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EFFECT OF EXTENDER SUPPLEMENTED WITH DATE PALM POLLEN GRAIN ON CAPRINE SEMEN QUALITIES

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

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DECLARATION

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

Ng Pui Sie

Student's Signature

Student's Name: Ng Pui Sie

Date: 26/01/2022

I certify that the report of this final year project entitled "Effect of Extender Supplemented with Date Palm Pollen Grain on Caprine Semen Qualities" by Ng Pui Sie, matric number F18A0105 has been examined and 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.

Approved by:

Supervisor's Signature

Supervisor's Name: Dr. Raja Ili Airina Binti Raja Khalif

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Effect of Extender Supplemented with Date Palm Pollen Grain on Caprine Semen Qualities

ABSTRACT

Date Palm Pollen Grain (DPPG) were proved to maintain semen characteristics and quality in bulls, equines and buffaloes. However, DPPG as supplementation in the extender for goats are scarce. Hence, the aims of this experiment were 1) to investigate the semen motility and viability and 2) to examine the functional membrane integrity of semen in different concentrations of DPPG supplemented in goat semen extenders. In this study, DPPG was added in tris-citrate-fructose-yolk (TCFY) semen extender with 0%, 4% and 8% of concentration and chilled at 5^{o}C for 0 hour, 24 hours and 48 hours. DPPG was absence in control group. The diluted semen samples were assessed for sperm motility, viability and membrane integrity. A total of four ejaculations from two Boer bucks were used in this study. The results indicated that the ability to maintain sperm viability was significantly higher in the extender supplemented with 8% DPPG in all storage times ($P<0.05$). Plus, 8% of DPPG in extender significantly improved sperm motility after 48 hours (56.67±5.57) compared to 0% DPPG and 4% DPPG (40.83±3.00 vs 43.33±5.24), respectively. Results also showed that sperm membrane integrity percentage in 8% DPPG (40.00±4.04) was significantly higher than 0% DPPG (30.33±2.19) at 0-hour storage time. Overall, the extender supplemented with 8% DPPG assisted in maintaining sperm quality after 24 hours of chilled storage ($P<0.05$). Thus, the findings revealed that 8% of DPPG supplemented in semen extenders proved to maintain viability, motility and membrane integrity compared to control.

Keywords: Boer goat, TCFY, DPPG, chilling, sperm viability, sperm motility, sperm membrane integrity

Kesan Extander Dengan Penambahan Serbuk Debunga Kurma Ke Atas Kualiti Semen Kambing

ABSTRAK

Serbuk Debunga Kurma (DPPG) telah dibukti untuk megekalkan ciri dan kualiti air mani lembu jantan, kuda dan kerbau. Walau bagaimanapun, penggunaan DPPG sebagai suplemen dalam extender untuk kambing adalah terhad. Oleh itu, matlamat eksperimen ini adalah untuk 1) untuk menyiasat motilitas dan daya maju air mani dan 2) untuk meneliti integriti membran berfungsi air mani dalam kepekatan DPPG berbeza yang ditambah dalam pemanjang air mani kambing. Dalam kajian ini, DPPG telah ditambah dalam pemanjang air mani tris-sitrat-fruktosa-kuning telur (TCFY) dengan kepekatan 0%, 4% dan 8% dan disejukkan pada suhu 5^{o}C selama 0 jam, 24 jam dan 48 jam. Kumpulan control tidak mempunyai DPPG. Sampel air mani yang dicairkan dinilai untuk motilitas sperma, daya maju dan integriti membran. Sebanyak empat ejakulasi daripada dua kambing Boer telah digunakan dalam kajian ini. Hasil kajian menunjukkan bahawa keupayaan untuk mengekalkan daya maju sperma adalah lebih tinggi dengan ketara dalam extender ditambah dengan 8% DPPG dalam semua masa penyimpanan ($P<0.05$). Tambahan pula, 8% DPPG dalam extender dapat meningkatkan motilitas sperma dengan ketara selepas 48 jam (56.67±5.57) berbanding 0% DPPG dan 4% DPPG (40.83±3.00 berbanding dengan 43.33±5.24), masing-masing. Hasil kajian juga menunjukkan bahawa peratusan integriti membran sperma dalam 8% DPPG (40.00±4.04) adalah lebih tinggi dengan ketara daripada 0% DPPG (30.33±2.19) pada masa penyimpanan 0 jam. Secara keseluruhannya, extender ditambah dengan 8% DPPG membantu dalam mengekalkan kualiti sperma selepas 24 jam penyimpanan sejuk ($P<0.05$). Oleh itu, penemuan kajain ini mendedahkan bahawa 8% DPPG yang ditambah dalam pemanjang air mani dibukti dapat mengekalkan daya maju, motilitas dan integriti membran berbanding kumpulan kontrol.

Kata kunci: Kambing Boer, TCFY, DPPG, penyejukan, daya maju sperma, motilitas sperma, integriti membran sperma

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curled tail indicates intact plasma membrane and (B) Sperm with uncurled tail indicates inactive membrane

LIST OF ABBREVIATIONS

AI	Artificial Insemination
ART	Assisted Reproductive Technology
AV	Artificial Vagina
CASA	Computer Assisted Sperm Analysis
CIDR	Controlled Internal Drug Release
DPP	Date Palm Pollen
DPPG	Date Palm Pollen Grain
EE	Electroejaculator
H_0	Null Hypothesis
H_1	Alternative Hypothesis
HOST	Hypoosmotic Swelling Test
IBM	International Business Machines Corporation
IoT	Internet of Things
LDL	Low-Density-Lipoprotein

MOET	Multiple Ovulation Embryo Transfer
ROS	Reactive oxygen species
SD	Standard Deviation
SEM	Standard Error Mean
SPSS	Statistical Package for the Social Sciences
SSL	Self Sufficiency Level
TCF	Tris-Citric-Fructose
TCFY	Tris-Citric-Fructose-Yolk
TPG	Tris Pollen Grain
TMR	Total Mixed Ration

LIST OF SYMBOLS

cm	Centimetre
°C	Degree Celcius
°F	Fahrenheit
g	Gram
mg	Milligram
µl	Microlitre
mL	Millilitre
%	Percentage
+	Plus
±	Plus Minus
P	Probability
n	Sample Size

CHAPTER 1

INTRODUCTION

1.1 Research Background

A National Agrofood Policy 2021-2030, or known as DAN 2.0 was launched by government of Malaysia to transform the agro-food sector into a sustainable, competitive and high technology sector in order to boost economic growth of the country (Bernama, 2021). In conjunction, the productivity of goat meat was planned to increase in order to meet the demand of goat meat in Malaysia. Meanwhile, one of the challenges facing by the livestock industry is the increasing cost of agricultural products annually. This is because the nation depends heavily on import of quality commodities to maintain the market demand. Hence, appropriate measures such as boosting the quality livestock production using assisted reproductive technology (ART) available, for example semen cryopreservation, artificial insemination, estrus synchronization and more along with modernized advanced farming system are taken to improve the livestock farming sector (Abdullah *et al.*, 2011).

At present, natural extracts from fruits such as pomegranate (Sheshtawy *et al.*, 2016a), watermelon juice (Jimoh & Ayedun, 2020), orange juice and apple juice

(Adekunle, 2018) as well as date palm pollen grain (Sheshtawy *et al.*, 2016b) had proven to improve semen quality in preservation due to their antioxidant properties. Jimoh *et al.* (2021) stated that the bioactive constituents of citrus fruit possess antioxidant enrichment on its juice and are potent in combating oxidative stress in poultry semen. Similar to that, natural extracts from herbs showed similar effect in improving semen quality parameters in animals due to high antioxidant properties. The continuous interest in utilizing herbal remedies in animal reproduction to counteract the deleterious action of reactive oxygen species (ROS) and oxidative stress on body cells or tissues is still developing presently (El-Sisy *et al.*, 2018).

For example, herbal extraction nano-formulations from curcumin (*Curcuma longa*) supplemented into Tris-extender were able to enhance post-thawed semen quality in terms of promoting cryotolerance and preventing cryodamage of spermatozoa (Ismail *et al.*, 2020). Other than that, Najafi *et al.* (2018) stated that loading of nanophytosome of *Achillea millefolium*, a type of common yarrow flower which formerly used in medicinal purpose at 3mg/ℓ dose added into basic extender can improve sperm viability, motility and oxidative stress value of post-thawed cock semen. Date palm pollen (DPP) is found to improve fertility of both men and women in ancient times (Tahvilzadeh *et al.*, 2016). Therefore, this research focused on the effect of post-chilled sperm quality using date palm pollen grain supplementation in an extender for goats.

1.2 Problem Statement

Enhancement and elevating self-sufficiency level (SSL) of meat is vital in the agro-food industry to reduce dependency on importation of the commodity. In this case, application of assisted reproductive technology such as artificial insemination (AI) will help to achieve rapid livestock production and genetic improvement. However, the successful rate of AI greatly depends on several factors including quality of chilled-thawed semen. Composition of preservation media or semen extenders also contributes to the success rate of pregnancy.

The most common semen extender is the tris-based egg yolk extender with other integral components such as buffer, cryoprotectant and antibiotics to provide protection for the spermatozoa collected. Usage of semen extender elucidate problems in semen storage such as protect sperm against bacterial contamination and cold shock. Although studies on semen preservation are well versed, yet there is little to no study on the best commercial extender supplementation from natural resources (Amsah *et al.*, 2020). Benefits of natural resources included should be easily obtained, available all year round and cost effective to ease local livestock farmers, for example, date palm pollen grain that is being used in this research study.

1.3 Hypothesis

This study comprises of one hypothesis:

H_0 = Different supplementation of Date Palm Pollen Grain (DPPG) to extender will not improve the post-chilled semen quality of male goat.

H_1 = Different supplementation of Date Palm Pollen Grain (DPPG) to extender will improve the post-chilled semen quality of male goat.

1.4 Research Objectives

a) To investigate the effect of different concentrations of Date Palm Pollen Grain (DPPG) supplemented to goat semen extender on semen motility, viability and functional membrane integrity.

b) To examine the effect of different concentrations of Date Palm Pollen Grain (DPPG) supplemented to goat semen extender on the length of chilled short-term storage time.

1.5 Research Scope

The scope of this study focuses on the effect of different concentration of DPPG supplemented to the tris-based extender before and post-chilling at 4°C on goat semen quality. Semen samples will be collected once a week from male goats from Agro-Techno Park at University Malaysia Kelantan Jeli Campus. Analysis of fresh raw semen quality serves as control while analysis of semen quality under compound microscope after supplementation of DPPG at 0%, 4% and 8% before and after chilling will be carried out on day 0 (0 hour), day 1 (24 hours) and day 2 (48 hours) of semen collection. A total of 3 replications of each concentration of analysis will be taken into account and an average value is obtained for further data analysis.

1.6 Research Significance

This purpose of this study is to evaluate the potential of DPPG as supplementation to increase semen quality after short term storage in goat semen diluent. The findings of the study may significantly contribute in the research of assisted reproductive biotechnology especially in the goat industry that is currently being highlighted in our country. Plus, the finding on the effect of different concentrations of DPPG supplemented in the extender would allow further investigation on improving the usage of natural resources specifically for goats.

1.7 Limitation of Study

The limitations in this study is low number of male goats available for semen collection. Since the studies were conducted at Agro-techno Park, University Malaysia Kelantan Jeli Campus, a limited number of male goats were available. Hence, it was difficult to obtain high amount of semen samples for analysis. Besides, the Covid-19 pandemic has restricted us from going to commercial goat farms in Kelantan for more semen samples. Furthermore, going to Faculty of Veterinary of University Malaysia Kelantan for computer assisted sperm analysis was time consuming, ineffective and sperm quality may be affected during the long journey.

CHAPTER 2

LITERATURE REVIEW

2.1 Goat Industry in Malaysia

Goat industry is considered as subsector in the ruminant industry other than the cattle industry. It is one of the highlighted industries in the agro-food subsector in the nation in providing nutrients to Malaysian through meats and milk supply. Breeds of meat goats in Malaysia including Boer and Katjang, whereas dairy goat breeds are Saanen and Jamnapari. The value of this industry reached about RM175.55 million in year 2017. (Bakar *et al.*, 2018). However, this industry is relatively still small compared to other livestock industry like the poultry industry.

Traditionally, goats and sheep were reared in small flocks by local farmers as a secondary income source and as a savings form to get over hard times. (Johan & Jamaludin, 1998). Statistics from Department of Veterinary Services Malaysia (2020), shows that the total population of the goats were 359,200 to supply around 4,500 million tonne of mutton in year 2018. Nevertheless, figure 2 indicates that the consumption of mutton in the country is around 38,000 million tonne in 2018 so that is why we still have

to depends on live goat and sheep as well as mutton import from other countries mainly New Zealand, Australia, South Africa and India.

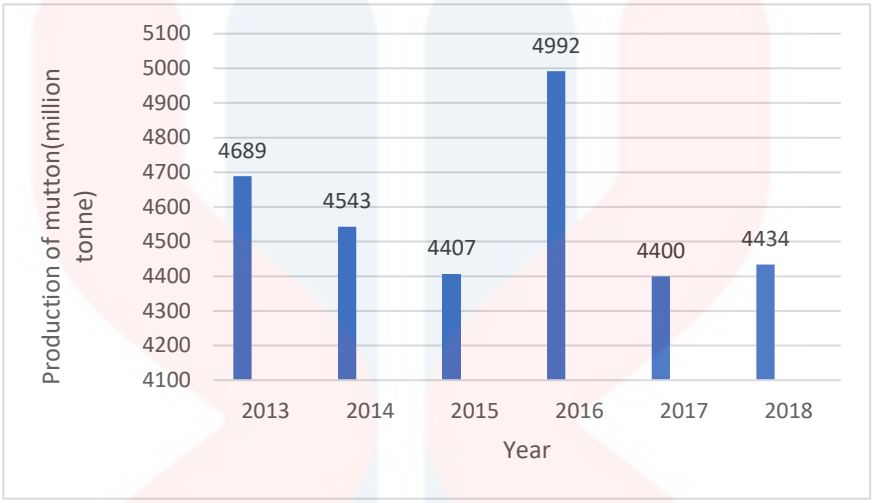

Figure 2.1: Total production of mutton (million tonne) in Malaysia from year 2013 to 2018 (source: Department of Veterinary Services Malaysia, 2013-2018)

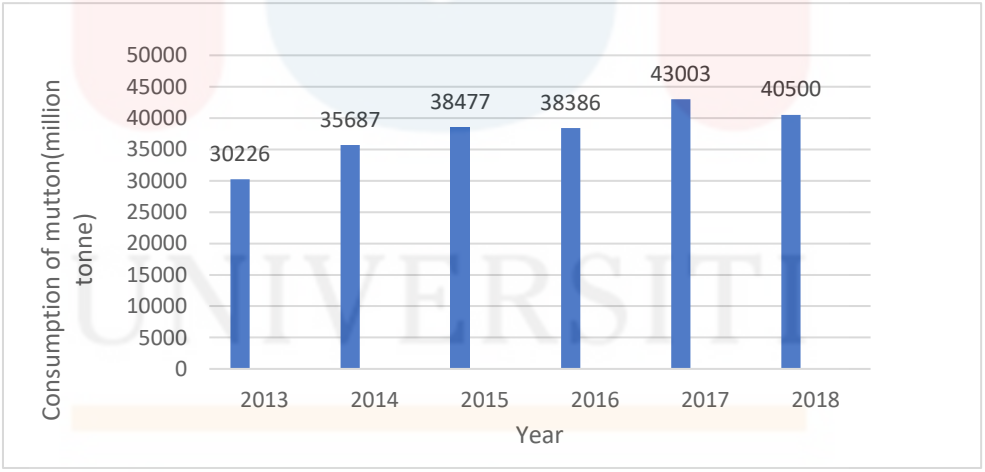

Figure 2.2: Total consumption of mutton (million tonne) in Malaysia from year 2013 to 2018 (source: Department of Veterinary Services Malaysia, 2013-2018)

Based on the Malaysian Development Plan and the National Agricultural Policy, production of mutton is targeted to increase in the effort of minimizing dependency of

meats from foreign country, and to improve self-sufficiency level of mutton to 35% which requires of 1.5 million heads increment of goats and sheep, said by Rafiu *et al.* (2012), as cited in Bakar *et al.* (2018) by implementing few plans, since the self-sufficiency level of mutton in Malaysia only ranged from 10% to 16% from year 2013 to 2018, which indicates low in local production. (Department of Veterinary Services Malaysia, 2020). In short, increasing the goat population is taught to be one of the ways in solving this problem in conjunction with importing selected goat breeds that have high productivity.

2.1.1 Boer Goat

Boer goat, originated from South Africa and it is one of the goat breeds producing meat commercially. White coat with red head, horned and roman nose were their general distinctive characteristics. Besides, they are kept extensively throughout Malaysia due to their fast-growing trait and produce high quality mutton. To illustrate, it is said to be able to grow 150 grams to 200 grams of weight per day. Commonly, mature male Boer goat and female Boer goat can weight an average of 172 kg and 120 kg respectively, with male reach puberty at 6 months old while female at between 10 to 12 years old. Boer goats are also well-known for their prolific traits where female can have 3 kidding in every two years and 2 kids per kidding. (Casey & Van, 1988).

2.2 Assisted Reproductive Technologies (ART)

Reproduction plays as a fundamental role for all living things and it guarantees the continued existence of every species (Bustani, 2021). In the modern livestock industry, assisted reproductive technique (ART) plays a crucial role in animal reproduction and being implemented especially during breeding season for reproductive performance enhancement, genetic improvement and most importantly, to boost the economy. Until year 2012, while ART are widely applied in the cattle industry, limitations are bigger in the goat industry due to restrictions in naturally occurring of anoestrus period, variable response to superovulatory treatments, failure of fertilisation and requirement of surgery for collecting and transferring of gametes and embryos. To highlight, high reproductive rates is also essential for a livestock farm to increase their operation productivity. In the effort to fulfil the increasing demand of animal products, various reproductive technologies have been developed. It is commonly known that artificial insemination (AI) technique, estrus synchronisation, multiple ovulation embryo transfer (MOET) and cryopreservation technique are part of the ART. (Amiridis & Cseh, 2012). Furthermore, semen collection from the male animals is one of the preliminary steps towards ART (Tarmizi, 2020). Not only that, as one of the vital tools in modern breeding practices for genetic improvement, the objective of AI could only be achieved with efficient semen extender. In short, semen extender is the only medium enabling us to exploit the reproductive potential of male animal with minimized spreading of venereal disease (Rehman, 2013).

2.3 Semen Collection Techniques

Nowadays, there are various ways to collect semen of different livestock species, such as bucks, rams, bulls or boars. Based on Nuti (2016), the most common methods used in ram and buck semen collection are artificial vagina (AV) and electroejaculator (EE). Among the two methods, AV is said to be the easier and faster technique to be performed and cause no pain and stress to the animal. A doe on heat will be bought into the pen for the buck to mount on it. It can be natural heat or man induced by using hormonal treatment of progesterone hormone contained in the controlled internal drug release (CIDR) device, which is a common protocol for estrus synchronisation or fixed time breeding technique. Doe in heat stands better during semen collection. This is because doe on heat emits an odour for the buck to ejaculate better. The buck will stick his nose into the urine stream of the doe to get a god whiff as its courting behaviour (Gail, 2021). Prior to ejaculation, the buck is allowed for a few false mounts, then the collector will direct the AV into the penis of the buck and a few thrust can be sensed. After done collecting semen, it should be protected from direct sunlight and cold temperatures (Nuti, 2016).

On the other hand, electroejaculator (EE) is more convenient in collecting semen when the bucks are not properly-trained for artificial vagina collection method previously, or unable to mount physically and no suitable doe is available. During semen collection using EE, the buck has to be well-restrained on a lateral position to avoid personnel injury as violent reaction of the buck may arise from the electric shock (Ramukhithi *et al.*, 2011b; Munyai, 2012). The application of three to five volts of electrical stimulation through rectal opening to the accessory glands cause the buck to ejaculate (Sundararaman,

Kalatharan & Edwin, 2007). Higher electrical volts will lead to vocalisation, muscle convulsion and even lying down of the buck sometimes (Ortiz-de-Montellano *et al.*, 2007).

Under comparison, AV is viewed as a better tool in collecting semen of domestic animals than the EE method (Wulster-Radcliffe *et al.*, 2001). The use of AV would ease the work of semen collection if only pre-training of the bucks in the presence of a doe on heat for a period of at least two weeks been carried out (Palmer, 2005; Marco-Jiménez *et al.*, 2005; Mahoete, 2010; Matshaba, 2010; Ramukhithi *et al.*, 2011b). However, the EE method has the advantage to be used when the bucks are reluctant to serve the AV even with doe on heat due to lack of libido or sexual desire, providing that the electrical volts for stimulation are not more than six volts or else it could stress the animal (Orihuela *et al.*, 2009; Ortiz-de-Montellano *et al.*, 2007; Sundararaman, Kalatharan & Edwin, 2007).

2.3.1 Artificial Vagina (AV)

The usage of AV is similar to natural service and most hygienic semen collection technique. The AV for goat resembles a car radiator hose and about 15cm in length. It has an inner rubber liner placed between the liver and hose that allows it to accommodate 37°C warm water, creating a stimulation of vagina of doe to provoke the buck to ejaculate. A cone-shaped latex rubber collector placed on the AV helps to direct the semen to the graduated semen collecting tube connecting to the end of the cone (Nuti, 2016). The volume of buck's semen collected with AV is 0.5mL to 2.0 mL while the concentration of the spermatozoa is $2.5–6\times10^9$ mL for each ejaculate (Liu & Troy, 2018).

Figure 2.3: Artificial vagina for sheep and goats (source: Minitube, 2021)

2.4 Semen Extender

Semen extenders are liquid diluents that are added to semen in order to preserve their fertilizing ability and desirable properties such as viability and motility in a long term besides increasing the volume of ejaculated semen. The main objective of extenders serve is to protect the sperms from a few detrimental factors such as osmotic and freeze shock, oxidative stress and cell damage from ice crystals (Bustani, 2021). Hence, it contains various protective substances that allow the survival of spermatozoa even it stays outside of the reproductive tract of female animal. The main material that protects spermatozoa against cold shock is lipoproteins, commonly found in egg yolk or milk. Tris, sodium phosphate and citric acid act as buffering agent to maintain the pH of the medium (Rehman, 2013). Besides, glucose acts as metabolizable substrates that supplies spermatozoa with abundant source of energy to keep them motile, whereas antibiotics such as penicillin or streptomycin that are added into the extender serves as a defender to eliminate any growth of unwanted bacterial microorganisms (Terry, 2003).

Extenders appear in two forms, preserving the sperms for an average of three days in chilled form or cryopreserved for up to years (Johnston, 2012). In the present, there are

various types of extenders that can be found useful in preserving sperms no matter during chilling or cryopreservation process. For example, the most common used tris-citrate-fructose-yolk (TCFY) extender, which is a type of egg-yolk based extender. Besides, skim-milk based extender, soy-lecithin based extender, Laiciphos, Beltsville Thawing Solution and Biociphos Plus are extenders that can be obtained in protecting sperms in a long run (Paulenz, 2005). The egg-yolk based extender is an example of animal source lipoprotein-based extender while the soy-lecithin based extender is plant source lipoprotein-based extender, both are commercially available. Different types of material sources are to better cater diverse problems in preserving sperms as it provides various features.

2.4.1 Tris-Citric acid-Egg Yolk Fructose (TCYF) Extender

The egg-yolk based extender used in this study consists of tris base, citric acid, chicken egg yolk, fructose, glycerol and penicillin. These materials help to preserve the fertility of animal sperm at high extension rates. Based on Swelum *et al.* (2018), chicken egg yolk is most suitable to be added in semen extender for buck semen preservation. Thus, it is commonly used as non-penetrating cryoprotectant source in protecting the sperm from freeze shock during chilling or freezing process. To further illustrate, egg yolk contains protein, lipid, carbohydrate and some minerals where it works as the reservoir for cholesterol and phospholipid to aid in protecting sperm cell membrane against freezing or chilling injury. Furthermore, the yolk possesses low-density lipoprotein (LDL) which maintains sperm membrane phospholipids throughout the

chilling or freezing process (Amirat *et al.*, 2005; Layek *et al.*, 2016). In most countries, a standard of 20% of egg yolk is used in extender as it is cheap and easily available (Rehman, 2013).

Other than that, glycerol was discovered by Polge *et al.* (1949) to be as an excellent cryoprotective agent for multiple animal species. It can penetrate the sperm cell membrane and act as anti-shock agent and prevent intracellular crystallization. This can be done by protect the sperms from the shock of sudden drop of temperature. As a result, post-chilled or post-thawed sperm quality can be improved. Fernández *e*t *a*l. (2006) found out that adding 6% of glycerol in egg-yolk semen extender could get better sperm quality outcomes compared to 3% glycerol following a rapid cooling rate.

Fructose or glucose serves as a primary energy source for the spermatozoa. Energy is much needed in sperm cell movement and metabolism. The sperm cell naturally contains fructose for self-utilization, however during extension of yolk extender with fructose, the components are also utilized. From that, sperm quality could be improved. Cantanhêde (2018) suggested that fructose is the best monosaccharide in maintaining sperms' functional membrane integrity and motility post thawing.

Tris base or known as hydroxymethyle aminomethane and citric acid are both common buffers used in various types of extenders. Tris-yolk extender containing glycerol was first developed in 1963 for both raw and frozen semen. (Foote, 1998; Iritani, 1980). During semen processing for preservation, cellular metabolic activities take place and cause lactic acid production. When the extracellular environment become more and more acidic, the pH drops and lead to decrement of spermatozoa energy production as well as influence the storage life of the semen (Rehman, 2013). Overall viability percentage declines from here.

On top of that, antibiotics such as penicillin and streptomycin added into semen diluent prevent microbial contamination and extend shelf life of the diluent. Normally, the fresh raw semen from the animal that are in good health and robust condition is free from microorganisms. However, bacteria may invade during semen collection and diluting with extender process, and since sugar such as fructose is added, the risk of bacterial contamination gets higher. When this situation occurs, it may interfere the semen parameters. *E. coli*, *Salmonella spp* and other types of bacteria are seen to be potential treat of contamination (Gadea, 2003).

2.4.2 Date Palm Pollen Grain (DPPG)

Phoenix dactylifera L., commonly known as date or date palm, belongs to the genus Phoenix, which has 14 species of mostly ornamental or wild palms, with only Phoenix dactylifera being cultivated for its fruit and date palm pollen is obtained from there (Torres *et al.*, 2021). In Malaysia, the dates fruit has gained popularity especially local Muslim consumers due to their benefits for health. In reality, the plantation of dates fruits had been undergone in past years to fulfil the increasing demand of the locals and it succeeded (Haris *et al.*, 2019).

It is well known that date palm pollen grain (DPPG) was used as dietary supplement in improving reproduction ability of both men and women since ancient times. In fact, DPPG is commercially used in enhancing male fertility rate by increasing sperm count, motility and DNA quality (Amsah *et al.*, 2020).

The protective properties of DPPG is due to the potent nutritive contents of DPPG that is rich in phytochemicals such as flavonoids estrone, estradiol, and crude gonadotrophic substance (Abedi *et al.*, 2014). Studies from El-Kashlan *et al.* (2015); Hassan (2011) and Bishr & Desoukey (2012) had proven that DPPG possess a high level of antioxidant compounds such as flavonoid, phenolic, carotenoid and vitamins like vitamin A, vitamin E and vitamin C. These compounds have contributed in the free radical scavenging activities which prevent the body cells from damage caused by oxidation. Furthermore, the pollen part had exhibited antibacterial, antifungal and antiviral activity which is believed to have useful effect in extender supplementation to avoid contamination and preserve the semen qualities (Abedi *et al.*, 2012; Mallhi *et al.*, 2014).

Few studies mentioned that DPPG has a beneficial effect on sperm qualities of animal's sperm. To further illustrate, study from El-Sisy *et al.* (2018) had demonstrated that supplementation of DPPG into extender is useful in preserving Arabian stallion sperm during chilling and freezing process. Besides, DPPG added into Tris-Citrate Fructose extender had proven its preserving capacity in maintaining sperm motility of chilled and thawed buffalo bull sperms (Sheshtawy *et al.*, 2016b). Meanwhile, another study by Amsah *et al.* (2020) stated that supplementation of DPPG in extender had showed the ability to protect viability of cryopreserved bovine sperm.

2.5 Semen Preservation

During the process to preserve semen, it is important to know which type of extenders to choose from in cryopreservation of either chilling or freezing the sperms. Based on Evans and Maxwell (1987), tris-based extender was suitable to be utilized prior to semen freezing to protect the sperms beside increasing semen volume, allowing more artificial insemination doses to be arranged.

Other than preserving semen using suitable extenders, the temperature used in cryopreservation at which the semen is stored is also important that have an impact on sperm survival rate (Bapope, 2015). Using a very low temperature to preserve structurally intact living cells and tissues such as sperm cells is known as cryopreservation (Pegg, 2007). While in animal semen cryopreservation, it is considered as a technique that is widely used to preserve sperms of livestock and wildlife to be used as supply for breeding purpose or genetic diversity maintenance in the effort of conserving endangered species. In a standard cryopreservation freezing protocol, the semen will be frozen in liquid nitrogen at −196°C after added with extender and cryoprotectant. Besides, semen samples will be kept at 5°C after added with extender under chilling protocol (Zampini *et al.*, 2020). In chilling protocol, the semen can be stored from hours to days when short term storage is preferred whereas it can be kept for months or even years under freezing protocol when long term storage is desirable before insemination.

Based on Blanchard (2003), storing the extended semen in temperature near refrigeration can maintain the viability or longevity of sperms better than storage at higher temperature due to reduced metabolic activity in high temperature. In short, extended semen cooled to 4°C to 6°C for storage is more superior compared to storage at room

temperature at 20°C to 25°C when the extended semen will be used for insemination 1 to 2 days later.

2.5.1 Thawing

The thawing procedure of sperm aims to recover the activities of previously chilled or frozen semen prior to insemination. From some researches, it is recommended that using warm water at 35°C or 95°F for sperm thawing for 40 to 90 seconds gives desirable result in improved sperm cells recovery compared to other methods of thawing such as using ice water at 5°C or 41°F. This is due to sperms are only exposed to critically dangerous temperature for only a small amount of time and the rise in temperature is fast enough to minimize damage of sperms in warm water thaw (Michael, 2016).

The major concern is the mishandling of semen after thawing, causing cold shock which is a sudden decrease of temperature. The effect of cold shock is irreversible while it can occur while preparing the thawed semen for insemination or sperm analysis. Thus, extra precaution should be taken to prevent cold shock of semen to fully realize the advantage of warm water thaw (Michael, 2016).

2.6 Semen Quality Assessment

In evaluating semen quality, there are two ways that can be done, namely the manual way and technological way. The manual way means that the semen parameters are observed with naked eyes of the evaluator and it is divided into macroscopic and microscopic technique. In macroscopic technique, the parameters are volume and colour of semen while viability, motility and membrane integrity can be observed through microscopic technique with the help of certain chemicals such as dyeing agent and computation procedure is required to obtain the final percentage of each parameter.

In the technological way of assessing semen quality, computer-assisted semen analyzer or known as CASA is utilized. It is actively used in animal reproduction field for quick and reliable reproductive data assessment using image analysis with a phase-contrast microscope and computer measurements of motion characteristics, concentration and morphology of sperms (Didion, 2008; Valverde & Madrigal, 2018). Usage of CASA system is able to substitute routine quality analysis that has the probability introducing biases and ultimately lead to various degrees of inaccuracy (Valverde & Madrigal, 2018).

2.6.1 Volume

The volume of ejaculate of a buck is generally 1.0 mL, ranging from 0.5mL to 1.2 mL (Ax *et al.*, 2016). It varies among individuals and also between every ejaculate of the same individual. The factors that affect the semen volume include age, body size, frequency of service and health. For example, the semen volume may increase following the rising of age for up to 6 to 8 years old. Other than that, in artificial animal reproduction, volume of teaser bull semen may be increased through regular practising to aid them acclimate to semen collection with minimal stress.

To obtain the volume of fresh raw semen precisely, it is better to read from the graduated semen collecting tube immediately right after the animal ejaculate. This is because delayed data reading may cause inaccuracy.

2.6.2 Colour

The colour of a buck's semen is usually greyish-white to yellow and the colour variation is more than ram semen. Semen colour of buck may appear milky white, creamy or opaque due to highly concentrated and less mobile. To add on, viscosity of fresh semen increases with sperm concentration. As a matter of fact, colour of ejaculates varies between each different buck, and also between ejaculates from the same buck (Ax *et al.*, 2016). However, if the semen colour turns out odd colour like dark red to pink, it might indicate that there is some health issue occurs to the animal.

In semen evaluation, the semen colour is observed visually and recorded down in each ejaculate for further analysis.

2.6.3 Viability

Viability in sperm quality assessment is defined as the ratio of live and dead sperm in the semen. The estimation of live and dead sperms is one of the crucial semen qualities that reflects the feasibility of the semen to be diluted and utilized in artificial insemination in livestock reproduction. Percentage of live sperms is directly related to sperm motility. Under normal circumstances, the higher the percentage of motile sperms, the higher the percentage of live sperms. However, semen with low sperm motility rate can still have normal live sperm percentage.

In sperm viability evaluation, staining with dye such as eosin-nigrosine is needed to differentiate the live sperms from the dead sperms. Dead sperms will be stained with the colour of the dye while live sperms will remain colourless. To further elaborate, plasma membrane is intact in live sperms but lost its integrity in dead sperms. Eosin is a stain that can pass through the loosely integrated plasma membrane of the dead sperms and stains it pink whereas nigrosine is a negative stain that gives the background colour so that the evaluator can easily spot the live and dead sperms. In addition, the eosin stain will not pass through the intact plasma membrane of the live sperms thus they still appear colourless or white.

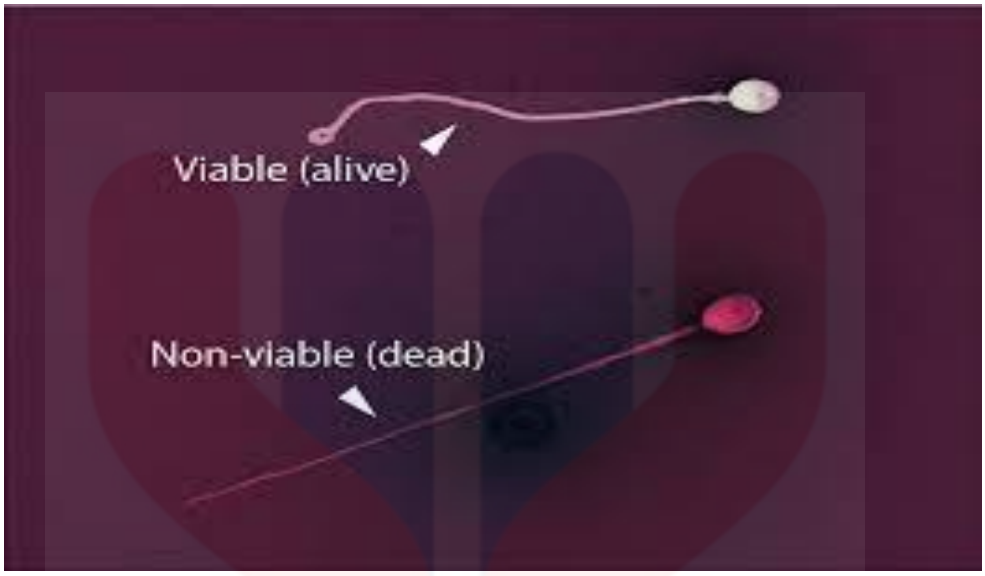

Figure 2.4: Schematic presentation of viable and non-viable sperm cells (source: Rothmann SA and Reese AA, 2010)

2.6.4 Functional Membrane Integrity

Hypoosmotic swelling test (HOST) is a well-known tool in evaluating membrane integrity of plasma membrane of spermatozoa of various domestic livestock such as horses, swine and cattle (Fonseca *et al.*, 2005). It is a useful indicator showing fertility potential of the spermatozoa.

The assay works based on the fact that fluid transport occurs across an intact cell membrane under hypoosmotic conditions until equilibrium is reached between the inside and outside of the cell. When the spermatozoa with intact cell membrane are exposed to hypoosmotic solution, the cell will expand, causing a bulging of the plasma membrane due to influx of fluid. The sperm tail is usually closely surrounded by plasma membrane. Thus, tail curling or bending occurs. Spermatozoan that shows a curled tail may also be

referred as swollen tail. Therefore, spermatozoa will only show curled tails when they have physically and chemically intact plasma membrane under hypoosmotic condition, whereas spermatozoa with an inactive membrane will not show curled or swollen tails (Jeyendran *et al.*, 1992).

Although HOST primarily identifies intact structural integrity of sperm cells which exhibit tail curling, it may also be a good indicator of intact head plasma membrane. This is because biochemical integrity of sperm plasma membrane is quite important in functioning of spermatozoa, not only for motility purpose, but also for events occurs during fertilization, for instance, acrosome reaction, capacitation and binding of sperm cell to the surface of egg cell (Jeyendran *et al.*, 1992).

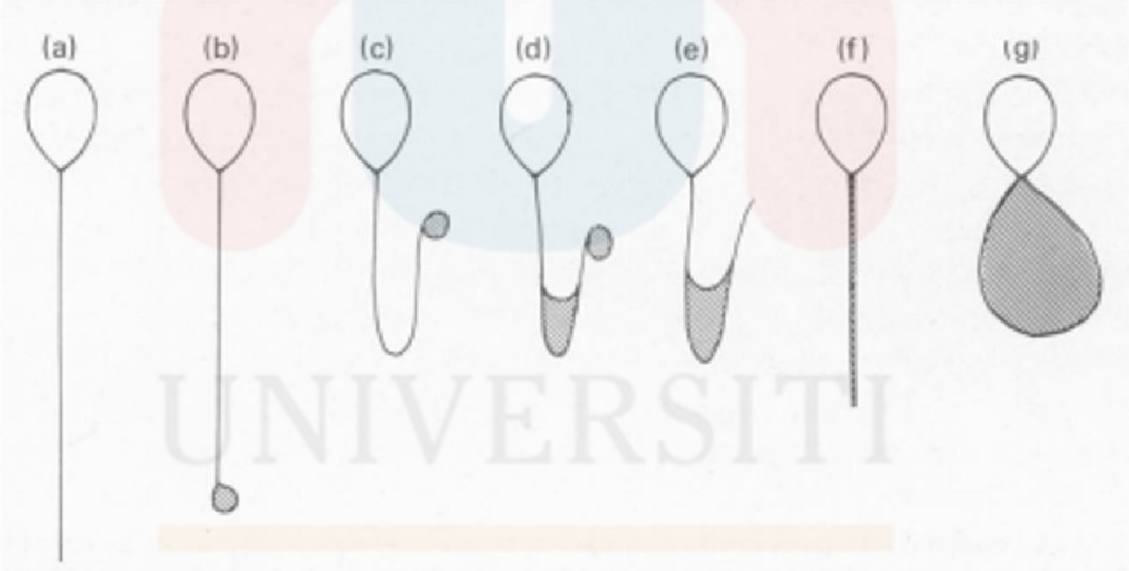

Figure 2.5: Schematic presentation of no reaction (a) and with mild (b) and severe (c-g) HOST reaction sperm cells. (source: Fonseca *et al.*, 2005 and Jeyendran *et al.*, 1984]

2.6.5 Individual Motility

According to Vijayaraghavan (2003), motility of in vivo sperms is the most essential indicator of fertilising ability. Motility evaluation involves subjective estimation of the spermatozoa's viability and their quality in terms of motility. Assessment of sperm motility can be conducted with either raw or extended semen. It indicates the sperm performance in its own accessory gland fluid in evaluation of motility of raw semen (Ax *et al.*, 2016).

When evaluating sperm motility, it is essential to avoid the semen from injurious condition such as excessive heat or cold prior to analysis as they are highly susceptible to extreme environmental temperature fluctuation. Thus, an experienced evaluator in using properly equipped microscope with built-in stage warmer is required to enhance the reliability of sperm motility assessment (Ax *et al.*, 2016). If the microscope used in sperm motility evaluation does not equipped with built-in stage warmer, the evaluator has to drop the semen in a pre-warmed glass slide and assessment should be done as quickly as possible.

2.7 Factors Affecting Semen Quality

Factors affecting semen quality are divided into extrinsic factors and intrinsic factors. Extrinsic factors include temperature, stress and nutrition whereas intrinsic factors comprise breed, age and daylight length.

2.7.1 Extrinsic Factors

Extrinsic factors that affect semen quality exert their influence from the outside or surrounding environment. For instance, temperature and stress as well as nutrition factors.

2.7.1.1 Temperature and Stress

According to Suyadi (2012), environmental temperature and season are said to be having an impact on sperm concentration and semen volume of Boer goats. Sheep and bucks are susceptible to high surrounding temperature and high humidity. When the environmental temperature is too high, it may affect fertility of the bucks while the risk of heat stress at any air temperature may increase when surrounding humidity is high (Bopape, 2015). This is because high environmental humidity traps the heat, causing the heat could not disperse easily. Subsequently, high surrounding temperature leads to rising of body temperature of the animal, bringing about some reproductive issues that could cause a loss to the farm. Other than that, elevated surrounding temperatures have an adverse effect on animal semen quality. To further illustrate, seminal characteristics such as spermatozoa viability and percentage of spermatozoa morphological defects are greatly affected (Catunda *et al.*, 2011). Moreover, sperm concentration and motility are also affected (Kastelic, 2013). This is due to exposure of the testis to high surrounding temperatures interferes the normal production of spermatozoa or spermatogenesis indirectly. Kastelic (2013) reported that when temperature of bull testes is 2°C to 6°C cooler than core body temperature, more fertile sperms can be produced. In short, the higher the testicular temperature, the more deterioration of the sperm quality causing infertility in bulls. Similar studies on bucks showed the same result, high ambient temperature increases the scrotal temperature and subsequently causes a decline in sperm quality (Al-Ghalban, Tabbaa & Kridli, 2004; Zarazaga *et al.*, 2009; Daramola & Adeloye, 2010).

2.7.1.2 Nutrition

Nutrition is one of the important factors making an impact on the reproductive performances of animals. An insufficient or imbalance diet can reduce the reproductive efficiency of bucks due to deficiency of certain nutrients required by the body for either maintenance, growth or reproduction. Under normal circumstances, for example, in intensive or closed-house rearing system, the feed given to the bucks are total mixed ration (TMR) or commercial pellets with some dry forages that are enough to meet the nutritional requirement such as dry matter, protein, fat and so on of the animals. However, if the bucks are raised under extensive or semi-extensive systems, where feed availability is highly dependent on the season and require proper management of the pasture for grazing, farmers may not able to control and record the exact feed intake, causing them hard to trace the nutrient content intake of the bucks (Zarazaga *et al.*, 2005). A good and balanced nutritional regime optimize the feed intake and improve average daily gain rate, that will directly further enhancing testicular development and sexual maturity in ram lambs, as reported by Bielli (1999). The heavier the testicular weight, the better the sperm quality, and that links the relationship between better nutrition intake and higher sperm-producing parenchyma in the testis of the animals, which in turn referring to higher reproductive function (Gebre, 2007).

2.7.2 Intrinsic Factors

Intrinsic factors act from within an individual that affect semen quality. For example, breed and age factors.

2.7.2.1 Breed

Scrotal circumference and semen quality of bucks differs not only among different breeds, but also among individuals of the same breed (Langford *et al.*, 1998; Noran, Mukherjee & Abdullah, 1998; Al-Ghalban, Tabbaa & Kridli, 2004). For example, the Gorno Altai bucks produce approximately 1.6 mL of semen, which make them having lower semen volume than the South African unimproved indigenous bucks which produce about 1.9 mL of semen per ejaculation, as stated by Webb, Dombo and Roets (2004). Despite that, the Gorno Altai bucks are reported to produce higher semen concentration compared to South African unimproved indigenous bucks. Similarly, Boer bucks are claimed to produce higher sperm concentration and higher semen pH than South African unimproved indigenous bucks (Ramukhithi, 2011a). Therefore, in order to look for the bucks producing desire semen quality, inspection and evaluation have to be made within the herd.

2.7.2.2 Age

Based on Suyadi, 2012, age of animals has an impact on the sexual behavior in bulls. In other words, age of animals is one of the major contributing factors that need to be take into account as it may affect the semen quality. For example, mature animal has a more efficient sexual behavior than young animal (Ahmad & Asmat, 2005). This may due to different age level indicates various semen characteristics and scrotal circumference (Toe *et al.*, 1994), and different testicular size that have an influence in total sperm productivity (Ahmad & Noakes, 1995). To further demonstrate, adult bucks in two to four years old have a larger scrotal circumference than yearling bucks of ten to twelve months old. Hence, adult bucks have a higher sperm concentration and motility as opposed to the yearlings. However, the percentage of abnormal sperms are found to be higher in adult bucks (28.8%) compared to yearling bucks (22.1%) (Al-Ghalban, Tabbaa & Kridli, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials & Apparatus

The materials and apparatus used in this study were male Boer goats, gred A eggs, Date Palm Pollen Grain (DPPG) powder, graduated Falcon tube, filter syringe, aluminium foil, dropper, tris buffer, citric acid, fructose, distilled water, pure glycerol, measuring cylinder, beaker, siever, filter paper, micropipette, tube, compound microscope, glass rod, eosin powder, 10% nigrosine stain, media bottle, sodium citrate dihydrate, sodium chloride, spatula, syringe, hot plate, magnetic stirrer

Figure 3.1: Date palm pollen grain in powder form

Figure 3.2: Tris-base buffer in crystalline powder form

Figure 3.3: Citric acid in crystalline powder form

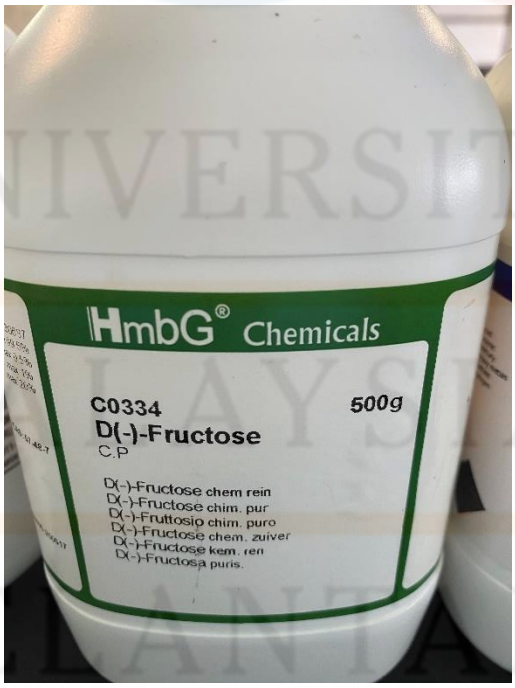

Figure 3.4: Fructose in powder form

3.2 Animals and Treatment

This study was conducted at the animal farm, namely Agro-Techno Park located in University Malaysia Kelantan Jeli Campus. A total of two male Boer goats of age around 1 and a half years old, reared under intensive management system equipped with IoT devices were selected. Thus, both the goats received the same nutrition and management practice in assuring we got the most accurate results. Besides, a total of three female Boer goats were selected for receiving Controlled Internal Drug Release (CIDR) in advance prior to semen collection. One day before semen collection, one CIDR was removed from one female goat for it to exhibit estrus sign so that we can utilize the female goat on heat to arouse the sexual desire of the male goats for ejaculation in order to have higher successful rate of semen collection. Same procedure was carried out with CIDR removed from one female goat at weekly interval for 2 weeks.

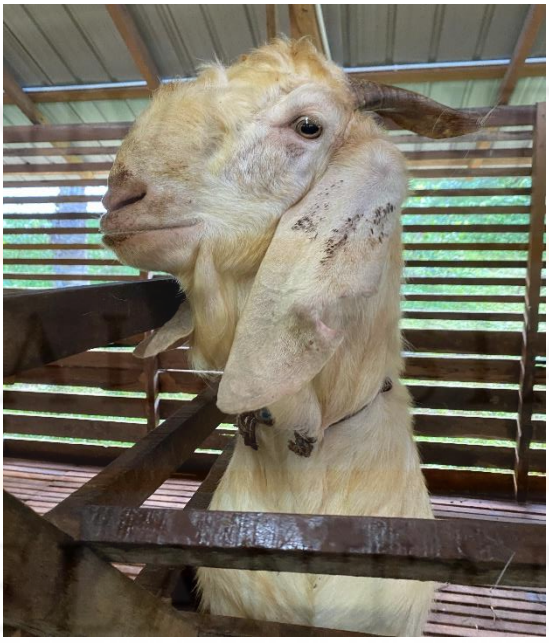

Figure 3.5: Male Boer goat A used for semen collection

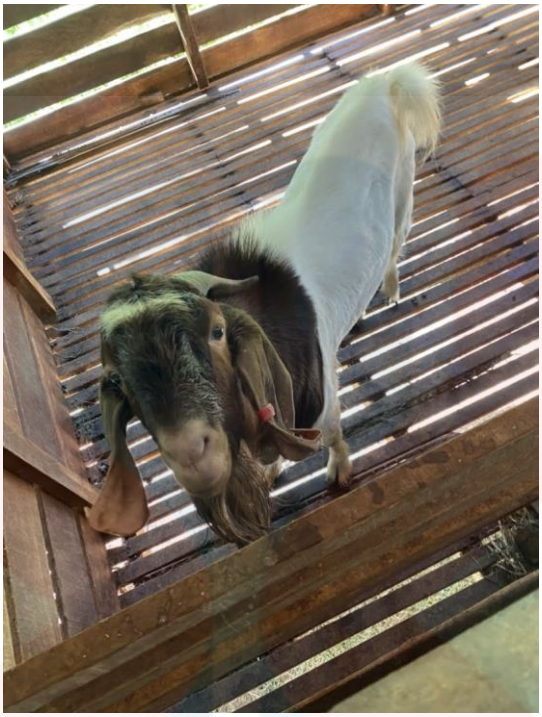

Figure 3.6: Male Boer goat B used for semen collection

3.3 Procedure, Method and Experimental Design

After semen was collected, it was diluted with Tris-Citric Acid-Egg Yolk Fructose (TCYF) extender and glycerol. Then, different amount of Tris Pollen Grain (TPG) stock solution was added into 2 different falcon tubes filled with diluted semen to obtain extender supplemented with 4% (treatment 1) and 8% of DPPG (treatment 2) respectively, whereas the control group is diluted semen without DPPG supplementation (0% DPPG). After that, semen assessments were carried out at 0 hour, 24 hours and 48 hours after chilling at 5°C with 3 replications for control group and each treatment groups in each assessment respectively.

3.3.1 Preparation of Stock Solution Tris Citric Fructose (TCF)

A total of 1.514g of tris base, 0.8375g of citric acid, 0.625g of fructose and 0.05g of penicillin were dissolved with 50mL of distilled water (Amsah *et al.*, 2020). The solution was mixed on a hot plate with magnetic stirrer for 10 minutes to get a more even dissolved solution.

3.3.2 Preparation of Stock Solution Tris Pollen Grain (TPG)

After preparing the TCF stock solution, 100mg of Date Palm Pollen Grain (DPPG) powder was dissolved in 40mL TCF stock solution to get TPG stock solution to be supplemented into the extender. The solution was mixed thoroughly using magnetic stirrer and hot plate. Then, the solution was filtered using filter paper. After that, the TPG stock solution was stored in chiller at 4°C until further use.

3.3.3 Preparation of 6% Glycerol

A total of 9.4mL distilled water was measured using a 10mL measuring cylinder and put it into a beaker. Next, 0.6mL of pure glycerol was taken out using syringe and put into the same beaker. Both solutions in the beaker were stirred until it is evenly mix. Finally, 10mL of 6% glycerol was obtained.

3.3.4 Preparation of Tris-Citric Acid-Egg Yolk Fructose (TCYF) Extender

A total of 3.028g of tris base, 1.675g of citric acid, 1.250g of fructose and 0.1g of penicillin were dissolved with 100mL of distilled water in a beaker. Then it was stirred on a hot plate for 10 minutes. Next, 2 eggs were cracked and filter the eggs with a filter paper until all egg whites are removed, left only egg yolks. The membrane of the egg yolk was broken to get 20mL pure egg yolk. The egg yolk was mixed into the solution in the same beaker and stirred on hot plate for another 10 minutes. The TCFY solution was first filtered using filter paper and then again second filtered using filter syringe. After filtering, he TCFY extender was stored in a chiller at 4°C until further use.

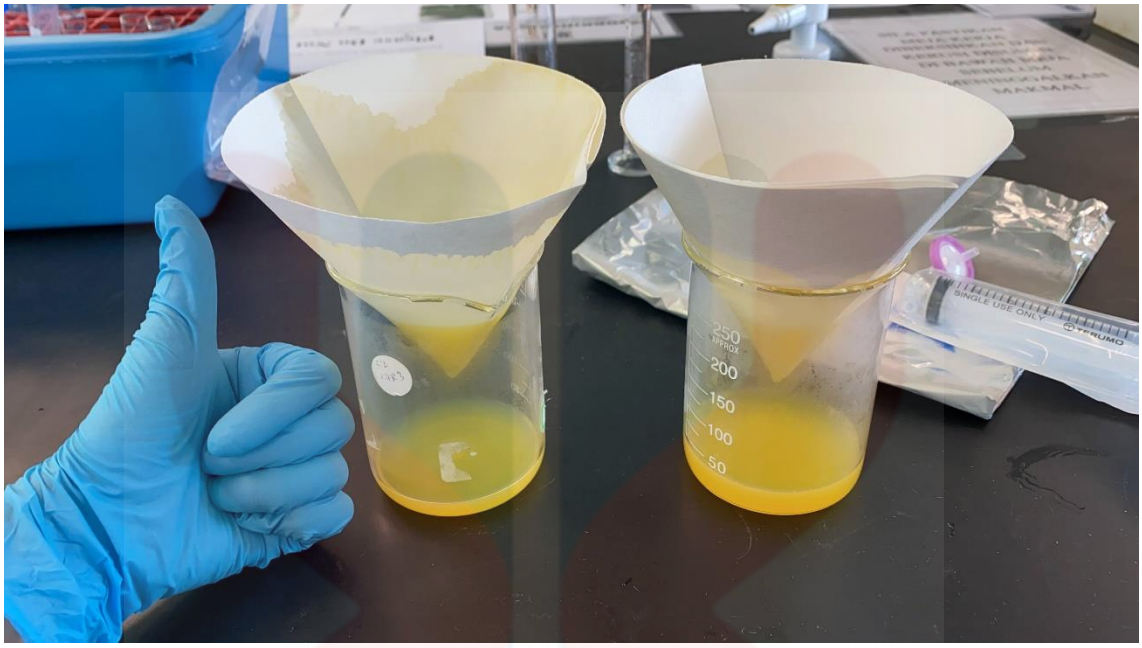

Figure 3.7: Filtering of TCFY solution using filter paper

Figure 3.8: Filtering of TCFY solution using filter syringe

Figure 3.9: Storing of TCFY solution in chiller at 4°C

3.3.5 Preparation of 1% Eosin Stain

1% of eosin stain was prepared by dissolving 0.5g of eosin powder in 50mL distilled water in a beaker. The solution was stirred evenly with a glass rod to make sure all the eosin powder dissolves completely in distilled water.

3.3.6 Preparation of Hypo-Osmotic Solution

In preparing hypo-osmotic solution, 0.735g of sodium citrate dihydrate and 1.351g of fructose were dissolved in 100mL of distilled water. Then, the solution was mixed well with a glass rod and stored in a media bottle.

3.3.7 Preparation of 0.85% Physiological Saline

To prepare 0.85% of physiological saline solution, 0.85g of sodium chloride was dissolved in 100mL of distilled water. The solution was stored in a media bottle and autoclaved for 15 minutes at 121°C. Then, it was cooled to room temperature.

3.3.8 Semen Collection

The experiment was conducted on non-breeding season. Controlled Internal Drug Released (CIDR) protocol was used in this study as mentioned above to bring the female goat on heat. One day before conducting semen collection, all tools of artificial vagina (AV) were autoclaved. Prior to collecting semen, the inner rubber sleeve was placed into the outer hard cylinder of the AV and both ends of inner sleeve were deflected over the cylinder, forming a water tight space. The space between the sleeve and cylinder of AV was filled with warm sterile water to provide an internal temperature of approximately 45°C before being closed with a rubber stopper. The open side of the AV was connected with a 15mL graduated semen collecting tube. The other open side of AV was lubricated with some K-Y jelly before collection took place. After done preparing the AV as shown in Figure 3.10, a doe on heat was driven into the pen and restrained with a neck clamp, then only bought in the buck into the pen for semen collection. When the buck in the pen started to mount the restrained doe, its penis was guided into the AV until it ejaculated. Immediately after ejaculation, reading of semen volume on graduated semen collection

tube was taken. The tube was removed from the SV, then labelled, covered with a layer of aluminium foil, followed by placing it in a thermoflask with water at 37°C (Bopape, 2014).

Figure 3.10: AV prepared for semen collection

Figure 3.11: Adding of warm sterile water into space between the sleeve and cylinder of AV

Figure 3.12: Application of K-Y jelly at open side of AV

Figure 3.13: Semen collection from buck using AV

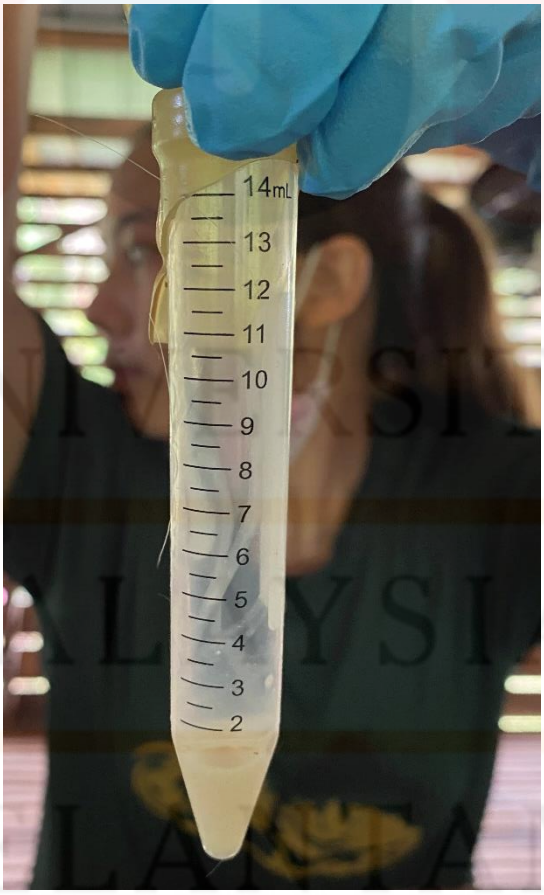

Figure 3.14: Fresh raw buck semen collected in a falcon tube

3.3.9 Semen Dilution

Semen samples collected using artificial vagina from 2 bucks were pooled together in order to have enough semen for dilution for 3 replications of each TPG concentration supplementation and to avoid individual differences (Sheshtawy *et al.*, 2016a). After that, 4mL of TCFY extender solution, 1mL of goat semen and 5mL of glycerol were taken using micropipette to make a total of 10mL of TCFY extender diluted semen. Then, the 10mL diluted semen was transferred to 3 different calibrated falcon tubes with 3mL of diluted semen each tube. Each of the falcon tubes were filled with different amount of TPG stock solution according to different concentration needed as showed in table 3.1.

Table 3.1: Amount of TPG needed for different concentration of DPPG in semen extender

Concentration (%)	Amount of TPG stock solution needed in 3mL semen extender
0	0µL
4	120µL
8	240µL

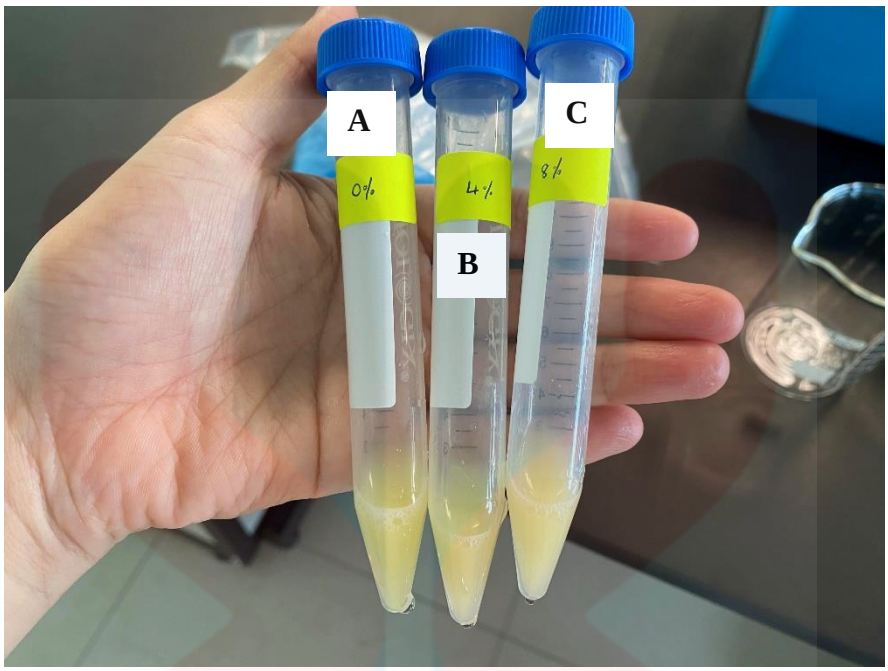

Figure 3.15: Semen extender with different concentration of TPG, namely (A) 0%, (B) 4% and (C) 8%

3.3.10 Semen Storage

All the diluted semen with TCFY extender were kept in water bath at 25°C for 30 minutes before being transferred into refrigerator at 4°C until 24 hours and 48 hours for semen analysis.

3.3.11 Thawing

After chilling in the refrigerator at 4°C for 24 hours and 48 hours, the diluted semen samples were carefully transferred into water bath at approximately 37°C for 30 seconds to be thawed to carry out sperm analysis (Sheshtawy *et al.*, 2016a).

3.3.12 Semen Sample Assessment

The semen samples collected were first be analyzed fresh, secondly, after diluted with extender on the same day of collection, then second analysis after 1st day (24 hours) and third analysis at 2nd day (48 hours) of chilling at 4°C and thawed. Macroscopic evaluation such as volume and colour were carried out using naked eyes while microscopic evaluation such as viability, functional integrity of sperm plasma membrane and individual motility were carried out. Each semen samples supplemented with different concentration of TPG had three replications of microscopic semen evaluation.

3.3.12.1 Volume

The volume of the freshly collected sperm in the calibrated semen collecting tube connecting the artificial vagina was observed using naked eyes and marked down before putting it in a 37°C water bath.

3.3.12.2 Colour

The colour of freshly collected sperm was observed using naked eyes and marked down.

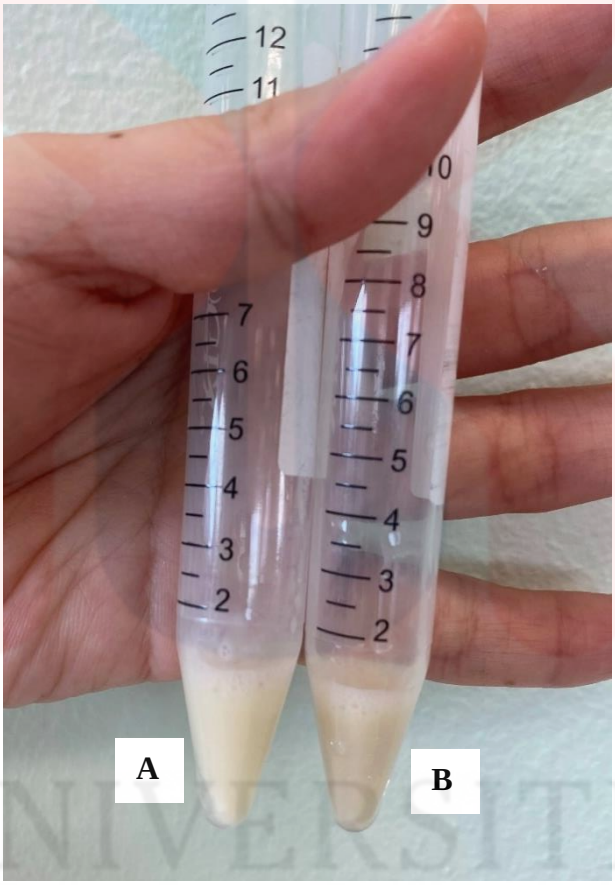

Figure 3.16: The comparison between fresh raw semen with different colours. (A) Semen in milky-white colour and (B) Semen with greyish-white colour.

3.3.12.3 Viability

To observe the viability of the semen samples, the samples had to be stained in advance. A drop of diluted semen was placed on a clean glass slide, followed by 2 drops of 1% eosin stain and 3 drops of 10% nigrosine stain. They were properly mixed using a needle and allowed them to stand for 10 to 20 seconds in order for the stain to be absorbed by dead spermatozoa. Then, another clean slide was taken, using one edge of the slide, some stain was taken and a smear was made on the third clean slide. The smear was allowed to air dry. The smear was observed under the compound microscope with 40x objective. 200 random sperm cells were observed and the number of dead and live sperm cells were recorded. Dead sperm cells were pink in colour whereas live sperm cells were colourless.

3.3.12.4 Functional Membrane Integrity

0.1mL of semen samples was taken using a micropipette and mixed with 1mL of hypo-osmotic solution evenly in a test tube. Then, the test tube was put in an incubator at 37°C for 45 minutes. After that, a drop of semen mixture was placed on a clean slide and covered with a cover slip. A total of 200 spermatozoa were randomly observed under 40x microscope, the number of sperm cells with curled tail as HOST reactive and non-curled tail were recorded and the percentage was calculated.

3.3.12.5 Individual Motility

A drop of liquefied or diluted semen samples with dilution ratio of 1:20 (semen: prewarmed physiological saline), was placed on a clean pre-warmed glass slide and gently covered with a cover slip. The slide was immediately observed under 40x power microscope. A total of 200 random sperm cells were observed one by one using 2 ways counting method without overlaps. Thus, microscopic view was changed frequently to prevent repeated assessment of the same sperm cells. The percentage of motile and non-motile sperm cells were computed.

3.4 Calculation of Percentage of Spermatozoa

Percentage of viable, HOST reactive and motile sperm cells were calculated using following formula:

$$\frac{Number\ of viable/\ HOST\ reactive/motile\ sperm\ cells}{Total\ sperm\ cells\ counted} \times 100$$

3.5 Statistical Analysis

Data was presented as mean ± standard deviation (mean ± S.D.). The statistical analysis was done by using the IBM SPSS Statistics version 21.0 for the different parameters between control and additives replications (Sheshtawy *et al.*, 2016). One-way ANOVA was used to determine significant differences between parameters. Tukey's post hoc test at 5% significant level was used for data analysis to test the significant differences among averages. P-values less than 0.05 was considered significant and null hypothesis was rejected.

CHAPTER 4

RESULTS AND DISCUSSION

Plant-based natural extract has gained popularity in supplementing into semen extender, resulting from the protective properties of the plants to improve reproductive performances in livestock. In recent years, date palm pollen grain (DPPG) was categorized as an herbal remedy which contains high level of antioxidant (Bishr *et al.*, 2012). In fact, DPPG places the second highest of plant extraction that possesses antioxidant activity among 28 fruits commonly consumed in China (Ghnimi *et al.*, 2017). Antioxidant is a compound that provides ability in minimizing the effect reactive oxygen species (ROS) from causing oxidative stress. Therefore, it is capable of protecting semen during preservation process from oxidative damage while maintaining the semen qualities. The statement was aligning with the studies shown by El-Sheshtawy *et al.* (2016b) and El-Sisy *et al.* (2018) where supplementation of DPPG into semen extender can improve sperm motility in buffalo semen.

This study showed that the effect DPPG supplementation with different concentration (TCFY, TCFY + 4% DPPG and TCFY + 8% DPPG) on chilled sperm characteristics such as motility, viability and membrane integrity at 0 hour, 24 hours and 48 hours. The extender with 8% DPPG has the highest mean value compared to control and another treatment group in sperm motility and viability. Additionally,

supplementation of 8% DPPG can help to preserve chilled semen quality for up to 48 hours. However, enrichment of 4% DPPG has no significant effect on sperm integrity and motility in all storage times.

4.1 Individual Motility, Viability and Membrane Integrity Assessment of DPPG Supplementation to TCFY extender on Chilled Goat Semen

4.1.1 Individual Motility

Table 4.1: Effect of diluents supplementation with different concentrations of DPPG on individual motility (%) of goat semen for different storage times (Mean ± SEM)

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	65.67±1.86	62.67±0.93	72.83±2.24
24	53.17±1.92	59.67±1.20	60.83±11.21
48	40.83±3.00[a]	43.33±5.24[a]	56.67±5.57[b]

[a,b] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

The results of individual motility in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 4.1.

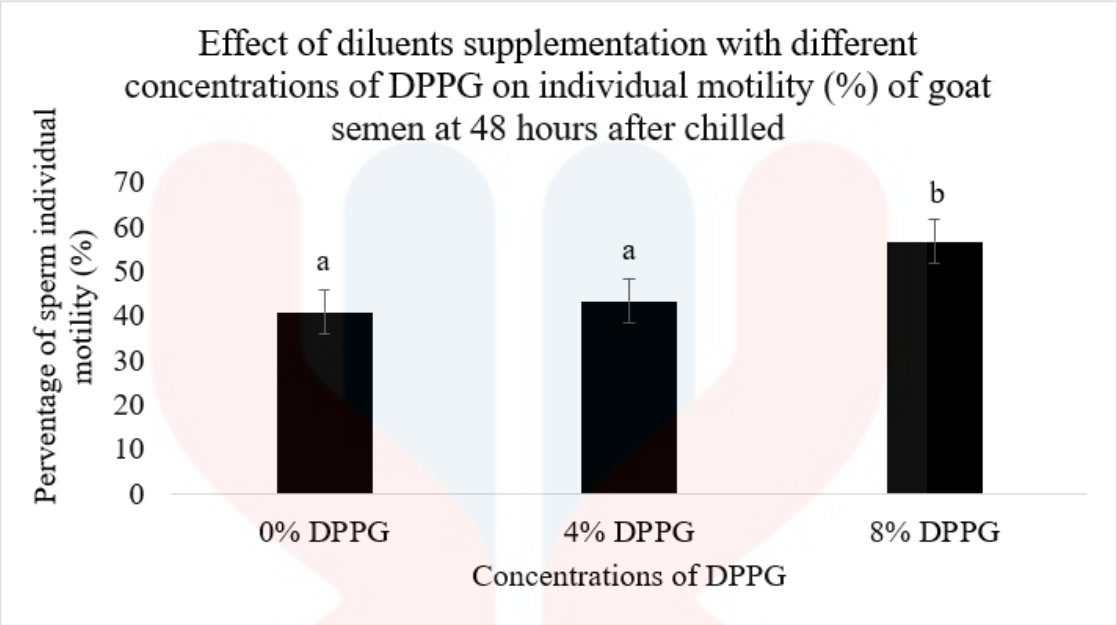

Figure 4.1: Effect of diluents supplementation with different concentrations of DPPG on individual motility (%) of goat semen at 48 hours after chilling

At 48 hours of chilled storage time, motility rate was recorded significantly highest in diluted semen supplemented with 8% DPPG (56.67±5.57) compared to diluted semen supplemented with 0% DPPG and 4% DPPG (40.83±3.00 and 43.33±5.24, respectively).

On top of that, El-Sheshtawy (2016b) stated that the motility was significantly highest in addition of 150mg and 250mg of DPPG into extender of buffalo semen after cooling for 2 hours and freeze thawing comparing to control group. To explain that, the motility rate could be maintained due to the interaction between species and antioxidant effect of DPPG via alleviation of reactive oxygen species (ROS) level. The antioxidant compounds in DPPG such as flavonoid and vitamins A, B, C provide the fertility maintaining capacity of chilled and after-thawed sperms in terms of motility rate.

Other than that, our finding was in line with Adikwu and Deo (2013), Ball *et al.* (2001) and Reza *et al.* (2011) that stated addition of vitamin C and vitamin E which are

antioxidants in preserved semen can improve sperm motility. Adekunle *et al.* (2018) also reported 10% of apple juice gave the highest motility rate in cooled goat semen at 216 hours of storage time. Moreover, motility rate in extender supplemented with 7.5% of apple juice was significantly higher than the control group (no supplementation added).

However, this is in contrast with the study from Aurich *et al.* (1997), who reported that the addition of ascorbic acid did not increase the motility rate of cooled horse semen during 72 hours of storage period. This may due to different species of animal such as equine and different extender such as skim-milk extender were used. To make it clear, the decreased of motility rate may be caused by a reduction of pH that masked the positive effect of ascorbic acid on sperm motility Aurich *et al.* (1997).

4.1.2 Viability

Table 4.2: Effect of diluents supplementation with different concentrations of DPPG on viability (%) of goat semen for different storage times. (Mean ± SEM)

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	74.17±2.46[a]	64.83±1.59[ab]	84.67±1.74[ac]
24	41.33±8.29[a]	59.33±2.19[b]	59.33±7.31[b]
48	30.00±2.65[a]	56.00±1.53[b]	58.00±2.31[b]

[a,b,c] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

The results of viability rate in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 4.2.

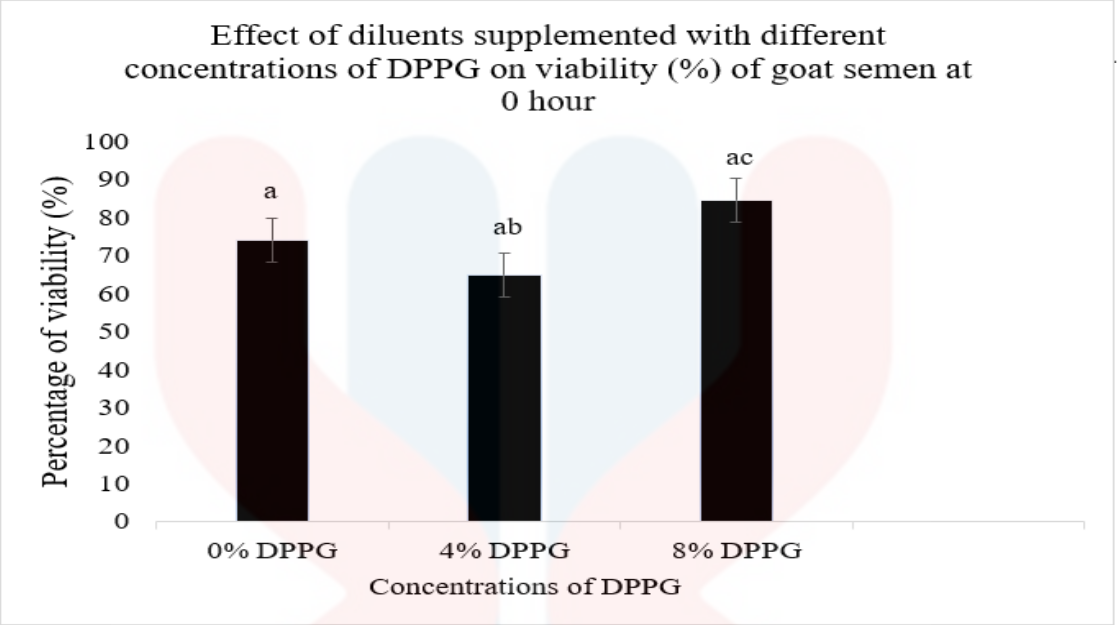

Figure 4.2: Effect of diluents supplementation with different concentrations of DPPG on viability (%) of goat semen at 0 hour

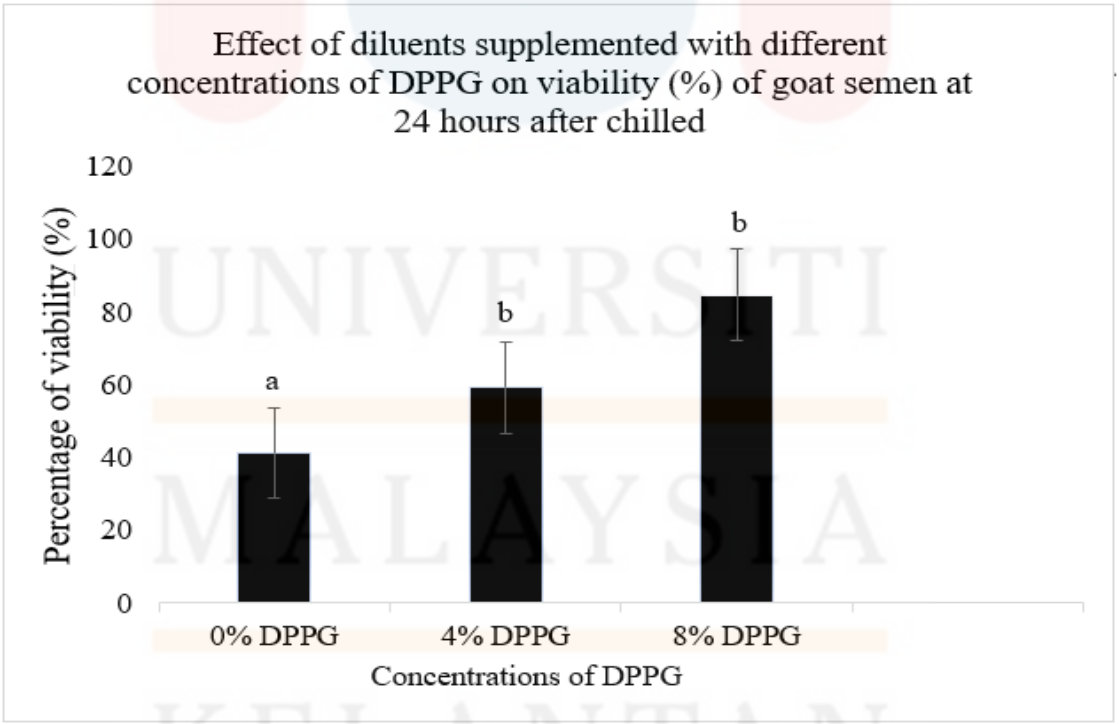

Figure 4.3: Effect of diluents supplementation with different concentrations of DPPG on viability (%) of goat semen at 24 hours after chilling

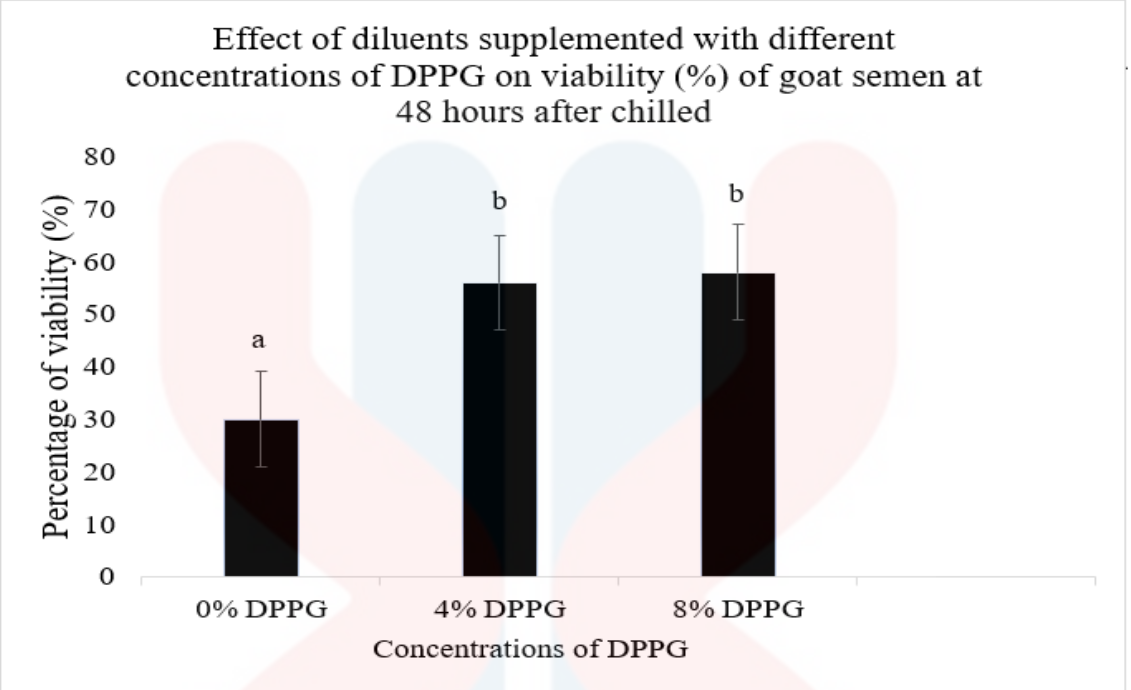

Figure 4.4: Effect of diluents supplementation with different concentrations of DPPG on viability (%) of goat semen at 48 hours after chilling

Figure 4.2 showed that viability rate was significantly higher in diluted semen supplemented with 8% DPPG (84.67±1.74) compared to 4% DPPG (64.83±1.59) at 0 hour of storage time. At 24 hours of storage time, the viability rate of diluted semen supplemented with 8% DPPG (59.33±7.31) is significantly higher than diluted semen supplemented with 4% DPPG (59.33±2.19) and control which is diluted semen supplemented with 0% DPPG (41.33±8.29). While at 48 hours of storage time, it gave similar results of viability rate with 24 hours of storage time where diluted semen supplemented with 8% DPPG (58.00±2.31) is significantly higher than diluted semen supplemented with 4% DPPG (56.00±1.53) and control which is diluted semen supplemented with 0% DPPG (30.00±2.65).

The results were similar with Al-Daraji (2014) that stated addition of 8% tomato juice into chicken semen extender can significantly increase the percentage of live spermatozoa significantly at 0-hour storage time compared to 2%, 4% and 6% of tomato juice supplementation. In addition, 8% tomato juice significantly increased the viability rate at all liquid storage times up to 36 hours when compared to diluted semen with no addition of tomato juice as control group and other treatments (2%, 4%, 6%) as well. Improvement of the sperm viability may be due to the positive effect of tomato juice as an antioxidant rich source.

Besides, Malik *et al.* (2016) also reported that inclusion of 0.4% and 0.3% of date palm juice can significantly increase the viability percentage of bull spermatozoa before freezing when compared to other concentrations. This may be contributed by date palm juice which is an outstanding material in producing refined sugar that provides protection for the spermatozoa against injury induced during freezing-thawing process.

On the other hand, the results of this study were aligned with El-Sisy *et al.* (2018), stating that the addition of 100mg and 150mg of DPPG to modified INRA-82 (mINRA-82) extender can significantly increase post-thawed stallion sperm viability index when compared to higher amount of DPPG inclusion, but did not significantly differ from control group without DPPG inclusion. Furthermore, this is supported by the study from El-Sheshtawy (2016b) who stated that inclusion of 150mg DPPG on post-thawed buffalo semen extender showed significant differences on the highest alive sperm percentage compared to control (no DPPG), 50mg, 100mg, 200mg, 250mg treatment groups.

The beneficial effects of DPPG to post-thawed semen viability may be attributed to components in DPPG that may protect the outer layer of spermatozoa against cold so that they may survive under low temperature. Natural antioxidant compounds in DPPG exert a protective effect in preserving the metabolic activity and cellular viability of

cryopreserved bovine spermatozoa. Besides, the antibacterial ability of DPPG improves the preservability of semen through minimization of bacterial growth in the extender (El-Sheshtawy *et al.*, 2016b).

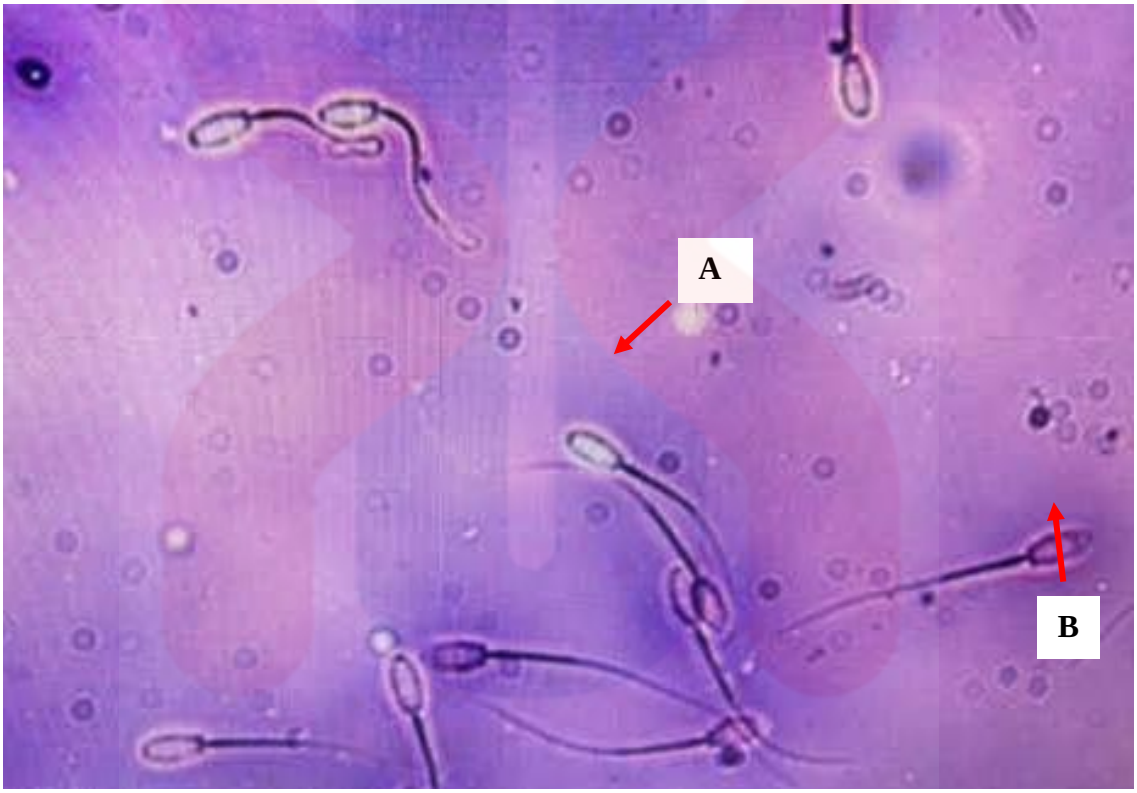

Figure 4.5: The comparison between live and dead sperm using eosin-nigrosine solution with staining method (400x). (A) Unstained sperm cells indicate viable sperm and (B) Stained sperm indicate dead sperm with a damage cell membrane

4.1.3 Membrane Integrity

Table 4.3: Effect of diluents supplementation with different concentrations of DPPG on functional membrane integrity (%) of goat semen for different storage times. (Mean ± SEM)

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	30.33±2.19[a]	35.33±3.28[ab]	40.00±4.04[b]
24	23.33±0.88	24.67±2.40	25.33±2.96
48	19.67±3.18	16.33±4.26	19.00±2.00

[a,b] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

The results of functional membrane integrity in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 4.3.

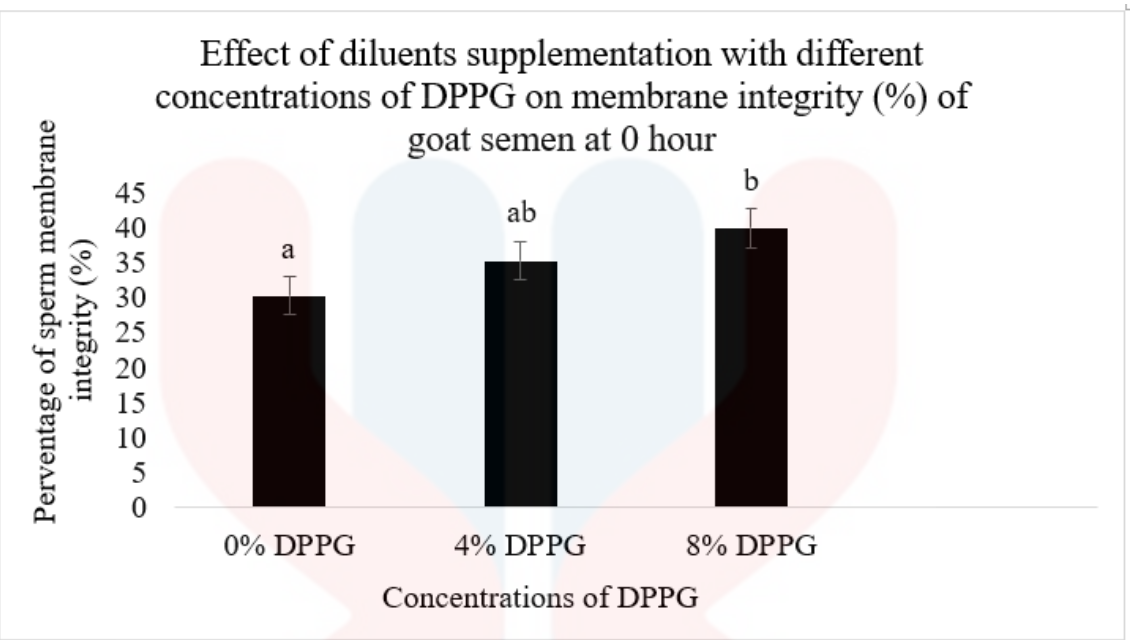

Figure 4.6: Effect of diluents supplementation with different concentrations of DPPG on membrane integrity (%) of goat semen at 0 hour

At 0 hour of storage, it was recorded that the functional membrane integrity rate of diluted semen supplemented with 8% DPPG (40.00±4.04) is significantly higher than control which is diluted semen supplemented with 0% DPPG (35.33±3.28).

The results were aligned with Adekunle *et al.* (2018) that concluded inclusion of 10% apple juice gave the highest membrane integrity rate of chilled bucks spermatozoa at 240 hours of storage compared to control, 2.5%, 5% and 7.5%. It could be linked to the presence of vitamin C and other antioxidant compounds such as ferulic acid in the juice (Mermeistein, 1999; Spanos and Wrolstad, 2004). The findings were corroborated with Zheng and Zhang (1997) that ferulic acid reduced lipid peroxidative damage to cell membranes which linked to the preservation of cell structural characteristics.

Moreover, El-Sheshtawy *et al.* (2016b) concluded that inclusion of 200mg of DPPG in extender showed the highest sperm membrane integrity percentage on post-thawed

buffalo semen when compared to the control group. This may on account of the antioxidant capacity of DPPG in scavenging free radicals that could harm the sperm cell membrane. Therefore, the antioxidant effect could increase fertility rate of post-thawed semen by maintaining the functional membrane integrity of spermatozoa (El-Sheshtawy *et al.*, 2016b).

However, this is in contrast with Malik *et al.* (2016) that reported inclusion of date palm juice did not significantly affect the membrane integrity rate of bull spermatozoa before freezing. This might due to the supplementation of date palm in juice form in extender and the study was carried out in frozen thawed bull spermatozoa.

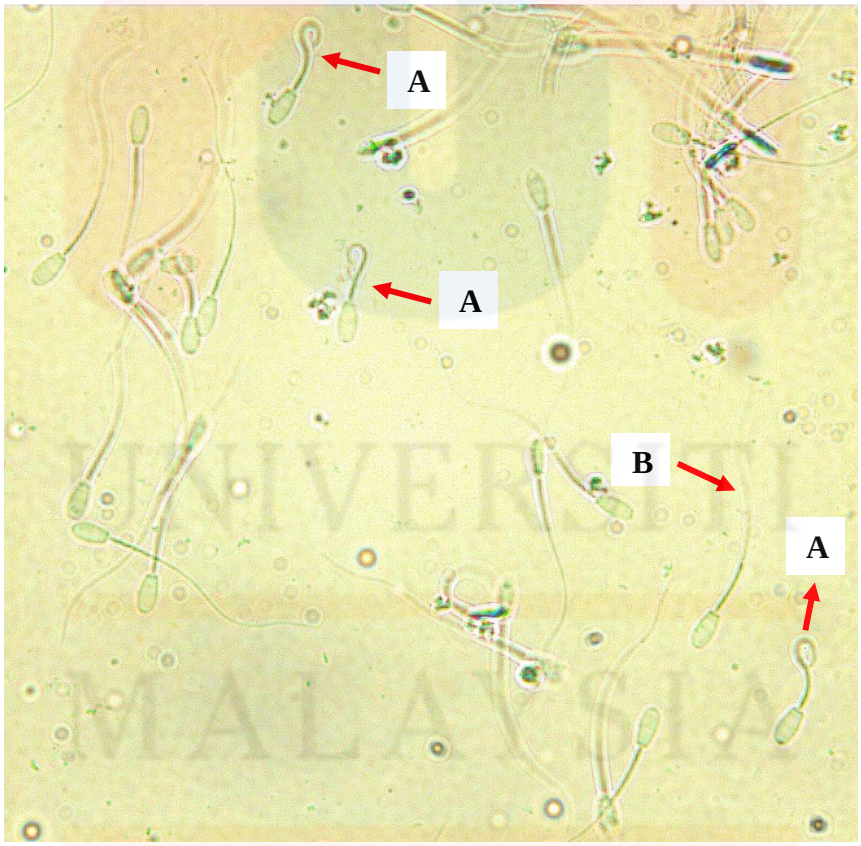

Figure 4.7: The comparison between sperms with curled tail and uncurled tail using hypoosmotic swelling test (HOST) (400x). (A) Sperm with curled tail indicates intact plasma membrane and (B) Sperm with uncurled tail indicates inactive membrane

4.2 Comparison of The Chilled Goat Semen Quality at Different Storage Times

Table 4.4: Effect of different storage times with different concentrations of DPPG on individual motility (%) of goat semen (Mean ± SEM)

Treatments	Storage time (hours)		
	0	24	48
Diluted semen with 0% DPPG (control)	65.67±1.86[a]	53.17±1.92[b]	40.83±3.00[c]
Diluted semen with 4% DPPG	62.67±0.93[a]	59.67±1.20[a]	43.33±5.24[b]
Diluted semen with 8% DPPG	72.83±2.24[a]	60.83±11.21[ab]	56.67±5.57[b]

[a,b,c] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

Table 4.5: Effect of different storage times with different concentrations of DPPG on viability (%) of goat semen (Mean ± SEM)

Treatments	Storage time (hours)		
	0	24	48
Diluted semen with 0% DPPG (control)	74.17±2.46[a]	41.33±8.29[b]	30.00±2.65[c]
Diluted semen with 4% DPPG	64.83±1.59	59.33±2.19	56.00±1.53
Diluted semen with 8% DPPG	84.67±1.74[a]	59.33±7.31[b]	58.00±2.31[b]

[a,b,c] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

Table 4.6: Effect of different storage times with different concentrations of DPPG on functional membrane integrity (%) of goat semen (Mean ± SEM)

Treatments	Storage time (hours)		
	0	24	48
Diluted semen with 0% DPPG (control)	30.33±2.19[a]	23.33±0.88[ab]	19.67±3.18[b]
Diluted semen with 4% DPPG	35.33±3.28[a]	24.67±2.40[b]	16.33±4.26[c]
Diluted semen with 8% DPPG	40.00±4.04[a]	25.33±2.96[b]	19.00±2.00[b]

[a,b,c] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (SPSS-post hoc test at $P<0.05$). (n=9)

Table 4.4 showed that semen diluted with 4% DPPG was managed to maintain sperm motility until 24 hours of chilling without a significant drop of motility rate whereas semen diluted with 8% DPPG managed to maintain the motility of sperm after 24 hours of chilled storage but below 48 hours. Besides, results indicated that the percentage of motility for semen after 48 hours of chilled storage was significantly lower than 0 hour.

For the viability aspect, semen can be stored until 48 hours when diluted with 4% DPPG as there were no significant decrease in viability rate across all storage time as shown in Table 4.5. Semen diluted with 8% DPPG was able to maintain viability rate after 24 hours of chilling. For membrane integrity aspect, semen diluted with 0% and 8% were able to maintain membrane integrity rate after 24 hours of chilled storage.

The results obtained from this study was aligned with Al-Daraji (2014) who reported that inclusion of 8mL per 100mL of tomato juice in extenders can preserve chicken sperm viability and motility for up to 36 hours. However, motility rate cannot be

maintained when less than 8mL of tomato juice was added. This could be due to lycopene, a type of organic pigment found in tomato that increased semen quality stored in vitro. It could also prolong the storage time while maintaining the semen characteristics.

On the contrary, El-Sheshtawy *et al.* (2016a) concluded that the use of 10% pomegranate juice enriched extender gave the highest motility percentage in chilled cattle semen all over 10 days compared to 40% and 50% pomegranate juice enriched extender. This proved that inclusion of 10% pomegranate juice is beneficial for maintaining sperm motility while higher levels of supplementation such as 40% and 50% did not make an impact on sperm motility. The positive results could be due to potent antioxidant activity of pomegranate juice.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, DPPG was able to increase goat sperm performance during short term chilling process when compared to control (without supplementation of DPPG). Extenders supplemented with 8% of DPPG showed the highest values to maintain sperm viability and motility. Plus, it managed to maintain the semen qualities for approximately 24 hours of chilled storage. This proved that 8% of DPPG had a beneficial effect on chilled Boer goat semen. Despite that, sperm membrane integrity was unable to be preserved after chilling at 5°C. Even though not all the sperm qualities showed significant difference ($P<0.05$) after post-chilled but the concentration of 8% DPPG had the highest mean value in sperm motility, viability and membrane integrity assessment compared to control group and 4% DPPG. Study indicated that the sperm qualities decreased following an increase in chilled storage time in all groups. However, the viability percentage of chilled goat semen supplemented by 8% DPPG is significantly higher ($P<0.05$) at all storage times.

5.2 Recommendations

To further enhance the work, there are a few improvements that can be made in this present study. Analysis on sperm characteristics such as sperm morphology and acrosome integrity can be analyzed. Since manual observation was used to determine sperm parameters in this study, human error or bias may occur. Thus, the sperm motion characteristics can be assessed using advanced technology such as computer assisted sperm analyzer (CASA) for detailed and accurate analysis.

Other than that, future work could investigate the effect of frozen semen in liquid nitrogen at -196°C using 8% supplementation of DPPG in extenders. The storage time can also be prolonged with an increase of supplementation of DPPG (10% DPPG or more). Lastly, the number of ejaculations and bucks should be increased for analysis.

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APPENDIX

Media Recipe

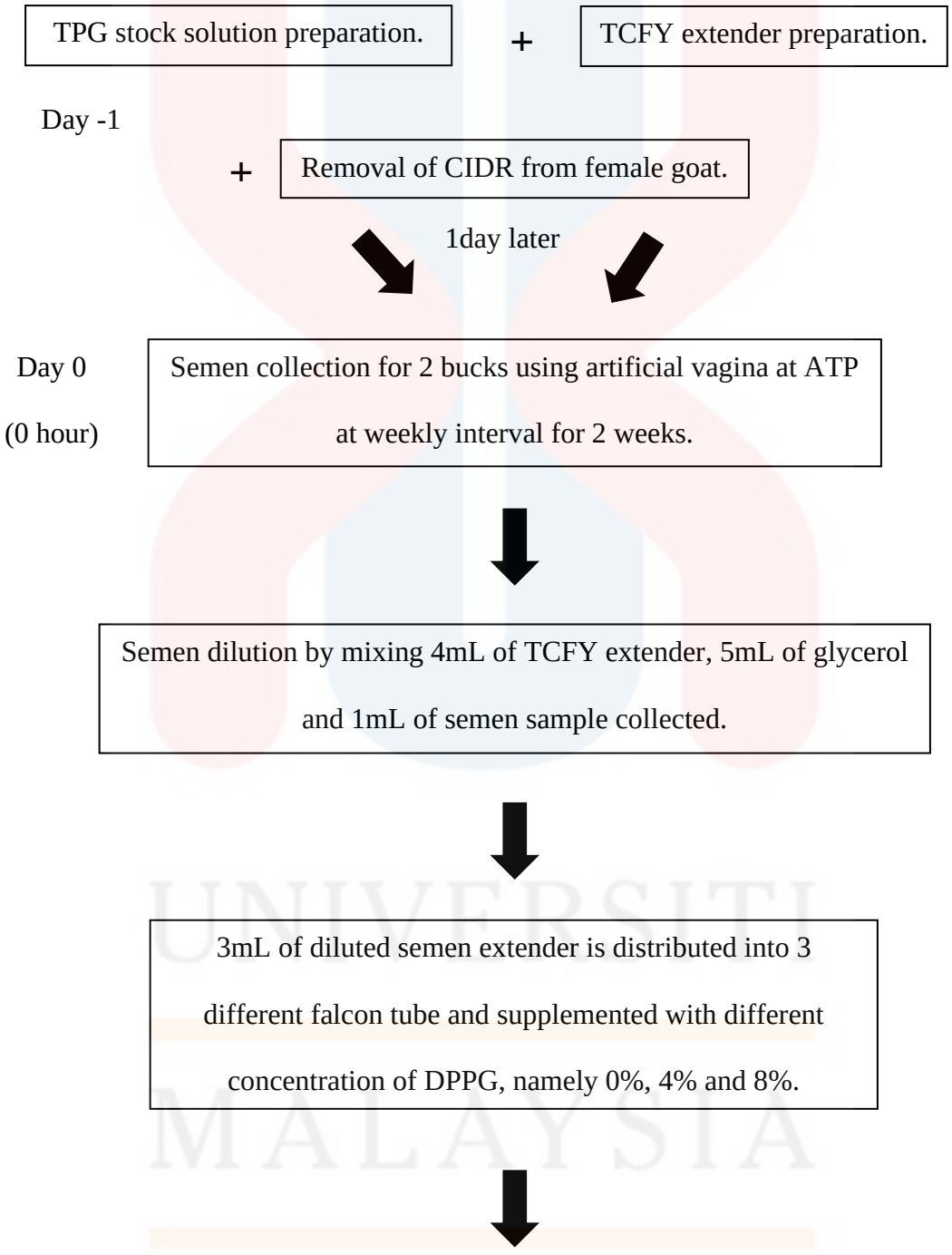
Table A.1: TCFY extender recipe

Components	Amount
Tris base	3.028g
Citric acid	1.675g
Fructose	1.250g
Penicillin	0.1g
Egg yolk	20mL
Glycerol	0.6mL

Table A.2: TPG stock solution recipe

Components	Amount
Tris base	1.514g
Citric acid	0.8375g
Fructose	0.625g
Penicillin	0.05g
DPPG	100mg

Research Flow Chart



Diluted semen microscopic analysis, with 3 replications for each parameter of the three different DPPG concentration:

- Individual motility
- Viability
- Functional membrane integrity

↓ After chilling at 4°C for 24 hours and thawed at 37°C for 40 seconds.

Day 1 (24 hours)

Repeated microscopic semen analysis process.

↓ After chilling at 4°C for another 24 hours and thawed at 37°C for 40 seconds.

Day 2 (48 hours)

Repeated microscopic semen analysis process.

↓

Data interpretation.

↓

Results and discussion.

↓

Conclusion and recommendation.