 2003; Sørensen et al., 2011). Conservation and maintenance of animal genetic resources are vitally important to ensure the potential to overcome the emerging disease, consumer demand changes, and most importantly is conserving the gene pool with available useful genes (Andrabi & Maxwell, 2007; Pereira & Marques, 2008).

Oocyte cryopreservation was first performed in 1958 by Sherman & Lin to determine the possibility of survival of post-thawed unfertilized mouse oocytes. In 1977, the first successful offspring from cryopreserved mouse oocytes was reported (Whittingham, 1977). Successful oocyte cryopreservation could assist in many ART by preserving the female genetic material of unexpectedly dead animals or superior breed stock. Oocytes are proven to be very hard to cryopreserve as they are very sensitive to

chilling. Their large size and low water permeability characteristic make the oocytes to have the tendency to retain water during chilling, and results in the formation of intracellular ice that would cause cryoinjury to the cell (Modello, Ricerca, & Veterinaria, 2011).

## 2.4 Oocyte Carrier

In order to increase the survival rate of oocytes after chilling, higher freezing and warming rates are needed by using a minimal volume of cryoprotectant. There are many types of oocyte carrier available during vitrification such as cryotop, cryoleaf, open-pulled straw (OPS). Higher freezing rate was proven to be able to facilitate vitrification with a lower concentration of cryoprotectants, while higher warming rate can prevent the occurrence of devitrification (S.-U. Chen & Yang, 2009). A high freezing and warming rates of oocyte carrier can bypass the cryoinjury by rapidly pass through the damaging temperature zone, which is in between 15ºC and −15ºC (Martino, Songsasen, & Leibo, 1996).

### 2.4.1 Open-pulled Straw (OPS)

OPS method was developed by Vatja and his team in 1988. This technique was reported to be able to reduce the cryoinjury on bovine oocytes and embryos. The OPS straw was derived from mini French straws that pulled and thinned to approximately half of the original diameter by heating. According to Vajta, Lewis, Kuwayama, Greve, & Callesen (1998), the freezing rates of OPS straw was enhanced to 20,000°C/min, which is 8 times higher than the original. This higher freezing rate with the reduced volume of cryoprotectant successfully minimized the cryoinjury towards oocytes. Besides that, the

fracture damage due to the changes of pressure was avoided due to the open end of OPS straw (G. Vajta, Booth, Holm, Greve, & Callesen, 1997). This low cost and simple technology give an alternative for the vitrification of oocytes (Vieira et al., 2002).

## 2.5 Cryoprotectant

Cryoprotectant is essential in cryopreservation process as the solution able to protect the living cells from damage during cooling or freezing process. The living cells are usually exposed to cryoprotectants before vitrification to avoid the formation of ice in the cell. In 1985, Rall and Fahy successfully vitrified 8-cell mouse embryos by using a medium consisting 20.5 % (w/v) DMSO, 15.5% (w/v) acetamide, 10% (w/v) propylene glycol and 6% (w/v) polyethylene glycol at the temperature of 4°C. During the treatment of cryoprotectants, the cryoprotectants used will penetrate into the cells and gained the access to all parts of the system.

However, the high concentration of cryoprotectant used in vitrification might cause toxicity to the vitrified living cells. There are several barriers to the free diffusion of membranes and resulting in changes of compartment volumes which is damaging the cells. Hence, the diffusion process of cryoprotectants and osmosis having important effects to the vitrification (David E. Pegg, 2015). Therefore, the optimal ratios of the equilibration time and toxic effect of cryoprotectant is very important before vitrification or cooling as well as the removal time of cryoprotectant after warming (D E Pegg & Diaper, 1988).

Ali & Shelton (1993) developed another formulation of cryoprotectant with EG based. The vitrification solution consisting of 5.5 M EG and 1.0 M sucrose to reduce

toxicity, and also permitted the equilibration steps to be performed at room temperature. In 2000, S. U. Chen et al. used this formulated solution for vitrification of human oocytes and recorded high survival rates by conventional straws. Ethylene glycol was commonly used as a basic permeable cryoprotectant due to its low toxicity level or combined with DMSO for vitrification (Kasai, 1996). The concentrations of EG and DMSO used in the present study were based on those used for the successful vitrification of bovine ova and embryos.

Two-step strategy had been developed and mainly used in cryopreservation recently. The living cells are exposed to two different concentration of vitrification solution where the pre-treatment solution is much less toxic due to lower concentration and followed by a higher concentration solution for a shorter time. The pre-treatment solution is used to reduce the time needed for exposure to the subsequent vitrification solution which is more toxic to oocytes. This two-step strategy had been demonstrated using human oocytes and recorded a significantly higher survival rate than those without pre-treatment.

### 2.5.1 Permeating Agent

Cryoprotectants are classified into two types, permeating and non-permeating agent according to their cell penetrating capacity (Brambillasca et al., 2013). Permeating agents are small molecules that able to form hydrogen bonds with water molecules, thus inhibiting the formation of ice crystal in a cell during vitrification. High concentration of permeating agent could solidify the water into glassy state and satisfy the goal of vitrification (Modello et al., 2011). DMSO and EG are the most common permeating

agents used in cryopreservation. DMSO is widely used in different protocols over decades and used in the combination with EG. This combination results a lower toxicity by reducing the concentration of single cryoprotectant (Chian et al., 2005; Hiraoka, Hiraoka, Kinutani, & Kinutani, 2004).

### 2.5.2 Non-permeating Agent

The most used non-permeable cryoprotectant in mammalian oocyte cryopreservation is sucrose solution. In contrast to the permeating agent, sucrose remains extracellular and facilitating dehydration. When sucrose is used in the combination of permeating agent, the water inside the cell will be drawn out and resulting dehydration. This process further assists the permeating agent in preventing the formation of ice crystal (Modello et al., 2011). Moreover, sucrose is also playing important role in thawing or warming process. It acts as an osmotic buffer to draw the water generated by melting ice rapidly, to prevent the excessive swelling, or even rupture of the cell during the removal of cryoprotectant (Martínez et al., 2002).

## 2.6 Transportation of oocytes

There are mainly four modes of transportation which are road, air, rail and marine transport. Generally, oocyte transportation will use road and air freight transportation to reach other facilities. Both modes are fast and precise, allowing the oocytes to be transported to other facilities in shortest time and minimum damage potential. However, there are few reports describing the effect of transportation on oocyte viability in positive and negative way. According to Vandenberg, Stevenson, & Levin (2012), the vibration

was suggested to have negative effect on embryo morphogenesis. McDonald et al. (2011) demonstrated that survival rate obtained 3 hours after thawing of shipped, vitrified oocytes was significantly lower than non-shipped vitrified oocytes (73.3% vs. 96%, $P<0.01$).

On the other hand, studies reported that 2 hours of transportation at 37°C does not give significant effect on the fertilization, cleavage, and implantation rates of oocytes (Alfonsín et al., 1998). The developmental competence of transported group was similar with the group of oocytes that cultured after aspiration. Isachenko et al. (2011) had also reported a low mechanical agitation of 6 hertz (Hz) had dramatically increase pregnancy rate of *in vitro* culture human embryos.

### 2.6.1 Vibration

According to Salvendy (1997), vibration can be defined as oscillatory motion or mechanical oscillation. It is also known as repetitive motion of an object and measurable in hertz (Hz). Regardless of the modes of transportation, the shipped oocytes will subject to one of the major dynamic hazards, vibration. The intensity of vibration experienced by shipped oocytes depends on the type of transportation used. Different modes of transport will produce different level of vibration (Dunno, 2014).

Inside a truck, vibration can be classified into shake, shimmy, and shudder. Shudder is known as brake vibration where the vibration transmitted across the hydraulic lines to the entire body during braking. When a truck is driving at a speed range of 60 –

80 kilometer per hour, the frequency of brake vibration will be in the range of 5 to 30 Hz (Matijević & Popović, 2017). Moreover, the road condition is also one of the factors of vibration. A low frequency of vibration (4 – 6 Hz) caused by rolling tires on rough road could increase the damage potential to vitrified oocytes (Nahvi, 2009). During transportation, the resonance frequency of whole-body vibration was recorded in between 4 – 5 Hz. According to Boyd, Cholewa, & Papas (2008), the vibration frequency that result the highest damage to the fruits shipped was in the range of 2 – 5 Hz. In order to simulate the transportation of truck using these values recorded, the vibration frequency of 5 Hz which equivalent to 300 rpm was used in this study.

As for airplanes, the frequency of vibration obtained during 13 flights under normal weather condition was referred. According to NASA (*1975)*, the average frequency of vibration is around 3 Hz. Generally, oocytes are transporting internationally using *two jet airplanes,* which is same with the commercial airplanes. During the flight, the aircraft might experience some fine vibration caused by the *cloud* or bad weather condition.

## 2.7 Liquid Nitrogen

Nitrogen is a non-toxic, odorless and colorless element. It is relatively inert and not flammable like oxygen. On April 15, 1883, two Polish physicists named Zygmunt Wróblewski and Karol Olszewski were first successfully liquefied nitrogen. The liquid nitrogen has a very low boiling point which is 77K (-195.8°C or −320.4°F) under normal pressure. Since the liquid to gas expansion ratio is very high, which is about 1:694, the liquid nitrogen will boil very quickly to fill a volume of nitrogen gas. Liquid nitrogen has to

be stored in special insulated containers that are vented to prevent pressure build-up and the storage duration is depends on the design of the containers called dewar or flask.

Liquid nitrogen has been widely used in cryopreservation as a cryogen where ultralow temperatures or rapid temperature reduction is required. The extremely low boiling point (– 195.8°C) makes it an excellent coolant as it evaporates when released and able to absorbs large quantities of heat. This characteristic enables liquid nitrogen to freeze the living cells upon contact. Many scientists prefer to store their biological sample by completely immersing it in liquid nitrogen due to the constant low temperature (– 196°C) characteristic (Lim et al., 2010).

## 2.8 Fluorescein Diacetate (FDA)

Fluorescein diacetate (FDA) is a non-polar, non-fluorescent fatty acid ester with the attachment of acetyl groups. The acetyl groups in the structure enable the FDA to diffuse through viable cell membranes passively. When FDA enters the cell, the intracellular esterase will de-acetylate it into fluorescein and acetic acid. The charged fluorescein is unable to pass through the cell membrane, hence retain in the cytoplasmic membrane and serve as an indicator of viable cells. FDA was first used by Rotman and Papermaster to evaluate the viability of mammalian cells (Kvach & Veras, 1982 ; Boyd, Cholewa, & Papas, 2008); now it is widely used in combination of propidium iodide (PI).

FDA is a very common assay probes used in cell viability evaluation. Stained cells will be incubated in the dark for 5 minutes and observed under fluorescence microscope with FITC filter. Under this filter, a blue light will be emitted to excite the fluorescent molecules in the stained cells. The maximal excitation and emission intensity

of FDA are 492 and 519 nanometre respectively (Hyka, Lickova, Přibyl, Melzoch, & Kovar, 2013). Green fluorescence will be observed on viable cells while dead cells will remain non-fluorescent.

# CHAPTER 3

# MATERIALS AND METHOD

## 3.1 Method

### 3.1.1 Oocyte Recovery

Ovaries were collected from Department of Veterinary Services (DVS) slaughterhouse at Shah Alam, Selangor, and transported to laboratory in a bottle containing 0.9% sodium chloride at 38°C. The collected ovaries were processed within 1 to 2 hours after collection to maintain their quality. First, the ovaries were washed using phosphate buffer solution (PBS) with 0.01 g/mL of streptomycin, followed by two times washing using PBS only. The washing procedure was done in water bath at the temperature of 38°C, followed by oocyte recovery using slicing technique. For each replicates, three ovaries were sliced using a No. 10 blade to retrieve oocytes. All visible cumulus-oocyte-complexes (COCs) with one or more complete layer of cumulus cells were selected under stereomicroscope (Nikon Instruments Inc.).

### 3.1.2 Modification of Open-pulled Straw

Oocytes were vitrified using the OPS method that described by Vatja et al. (1998). Each 0.25 mL capacity semen straw was softened over a hotplate and pulled from both ends to reduce its diameter evenly to approximately half of the original. The lengthened straw was cut at the opposite end of cotton plug to make it approximately 13 – 15 cm

long. The thinning process increases the volume rate and hastens the cooling rate of the droplet that containing the oocytes (Grizelj et al., 2009).

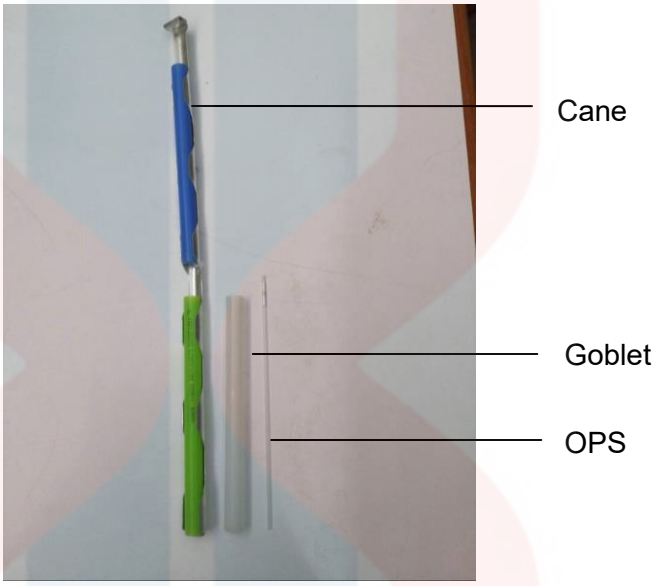


**Figure 3.1: Cane setup with OPS**

### 3.1.3 *In vitro* Maturation of COCs

Selected COCs were washed with PBS and maturation medium twice each before placing in pre-incubated maturation medium. The procedure of *in vitro* maturation was from those of Lee & Fukui (1995) with modification. The maturation medium consisting of TCM-199 (with Earle's salt) supplemented with 10% fetal calf serum (FCS), 5 µg/ml follicle-stimulating hormone (FSH) and 1 µg/ml oestradiol. Before placing selected oocytes, the maturation medium was covered with mineral oil and placed into carbon dioxide ($CO_2$) incubator (Heraeus® HERAcell®, Thermo Fisher Scientific, US) for 1 hour at 38°C. After washing, the selected COCs were incubated for 24 hours in $CO_2$

incubator at 38°C. At the end of IVM, the oocytes were denuded by repeat pipetting in PBS that containing 1 mg/ml of trypsin for 1 minute.

### 3.1.4 Vitrification of Mature Oocytes

Denuded oocytes were first equilibrated using equilibrium solution composed of 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycerol (EG) in PB1 for 2 minutes, then transferred to vitrification solution composed of 15 %DMSO/EG, and 0.5 mol/L sucrose for 45 seconds. Oocytes were loaded into the straw via capillary action, by placing the end of the OPS into the droplet, and immediately plunged into liquid nitrogen ($LN_2$) for vitrification.

### 3.1.5 Transportation using Liquid Nitrogen

Vitrified oocytes were being divided into three groups; one serves as control group and two experimental groups. The control group remained in the cryotank and not transported while experimental groups were given vibration load of 300 and 180 rpm on shaker (Edmund Bühler) for 1 hour to mimic transportation of truck and airplane, respectively. $LN_2$ was replenished every 30 minutes to ensure the OPS were fully immersed.

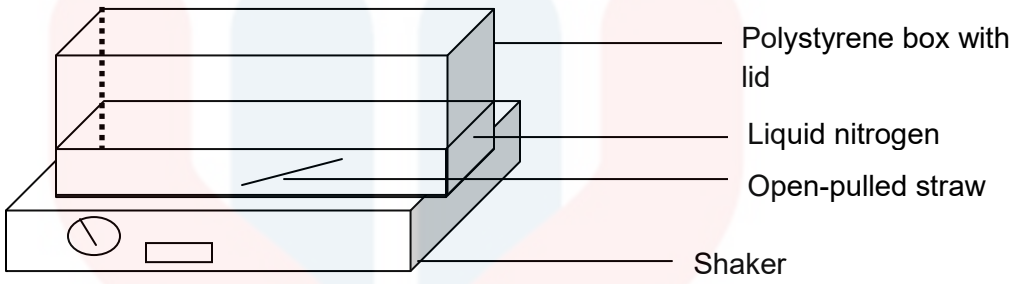


**Figure 3.2: The setup of transportation simulation**

### 3.1.6 Warming of Vitrified Oocytes

After 1 hour, the straws were taken out from the polystyrene box and held in the air at room temperature for a few seconds. The cotton plug end of OPS was cut to unload the oocytes into the droplet of 0.5 M sucrose in PB1 for 10 minutes, followed by 0.25 M sucrose for 3 minutes immediately.

### 3.1.7 Evaluation of Oocyte Viability

The viability of post-warmed oocytes was evaluated by using fluorescein diacetate (FDA) staining. Oocytes were washed several times using PBS and stained with FDA solution followed by incubation at room temperature for 10 minutes in the dark. Then, oocytes were evaluated using fluorescent microscope (Nikon Eclipse T*i*-S) after

washing with PBS. The numbers of oocytes that show green fluorescence under fluorescent microscope were recorded as viable oocytes.

### 3.1.8 Statistical Analysis of Data

Statistical analysis was performed using IBM SPSS Statistic Program (IBM Corporation, UK). One-way ANOVA test was used to evaluate the significance of differences among treated groups. P values of $<0.05$ were considered statistically significant.

# CHAPTER 4

## RESULTS

In this study, three ovaries were sliced to retrieve oocytes for each replicates. Table 4.1 shows a total of 110 oocytes which were retrieved from 9 ovaries indicating an average of 12 oocytes per ovary.

**Table 4.1: Number of oocytes retrieved from ovaries collected from slaughterhouse**

| Replicates | Ovaries collected (N) | Oocytes retrieved (n) |
|---|---|---|
| **I** | 3 | 33 |
| **II** | 3 | 38 |
| **III** | 3 | 39 |
| **Total** | **9** | **110** |

As shown in Table 4.2, all groups in replicate I had 0% viability. According to replicates II and III, there was no significant differences ($P > 0.05$) in the viability of oocytes from the control group and experimental groups that were given vibration loads of a truck and an airplane. The oocytes from the control group were remained vitrified in the cryotank (not exposed to any transportation condition for 1 hour) gave a high

viabilities of 100% and 90%. The vibration load of 300 rpm and 180 rpm that mimicked transportation by truck and airplane respectively did not affect the viability of post-warmed oocytes. Both groups of post-warmed oocytes gave 100% of viability in replicates II and III.

**Table 4.2: Viability of post-warmed oocytes from each group after 1 hour of mimicked transportation**

| Group | Replicates | Vitrified oocytes[a] (n) | Retrieved oocytes[b] (n) | Degenerated oocytes[c] (n) | Viability[d] | |
|---|---|---|---|---|---|---|
| Control (Static) | I | 5 | 5 | 2 | $\frac{0}{3}$ | (0%) |
| | II | 7 | 6 | 0 | $\frac{6}{6}$ | (100%) |
| | III | 10 | 10 | 0 | $\frac{9}{10}$ | (90%) |
| Truck (300 rpm) | I | 6 | 6 | 0 | $\frac{0}{6}$ | (0%) |
| | II | 7 | 7 | 1 | $\frac{6}{6}$ | (100%) |
| | III | 11 | 11 | 0 | $\frac{11}{11}$ | (100%) |
| Airplane (180 rpm) | I | 10 | 0 | 0 | $\frac{0}{0}$ | (0%) |
| | II | 12 | 12 | 0 | $\frac{12}{12}$ | (100%) |
| | III | 12 | 1 | 0 | $\frac{1}{1}$ | (100%) |

[a] Number of oocytes vitrified
[b] Number of oocytes successfully retrieved after warming
[c] Number of oocytes with broken zona pellucida
[d] Number of retrieved oocytes that show positive staining with fluorescein diacetate (FDA)

Since all oocytes in the first replicate did not show any green fluorescence, the viability was calculated using replicates II and III only. The viability of post-warmed oocytes under different vibration loads are summarized in Table 4.3. The retrieval rate of the airplane group was lower than the control and truck group due to technical errors. However, the average viability of post-warmed oocytes of control, truck and airplane groups were similar, 95%, 100% and 100%, respectively. Result showed there was no significant difference ($P > 0.05$) in between the viability of post-warmed oocytes of control, truck and airplane group even though the transportation conditions were different.

**Table 4.3: Viability of post-warmed oocytes under different vibration frequencies**

| Group | Control (Static) | Truck (300 rpm) | Airplane (180 rpm) |
|---|---|---|---|
| **Total number of vitrified oocytes (mean ± SD)** | 8.50 ± 2.12 | 9.00 ± 2.83 | 12.00 ± 0.00 |
| **Retrieval rate[e] (%)** | 94.12 | 100.00 | 54.17 |
| **Viability[f] (%)** | 95 | 100 | 100 |

[e] % of vitrified oocytes that successfully recovered from the OPS after warming
[f] % of viable oocytes that show green fluorescence after staining

Figure 4.1 shows the comparison of post-warmed oocytes with bright field and with FITC filter. Oocytes that show green fluorescence despite the light intensity were considered as viable oocytes. In the control group, there was one non-viable oocyte (arrowed) that did not show any green fluorescence.

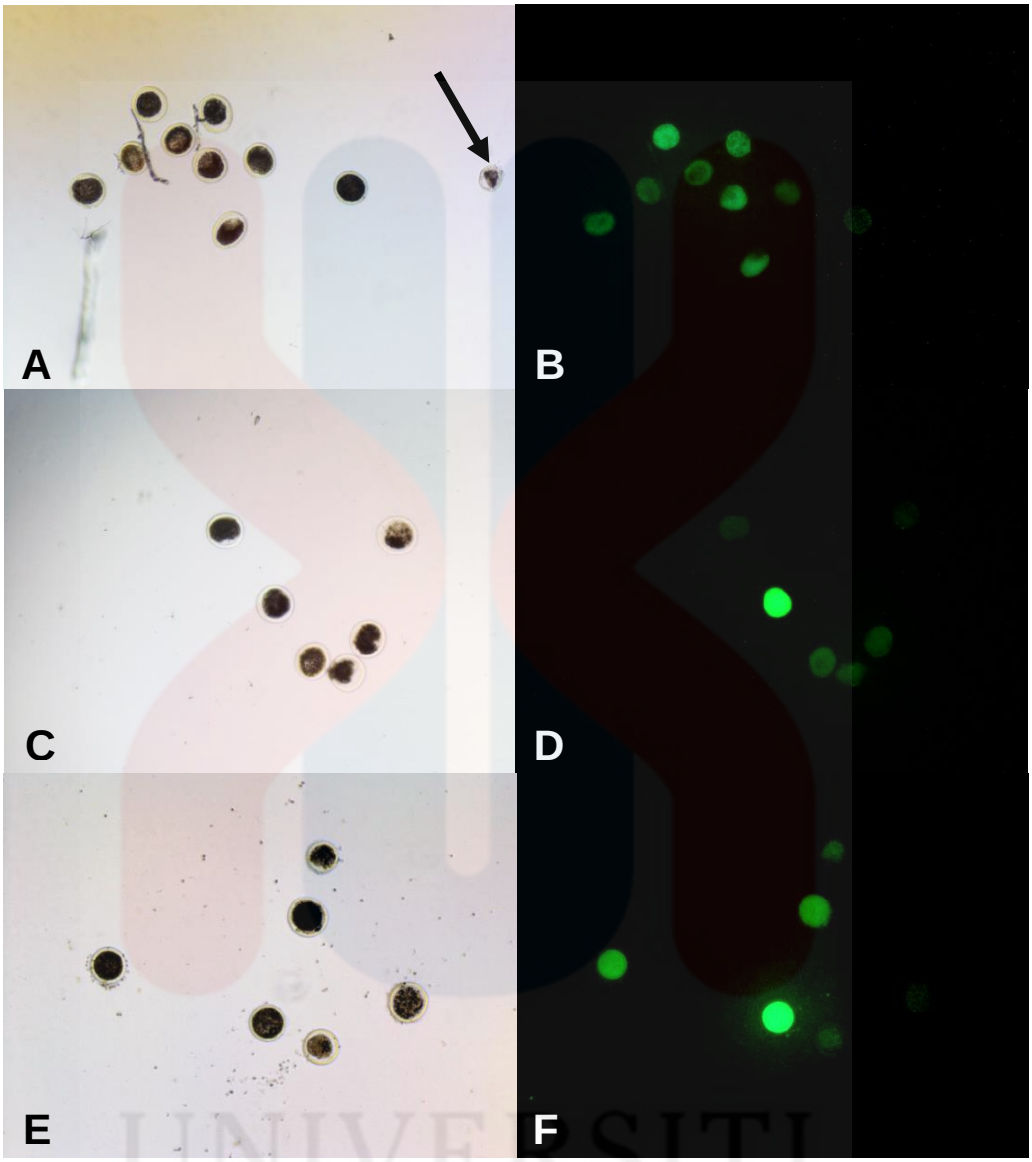


**Figure 4.1: Image of post-warmed oocytes captured under fluorescence microscope with bright field and FITC filter.** (A,B) Control group. (C,D) Truck group. (E,F) Airplane group

# CHAPTER 5

## DISCUSSION

Oocyte cryopreservation provides a greater flexibility in animal breeding compared to embryo cryopreservation. The ability to preserve maternal genome routinely enables researchers and breeders to produce offspring via *in vitro* fertilization of vitrified oocytes and selected male germplasm. However, some facilities are not able to perform *in vitro* fertilization such as intracytoplasmic sperm injection (ICSI) due to the limitations of specialized equipment and the presence of a skilled embryologist. Hence, the vitrified oocytes need to be transported to other facilities while maintaining the quality of oocytes upon arrival. By using the various modes of transportation, vitrified oocytes are subjected to a dynamic hazard which is vibration. Vibrations could cause damage to the complex structure of oocytes and result in a drop of viability. In this study, vibrations were concluded to have no significant effect on the viability of transported vitrified bovine oocytes.

In order to simulate transportation by truck and airplane, the vitrified oocytes were divided randomly into two experimental groups, and subjected to vibrations of 300 rpm and 180 rpm respectively. The transportation simulation was conducted for 1 hour to represent the transport motion. The transport time in this study is the actual ‘in motion’ time but not the duration of the travel. If, for example, the vehicle needs to travel for 1.5 hours to reach another facility, the accumulated ‘in motion’ time would be about 1 hour.

The vibration might be caused by poor road conditions at certain parts of the journey which results in a noticeably higher vibration level.

Among the four modes of transportation (rail, road, marine and air), road transport is the most common mode of shipping goods and materials. Many facilities use land transport to deliver their vitrified oocytes as it is quick and flexible. Moreover, the cost is cheaper compared to air cargo. This makes road transport the first choice for local transportation. However, in the same amount of travel time, a truck cause higher vibrations compared to an airplane that is flying in smooth air (Kipp, 2008). During a one hour journey, a truck can transmit an average of 5 Hz (300 rpm) of vibration (Boyd et al., 2008; Matijević & Popović, 2017; Nahvi, 2009) while airplanes produce a lower vibration frequency of around 3 Hz in average (Catherines, Mixson, & Scholl, 1975).

Thus, a mechanical vibration at the frequency of 300 rpm was applied to the vitrified oocytes for 1 hour to mimic truck transportation. The vibrations in vertical direction were not monitored in this study due to the limitations of the equipment. The shaker used in this study has an orbital movement, which means that it moves not only laterally but also longitudinally. This is similar to the vibration of a truck that is steadily driven and the road is on average in good condition. Thus, it can correlates with our results. In the present study, the vibration of 300 rpm (5 Hz) does not give significant adverse effect to the oocytes viability ($P > 0.05$). The post-warmed oocytes from the truck group gave a high viability (100%) and showed green fluorescence after staining with fluorescein diacetate (FDA).

As for airplanes, NASA (1975) reported more than 90% of the vibration energy measured on the airplanes occurred in the range of 0 – 3 Hz. The vibration frequency was obtained during 13 flights under normal weather conditions using two jet aircrafts that were widely used in commercial airlines. Generally, a central laboratory will utilize direct airline flights to transport oocytes to their destination in order to minimize transportation risks and potential hazards. Furthermore, air freight transportation is preferable when it comes to international collaboration or trading. The barriers of country boundaries or long distance could be overcome by using air freight transportation.

In this study, the high viability (100%) was recorded from the oocytes in the airplane group. This indicating the vibration frequency of 180 rpm does not cause significant harm or damage to the oocytes viability. Recently, mechanical vibration was proven to have beneficial effects on oocyte and embryo development. A study on the effect of mechanical vibration reported that cytoplasmic maturation of *in vitro* matured pig oocytes could be enhanced after the vibration treatment. The mechanical vibration did not give any effect on the maturation rate but the blastocyst formation rate of oocytes matured with mechanical vibrations were significantly higher than the oocytes matured without vibration treatment (Mizobe, Yoshida, & Miyoshi, 2010).

In addition, Isachenko et al. (2011) indicated that short intervals of mechanical vibrations could increase the developmental rates of human embryo cultured *in vitro* while not affecting the fertilization rate of oocytes. The mechanical vibration serves as a stimulation that induces or activates cell-to-cell communication and this intercellular communication was proposed to have the ability to establish cell co-operation by co-ordinating the cell's activity. In effect, the cell system will be able to respond to the stimulus which is essential in cell differentiation.

The results obtained from the present study showed that there were no significant differences (P>0.05) between viability of control group and experimental group. The oocytes that were exposed to different vibration frequencies gave similar percentages of viability as the control group (95% vs 100% vs 100%, P>0.05). These findings demonstrated that vibration occurred during truck and air freight transportation does not have a detrimental effect on the viability of vitrified bovine oocytes. This statement was supported by Alfonsín et al. (1998) that suggested transportation do not contribute harmful effects on oocyte quality. However, lower viability was recorded in the control group compared to the truck and airplane groups. This could be due to technical errors during the experiment. As mentioned earlier, oocytes are extremely sensitive especially in *in vitro* conditions. Several factors such as repetitive pipetting that is too frequent, overexposure to cryoprotectant or fluctuation of temperature could harm the complex structure of oocytes and causing it to lose its viability.

In order to evaluate the viability of post-warmed oocytes, each group of oocytes were stained with FDA and incubated in the dark. FDA stain is a fluorescent dye commonly used for rapid evaluation of mammalian oocyte viability. During staining, the non-fluorescent substrates in FDA will diffuse into oocytes passively and hydrolysed by esterase to produce polar fluorescent products called fluorescein. These fluorescein will accumulate in the cytoplasm as they are unable to pass through the intact cell membrane, therefore giving a green fluorescent appearance to the viable oocyte as shown in Figure 4.1 (Hyka et al., 2013). However, the oocyte (indicated with arrows) did not show any green fluorescence after staining (Figure 4.1). This indicates that the oocyte is no longer viable or is dead. A dead oocyte will show distinct loss of polar fluorescent products and remain non-fluorescent.

Different intensities of green fluorescence were observed after staining (Figure 4.1). Three factors affecting the intensity of the fluorescence are the influx of FDA, cell membrane integrity and esterase activity (Boender, 1984). From the result, the low level of green fluorescence could be interpreted as poor esterase activity in the oocytes. As mentioned above, esterase is needed to produce the polar compounds called fluorescein. When the esterase is not able to produce certain amounts of fluorescein, a lower level of green fluorescence will be observed. However, the oocytes were technically not dead but not quite as robust due to the poor esterase activity (Boyd et al., 2008).

The present study had recorded 0% of viability for all groups of oocytes from the first replicate as these oocytes showed non-fluorescence during the evaluation. The failure of staining might be due to the FDA working solution that was prepared a few days earlier. A study demonstrated that the time duration after preparing FDA working solution will affect the staining result. In their experiment, the viability score of the sample using a two hour old solution was recorded less than half of the score of those using a newly prepared solution. Pinillos & Cuevas (2008) stated that properties of FDA will be lost 1 hour after preparation and recommended for it not to be used for viability evaluation. In order to solve this problem, a newly prepared FDA working solution was used for each staining and positive staining results were obtained.

Before vitrification, matured oocytes were expected to be produced after 24 hours of incubation. However, all oocytes were remained immature and did not show polar bodies after *in vitro* maturation (IVM). There are three factors affecting the IVM which are the presence of cumulus cells, culture conditions and the composition of IVM media (Le Du et al., 2005; Papanikolaou et al., 2005). The factor that most likely caused the failure

of maturation was the culture environment. When the media or culture environment is contaminated by microorganisms, the maturation rate will be affected. However, there are studies that reported that the survival rate of vitrified immature and matured oocytes is similar (Cao & Chian, 2009; Wang, Racowsky, & Combelles, 2012), thus, the changes at the developmental stage of oocytes during vitrification was assumed to have no effects on the viability after transportation.

Even though the oocytes failed to mature, the results obtained in this study could contribute to the data on immature oocytes. The number of experiments using immature oocytes is low, especially for transportation after vitrification. Vitrification of oocytes is usually performed at two stages: germinal vesicle (GV) and metaphase II (MII). The developmental stages of oocytes during vitrification is still a matter of debate, some reports concluded that oocyte vitrification should be performed at the MII stage due to the high membrane stability during chilling (Le Gal & Massip, 1999; Ledda et al., 2007). However, cryopreserving mature oocytes could damage the microtubular spindle and result in chromosomal aberrations, increasing polyploidy, and fertilization impairment (S. U. Chen et al., 2003; Tharasanit, Colenbrander, & Stout, 2006).

Cryopreserving immature oocytes becomes one way of circumventing this problem as the spindle system is not yet organized and genetic material are protected within the nucleus (Rodrigo Marques dos et al.,2006; Prentice & Anzar, 2010). Nevertheless, IVM is required after warming. Previous reports reported that cryopreservation will result in damage and loss of cumulus cells in immature oocytes (Hochi, Fujimoto, Braun, & Oguri, 1994). Cumulus cells are crucial in cell maturation especially in the first hour of IVM (Kastrop, Hulshof, Bevers, Destrée, & Kruip, 1991; Bruynzeel, Merton, Wijst, Hazeleger, & Kemp, 1997; Gilchrist, Ritter, & Armstrong, 2004).

Without the presence of cumulus cells, the maturation rate would be affected. Cryopreserving oocytes have been proven to be very difficult regardless of the developmental stages. If IVM needs to be done after vitrification, the best cryopreservation protocol is needed to maintain the quality of oocytes and cumulus cells. Thus, up to date, MII is still the preferable stages for vitrification (Modello et al., 2011).

Furthermore, the quality of ovaries could also contribute to the maturation process. In this experiment, the oocytes were recovered from ovaries that were collected from slaughterhouse. This is the most common source of oocytes as it is economical and allows for large scale embryo production. However, the sources of ovaries are highly variable in terms of breed, age, reproductive ability and disease background. These factors could contribute to the quality of oocytes. Besides that, the condition of the ovary could also affect the quality of oocytes. In 2015, Karamishabankareh, Hajarian, Shahsavari, & Moradinejad proven that the oocytes retrieved from the right ovary had greater developmental competence than the left side. Different follicle sizes and diameters would lead to different developmental competence especially in ovaries that were collected from the slaughterhouse (Priscilla & Balakrishnan, 2011).

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# CHAPTER 6

# CONCLUSION AND RECOMMENDATION

## 6.1 Conclusion

Throughout this study, analysis of results suggested that the vibrations that occurred during truck and air freight transportation did not have harmful or adverse effects on the viability of oocytes. There was no significant difference between the viability of oocytes in the control group and vitrified oocytes that were exposed to vibrations for one hour ($P>0.05$). This study contributes beneficial information to researchers to feel secure and comfortable in transporting vitrified oocytes. Moreover, the viability of both groups of oocytes was recorded to be the same which is 100%. This high percentage of viability showed that mechanical vibration was not a limiting factor in transport for assisted reproductive technologies. In contrast, it was suggested to have beneficial effects on oocytes and embryo development. The mechanical vibration seems to be able to induce cell-to-cell communication and establish cell co-operation. However, the mechanism of transforming the extracellular mechanical signal into intracellular signals is still unknown and requires further study.

## 6.2 Recommendation

In order to improve the effectiveness of transport assisted reproductive technology, further investigation on the fertility of transported vitrified oocytes is needed. *In vitro* fertilization could be conducted in the future using transported vitrified oocytes to determine the fertilization, cleavage and blastocyst development rates. Furthermore, the source of oocytes for further study could be changed from slaughterhouse to live animals using the ovum picked-up (OPU) technique. By using OPU technique, the intrinsic factors of the oocytes such as age and breed of the donor can be manipulated.

In addition, the effect of vibration on the viability of mature oocytes could be investigated in future work. In this study, vitrification was done using immature oocytes due to the failure of *in vitro* maturation (IVM). Even though immature oocytes were proven to be more resistant to cryoinjury, the blastocyst formation rate of immature oocytes was much lower than mature oocytes. Hence, taking into the consideration of the developmental competence, it is better to perform vitrification in mature oocytes. Moreover, vitrification procedures might also cause detrimental effects on the maturation capacity of the oocytes. In order to prevent development failure, IVM should be carried out before vitrification to ensure all groups of oocytes are matured.

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## APPENDIX A

Table A.1: One-way ANOVA

**ANOVA**

VIABILITY

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 33.333 | 2 | 16.667 | 1.000 | .465 |
| Within Groups | 50.000 | 3 | 16.667 | | |
| Total | 83.333 | 5 | | | |

## APPENDIX B

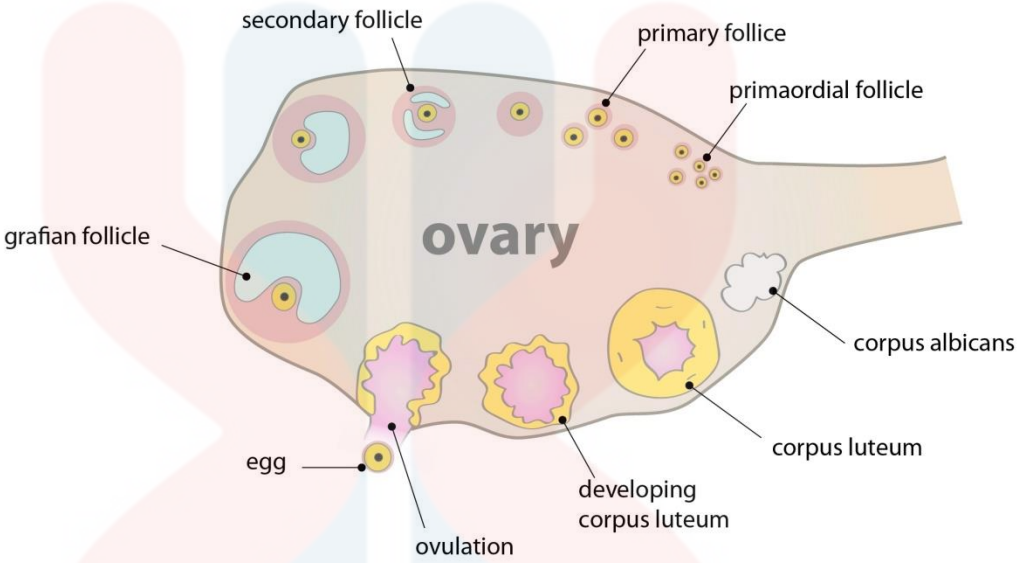


Figure B.1: Developmental stages of oocyte

Taken from: Lucie Nováková & Bětka Blanková. (n.d).


Figure B.2: Transportation setup