Effect of Extender Supplemented with Olive Oil on Bovine Semen Qualities

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Faculty of Agro-Based Industry
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DECLARATION

I hereby declare that the work embedded 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 Extender Supplemented with Olive Oil on Bovine Semen Qualities" by Nur Farihah Binti Abd Manan, matric number F15A0141 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:

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Date:

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ABSTRACT

Malaysia's development of artificial insemination (AI) programme in the assisted reproduction technologies during the past decades has been significant. Meanwhile supplementing olive oil in humans seems applicable since olive oil contains several potent bioactive compounds of antioxidant that aids in protected the spermatozoa. The aim of this study was to evaluate the effects of using olive oil as an antioxidant supplement into Tris based extender in comparison with different concentration on bull semen cryopreservation quality of progressive motility, membrane integrity and viability. A total of three ejaculation semen samples from a fertile bull of Brahman breed was collected using an electro-ejaculation method. Collected samples was freshly evaluated physically and microscopically before divided and diluted using four Tris based extender containing 0% (control), 0.25%, 0.50% and 0.75% olive oil. The processed semen was equilibrated at 5°C for 3 h (chilling), packed into straws (0.25 ml), frozen and stored in a liquid nitrogen tank for 7 d before thawing and assessment of quality parameter. Semen quality parameters used for evaluation and comparison after chilling and freezing included progressive motility, membrane integrity (hypo-osmotic swelling test) and viability (eosin-nigrosin staining). The experiment showed that Tris based extender on chilling for semen quality was highest from Tris+0.75% olive oil, which was 82.62% (progressive motility) and 91.51% (membrane integrity). Contrast to chilling, Tris+0.25% olive oil in freezing gave the best value, which was 95.96% (membrane integrity) and 91.71% (viability). Results from chilling and freezing showed insignificantly different. The use of olive oil at different concentration on different temperature however influenced the semen quality parameters following cryopreservation. In conclusion, Tris based extender with olive oil can be considered as a cheaper alternative to commercial extender as it is cost effective to the farmers.

Keyword: Artificial insemination, Tris based extender, semen cryopreservation, olive oil, antioxidant

Kesan Penambahan Minyak Zaitun dalam Extender pada Kualiti Air Mani Lembu

ABSTRAK

Pembangunan program permanian beradas (AI) dalam teknologi reproduktif bantuan (ART) telah menjadi kepentingan di Malaysia setelah beberapa dekad. Sementara itu, penggunaan minyak zaitun kelihatan sesuai diaplikasikan kepada manusia memandangkan minyak zaitun mengandungi beberapa campuran daya bioaktif antioksidan yang membantu melindungi spermatozoa. Tujuan kajian ini dijalankan adalah untuk menilai kesan minyak zaitun sebagai antioksidan tambahan ke dalam extender berasaskan Tris ke atas kualiti krioawetan sperma lembu dari segi motiliti progresif, integriti membran dan daya maju dengan menggunakan kepekatan yang berbeza untuk perbandingan. Sejumlah tiga sampel ejakulasi air mani dari baka Brahman lembu penjantan yang subur telah dikumpul dengan menggunakan kaedah elektro-ejakulasi (EE). Sampel yang terkumpul akan segera dinilai secara fizikal dan mikroskopik sebelum dibahagikan dan dicairkan ke dalam empat extender berasaskan Tris yang mengandungi 0% (kawalan), 0.25%, 0.50% dan 0.75% minyak zaitun. Air mani diproses dengan mengimbangi suhu pada 5°C selama 3 j (penyejukan), dimasukkan ke dalam straw (0.25 ml), dibekukan dan disimpan di dalam tangki cecair nitrogen selama 7 h sebelum pencairan dan penilaian parameter kualiti dibuat. Parameter kualiti air mani yang digunakan untuk membuat penilaian dan perbandingan selepas penyejukan dan pembekuan adalah motiliti progresif, integriti membran (ujian hypo osmotic swelling) dan daya maju (eosin nigrosin staining). Kajian menunjukkan Tris+0.75% pada suhu penyejukan (chilling) mempunyai nilai kualiti air mani yang tertinggi dalam extender berasaskan Tris iaitu 82.62% (motiliti progresif) dan 91.51% (integriti membran). Berbeza dengan suhu penyejukan, kepekatan 0.25% minyak zaitun pada suhu pembekuan (freezing) menunjukkan kualiti parameter air mani adalah yang terbagus iaitu 95.96% (integriti membran) dan 91.71% (daya maju). Kesan pada kedua-dua suhu penyejukan dan pembekuan kualiti air mani memberi nilai perbezaan yang sama. Penggunaan minyak zaitun pada kepekatan yang berbeza mempengaruhi parameter kualiti air mani diikuti oleh krioawetan. Extender berasaskan Tris dengan minyak zaitun boleh dipertimbangkan sebagai alternatif yang lebih murah berbanding extender yang komersial memandangkan ianya boleh disediakan di dalam makmal apatah lagi dapat mengurangkan kos kepada penternak.

Keyword: Permanian beradas, extender berasaskan Tris, krioawetan air mani, minyak zaitun, antioksidan

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION AND SYMBOL

ABBREVIATION

AI	Artificial insemination
ART	Assisted reproductive technology
SEM	Standard mean of error
DVS	Department of Veterinary Services
NIVB	National Institute of Veterinary Biodiversity
EE	Electro-ejaculation
AV	Artificial vagina
ATP	Adenosine triphosphate
OXPHOS	Oxidative phosphorylation
CPA	Cryoprotectant agent
MES	2-(N-morpholine) ethanesulfonic acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
OO	Olive oil
ANOVA	Analysis of variance
PUFA	Polyunsaturated fatty acids
LPO	Lipid peroxidation
tROS	Total reactive oxygen species
OS	Oxidative stress
CASA	Computer-assisted sperm analysis

SYMBOL

°C	Degree Celcius
%	Percentage
g	Gram
cm	Centimetre
mm	Millimetre
µl	Microlitre
mL	Millilitre
±	Plus minus

CHAPTER 1

INTRODUCTION

1.1 Research Background

Production of beef is one of Malaysia's vital agricultural industries since beef consumption has been on an increasing trend (Ariff, Sharifah, & Hafidz, 2015). However, Malaysia lost to kept pace in the beef production with the increasing demand from consumer for fresh beef and processed beef product (Sahar & Chamhuri, 2016). Therefore, in order to meet the domestic demand, Malaysia had imported 75 – 80% of beef from different parts of the world such as India, Australia and New Zealand (Mohamed, Hosseini, & Kamarulzaman, 2013).

An effort to meet the demands of meat is to grow more beef cattle farms (Sitepu, Zaituni, Jaswandi, & Hendri, 2018). The current limits are the low population and genetic quality of local cattle. The growth of local beef cattle is still very low, but it has the

advantage of environmental condition tolerance and low quality food required. It is needed to consider ways to increase the population and genetic quality of local cattle.

Livestock production have come about a major changes during the past few decades due to the introduction of artificial insemination (AI) in new technology application (Isa, 2016). Effort that can be taken to increase the livestock population and quality are to create improvement on artificial insemination by using sperm from superior cattle such as stated in eleventh Malaysian plan. This effort is considered as an alternative strategy to enhance the self-sufficiency of beef from 27% in 2014 to 50% in 2020 (Abdulla, Arshad, Bala, Bach, & Mohammadi, 2016) where artificial insemination is a widely applied tool aiding extensive application of frozen semen from genetically superior male animal (Lemma, 2011).

Artificial insemination (AI) in assisted reproductive biotechnology (ART) is a process of collecting sperm cells from a sires and manually depositing them into the reproductive tract of a female (Yimer et al., 2015). It has been applied in livestock management where sperm donor breeding males presenting a series of desirable traits are used in order to obtain more offspring with economically desirable traits and high yield (Ehmcke & Schlatt, 2008; P. Rédei, 2008). This assisted reproductive technology is introduce mainly to aid for speed up genetic progress and also to lessens the risk of diseases spreading and enlarges the number of animals that can be produced from a superior parent (Patel et al., 2017). Usually the cattle are able to transmit the diseases when one bull is used to be exploited by many farmers (Bayemi & Mbanya, 2007). Moreover, the successful of artificial insemination depends on few factor, include the quality of semen used for cryopreservation. In order to produce good chilling and freezing semen cryopreservation

quality, many other factors should be considered, which is include the semen extenders composition of the cryopreservation media used.

Generally, the frozen semen cryopreservation can be obtained from an artificial breeding centre such as National Institute of Veterinary Biodiversity (NIVB) and Department of Veterinary Services (DVS) as they provided with such a complete equipment and facilities from semen collection until artificial insemination is able to apply.

1.2 Problem Statement

Since, the beginning of artificial insemination (AI) services in the 1960's, the country already came to a long way for the improvement of cattle breeding in Malaysia (Haron, 2013). Assisted reproductive technology (ART) will continues to be one of the most required employed technologies for use in improving breeding programme in Malaysia in future. Furthermore, the National Institute of Veterinary Biodiversity (NIVB) has its own program of producing semen cryopreservation for cattle (Raymond & Saifullizam, 2010). Even so, Malaysia still imported stock of the frozen semen even though it is expensive (Bayemi & Mbanya J N, 2007). The costly of imported frozen semen and inaccessibility of Department of Veterinary Services (DVS) in providing frozen semen due to cost of production are problem that become an obstacle in improving and promoting the artificial insemination among farmers (DVS, 1980).

Locally bovine frozen semen production should be rebuilt and better promoted so that the dependence on imported semen will be reduced and increasing the uptake by farmers. Thus, the aim of this research is to produce cryopreserve bovine semen by using Tris-yolk based extender supplemented with olive oil. This research as an incentive to give an idea for artificial breeding centre such as NVIB and DVS could be prepared in laboratory and strengthen the cattle breeding development in Malaysia.

1.3 Hypothesis

H_o = Tris extender supplement with different concentrations of olive oil will not affect to the progressive motility, membrane integrity and viability of bovine sperm after cryopreservation.

H_1 = Tris extender supplement with different concentrations of olive oil will affect to the progressive motility, membrane integrity and viability of bovine sperm after cryopreservation.

H_o = Bovine chilled and frozen semen quality will not affect to the effects of olive oil supplemented to Tris extender.

H_1 = Bovine chilled and frozen semen quality will affect to the effects of olive oil supplemented to Tris extender.

1.4 Objectives

1. To evaluate the progressive motility, membrane integrity and viability of bovine sperm after cryopreservation using Tris extender supplement with different concentrations of olive oil.
2. To determine the effects of olive oil supplemented to Tris extender on bovine chilled and frozen semen quality.

1.5 Scope of Study

This research will consist of two scopes of study to justify the objectives and prove the validity of the hypotheses stated. Firstly, this research will focus of evaluating parameters of the bovine sperm which are progressive motility, membrane integrity and viability after cryopreservation using Tris based extender supplement with different concentrations of olive oil. Secondly, this research will study on the potential antioxidant activity of olive oil to the Tris based extender for chilling and freezing of bovine semen. These results will be obtained by using methods which involves entire process of semen cryopreservation such as collecting, diluting, cooling, equilibrating, freezing, thawing and evaluating at the respective stage. The entire study will be used cattle breed from Brahman located at the University of Princess Naradhiwas's beef cattle farm for electro-ejaculation (EE) for semen collection, chilling, freezing, storing and thawing for semen

cryopreservation, and will be analyzed with hypo-osmotic swelling test (HOST) and eosin-nigrosin staining under a light microscope magnification of 40x for semen assessment to study the effectiveness of olive oil encounter as supplemented antioxidant to Tris based extender. When the evaluation is done, the data is taken for recording purposes.

1.6 Significance of Study

The findings of this study will aware the benefit of the ability in locally producing bovine chilled and frozen semen that contributes significantly in the artificial breeding centre related to the artificial insemination (AI) in assisted reproductive technology (ART). Further study into this field justifies the need for more accessible, economical and potent instruments in order for better improvement in producing chilled and frozen semen. Therefore, this proposed study aims to evaluate the ability of an olive oil which is more affordable and more closer with people to present for its suitability to be substitute as an alternative source of cheaper extender compared with commercial extender instead reduce the imported semen as an antioxidant in bovine chilled and frozen semen quality.

1.7 Limitation of Study

As to our knowledge, the usage of olive oil as an antioxidant supplement to extender is new in bovine yet had implied to other mammals such as boar. The fact that the

focus of many health investigation into olive oil had done by several researchers to explored the effectiveness of olive oil on semen quality and sperm physiology via intentional modification of dietary habits (Banihani, 2017). Thus, these is limiting the source of study of the implementation of olive oil to the bovine semen extender yet have to refer with other researches that had carried out the studies of the implementation of several antioxidants as supplement to bovine semen extender .

CHAPTER 2

LITERATURE REVIEW

2.1 Assisted Reproductive Technology (ART)

Over the last two decades, several assisted reproductive technologies (ART) are commonly employed and refined to enhance their reproductive capacity from genetically superior animals, improve and preserve livestock genetics and build new products such as cloned animal or transgenic for genetic improvement of farm animals as well to disease control (Widayati, 2012).

The use of ART in livestock industry has become progressively widespread with the development of artificial insemination, cryopreservation of embryo and semen, in vitro embryo production, estrus synchronization, and embryo transfer have made it possible to manipulate the reproductive processes in many ways to sustain agricultural production (Verma, Kumar, Kumar, & Chand, 2012; Hafez, 2015).

This is mostly adapted in the dairy and beef cattle industry, where advancements of semen and embryo technological have been the most successful and widely used by using sperm of selected bulls with superior genetic traits for artificial insemination (Ombelet & Van Robays, 2015). However, the quality of cryopreservation in initial specimen collected is not similar to the many species and face with many challenges (Haron, 2013).

2.2 Semen collection

Semen collection is widely practiced in modern breeding for use in artificial insemination or for the purpose of reproductive evaluation (Waelchli, 2007). There are several methods can be practiced to collect semen. Nevertheless, electroejaculation (EE) and artificial vagina (AV) are the most methods that always being used to obtain ejaculates that are suitable for semen evaluation and processing (Bravo, 2014).

2.2.1 Artificial vagina method

Semen collection with an artificial vagina (AV) is conducted by allowing the trained male cattle to mount a female cow or dummy cow to ejaculate with an AV or manual stimulation while standing on the ground (Brinsko et al., 2011). The AV for cattle consist several equipment as shown in figure 2.1:

1 – a graduated test tube for semen with a avolume scale and a water jacket,

2 – an elastic, thin latex sleeve,

3 – a joining together of latex cone with the collection test tube at the end of the vagina,

4 – a rubber cylindrical is casing with a valve for blowing air and pouring water,

5 – a bag for thermal protector and a mechanical container.

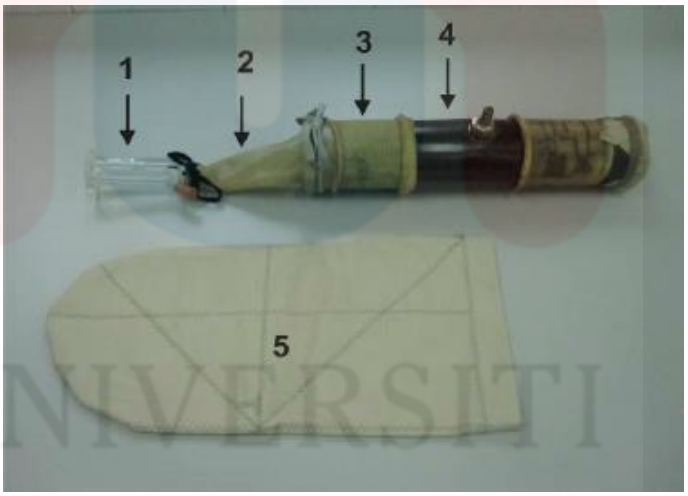

Figure 2.1 The structure of AV use on bull

Source: (Barszcz et al., 2011)

2.2.2 Stimulation electro-ejaculation method

Electro-ejaculation involves the electrical current of emission, erection and ejaculation for the stimulation by (Ball, 1986). The current stimulates accessory sex glands and the muscles of ejaculation by slowly increasing voltage in rhythmic fashion with a rheostat within a short period (Dunn, Fawcett, Fahey, Boothby, & Fordyce, 2005). When the animals are really slow in serving the AV or physically not capable of mounting, EE become an alternative method to be used (Memon, Bretzlaff, & Ott, 1986).

Distinct with artificial vagina (AV), stimulation electro-ejaculation (EE) can be used to collect semen from almost any mammals, wild or train with a little exception like the horse (Sarsaifi et al., 2013). However, Sarsaifi et al., (2013) also stated that collecting semen from wild animals requires the males to be anesthetized before the start of the procedure for protection. Besides, with EE, the volume of the semen sample is larger compared with other methods which have a lesser volume (Greyling & Grobbelaar, 1983). Moreover, repeatedly used of EE in collecting ejaculates from several bulls in large number will not cause death (Santiago-Moreno et al., 2009). The stimulation electro-ejaculation equipment is shown in figure 2.2:

1 – Stimulation for control the electrical pulse of probe,

2 – Probe to be inserted into bull's rectum.

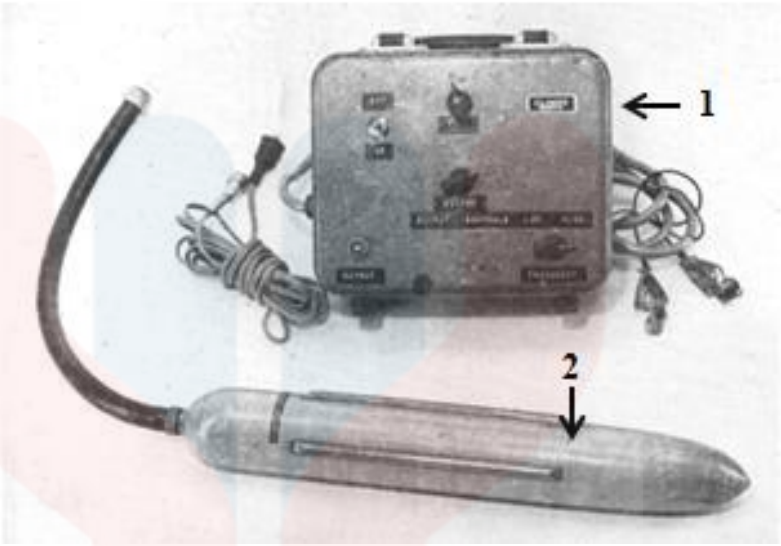

Figure 2.2 Equipment used in stimulation EE

Source: (Andrews, 1967)

2.3 Brahman cattle

Bos indicus cattle or referred to as Brahma in United States and Zebu as in South America and Europe have several types and breed that origin from some part of India depending on the use of the cattle (Kanwal, 2010). Malaysia had imported Brahman since the 1970's as breeder animals (Isa, 2016). In Malaysia, Brahman are primarily reared by smallholders in the villages or integrating cattle with oil palms since Malaysian beef industry is largely based on semi-intensive and extensive production system (Ariff et al., 2015). Since Malaysia is a tropical country, Brahman is selected by nature for traits such as ruggedness, survivability, parasitic and heat tolerance, and most importantly ability to survive under unfavorable and harsh climatic conditions (Abeygunawardena &

Dematawewa, 2004; Ariff et al., 2015). Brahman also used for working, dairying, and some religious sects besides for beef production alone and considered it as a vital part of agriculture for some country such as India (Verser Parr, 1923).

Figure 2.3 Brahman cattle
Source: (Gillespie & Flanders, 2010).

2.4 Spermatozoa

Sperm which is has its own specific word based on species is a broadly any male gamete or reproductive cell (Fitzpatrick & Lüpold, 2014). Fitzpatrick & Lüpold, (2014) also stated that a viscid whitish fluid of the male reproductive tract consisting of spermatozoa suspended in secretions of accessory glands; as of the prostate and Cowper's glands in the motile male gametes of animals are known as spermatozoa. Sperm are made up of several compartments surrounded within plasma and mitochondrial membranes and these membranes must remain intact and functional to allow cell survival (Connell, Mcclure, & Lewis, 2002). Energy is supplied in the form of ATP synthesized either by

glycolysis in the cytoplasm (Ford and Rees, 1990) or through oxidative phosphorylation (OXPHOS) in the mitochondria (Mahadevan, Miller, & Moutos, 1997) is also necessary both for sperm motility and fertilization.

Semen can be collected by using either artificial vagina (AV) or electro-ejaculation stimulation (EE) method as mention before. Minimal quality control standards of all ejaculates is important in order to assure that reproductive efficiency is not limited by the bull's semen fertility when using artificial insemination (AI) (Knox, 2010). Knox, 2010 also suggested to undergo several criteria that are used to pass an ejaculate since factors such as stress, disease and environmental factors, can significantly reduce the quality of a bull ejaculate. The semen or the ejaculates should be initiated with macroscopic analysis based on weight (volume), capacity (clarity), color, odor, and turbidity (swirling) before further processing (Connell et al., 2002). Semen fertility will not be extended over days since raw semen is only fertile for hours after collection (Knox, 2010).

2.5 Cryopreservation

Cryopreservation of semen for assisted reproduction is routinely used in a variety of circumstances as an important tool for breed improvement or conservation programs in various species including small ruminants (Connell et al., 2002). However, cryopreservation has been reported to cause cell damage in sperm morphology, including mitochondria, the acrosome and the sperm tail (Wooley & Richardson, 1978). Moreover, Watson, (1995) claimed that 'good and bad freezers' concept extends beyond variations in

semen from different individuals to the ability of individual sperm from within one sample to survive cryopreservation (Watson, 1995).

2.6 Extender

Semen extenders contain protective ingredients or liquid diluent added mixed with semen to preserve its fertilizing ability outside the reproductive tract (Gitta, 2011; Knox, 2010). The composition of extender may have an effect on spermatozoal response to cooling (Katila, Combes, Varner, & Blanchard, 1997). Extenders facilitate semen preservation which allows for long term storage, maintains a packaged processed semen product that allows for worldwide distribution and utilization. The extender able to made the semen to be shipped to the female, rather than necessitating the male and female to be in a one place. An extender consist energy source for the sperm, antimicrobial while maintaining the osmotic pressure and the pH diluents. The specialty of freezing extender use also allows cryogenic preservation of sperm (frozen semen), which may be transported for use, or used on-site later. The sperm will be defensed against possible damage by toxic seminal plasma by supplementing an extender to the semen. Extender also helps to provide essential nutrients and cooling buffers for cooling. While for freezing extenders, addition one or more penetrating cryoprotectants are needed.

2.6.1 Cryoprotectant

Addition of cryoprotectant agent (CPA) such as glycerol, or of other components such as egg yolk, milk, bovine serum albumin, polyvinyl alcohol and liposomes in extenders have been used in a way to giving some defense to spermatozoa and reducing the adverse impacts of cryopreservation (Katila et al., 1997).

However, glycerol remains to be one of the most beneficial penetrating cryoprotectant, acting as a solvent and readily occupied up by spermatozoa by depositing the cell within one minute of addition to the surrounding medium (Pickett and Amann, 1993). It is acts to lower the freezing point of the medium to a temperature much lower than that of water as it is presence in both intra- and extracellularly. This in turn minimises the proportion of the medium which is frozen at any once, decreasing the effect of low temperature on solute concentrations and hence on osmotic pressure changes (Amann and Pickett, 1987; Medeiros et al, 2002; Watson and Duncan, 1988). It also provides space of unfrozen medium, between ice crystals, in which spermatozoa may exist while at low temperatures. The suitable concentrations use is approximately -4 – 10% v/v due to extreme sensitivity even though glycerol is most favors CPA especially with bovine semen however, it is toxic for spermatozoa of many species (Watson & Holt, 2001). Egg yolk, which has cryoprotective properties, is also a common component (Gitta, 2011).

2.6.2 Sugar

Non-penetrating CPAs include sugar like glucose, fructose, mannose and protein such as egg yolk lipoprotein is a metabolizable substrates provided by sperm to give a plentiful source of energy for sperm during incubation besides maintaining the osmotic balance of the diluents (Lemma, 2011). These CPAs are believed to contribute in reducing the risk of formation of ice crystals and hence physical damage by increasing the osmotic pressure of the extracellular fluid and hence drawing water out of the spermatozoa (Yimer et al., 2015).

Species differences in the rate at which these sugars are metabolized by govern which is optimal for any given species (Fuller, 2004). Sugars also having a cryoprotective effect beside functions within the diluent contributing to the osmotic strength. However, which combinations of sugars may prove to be most beneficial under any given set of conditions remains unclear (Day & Stacey, 2007).

2.6.3 Antibiotics

Antibiotics are chemical product produced by living organisms, which is added to extenders to retard or eliminate growth of bacterial organisms arising during collection and processing as a precautionary measure (Dunn, Fawcett, Geoff, Dennis, & Fordyce, 1953). Gentamicin, was shown to have an adverse effect on

sperm motility and viability as it is commonly-used antibiotic in commercial semen extenders (Aurich & Spergser, 2007). In addition, it would be safe to reduce antibiotic usage as much as possible because a small amount of antibiotic usage could develop antibiotic resistance within an animal species (Johansson, Greko, Engström & Karlsson, 2004). Moreover, artificial insemination would be beneficial both to improve sperm quality and survival and to slow the development of antibiotic resistance with finding alternatives to antibiotics to control microorganisms in semen doses (Morrell & Wallgren, 2014).

2.6.4 Buffer

Buffer is used as a major component in the diluent serving to control pH and osmolarity (Day & Stacey, 2007). There are several types of buffer in preparing as a based extender such as Tris and citrate (Ferandes e Silva et al., 2016; Chuawongboon et al., 2017). Tris-based extenders are frequently used for semen cryopreservation in domestic animals as also in buffaloes (Purdy, 2006). Citrate, is one of the earliest and most extensively used buffers, that acts as a major component while other buffers such as MES or HEPES may be used as minor components only controlling pH (Day & Stacey, 2007).

2.7 **Olive oil** (*Oleo europaea L.*)

Olive is the common name for about 35 species of evergreen shrubs and trees of the genus Olea in the olive family (Preedy & Watson, 2010). The olive tree is a familiar feature of the Mediterranean landscape that may originate in Syria, Asia Minor, Ethiopia, Egypt, or India and the first that were possible to produce olive oil is from the Phoenicians (Boskou, 2006).

About 77% of olive oil is mono-unsaturated fatty acids such as oleic acid and also contains saturated fatty acids (~14%), polyunsaturated fatty acids (~9%), alpha-tocopherol (Waterman & Lockwood, 2007; Lancellotti et al., 2013; Al-Bachir & Sahloul, 2016). Sperm vitality can be improved by oleic acid that extinguishes free radicals, which may attack the sperm plasma membrane and reduces lipid peroxidation (Banihani, 2017). Oleic acid contain in olive is the highest compare to the soybean, canola, palm and fish which is 81.6 compared to 22.1, 56.3, 39.6 and 18.0 respectively (Preedy & Watson, 2010).

Olive oil is also rich in several potent antioxidant compounds such as polyphenols and vitamin E (O'Flaherty, 2014). Several studies have been conducted and published showing the influence of antioxidants in the olive oil. Significantly reduction of lipid peroxidation in seminal plasma, improve sperm motility and higher pregnancy occurrence had discovered Suleiman et al. (1996) with vitamin E supplementation in diet.

Olive oil has several types of product distinguish by the processing such as cold-pressed extra-virgin olive oil, which is the purest olive oil and the first oil that gushes out of just-picked and gently pressed fruit (Falcinelli, Castronovo, & Meehan, 2010).

2.8 Semen chilling

Chilling semen provides an efficient and successful means of short-term storage (Watson, 2000). Semen stored after cooling (5-8°C) will survive for 24-48 h without a significant drop in motility and even up to 96 h without a significant decline in fertilization rates. However, cryopreservation that undergo chilling is known to have some adverse effects on the spermatozoa manifested as a depression in viability rate, structural integrity, depressed motility and conception rates even with the most up to date techniques (Batellier et al, 2001; Medeiros et al, 2002). Generally, sperm viability is decreased by 50%, whereas fertilizing capacity is affected by a factor of sevenfold after cryopreservation (Lessard et al, 2000).

2.9 Semen freezing

Preferably, the spermatozoa of many species can now freezing to be stored indefinitely at -196°C in liquid nitrogen for future use, while still retaining acceptable fertilization rates postthaw (Lemma, 2011). Freezing and maintaining samples at such temperatures stops their 'biological clock' and allows the samples to remain alive for very long periods (Dunn et al., 1953).

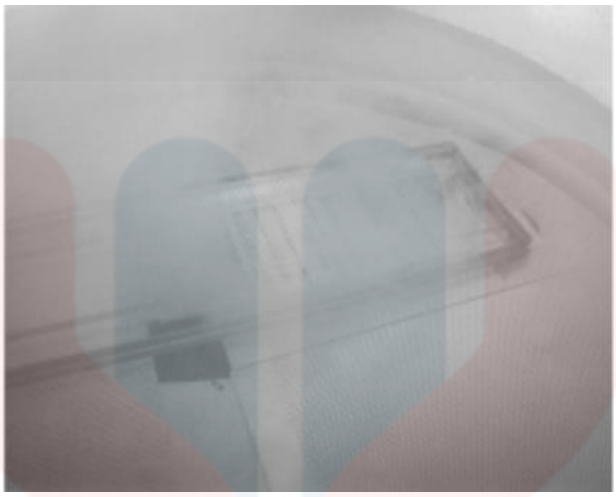

Figure 2.4 Straws are placed in vapour for freezing in liquid nitrogen vapour.

Source: (Dunn et al., 1953)

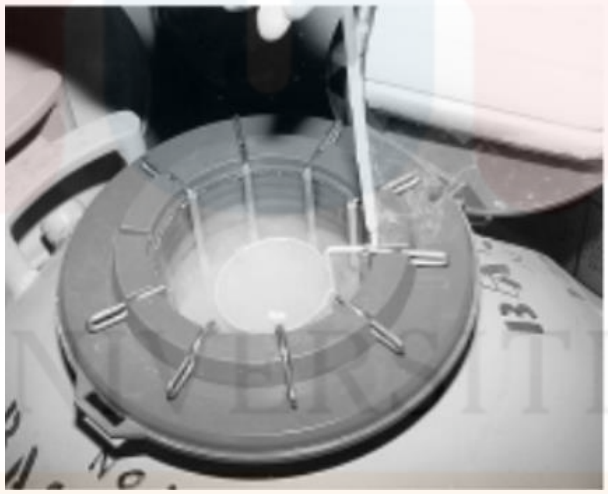

Figure 2.5 Freezing is completed in liquid nitrogen

Source: (Dunn et al., 1953)

2.10 Semen assessment

In vitro tests are important to be applied in order to determine the quality of semen for its approval and use in both AI and other biotechnology procedures since semen quality and its relationship with fertility have great importance in animal production (Lemma, 2011). Conventional laboratory tests for assessment of semen quality include light microscopic study of spermatozoal morphology, and estimation of spermatozoal motility, which in turn include percentages of motile and progressive motility, membrane integrity and viability following in vitro storage (Yimer et al., 2015). Other characteristics of semen quality that mostly taken into consideration include are concentration, volume and colour (Clement, 2001; Love et al, 2000).In addition, the quality and quantity of semen varies according to breed/type, age, body size, health, frequency of collection and environment (Hafez, 2015).

CHAPTER 3

METHODOLOGY

3.1 Experimental Designs

Experimental 1: Potential antioxidant effects of different concentration of olive oil supplement to Tris extender on progressive motility, membrane integrity and viability for chilling semen.

This experiment aimed to studied the effect of potential antioxidant in olive oil supplement to Tris extender for chilling semen at 5°C to improved frozen-thawed sperm progressive motility, membrane integrity and viability. Four treatments were designed; Tris extender supplementation with 0% olive oil as control (Treatment 1: Tris+0% OO), 0.25% olive oil (Treatment 2: Tris+0.25% OO), 0.50% olive oil (Treatment 3: Tris+0.50% OO) and 0.75% (Treatment 4: Tris+0.75% OO), respectively. The semen were thawed evaluated after 7 days by the methods described above. Three replications were performed for each treatment.

Experiment 2: Potential antioxidant effects of different concentration of olive oil supplement to Tris extender on progressive motility, membrane integrity and viability for freezing semen.

This experiment aimed to studied the effect of potential antioxidant in olive oil supplement to Tris extender for freezing semen at -196°C to improved frozen-thawed sperm progressive motility, membrane integrity and viability. Four treatments were designed; Tris extender supplementation with 0% olive oil as control (Treatment 1: Tris+0% OO), 0.25% olive oil (Treatment 2: Tris+0.25% OO), 0.50% olive oil (Treatment 3: Tris+0.50% OO) and 0.75% (Treatment 4: Tris+0.75% OO), respectively. The semen were thawed evaluated after 7 days by the methods described above. Three replications were performed for each treatment.

3.2 Animal

Semen sample from one sexually mature and healthy Brahman bull belonging to the University Princess Naradhiwas beef cattle farm was used in this research. The age of the bull was within 2 to 3 years old and with body weight between 300 to 400 kg.

Figure 3.1 Brahman male cattle was prepared for semen collection

3.3 Semen Collection and Evaluation

A total of three ejaculates are collected from a bull is used. Ejaculates were collected once a week from the Brahman bull with the aid of an automatic electro-ejaculation (EE). Immediately after collection, the ejaculate that was placed in 15 ml of insulated graduated test tube was immersed in warm water bath at 38°C and semen assessment was performed. The average time recorded for each collection cycle was 5 to 6 minutes. The bull was allowed to rest for one week before the next collection.

Figure 3.2 Probe was deposited into sire's rectum

Figure 3.3 Semen was collected via electro-ejaculation

Evaluation occurred in the lab of University of Princess Naradhiwas. The semen samples were evaluated based on macroscopic and microscopic characteristics. Macroscopic evaluations included volume and colour. Microscopic evaluation included spermatozoa concentration, mass motility, progressive motility, membrane integrity and viability. Only fresh semen between 5 and 10 ml in volume, cloudy in colour, had at least 90% sperm concentration and 100% mass motility were used for the chilling and freezing process.

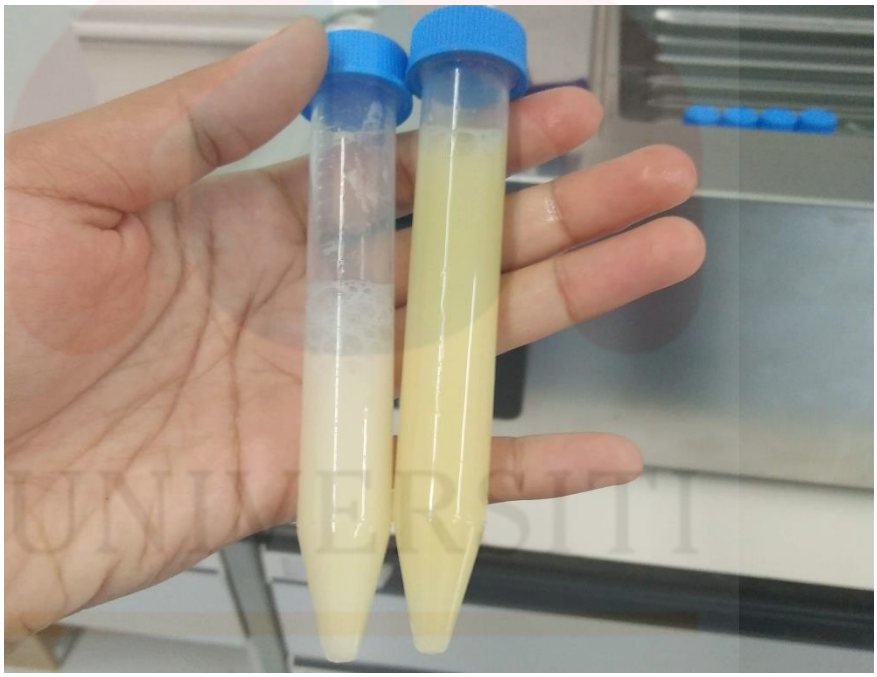

Figure 3.4 Volume and colour were evaluated before proceed for cryopreservation

The volume of each ejaculate was measured in a 15 ml of insulated graduated test tube. The mass motility of fresh semen was observed under an optical microscopy within score of 0 to 5. The sperm concentration of each ejaculate was determined by means of a

haemocytometer. Viability was assessed using eosin nigrosin stain technique. A total of 300 spermatozoa were examined under a light microscope for evaluation of live or dead spermatozoa based on their staining characteristics. Spermatozoa that stained dark purple due to absorption of eosin nigrosin were considered dead while spermatozoa that did not take up the stain were considered live. The evaluation of the membrane of the plasma membrane of the spermatozoa was done using methods hypoosmotic swelling test (HOST) for which 300 sperm cells were examined for live or dead spermatozoa based on their tail characteristics. Spermatozoa that had straight tail were considered dead while spermatozoa presented otherwise were considered live. The progressive motility was evaluated by the percentage of spermatozoa moving progressively forward. Evaluation was done by observing spermatozoa with a light microscope. The percentage of the parameters of spermatozoa for each different concentrations of olive oil was evaluated under a light microscope at x400 magnification by placing a 5µl drop of diluted semen on a slide covered with a glass cover slip (22 mm x 2 mm) for three replications. The mean of the three replications of each different concentration of olive oil were recorded as the final reading.

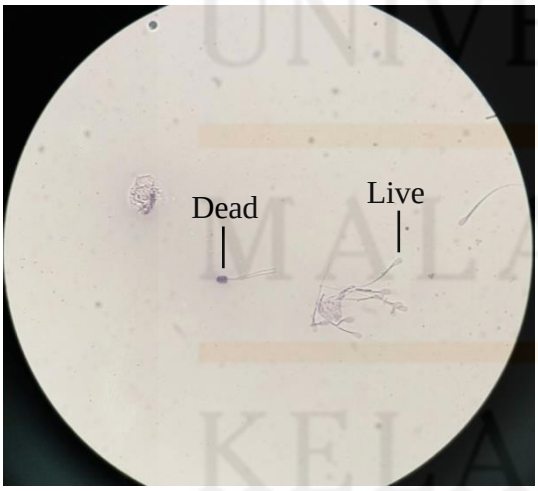

Figure 3.5 Microscopic evaluation on sperm viability quality using eosin nigrosin staining at x400 magnification

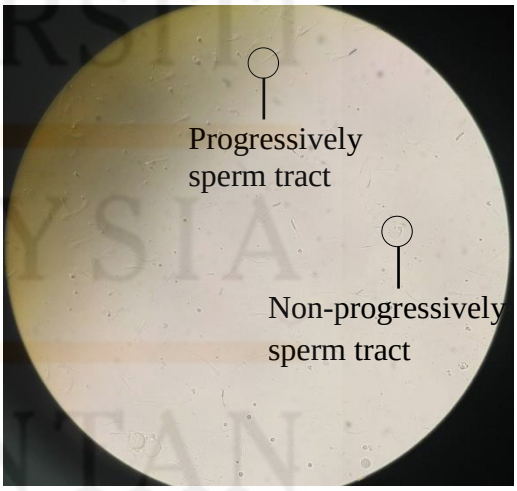

Figure 3.6 Microscopic evaluation on sperm progressive motility quality at x400 magnification

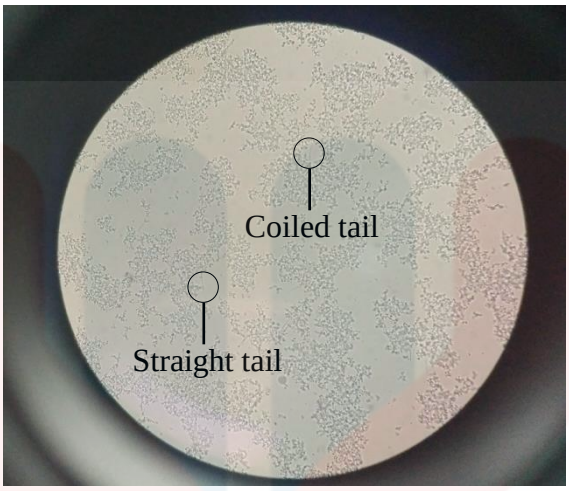

Figure 3.7 Microscopic evaluation on sperm membrane integrity using HOST at x400 magnification

3.4 Extender Preparation

According to Chuawongboon et al., (2017), to prepare the Tris buffer diluents, 3.028 g of Tris (hydroxymethyl), 1.675 g of citric acid, 1.250 g of fructose and 0.1 g penomycin (Sigma-Aldrich®) were weighed and dissolved in 100 mL of distilled water with the aid of a magnetic stirring device. Then, 20 mL of egg yolk (20%) freshly separated from the egg albumin was dissolved in 80 mL of filtered Tris buffer diluents and was centrifuged at 1000 unit speed for 10 minutes. Then, the diluent was distributed into four different tubes, which are 10.28 mL, 10.25 mL, 10.22 mL and 10.19 mL for treatment control, treatment 2, treatment 3 and treatment 4 respectively and 0.72 mL of glycerol were added for each treatment. Lastly, the three treatments were supplemented by respective amount of olive oil to have a final 0.25%, 0.50% and 0.75% olive oil concentrations while

the first tube was left as a control. The overall experimental treatments were used for comparison are control (Tris+0% OO), Tris with 0.25% olive oil (Tris+0.25% OO), Tris with 0.50% olive oil (Tris+0.50% OO) and Tris with 0.75% (Tris+0.75% OO). All the chemicals were reagent grade and were purchased from Sigma-Aldrich, St. Louis, Mo.

Figure 3.8 Tris extender was thoroughly mixed with olive oil

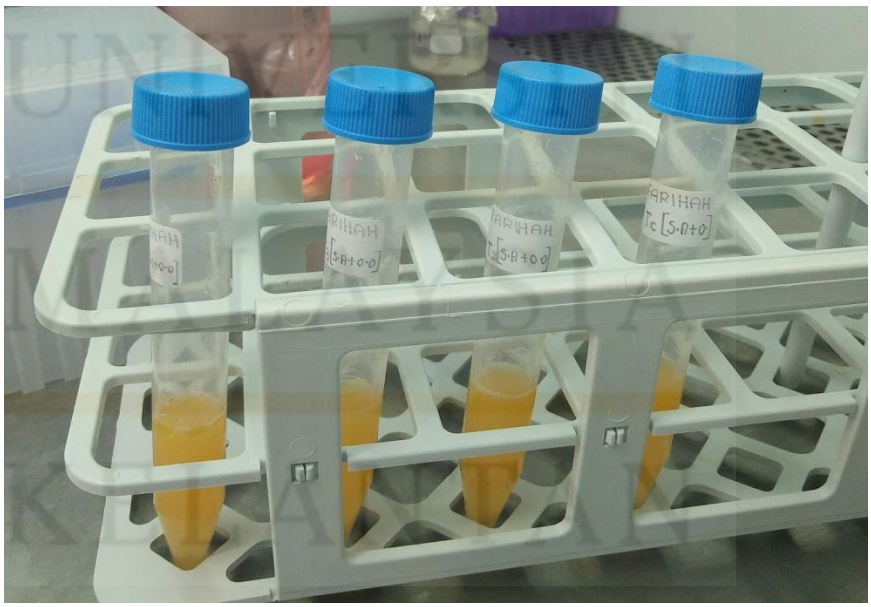

Figure 3.9 Four treatments with different concentration of olive oil

3.5 Cooling, Freezing and Thawing of Spermatozoa

Fresh semen sample was diluted in four different treatments groups (1:12 at 38°C): 0% (control), 0.25%, 0.50% and 0.75% olive oil groups respectively. Then the diluted semen was subjected to chilling at 25°C for 10 minutes before cooling in the refrigerator for three hours to make the temperature decelerate until 5°C and was equilibrated at 5°C for another for two hours. Each diluted semen samples was then loaded into 0.25 mL straws and the open end was sealed with a polyvinyl powder. The straws were subject to a slow gradual freezing process manually before they were stored in a liquid nitrogen tank. After sealing, the straws were placed horizontally on a cold rack (5°C) and lowered into nitrogen vapors, at 3 – 4 cm above the surface of liquid nitrogen contained in a styrofoam box (25 x 35 x 30 cm) for 15 minutes, and then the straws were fall into the liquid nitrogen for another 15 minutes. The straws from liquid nitrogen in styrofoam box were then transferred into a goblet of appropriate size and plunged directly into a liquid nitrogen tank (-196°C). Lastly, thawing of semen was performed in a water bath at 38°C for 30 seconds after 7 days.

Figure 3.10 The semen was chilled before cooled down to 5°C

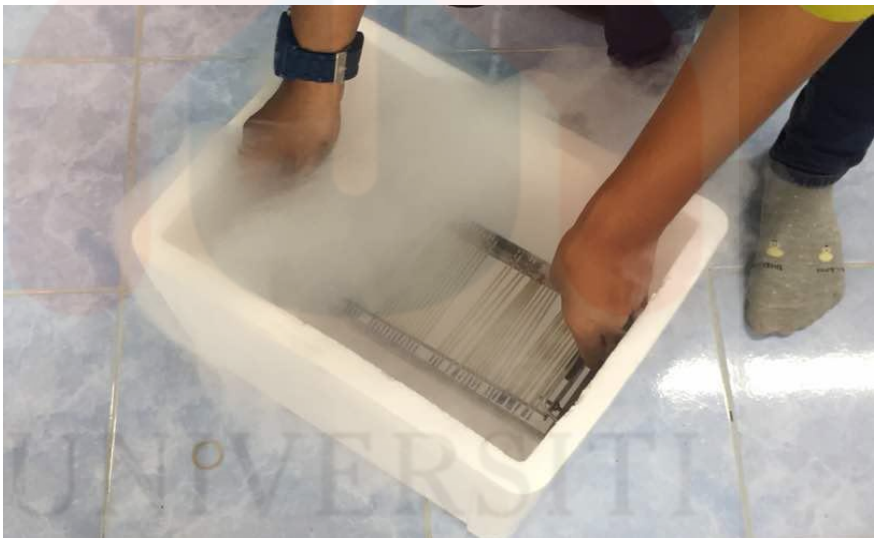

Figure 3.11 The cold rack was lowered filled into nitrogen vapour

3.6 Statistical Analysis

The chilling and freezing semen quality parameters of progressive motility, membrane integrity and viability were analysis using one-way analysis of variance (ANOVA) in SAS Statistical Software. The data were expressed as Mean±SEM, where follows by Duncan's multiply range tests with significance of ($P < 0.05$).

CHAPTER 4

RESULT AND DISCUSSION

Antioxidants are important in semen cryopreservation research in protecting spermatozoa from oxidative damage due to oxidative stress/reactive oxygen species (Kim & Parthasarathy, 1998; Bansal & Bilaspuri, 2008; Bansal & Bilaspuri, 2011). Research on antioxidants has been intense in recent years to improve the artificial insemination (AI) techniques using frozen semen (Ferandes e Silva et al., 2016). To our knowledge, this is the first report of olive oil as antioxidant for chilling and freezing bovine semen. Olive oil is known to be a natural antioxidant with superior safety and potential toxicity lower than synthetic antioxidants (Anbudhasan, Surendraraj, Karkuzhali, & Sathishkumaran, 2014). Banihani, (2017) reported that the majority of research studies that have directly interrelated olive oil with semen quality were evaluated usually from feed formulation. Olive oil in feeding appears to preserve sperm quality. Taken this together, these studies revealed consistent effects of olive oil on sperm quality when they are used in diet or extender.

4.1. Progressive Motility, Membrane Integrity and Viability Assessment of Olive Oil Supplement to Tris Extender on Bovine Chilling and Freezing Semen

Table 4.1 shows there was an increase in chilling semen quality in parameters compared to Tris+0% OO for all extenders. Among Tris based extender supplemented with olive oil, the highest chilling semen quality was obtained using Tris+0.75% OO extender to the parameters of progressive motility and membrane integrity. In chilling, Tris+0.75% OO continued to show its dominance significantly ($P<0.05$) in all parameters compared to Tris+0% OO, Tris+0.25% OO and Tris+0.50% OO.

Table 4.1 Mean and standard error of mean (SEM) of chilling semen quality parameters using four extenders with different concentrations of olive oil

Parameters (%)	Extender Type			
	Tris +0% OO (control)	Tris +0.25% OO	Tris +0.50% OO	Tris +0.75% OO
Progressive motility	72.17±7.38[b]	79.06±11.39[ab]	74.09±10.09[ab]	82.62±11.17[a]
Membrane integrity	89.70±3.51[a]	91.21±2.72[a]	91.07±2.55[a]	91.51±2.83[a]
Viability	81.00±4.67[a]	86.22±6.07[a]	86.52±7.90[a]	81.67±12.00[a]

[ab]Values with different superscript across the row indicate significant differences ($P<0.05$)
OO – Olive Oil

Contrast to chilling, table 4.2 shows that there was a general decrease in freezing membrane integrity and viability of semen quality parameters for all extenders with olive oil after cryopreservation in liquid nitrogen and thawing. Among Tris based extender supplemented with olive oil (Tris+0.25% OO, Tris+0.50% OO and Tris+0.75% OO), 0.25% olive oil showed higher freezing semen qualities compared to 0% olive oil in all semen parameters (Table 4.2). After thawing, the different Tris+0.25% OO compared to Tris+0% OO, Tris+0.50% OO and Tris+0.75% OO resulted in insignificant readings ($P>0.05$).

Table 4.2 Mean and standard error of mean (SEM) of freezing semen quality parameters using four extenders with different concentrations of olive oil

Parameters (%)	Extender Type			
	Tris +0% OO (control)	Tris +0.25% OO	Tris +0.50% OO	Tris +0.75% OO
Progressive motility	68.29 ± 6.74^{a}	72.26 ± 6.42^{a}	68.26 ± 7.77^{a}	74.15 ± 6.62^{a}
Membrane integrity	95.37 ± 0.16^{a}	95.96 ± 0.84^{a}	95.89 ± 0.34^{a}	95.78 ± 0.33^{a}
Viability	85.52 ± 5.16^{a}	91.71 ± 0.88^{a}	85.81 ± 4.14^{a}	85.15 ± 2.10^{a}

[a]Values with different superscript across the row indicate significant differences ($P<0.05$)
OO – Olive Oil

The first novel finding of this study was Tris+0.25% OO proved to be significantly higher compared to control for the progressive motility, membrane integrity and viability on freezing semen. This is similar to (Ferandes e Silva et al., 2016) that suggested 0.25% OO significantly higher than control for freezing using boar semen. This similarity could of all be explained by fact that the experimental animals used in both studies are come from mammalian and the effectiveness of olive oil itself.

Mammalian spermatozoal membrane contains a large surface of plasma membrane with high quantities of polyunsaturated fatty acids (PUFAs) (Ochsendorf, 1999; Storey, 1997). Therefore, it is vulnerable to per oxidative damage (Mansour, Sangi, Harsha, Khaleel, & Ibrahim, 2013). Lipid peroxidation (LPO) could destroy the structure of the lipid matrix of the membranes in mammalian spermatozoa and could be associated with impaired motility (Rao, Soufir, Martin, & David, 1989). These attacks ultimately lead to the impairment of sperm function, such as sperm motility and functional membrane integrity through the oxidative stress (Agarwal, Virk, Ong, & du Plessis, 2014; Guérin, El Mouatassim, & Ménézo, 2001). Result from previous study by Mansour et al., (2013) revealed that there is a positive correlation between olive oil consumption and enhanced sperm parameters using on healthy rats when sperm quality could be improved by supplementation with antioxidants to protect spermatozoa against per oxidative damage (Sikka, 2004). El-Sheshtawy, El-Sisy, & El-Nattat, (2016) also suggested that the polyphenols used in recent study in cryopreservation of human semen can acts as antioxidants and exhibit potential for sperm protection against oxidative stress. This can be supported since olive oil contains several potent bioactive compounds such as antioxidant polyphenols.

4.2 Comparison Bovine Chilling and Freezing Semen Quality on Olive Oil Supplement to Tris Extender

Figure 4.1 shows comparison of the percentage of mean and standard error of mean (SEM) of the sperm progressive motility between chilling and freezing semen in four Tris extender supplemented with different concentrations of olive oil. The figure shows Tris+0.75% OO in chilling has the highest sperm progressive motility compared to freezing semen while control was the lowest in both chilling and freezing semen.

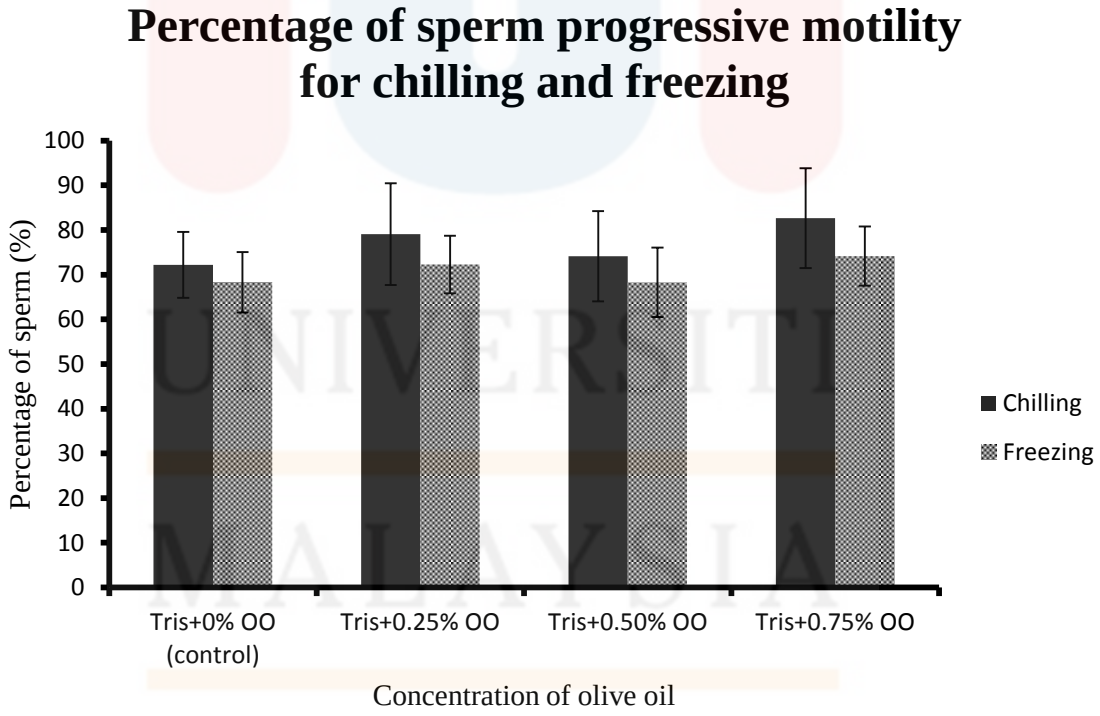

Figure 4.1 The percentage of Mean±SEM of sperm progressive motility from chilling and freezing semen after 7 days

Figure 4.2 shows comparison of the percentage of mean and standard error of mean (SEM) of the sperm membrane integrity between chilling and freezing semen in four Tris extender supplemented with different concentrations of olive oil. The figure shows sperm membrane integrity in all Tris extender for freezing semen was steadily higher compared to chilling while control was the lowest in both chilling and freezing sperm progressive motility.

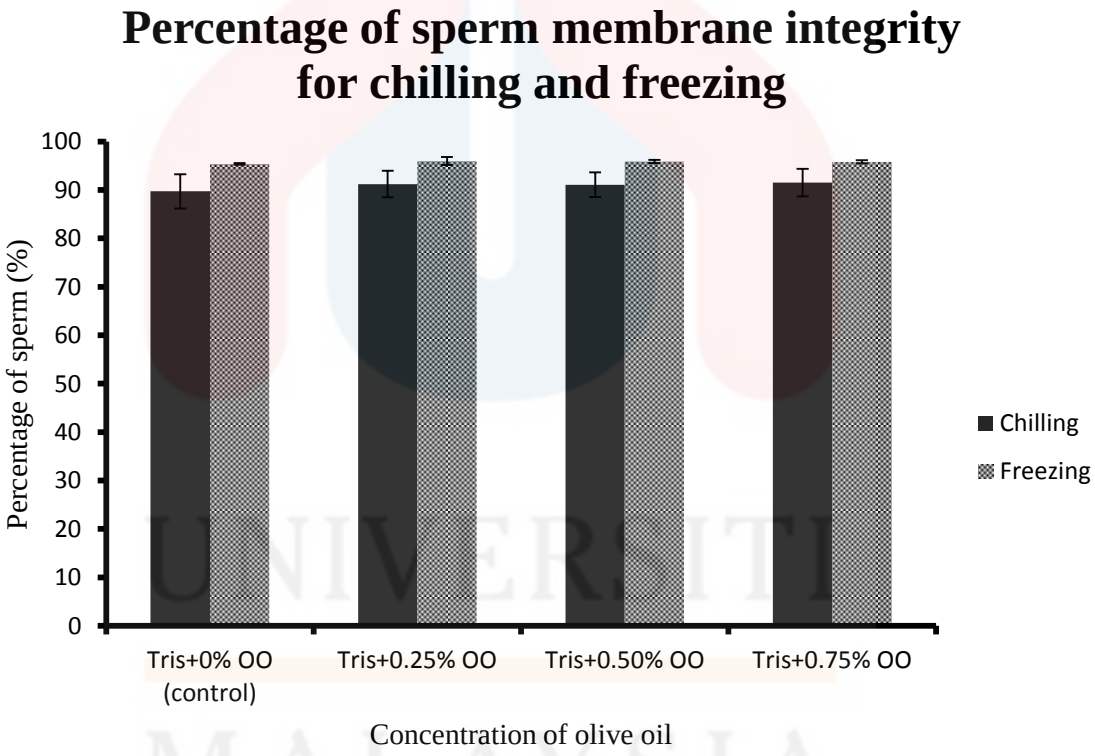

Figure 4.2 The percentage of Mean±SEM of sperm membrane integrity from chilling and freezing semen after 7 days

Figure 4.3 shows comparison of the percentage of mean and standard error of mean (SEM) of the sperm viability between chilling and freezing semen in four Tris extender supplemented with different concentrations of olive oil. The figure shows sperm viability in Tris+0.25% OO for chilling semen was the highest compared to chilling while control was lower compared to all Tris extender with olive oil in both chilling and freezing semen.

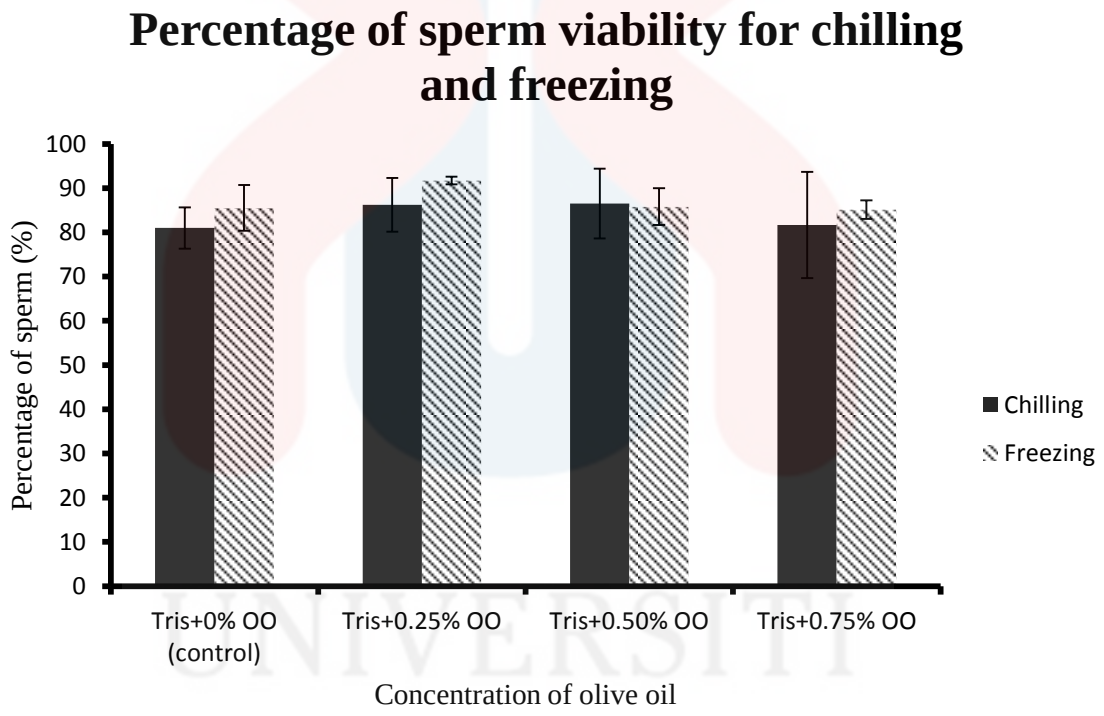

Figure 4.3 The percentage of Mean±SEM of sperm viability from chilling and freezing semen after 7 days

The second key point was 0.75% olive oil supplement gave the highest progressive motility and membrane integrity in chilling. In contrast, the semen quality for membrane integrity and viability in 0.25% olive oil was the highest and tended to deteriorate when the

concentration of olive oil increased to 0.50% and 0.75% in freezing implying that the change in temperature is one of the potentially damaging stresses that affect sperm quality. This may be explained that reduced semen quality parameters in the freezing extender are due to viscosity of olive oil. Ferandes e Silva et al., (2016) described that higher viscosity of olive oil in extender may reduce sperm metabolic demand prolonging the viability of gametes after thawed, as demonstrated by Nagy et al. (2002) and Lopez-Gatius et al. (2005). The trends of semen protection are shown in chilling results helps to prove the fact that increasing concentration of olive oil in extender is not due to toxic effect but to temperature changing during cryopreservation.

Moreover, chilling semen provides an efficient and successful means of storage compared with frozen semen because of the freeze-thaw process causes significant sperm damage, and it may be preferable to cool and rewarm samples, although cooled spermatozoa has a limited lifespan (England & Ponzio, 1996; Bansal & Bilaspuri, 2011). Still, effect of temperature change in cryopreservation for long-term and short-term storage of sperm may lead to membrane deterioration due to membrane phase transition occurring in the regions of highly specialized sperm plasma membrane (Maxwell & Watson, 1996; Bucak, Tuncer, Sarıözkan, & Ulutaş, 2009).

In addition, decreasing the amount of Tris extender with the increasing of supplementation of olive oil in chilling might be attributed to the combined effect of other protectants (Anand, Yadav, & Shukla, 2014) and antioxidant that may have benefited the sperm by protecting the sperm from cold shock, maintaining plasma membrane integrity and improving the protection of sperm against oxidative damage.

4.3 Comparison Bovine Chilling and Freezing Semen Qualities Effect of Extender Supplemented without Olive Oil

Figure 4.4 shows the percentage of the mean and standard error of mean (SEM) of the sperm progressive motility, membrane integrity and viability from chilling semen in Tris extender of four treatments supplemented with different concentrations of olive oil. The following figure shows semen qualities in treatment Tris+0.75% OO shows the best percentage mean while Tris+0.0% OO (control) has the lowest semen qualities compared to all extender supplemented with olive oil.

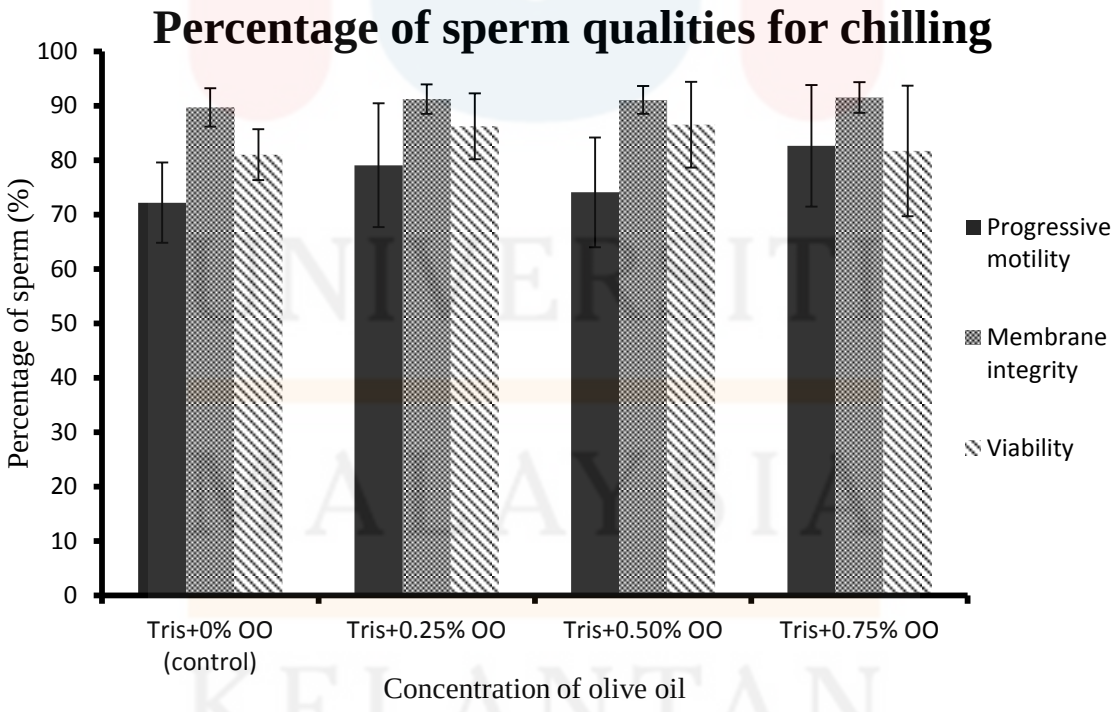

Figure 4.4 The percentage of Mean±SEM of sperm quality parameters from chilling semen after 7 days

Figure 4.5 shows the percentage of the mean and standard error of mean (SEM) of the sperm quality from freezing semen in four Tris extender supplemented with different concentrations of olive oil. From the figure, sperm membrane integrity from Tris+0% OO shows the lowest percentage mean among Tris extender. However sperm progressive motility in Tris+0.25% OO and viability in Tris+0.75% OO show the lowest percentage mean compared to Tris+0% OO with just a slightly different.

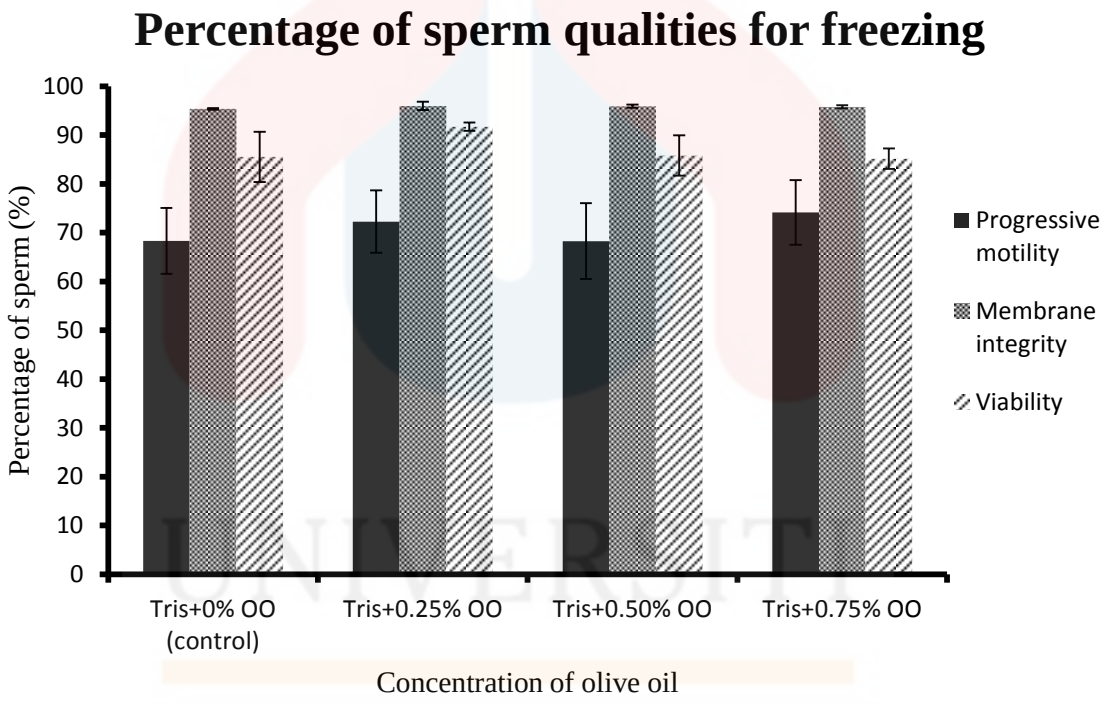

Figure 4.5 The percentage of Mean±SEM of sperm quality parameters from freezing semen after 7 days

Next, control group appeared to be significantly lower compared to extender with olive oil in both chilling and freezing semen (Tables 4.1 and 4.2). However, when the

amount of olive oil added, the semen quality parameters tended to improve significantly both chilling and freezing implying that by adding olive oil in extender to be incorporated to see positive effect.

This is similar to a previous study reported by Michael et al., (2009), suggested that from all the antioxidants tested vitamin E supplemented in semen extender was greater benefit using on dog. (Michael et al., 2009) also had observed effect of antioxidant supplementation in semen extenders on reactive oxygen species (ROS) stated that vitamin E gave satisfactory results in total reactive oxygen species (tROS) inhibition by having always values lower than those of the control group have. ROS production that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress (OS), which is harmful to spermatozoa (O'Flaherty, 2014). All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Thus, low in amount of several potent antioxidant compounds such as polyphenols and vitamin E that in 0% OO could reduce the sperm quality (Adewoyin et al., 2017).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

In conclusion, the present study revealed that the concentration of olive oil supplemented to Tris extender is contrast in both chilling and freezing, which are at 0.75% and 0.25% respectively. Although the optimum concentration of olive oil is different for both chilling and freezing, the present study reveals that Tris extender without olive oil (Tris+0% OO) resulted in significantly poor quality semen following cryopreservation in both chilling and freezing. In addition, commercial extender has been reported to be successful utilized for cryopreservation as revealed in previous study by Yimer et al., (2015) stated that commercial extender produced the best semen quality compared to all Tris extender when supplemented by honey. Nevertheless, its result was not significantly different from Tris+0.25% OO in all of the parameters, reflecting their comparable protective effect to the spermatozoa during cryopreservation. However, commercial extender is a costly product and usually has a short shelf life that makes it difficult to use it

effectively especially for AI centers processing semen at small scale (Yimer et al., 2015). Therefore, Tris extender supplemented with olive oil should be consider as a cheaper alternative to commercial extender.

5.2 Recommendations

The present study indicates several recommendations for additional research. As to our knowledge, research on supplementation with olive oil in extender for bovine is a novelty. Information on the effectiveness of potential antioxidant in olive oil for the quality of spermatozoa is scarce. In the present study, evaluation was carried out using manual evaluation and observation. Thus, more studies should be conducted using advanced technologies such as computer-assisted sperm motility analysis (CASA) to analyze sperm motion characteristics for in depth analysis.

In addition, the freshness of ingredient for an extender is also important to investigate. Due to restraint of time and manpower, the present study did not focus on utilizing fresh eggs for extender. Thus, a recommendation on the effect of fresh eggs for extender is highly recommended.

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APPENDIX A

Table A.1 Tris based extender recipe

MEDIA RECIPE

Components	Amount
Tris (hydroxymethly) amino-methane	3.028 g
Citric Acid	1.675 g
Fructose	1.250 g
Penomycin	0.1 g
Glycerol	0.72 mL
Egg yolk	20 mL
Distilled water	Up to 100 mL

Table A.2 Dilution of fresh semen to Tris extender supplement with olive oil (1:12)

DILUTION

Treatment	Tris based extender	Concentration of olive oil	Fresh semen
Tris+0% OO(control)	11	-	1
Tris+0.25% OO	10.97	0.03	1
Tris+0.50% OO	10.94	0.06	1
Tris+0.75% OO	9.47	0.09	1

Table A.3 Sperm concentration analyses by means of a haemocytometer method

CONCENTRATION SOLUTION

Component	Amount
Eosin	0.99 mL
Fresh semen	0.01 mL

Table A.4 Sperm membrane integrity analyses by hypo-osmotic swelling test (HOST) method

HOST SOLUTION

Component	Amount
Solution 1:	
Hos	0.20 mL
Preserve semen	0.10 mL
Solution 2:	
Hos	0.95 mL
Formalin	0.05 mL

Table A.5 Sperm viability analyses by eosin nigrosin staining method

EOSIN NIGROSIN SOLUTION

Component	Amount
Eosin	0.2 mL
Fresh semen	0.2 mL

APPENDIX B

Figure B.1 Verge extra virgin olive oil

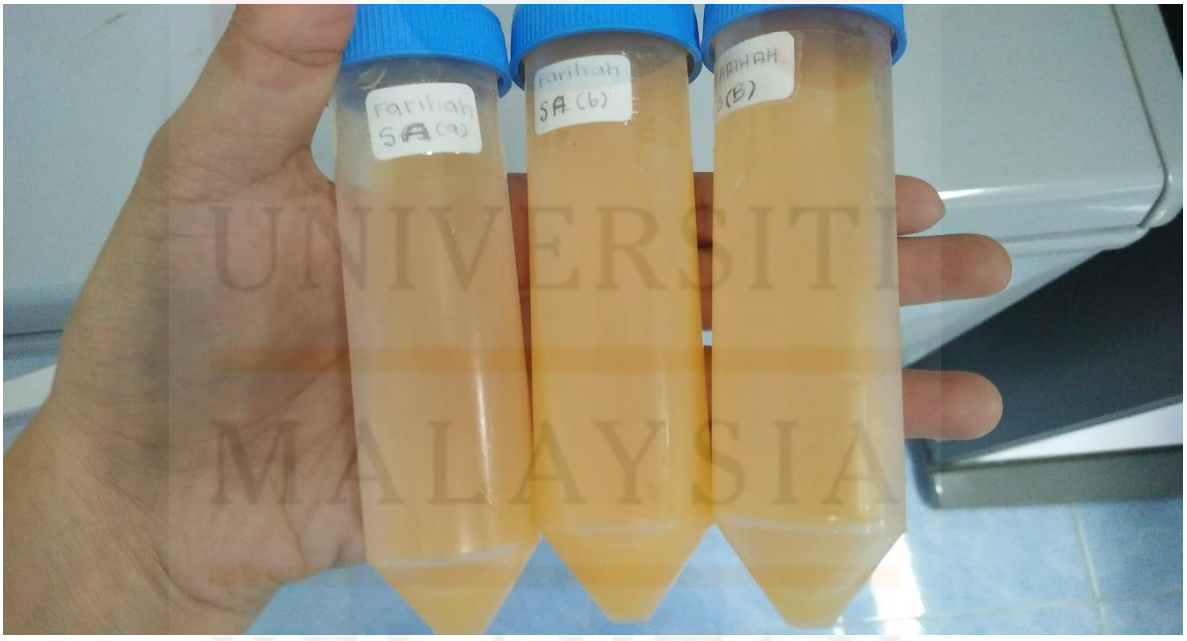

Figure B.2 Tris based extender solution

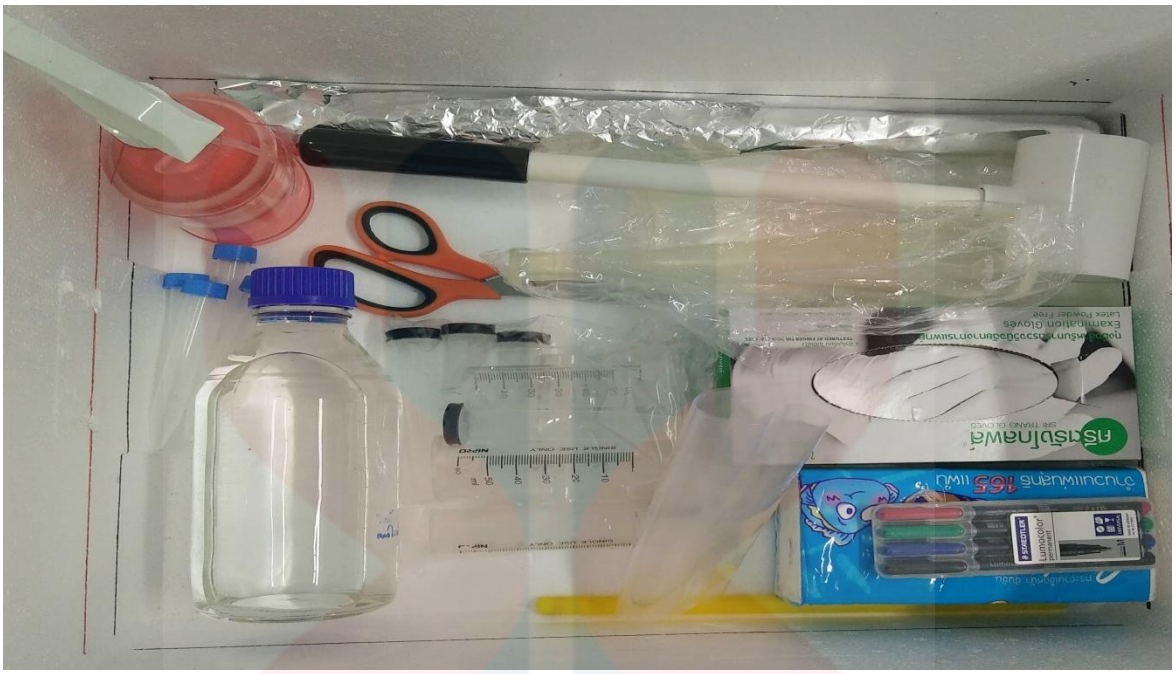

Figure B.3 Materials and apparatus use for semen collection

Figure B.4 The Brahman bull was tied up with a rope around his head in order to restrain and guard the bull for safety precaution when collect the semen

Figure B.5 Performing rectal palpation before collected the semen

Figure B.6 Collecting the semen via stimulation electro-ejaculation technique

Figure B.7 Electrojac was controlled to stimulate the current flow use

Figure B.8 Loaded the diluted semen into straw at 5°C

Figure B.9 Liquid nitrogen tank at -198°C for freezing semen treatments storage

Figure B.10 In line with president of PNU and her staffs