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

**AMIRAH BINTI AMSAH**

**F15A0013**

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

**Faculty of Agro Based Industry**

**Universiti Malaysia Kelantan**

**2019**

## DECLARATION

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

_____

Student

Name: Amirah binti Amsah

Date:

I certify that the report of this final year project entitled "Effect of Extender Supplemented with Date Palm Pollen Grain on Bovine Semen Qualities" by Amirah binti Amsah, matric number F15A0013 have been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

_____

Supervisor

Name:

Date:

**ACKNOWLEDGEMENT**

First and Foremost, praise and thanks to the God, the Almighty, for His showers of blessings throughout my research. I am grateful to God for my health and well-being which are vital in finishing the research successfully.

I would like to express my deep and sincere gratitude to my research supervisors, Dr Raja Ili Airina binti Raja Khalif for all her guidance and dedicated involvement throughout my final year project. This research would have never been accomplished without her support and encouragement. Her advice and motivation have deeply inspired me because she always remind me that is nothing impossible in the world if we are willing to put extra effort. I am extremely thank you for have a great supervisor.

I would like to show gratitude to my co-supervisor, Dr Jakkphan Pitpayapitpakul for providing invaluable guidance throughout my research. He has taught me on how to carry out the research experiment step by step and how to make the research work possible. His dynamism and sincerity which willing to spend the time for teaching me. Besides, he is very passionate and enthusiastic to make sure that I can exposed as much as I can during conducting the experiment.

I would also like to thank to Rossukon Sangmanee, the president of University of Naradhiwas, Thailand for allowed me to do my research and I would express my thank you to Prof. Tawee Boonpirom, the dean of Faculty of Agricultural because allowed me to using the laboratory, facilities, chemicals and equipment for three month there. I gained many extraordinary experience while being there.

Additionally, I would like to thank to my FYP teammate, Tai Cheah Jie and Nur Farihah binti Abd Manan for supporting me through this entire process and willing to

listen all of my problems during conducting my final year project. A big thanks to Tai Cheah Jie for volunteer to provide a car for us for three month at Princess of Naradhiwas University (PNU), Thailand and always have a kind heart for sharing all the knowledge to me. Special thanks to my friends at PNU: Asleena, Suhaila and Azam for well wishes, patient and willing to accompany me to stay up in the laboratory until my project completed. Thanks for being with me whenever I needed a friends. Thanks also to nursing student of PNU for accepted me to stay at nursing hostel until my project completed. Thank you for your attention, caring and loved to me.

Last but not least, I am grateful to my parents for their love, prayer, sacrifices, financial support and understanding for allowing me to complete my research outside from Malaysia.

# TABLE CONTENT

**CHAPTER 2 LITERATURE REVIEW**

**CHAPTER 3 MATERIALS AND METHODS**



**CHAPTER 4 RESULTS & DISCUSSION**

## LIST OF TABLES

## LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AI | Artificial Insemination |
| ART | Assisted Reproductive Technology |
| AV | Artificial Vagina |
| CPA | Cryoprotectant Agent |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DPPG | Date Palm Pollen Grain |
| EE | Electro Ejaculation |
| g | Gram |
| HOST | Hypo-osmotic Swelling Test |
| lb | Libra which is pound by weight |
| $LN_2$ | Liquid Nitrogen |
| mm | Millimetres |
| mOsm/kg | Milliosmoles per kilogram |
| Nacl | Sodium Chloride |
| NIFA | National Institute Food and Agriculture |
| NM | Natural Mating |
| OS | Oxidative Stress |

| | |
|---|---|
| PNU | Princess of Naradhiwas |
| PUFA | Polyunsaturated Fatty Acid |
| RCBD | Randomized Complete Block Design |
| ROS | Reactive Oxidative Stress |
| Rpm | Rotation per Minute |
| TCF | Tris Citric Fructose |
| TCFY | Tris Citric Fructose Yolk |
| TPG | Tris Pollen Grain |
| *ul* | Microlitre |
| SAS | Statistical Analysis System |
| SEM | Standard Error Mean |

**Kesan Extander Dengan Penambahan Serbuk Debunga Kurma Keatas Kualiti Semen Lembu**

## ABSTRAK

Kajian ini bertujuan untuk menilai (1) kesan penambahan extander dengan kepekatan sebuk debunga kurma (DPPG) yang berbeza keatas parameter kualiti sperma terhadap lembu Brahman dan (2) melihat kualiti sperma dengan suhu yang berbeza di antara air mani sejuk dan pembekuan beku air mani selama tujuh hari penyimpanan. Air mani dikumpulkan melalui rangsangan elektrik dan dibahagikan kepada empat kumpulan rawatan. Air mani dicairkan di dalam Tris sitrat fruktosa (TCF) (kumpulan kontrol; CG) dan Tris dengan penambahan kepekatan DPPG yang berbeza (G1=2% DPPG; G2=4% DPPG dan G3=6% DPPG). Dalam eksperimen pertama, sampel air mani (n=12) disimpan di dalam peti sejuk (4°C) manakala dalam eksperimen kedua, sampel air mani (n=12) dibekukan didalam nitrogen cecair ($LN_2$) dan eksperimen mempunyai tiga replikasi. Sampel dicairkan di dalam air suam (37°C) dan dianalisis untuk pergerakan, integriti membran dan daya maju. Penambahan 6% DPPG menghasilkan penyelenggaraan sperma yang lebih baik dalam proses penyejukan dan beku. Tiada perbezaan yang ketara diperhatikan ($p>0.05$) diantara kumpulan kawalan dan rawatan untuk semua parameter sperma dalam eksperimen pertama. Walau bagaimanapun, dalam eksperimen kedua, penambahan 6% DPPG menghasilkan peningkatan yang ketara ($p<0.05$) dalam daya maju (71.25±1.04) berbanding dengan kumpulan kawalan (56.47±4.69). Selain itu, tiada perbezaan yang signifikan ($p>0.05$) daripada kepekatan terhadap pergerakan sperma. Kesimpulannya, penambahan 6% dalam 20 mg DPPG mempunyai keupayaan yang lebih baik untuk melindungi daya maju sperma lembu manakala, penambahan 20 mg DPPG untuk kesemua kepekataan tidak cukup berkesan untuk melindungi pergerakaan sperma.

**Kata kunci:** Simpan sejuk, sebuk debunga kurma, sejuk, pembekuan, lembu brahman

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

## ABSTRACT

This study was to evaluate (1) The effect of extender supplementation with different concentration of date palm pollen grain (DPPG) on sperm quality parameter in Brahman bull and (2) To observe the quality of sperm with different temperature between chilled and frozen semen after seven days of storage. The semen were collected through electrical stimulation and assigned to four treatment groups. The semen were diluted in Tris citric fructose (TCF) based extender (control group;CG) and Tris based extender supplemented with different concentration of DPPG (G1=2% DPPG; G2=4% DPPG and G3=6% DPPG). In the first experiment, the semen samples (n=12) were preserve in refrigerator (4°C) while in second experiment, the semen samples (n=12) were cryopreserve in liquid nitrogen and the experiment have three replication. The samples were thawed in water bath (37°C) and analysed for motility, membrane integrity and viability. The addition of 6% DPPG resulted in improved maintenance of sperm in chilling and frozen process. No significant different was observe among treatments groups for all the parameter ($p>0.05$) in first experiment. However, in the second experiment, the addition of 6% DPPG resulted significantly higher ($p<0.05$) in viability (71.25±1.04) compare to control groups (56.47±4.69). Besides, there is no significant different ($p<0.05$) of the concentrations on sperm motility. In conclusion, the supplementation 6% in 20 mg of DPPG had better ability to protect the bovine sperm in viability only while the supplementation of 20 mg for all concentration not quite effective to protect the sperm motility.

**Keyword:** Cryopreserve, Date palm pollen grain, chilling, frozen, brahman bull

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

The first recorded history of semen cryopreservation was created 200 years ago, when Lazaro Spallanzani (1776) tried to preserve spermatozoa by cooling it in the snow (Royere, Barthelemy, Hamamah, & Lansac, 1996). Continuous further research made by Polge to discovery the glycerol's cryoprotectant properties (Polge, Smith, & Parkes, 1949). This marked a turning point in the field of fertility preservation which it shows the improvement in techniques of semen cryopreservation with different species. Curry (2000) reported, the earliest offspring successful produced from sperm cryopreservation started in 1951 by cow, followed by human in 1953, pig and horse in 1957 and sheep in 1967. The development of sperm cryobanks started in the 1960s for cattle and in the 1970s for human (Kopeika, Thornhill, & Khalaf, 2014) which on that era, semen cryopreservation very synonym in their life. The continuous research of semen cryopreservation lead to new discovery of egg yolk based extender and followed with the addition of antibiotics in the extender to minimize bacterial contamination and provide suitable medium for sperm.

In this modern day, cryopreserved semen rapidly used by artificial insemination (AI) and human assisted reproductive technology (ART) as a method to produce offspring from selected superior animals and infertile or sub fertile animals (Evans, 2011). The beginning of (ART) started at 1890s when the first cases reported of embryo transplantation in rabbit (Kamel, 2013). Sperm cryopreservation, AI and ART help to protect the endangered species and cut the time for fertilisation (Cardoso, Silva, Uchoa, & Da Silva, 2003). AI become the most important reproductive technologies implemented by dairy industry especially in India because the availability of quality sires in India is inadequate (Gupta and Singh, 2012). National Institute of Food and Agriculture (NIFA) from United States Department of Agriculture support the technologies of AI can improve the quality of the animal and increase the production. Despite the substantial benefit of semen cryopreservation, there are some disadvantage that caused chemical, physical and mechanical injuries to sperm membranes of all mammalians species (Watson, 2000).

Nowadays, natural extract from plant-based has grown in popularity as protective properties in extender for preserving animal semen such as bovine and caprine semen (Sansone, Nastri, & Fabbrocini, 2000). The innovation of the ingredient in the extender help resolved problems in semen cryopreservation such as protect bacterial contamination in the semen extended and protect sperm against cold shock. Natural extract from plant based has been proven that give positive improvement in semen preservation such as coconut oil (Tarig et al., 2017) tomato (Al-Daraji, 2014) and pomegranate (El-sheshtawy, El-sisy, & El-nattat, 2016). Besides, the use of natural antioxidant from plant sources help to minimize the reactive oxygen species (ROS) and oxidative stress (OS) on the cell that has catch the researcher's attention today. From ancient times, date palm pollen grain

(DPPG) is a fine powder produced from male flowering which contain herbal antioxidant to against the action of ROS and OS of sperm membrane in cryopreservation process. Recent evidence mentioned that, DPPG is rich source of natural antioxidant which content high of phenolic, carotenoid, flavonoid compound and vitamin A, E and C. Besides, DPPG used as dietary supplement to improve reproductive performance of male and women. (El-Sisy, El-Badry, El-Sheshtawy, & El-Nattat, 2016). In fact, DPPG is the second highest contain antioxidant activity among 28 fruit commonly consumed in China (Ghnimi, Umer, Karim, & Kamal-Eldin, 2017).

## 1.2 Problem Statement

Cryopreservation is the process of freezing the biological material in the nitrogen liquid ($LN_2$) in -196°C to stop the biological chemical reaction. However, the process of cryopreservation bring harmful to the cell which may lead to the cell destruction. The major problem happen in slow cooling is the formation of ice crystals in intracellular and extracellular cell. Many of researchers have studied about the effect of natural supplementation in extender in order to protect the cell from the ice formation and to reduce the cell damage in semen cryopreserved.

Even though, the study of sperm preservation are accessible worldwide but there were still lack of the development regarding the best commercial of supplementation in the extender based on natural resources which are more efficient to protect the sperm cell during freezing, cost-effective and convenient to the farmer. Furthermore, the existing supplementation nowadays, still not help to improve the quality of the semen. There a few studies have been conducted to investigate the effect of semen preservation in DPPG

and the previous studies has been proved that the addition of DPPG in the semen extender can improved the quality of the sperm (El-sheshtawy, El-nattat, Sabra, & Ali, 2014; El-Sisy, El-Badry, El-Sheshtawy, & El-Nattat, 2016) The aim of the research is to investigate the efficiency of Tris citric-acid fructose (TCF) supplemented with different concentration of DPPG on bovine semen qualities after seven days of storage.

## 1.3 Hypothesis

The study comprises two hypothesis:

$H_0$: Supplementation of date palm pollen grain will not improve the quality of sperm.

$H_1$: Supplementation of date palm pollen grain will improve the quality of sperm.

$H_0$: The quality of sperm between chilled and frozen semen have no differences after seven days of storage.

$H_1$: The quality of sperm between chilled and frozen semen have differences after seven days of storage.

## 1.4 Objective

1. To investigate the effect of extender supplemented with different concentration of date palm pollen grain on motility, progressive motility, viability and membrane integrity.
2. To observe the quality of sperm with different temperature between chilled and frozen semen after seven days of storage on motility, progressive motility, viability and membrane integrity

## 1.5 Scope of Study

The scopes of this research focus on two different objectives which to prove the validity of hypothesis stated. Firstly, this research focus on the effect of extender supplementation with different concentration of date palm pollen grain (DPPG) on bovine semen qualities. The concentration used in this study is lower than the concentration carried out from the previous studies. This is because, the research want to determine the most effective concentration of DPPG that can protect the quality of the sperm.

Secondly, to observe the quality of the sperm with different temperature between chilled and frozen on motility, progressive motility, viability and membrane integrity after seven days of storage. The research want to determine the significant changes of the sperm quality in chilled and frozen. These result will follow several techniques to preserve the sperm which followed the protocol of freezing and thawing process. Along the study, the supplemented DPPG is suitability to be used as an alternative source to preserve the sperm.

## 1.6 Significant of Study

The finding of this study will help to determine the effectiveness of supplementation of DPPG in the extender towards the sperm quality such as motility, membrane integrity and viability during freezing and post-thawing. The farmers can use the supplementation of DPPG in the extender which is it can help to provide nutrient and source of energy to the sperm. Furthermore, the DPPG can be replaced with other supplements as it is most convenient for farmers. Besides, it contain natural antimicrobial

agents to control the pathogens and also contain antioxidant agent such as rich in vitamin A, C and E to protect the cell damage. From this research, the researcher can determine the best concentration of DPPG to add into the extender which can help to protect the quality of sperm after freezing. The significance of this research is to determine whether lowering the concentration of DPPG can give protection to the sperm cell or give toxicity which may bring harmful to the sperm cell.

## 1.7 Limitation of Study

The limitation in this study such as the fresh semen was not high quality. The fresh sperm must reach >80% of mass motility. This is because the quality of the fresh semen will affect the quality of semen cryopreserved. Furthermore, the fresh eggs were difficult to find since Narathiwat have lack of poultry factory. Unfresh eggs have high risk of microorganism's contamination which can reduce the fertilisation capacity of sperm. Besides, the schedule of semen collection should be done one times per week to avoid the low of sperm quality produced by the bull if the sperm was collected frequently.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Artificial Insemination

Artificial Insemination (AI) is a techniques of semen deposited into reproductive tract when the female express oestrus (Mohammed, 2018). AI is a process of collecting the semen from male and stored it by preserving the semen into the liquid nitrogen ($LN_2$) until when it wants to be used, the semen will artificially inserted into the female reproduction (Temesgen, Tibebu & Usman, 2017). Foote (2002) stated that AI technology is the first of biotechnology established in 1936 which help to improve the production of superior genetic of domestic animals. Before the Second World War, Europe and North America widely practiced natural mating method. Since, most of the cows mated from different farms, the disease easily dispersed rapidly and reduced the fertility of the cow (Vishwanath, 2003). The success of AI as an alternative method for animal breeding has replaced the natural breeding method due to the weakness of the method. Furthermore, it also provided the opportunity to the other development of technology such as cryopreservation. Despite of many advantages, AI also have disadvantages in terms of inaccurate prediction of heat cycle. So, this is the reason mostly farmers all over the world still used natural mating to breed their cattle.

AI has many advantages such as it can reduce the physical contact between the other animals, limit the transmitted disease (Bailey, Bilodeau, & Cormier, 2000) and maintain the quality and performance of the animals. In most cases, natural mating transmit venereal disease such as brucellosis, listeriosis, leptospirosis and trichomoniasis (IAEA, 2005). Furthermore, AI provide opportunity to select the best sire to produce the desirable trait to the next generation which it can help to increase the selection intensity (Tadesse, 2010). Besides, AI enhance the animal performance such as milk and meat to achieved more profitable (Valergakis, Arsenos, & Banos, 2007). The main factor of disadvantages of AI is it required highly cost compare to NM such as trained labour, advance equipment and liquid nitrogen (Valergakis et al., 2007). Furthermore, AI also have poor heat detection which it is difficult to detect the exact time to deposited the semen to the female reproductive (Mohammed, 2018).

## 2.2 Method of semen collection

The development of the technologies drives many researcher to introduce new method of sperm collection which can help to reduce the disease among the animal during the sexual contact. Williams, Stellflug, & Lewis (2001) stated that there have two principle method used for semen collection: (i) Artificial vagina (AV) and (ii) Electrical stimulation (EE). The different method of semen collection will affect the quality of the sperm.

### 2.2.1 Artificial Vagina

The artificial vagina (AV) method is stimulates similarities of natural mating which induces natural ejaculation for the collection of semen and preferable animals that have been trained. AV method is painless because the method is not forced the animals to ejaculate. AV is a clone vagina which help to provide internal stimulation of male without using physical sexually contact. Besides, AV provide same body temperature of female reproductive to ensure that the male animal will stimulate the sperm through the AV. The advantages of AV can reduce the spread of disease and limit the sexual physical contact of the animals (Bopape, Lehloenya, Chokoe, & Nedambale, 2015).

### 2.2.2 Electrical Ejaculation

Electrical Ejaculation (EE) method was introduced in sheep in 1936s. The electrode will be inserted into the sire's rectum then the electric current will directly attack the rectum. The method is used when the male animal is unable to mount to the female animal (Williams et al, 2001). EE is more easy and convenient than the AV. However, EE is stressful procedure because the animal will feel discomfort and pain (Bopape et al., 2015). The risk of this method is it will cause the contamination of urine in the semen during the collection. Besides, the advantage of EE method is can obtained greater volume of seminal plasma than AV.

## 2.3 Cryopreservation

Cryopreservation is the process of preserve the living cells and tissues under extreme temperature in the nitrogen liquid ($LN_2$) (-196°C) for a long period time (Hoon et al., 2017). Cryopreservation is an essential tools to maintain the genetic diversity of species populations, kept the genetically superior species and help to preserve the living cell for a long-term storage. The major steps in the cryopreservation process are (1) Cryoprotective Agents (CPAs); (2) Cooling process at low temperature and (3) Thawing. Nowadays, the application of cryopreservation widely practised which it can be categories into: (1) cells and organs; (2) cryosurgery; (3) biochemistry and molecular biology; (4) food science; (5) ecology and plant physiology; (6) medical applications such as artificial insemination (AI) and *in vitro* fertilisation (IVF) (Hoon et al., 2017).

Semen cryopreservation is an effective technology to improve the animal breeding program. The successful of cryopreservation was discovered by Polge and coworkers which at the time the spermatozoa is the first mammalian that successfully cryopreserved (Polge, Smith, & Parkes, 1949; Baust, J. G., Gao, D., & Baust, J. M., 2009). The starter marked of semen cryopreservation in 1938 which the sperm can survive in the freezing condition and it became successful when it can increase the improvement in the dairy industry in the 1950s (Walters, 2009). Cryopreserved semen are commercially practised in dairy cattle industry and it showed a better result compared to natural mating. However, cryopreservation can reduce the sperm fertility which approximately 40-50% cannot survive in cryopreservation process even with optimised protocols (Watson, 2000).

### 2.3.1 Challenges of cryopreservation

Eventhough cryopreservation have numerous of beneficial usages but there still have some limitations. Mostly, cells that preserved at lower temperature (-196°C) has inevitable side effect such as structural changes and genetic damage. The addition of cryoprotective agents (CPAs) in the extender can help to protect the cell from damage. However, CPAs themselves brings harmful to the cell when used in high concentration. Sperm is highly sensitive and fragile cell which it can easily susceptible to damage. The most challenge in semen cryopreservation is during freezing and thawing procedures. Ozkavukcu, Erdemli, Isik, Oztuna, & Karahuseyinoglu (2008) stated that cryopreservation can affect the morphology of the sperm such as the damage of the mitochondria, acrosome and the sperm tail due to the effect of freezing and thawing process. Sperm will not available to survive in low temperature. The cold shock is cause by the ice formation in semen which can decrease the semen quality. This can destroy the selective permeability membrane of calcium such as loss of motility, reduce the function of membrane integrity of spermatozoa and the sperm cell will become necrosis (Bailey, Bilodeau, & Cormier, 2000). Commonly problem happened during freezing is the formation of ice crystal in the intracellular of sperm cell which can cause injury to the cell and damage the cell and also decrease the fertility of sperm.

### 2.3.2 Freezing

Freezing is a process to stop the biological activity of sperm cell. Frozen semen can become more convenient and economical for animal breeding because the semen can remain viable in the ($LN_2$) for many decades and available for insemination at the most appropriate time (Loomis and Squires, 2005). There have many factors that may affect

the qualities of sperm preservation during freezing process include temperature changes, ice formation and osmotic stress (Hezavehei et al., 2018).

There have two types of freezing method which are slow freezing and fast freezing. Slow freezing is a process that the cells are slowly cooled by decrease the temperature until reaches the temperature of -196°C in a long period time. The protocol of slow freezing include cooling rate of 1°C/min, CPAs less than 1.0M and high cost controlled rate freezer. The advantages of this method is low risk of contamination during the process and not required high skills. However, this method also bring harmful to the cell which it has high risk to injury the cell due to the extracellular ice formation. Next, fast freezing is a process that cells are cooled quickly in -196°C until become glass like. The advantages of this methods is it has low risk of freeze injury which can ensure the high survivability of the cell. However, this method have high potential of pathogen contamination and required high skills (Watson, 2000)

### 2.3.3 Thawing

Thawing is a process involves a changes from frozen solid to liquid by gradual warming. Many studies have been conducted to determine the optimal thawing temperature and duration time which can give highest percentages of viable spermatozoa after post thawing process (Lyashenko, 2015). Thawing solution can increase the fertility and survivability of sperm. The practical thaw that mostly recommended by AI organisation for bull spermatozoa at 35°C for 30 seconds (Abdolreza Rastegarnia, Abdolhossein Shahverdi, Tohid Rezaei Topraggaleh, Bita Ebrahimi, 2013).

## 2.4 Cryoprotective agents (CPAs)

CPAs contain highly permeable chemicals and have low molecular weight which helps to minimize the physical and chemical effect of the cooling and freezing process of the sperm cell (Santo, Tarozzi, Nadalini, & Borini, 2012). CPAs help to decrease the freezing point of a substance and reduce the amount of salts and solutes in the liquid phase which may lead to the ice formation in the spermatozoa. CPAs can be classified into two types which are penetrating or non-penetrating.

A penetrating cryoprotectant is a membrane permeable which can pass through intracellular and extracellular. Penetrate CPAs is a solvents which can help the sugar and salt dissolve in cryopreservation medium (Holt, 2000). The example of penetrating CPAs such as glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), dimethyl acetaldehyde and propylene glycol (Santo et al., 2012). Glycerol is commonly used in cryopreservation medium because the glycerol can reduce osmotic damage of sperm cell. Previous studies shown that glycerol is preferable as a CPAs compare to DMSO to protect the sperm structure cell (Hoon et al., 2017).

Next, non-penetrating is a non-membrane permeable which cannot cross the sperm plasma membrane and only can act extracellularly. Thus, non-penetrating will alter the plasma membrane of the cell or lower the freezing temperature of the medium and act as a solute. The example of non-penetrating CPAs such as egg yolk citrate, raffinose, sucrose, albumin, polyethylene glycol and polyvinyl pyrolidone (Santo et al., 2012).

### 2.4.1 Extender

Extender is a chemical medium that can increase the ability of sperm survival (Mishra, Alam, Khandokar, Mazumder, & Munsi, 2010). The function of extender is to stabilise the plasma membrane, protect the semen against thermal shock, provide energy and maintain optimal environment for survival of sperm (Futino et al., 2010). Extender also can help to reduce the damage during freezing, effect of change in pH and osmolality and bacteria growth during freezing and thawing (Vidal et al., 2013).

Extender is an alternative way to increase the efficiency of semen cryopreservation in animals. The most common extender used in semen preservation is egg yolk. Egg yolk can help to protect the sperm from damage during freezing and thawing process but it also have major problem which it contain high risk of microbial contamination (Becaglia et al., 2009). Besides, egg yolk can cause coagulation of enzyme which can bring harmful to the sperm cells (Leboeuf, Restall, & Salamon, 2000; Purdy, 2006).

### 2.4.2 Sugar

Sugar provide sources of energy to the sperm and helps to maintain the osmotic pressure of the diluents by induce cell dehydration, reduce the formation of ice crystal into the spermatozoa and increase the viable sperm cells. There have two types of sugar molecular weight molecules which are low molecular weight molecules and high molecular weight molecules. Low molecular weight molecules can pass through the plasma membrane of spermatozoa and provide energy for metabolism and physiological

while high weight molecules cannot across a plasma membrane then it will produce osmotic pressure to induce intracellular ice formation (Naing et al., 2010).

Sugar is very important in order to optimize extender medium to achieve the best of post-thaw semen characteristics which will drive to high fertility. There have many types of sugar such as sucrose, lactose, fructose, glucose and trehalose. Glucose is the most excellent sugar in cryopreservation media which can increase 10% to 30% of sperm motility (Aboagla, 2003). From various studies shows that, supplementation of sugars can improved sperm counts, motility and increase the concentration of testosterone (Rasekh, Jashni, Rahmanian and Jahromi, 2015). The addition of sugar from natural plant extract give a positive effect to the sperm preservation which it can provide nutritive, antioxidant and antibacterial (Sansone, Nastri and Fabbrocini, 2000) such as DPPG presence of higher natural sugar and bioactive compound.

## 2.5 Date Palm Pollen Grain

**Figure 2.1:** Male date palm flower from source of date palm pollen grain

Source: Shutterstock (2016)

Date palm (*Phoenix dactylifera L.*) is from Arecaceae family. Date palm is the largest cultivation in Saudi Arabia and used by the early Egyptians and the ancient Chinese as dietary supplement (Bishr, Desoukey, & Palm, 2012). Commonly, Date palm pollen grain (DPPG) are used in Middle East as medicine for curing and promote male and female fertility (Bahmanpour, Talaei-khozani, Vojdani, & Poostpasand, 2006). DPPG is the part of date plant tree which produce by male flowering. Date palm is dioecious which it contains both male date and female reproductive. At the maturity level, the male date palm will harvest the flower and become the pollen while the female date palm will harvest the flower and become dates. According to author Lobo & Yahia (2014) estrogen is the one of hormone which help to regulate the renewal of spermatogonial stem cell. Many of investigations have been proven that the extraction of DPPG contain estrogenic material such as estrogen and sterols which can improved male infertility (Abed El-Azim, Yassin, Khalil, Amani, & El-Mesalamy, 2015).

Date palm fruit is well known as a nutritious fruit because rich in nutritional value such as carbohydrates which contain glucose (Sola Agboola & Lateef Adeju, 2013), dietary fibres, essential vitamins and minerals. In fact, DPPG were found to have the second highest antioxidant activity among 28 fruit commonly consumed in China (Ghnimi et al., 2017). The results from the previous studies mentioned that DPPG contain moisture (28.80%), ash (4.57%), crude fiber (1.37%), crude fat (20.74%), crude protein (31.11%) and carbohydrates (13.41%) (Abed El-Azim et al., 2015). Besides, DPPG contain more nutrient such as mineral salts, sugars, vitamins and lipids. The DPPG was used widely in a traditional medicine for improve the fertility of the male and it also used as herbal medicine which it contain antioxidant, antimicrobial, anti-oxidative and anti-toxicant which can protect the body cell. DPPG have many beneficial such as it also

contain flavonoids which can increase the level of testosterone and increase the number of male sperm (Anjum et al., 2012).

## 2.6 Brahman cattle breed

Brahman is the one of the most popular breed of meat beef in the world due to their fast growth rate and the meat qualities. Brahman breed cattle is originated from Bos Indicus group which originate from India. Brahman size category as medium size which the weight of Brahman bull can achieve from 1600 lb up to 2200 lb. Furthermore, Brahman bull have high resistance to extreme climate, disease and high rapid growth. Besides, Brahman have high motility of sperm during at puberty stage. Bos indicus have high testicular blood supply compared to Bos taurus which it effect the morphology of testicular vascular cone and can improve thermoregulatory capabilities with positive effect on semen quality and sperm production (Aponte, Rooij, & Bastidas, 2005).

## 2.7 Bovine spermatozoa

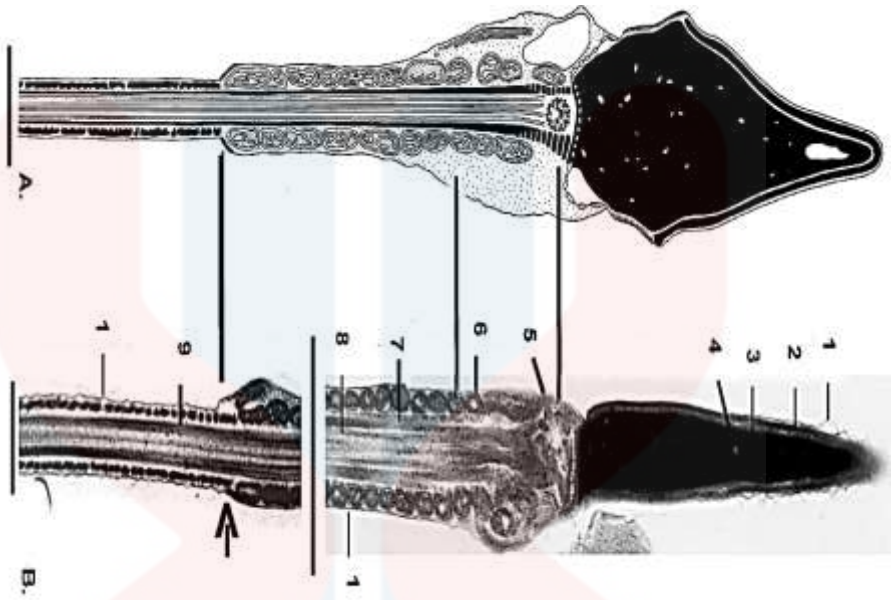


**Figure 2.2:** (A) Schematic drawing of human sperm and (B) Longitudinal section of a normal stallion 1) Plasma membrane, (2) acrosome, (3) nucleus, (4) small nuclear vacuole, (5) proximal centriole, (6) mitochondria, (7) outer dense fibre (ODF), (8) outer tubule doublet, (9) fibrous sheath; ( ← ) junction of midpiece with mitochondrial helix and principal piece with fibrous sheaths.

Source: Holstein and Roosen-Runge (1981)

Spermatozoa can categories as highly differentiated cell. This is because the structural organisation of spermatozoa are complex which are include acrosome formation derived from golgi vehicle, axoneme formation from the distal centriole, the migration and development of mitochondria in the mid piece of acrosome, anisotropic head sperm formation and the loss of cytoplasm (Auger, 2010). Spermatozoa is produced by spermatogonia meiosis in seminiferous tubule of the testis. Spermatogonia develop and become mature spermatozoa or called sperm cell. Sperm cell is the male sex which

function to fertilize with the ovum during fertilisation and will create a new organism from combination of DNA carries by sperm and the ovum. The normal sperm consist of head, mid piece and tail which help the sperm to motile through the ovum. The mid piece of spermatozoa is contain mitochondria which provide energy for motility while the head carries the parental DNA and the tail for movement (Auger, 2010).

### 2.7.1 Reactive (ROS) production in sperm

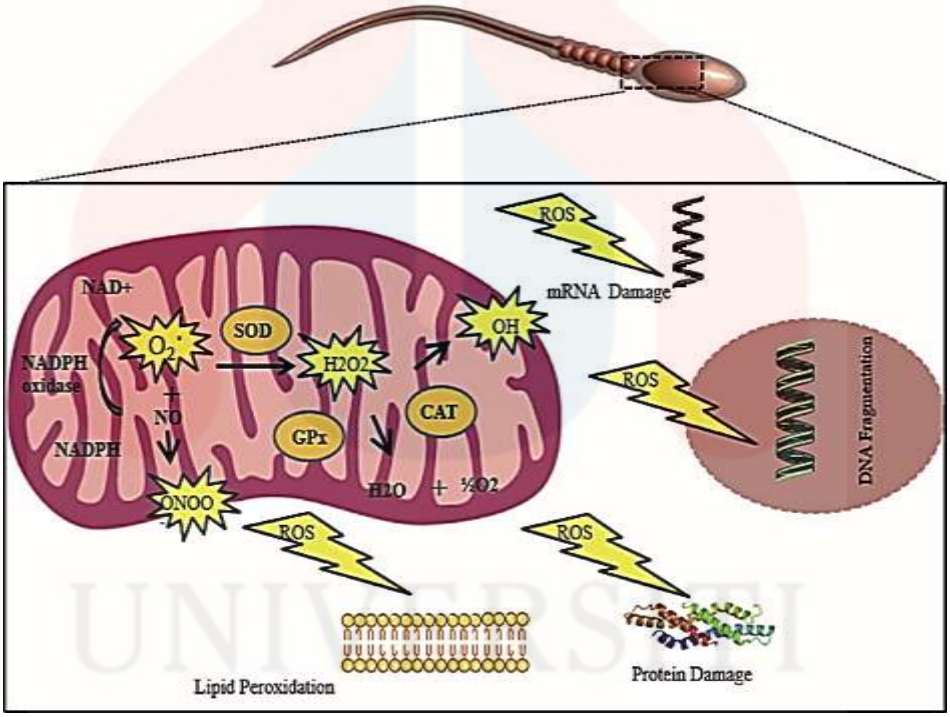


**Figure 2.3:** Summary of reactive oxygen species (ROS) production in cryopreserved sperm. NADPH = dihydronicotinamide-adenine dinucleotide phosphate; NAD = nicotinamide adenine dinucleotide; O2° = superoxide; H2O2 = hydrogen peroxide; SOD = superoxide dismutase; ONOO– = peroxynitrite; GPX = glutathione peroxidase; CAT = catalase.

Source: Hezavehei et al., (2018)

Commonly, in normal physiological process sperm will produce optimal level of reactive oxygen species (ROS) which play significant role for sperm capacitation, acrosome reaction, fertilisation ability and stabilisation of the mitochondria capsule in the mid pieces in bovine (Bansal & Bilaspuri, 2011). However, imbalance of ROS production and scavenging activity may lead the sperm damage which affect to the male infertility. Sperm are protected by its natural antioxidant which is seminal plasma. However, the excessive production of ROS that exceed the antioxidant capacity of seminal plasma may activated the oxidative stress (OS) in all cellular component such as lipid, protein, nucleic acids and sugars. In bovine semen, ROS are generated from dead spermatozoa via an aromatic amino acid oxidase catalysed reaction (Sarıözkan, Numan, Barbaros, Ulutas, & Bilgen, 2009). There have two main source of ROS which are leukocytes and immature spermatozoa. Leukocytes associated with excessive ROS production which conclusively cause abnormal functioning (Saleh et al., 2002). Mammalians spermatozoa contain abundance of polyunsaturated fatty acids (PUFAs) which may lead to the lipid peroxidation damage. During cryopreservation process, PUFAs in the sperm plasma membrane undergo peroxidation which results in the ROS formation (Sarıözkan et al., 2009). Lipid peroxidation is determine by the concentration of ROS which generate by the contaminated leukocytes mixed with sperm (Williams & Ford, 2005). Noted by the author Lushchak (2014), more than 90% of ROS are produced from the mitochondria. Lipid peroxidation occur when free radicles steal electron from the lipid membrane which may disrupt DNA integrity and reduce the quality of the sperm.

## 2.8 Semen Assessment

### 2.8.1 Assessment of motility

The assessment of sperm motility is the ability of the sperm movement properly. The pattern of the sperm movement is important to determine the sperm in normal forward progression. The effect of immotile spermatozoa were rapidly happened on post thawed which may reduce the sperm motility (Ozkavukcu et al., 2008).

### 2.8.2 Assessment of viability

Viability is refers to the percentage of live sperm in the semen sample. The sperm viability can be observed using the eosin-nitrogen stain procedures as stated by (Chauhan & Anand, 1990). The dead sperm will appeared stained because the cell have an incomplete cellular membrane which the dye easily can penetrate into the cell membrane while the viable have complete sperm membrane cell which makes the dye difficult to penetrate the cell membrane.

### 2.8.3 Assessment of membrane integrity

Membrane integrity is important to ensure the success of fertilisation and capability of sperm towards the osmotic inside the female reproductive tract (Fleash & Gadella, 2000). Salvador, Yaniz, Viudes-de-Castro, Gomez, & Silvestre (2006) stated, membrane integrity was observed by using hypo-osmotic swelling test (HOST). HOST is to investigate the functional integrity of the mammalian sperm membrane. The principle of the HOST is based on fluid transport across the sperm tail membrane under

hypo-osmotic conditions until equilibrium is reached. The influx of fluid will result the tail expands which considered as hypo-osmotic response. Undamaged sperm tail membrane permits the fluids to passage into the cytoplasmic space which cause swollen and the pressure generated makes tail fibres curl while the damaged tail membrane allows fluid to pass across the membrane without occur the cytoplasmic swelling and tail curling. The result, swelling of the tail indicate an intact membrane and presumably the spermatozoa are functioning normally (R.Jothipriya, S.Sasikumar, EK.Madhankumar & Nova, 2014).

# CHAPTER 3

# MATERIALS AND METHODS

All the chemicals used in this experiments were purchased from Sigma – Aldrich (U.S.A), unless otherwise will be stated.

## 3.1 Experimental Design

This study is comparison of the different extenders on quality semen of bovine. The experimental design was used randomized completely block design (RCRD), block with different temperature on the sperm quality and operators for four treatment groups as Tris citric fructose yolk glycerol (TCFY; control), (TCFY + 2% DPPG; G1), (TCFY + 4% DPPG; G2), (TCFY + 6% DPPG; G3). The study were replicate three times for each of the experiment.

Experiment 1: Investigated the effect of semen extender (TCFY, TCFY + 2% DPPG, TCFY + 4% DPPG and TCFY + 6% DPPG) on sperm quality in chilled (-4°C) after seven days of storage. The straw semen were placed in the water bath at 37°C for 30 second and were assessed of sperm motility, sperm viability and membrane integrity.

Experiment 2: Investigated the effect of semen extender (TCFY, TCFY + 2% DPPG, TCFY + 4% DPPG and TCFY + 6% DPPG) on sperm quality in frozen (-196°C) after seven days of storage. The straw frozen semen were placed in the water bath at 37°C for 30 second and were assessed of sperm motility, sperm viability and membrane integrity.

## 3.2 Experimental Animal

**Figure 3.1**: Brahman Bull

The study conducted at the Princess of Naradhiwas University, Thailand. (6.4557º N, 101.7898º E). A Brahman bull cattle (Bos indicus) aged between three to six years old was utilise in this experiment. The animal was raised on a cattle farm of Faculty of Agriculture, PNU. The semen was collected by using ElectroJac IV ejaculator (Neogen, Lexington, KY, USA). The bull ejaculated once times per day which on the early morning 0700 and the collection of semen done twice a week for a four weeks.

## 3.3 Preparation of extender

Tris buffer: Tris citric acid fructose with egg yolk (TCFY) was prepared according to (Homa, Vessey, Perez-Miranda, Riyait, & Agarwal, 2015) and was used as control extender in the experiment. The extender consists of 3.028 g Tris, 1.675 g citric acid, 1.250 g fructose, 0.1 g penomycin (M & H manufacturing Co., Ltd, Samutprakam, Thailand) and 20% of egg yolk. All the ingredients of Tris buffer were dissolved in one litre of distilled water. The extenders of TCFY were supplemented with different concentration of DPPG followed by adding 6% of glycerol. A total of semen extender solution was prepared in 12 ml of each treatment groups.

## 3.4 Preparation of stock solution

Date palm pollen grain (DPPG) was purchased and imported from the Saudi Arabia. The stock solution was prepared according to (El-sheshtawy et al., 2014). The Tris citric acid fructose (TCF) solution were prepare (Tris = 1.514 g; citric acid = 0.8375 g; fructose = 0.625 g and penomycin = 0.05 g) and dissolved in 50 ml of distilled water. Then, 20 mg of DPPG were supplement and dissolved in 40 ml of TCF. Thus, 40 ml of 0.02 g of DPPG (0.02g/40ml) served as stocks solution. Next, stock solution was filter by using syringe filter (Whatman plc, UK) to remove the precipitated at the bottom and centrifuged for 30 minutes to make sure all the DPPG powder were completely dissolved with the TCF. Then, kept the stock solution in the refrigerator (4°C).

### 3.5 Semen collection and initial evaluation

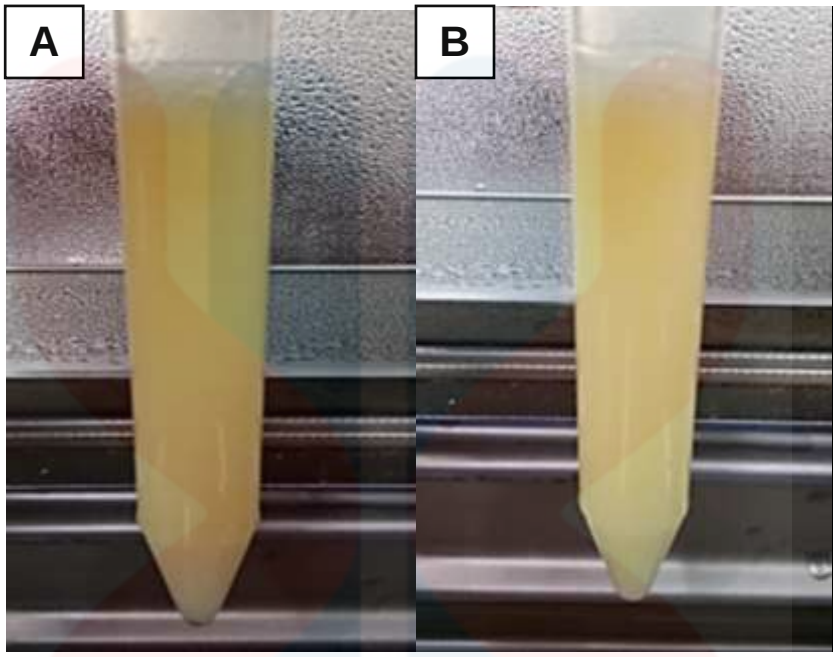


**Figure 3.2:** The comparision of the sperm quality based on the colour of the sperm:

(A) Low quality (B) High quality

The bull was ejaculated using electroJac VI ejaculator (Neogen, Lexington, KY, USA) once a week for four weeks. Before the semen was collected, the fur of the bull penis were trim to avoid the contamination of microorganism and dust into the semen. Then, the penis were cleaned by washing four times of sodium chloride (NaCl) solution. The semen samples were kept in 37°C in water bath. Then, the semen samples were directly evaluated for mass motility and semen concentration. Mass motility of fresh sperm was evaluated (40x) under phase contrast microscope (CH30; Olympus, Tokyo, Japan). A sperm was placed on pre-warmed glass slide 38 °C (El-Sisy et al., 2016) and aliquot of 10 $\mu l$ for the evaluation.

**Table 3.1:** Mass motility rating system for ejaculated ram sperm

Source: David (2015).

| Rating | Microscopic appearance |
|---|---|
| 0 | No swirl – Sporadic oscillation of individual sperm |
| 1 | No swirl – Generalized oscillation of individual sperm only |
| 2 | Very slow distinct swirl |
| 3 | Slow distinct swirl |
| 4 | Moderately fast distinct swirl |
| 5 | Fast distinct swirl |

## 3.6 Sperm concentration

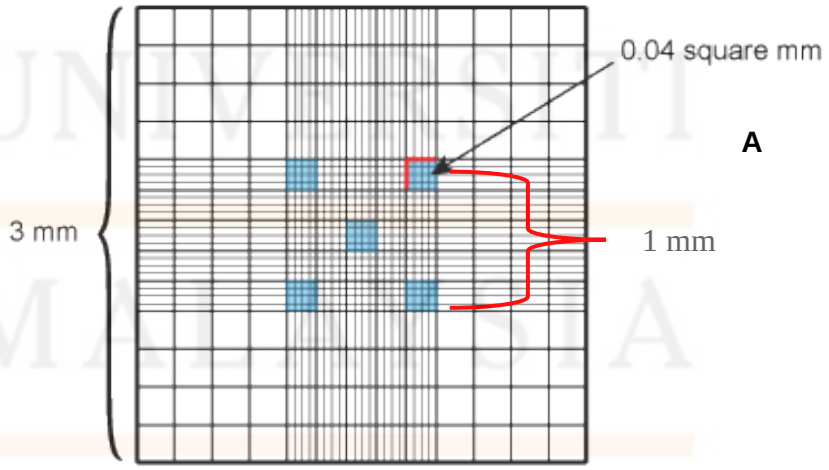


**Figure 3.3:** Counting sperm cell using haemocytometer

Source:Saadat Parhizkar (2014)

The semen concentration was determine using a Neubauer hemocytometer (Boeco, Hamburg, Germany) with added the original semen sample 1 ml of semen to 99 ml of diluent (1:100 dilution factor). Semen and NaCl were put into the micro centrifuge tube (Molecular products lnc, San Diego, USA) for mixing using vortex (Scientific Industries, Inc, USA). Aliquot 10 ul of solution and put on the haemocytometer and then observed using microscope under 400x (CH30; Olympus, Tokyo, Japan). The sperm head were counted in the five squares (blue square) which each of the square contain 16 smallest squares (0.04mm). Counted all the sperm heads that was half bounded on the middle line (red line). Sperm count was done by counting 4x4 squares.

The deep of the haemocytometer grid is 0.1 mm which represent 1 mm of the centre square which contain 25 smaller square inside. Thus, the volume of 25 squares which located at the centre is 0.1 µl. As the sperm only counted in five small squares (blue square) from 25 squares. Thus, the total volume per square is (0.1/5=0.02µl). The sperm used in this experiment was 1.0ml (1000µl/0.02ul=50,000 concentration of squares)

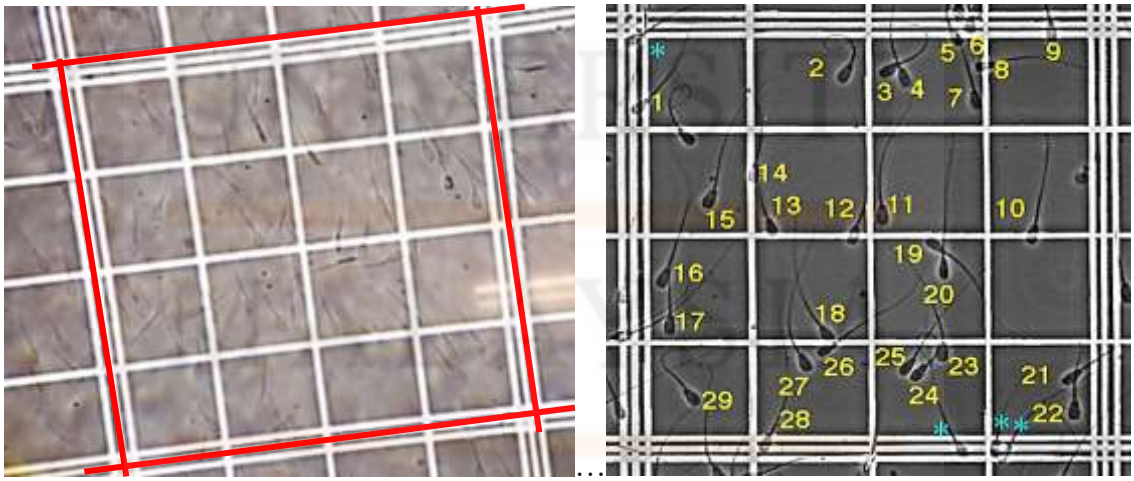


**Figure 3.4:** Sperm head at the edges were included from counting (400x)

In one small square (blue square) contain 16 smallest squares. At the figure 3.4, there were 29 sperm present on the 16 smallest square. The sperm indicate * were not counted because these sperm located half way outside from the counting area. Noted that, sperm 6 was counted because the sperm still in the middle of the triple line. The concentration of sperm was calculated by the following formula according to (Saadat Parhizkar, 2014).

Sperm concentration/ml: (Sperm count in 5 square) (Dilution factor) (0.05 X $10^6$)

## 3.7 Semen processing

### 3.7.1 Semen Dilution

The semen was diluted with sodium chloride (Nacl) with added 1 ml of semen to 99 ml of diluent (1:100 dilution factor). Then, the semen samples were diluted with TCFY extender and Tris pollen grain extender (TPG) which the total of semen and extender is (1:12). Only the fresh sperm that ejaculates (>70%) of progressive motility and (>50 x $10^6$) spermatozoa (spz/ml) of sperm concentration were acceptable in this experiment.

### 3.7.2 Semen cooling

The diluted semen were kept at 25ºC for 30 minutes. Then, all the diluted semen of all the groups were slowly cooled to 5ºC which equilibrated for 5 hours. After equilibration, all the treatment groups were packed into paillette 0.25 ml orange pastel (Imv technologies, L'Aigle, France) and sealed with the polyvinyl powder at the end of the straws.

### 3.7.3 Semen Freezing

The straws were horizontally placed on a rack and pre-frozen in liquid nitrogen ($LN_2$) vapour at 4 cm above the liquid nitrogen (8 cm) level in a styrofoam box (25 x 35 x 30 cm) for 15 minutes and then the straws were plunged directly into the $LN_2$ for 15 minutes. The sample divided into two experiment. First experiment, all treatment groups was chilled in the refrigerators for seven days and inspected all the semen quality parameters include motility, membrane integrity and viability. Second experiment, all the straws were store in $LN_2$ for one weeks and evaluated all the sperm quality.

## 3.8 Thawing

Thawing was performed at water bath and frozen straws were thawed at 38°C 30 seconds (Vidal et al., 2013) by after storage.

## 3.9 Assessment of semen quality parameters

The assessment was test on chilled and frozen sperm parameter up to seven days of storage. The experiments were analyse the mass motility, motility, viability and membrane integrity. A total of sperm cell of each parameter must achieved 300 counted sperm cell to find the average.

### 3.9.1 Assessment of motility

Aliquot 10 µl of diluted semen and put on a warm slide and then covered with cover glass. Then, observed the sperm motility using microscope under 400x and counted the sperm which show normal forward progression.

### 3.9.2 Assessment of viability

The sperm viability can be test using the eosin-nitrogen stain method as stated by (Chauhan & Anand, 1990). The eosin nigrosine was prepared according to (Malik et al., 2016). The eosin nigrosine solution (0.2 g of eosin and 2 g nigrosin were dissolved in buffer saline solution and mixed for two hours at room temperature. Then, aliquot 200 µl of diluted spermatozoa and 200 µl eosin-nigrosin stains were mixed using vortex and smeared on a warm slide by using hot plate. Then, the samples were observed using microscope (400x).

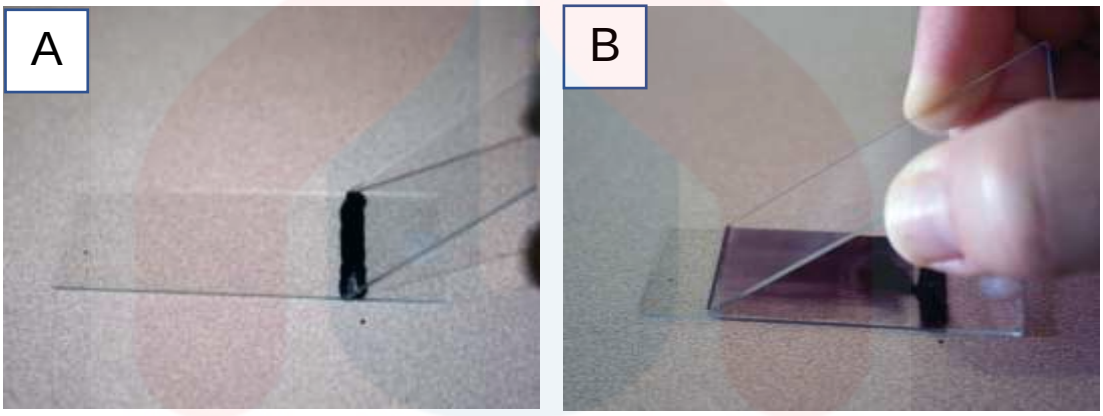


**Figure 3.5:** Semen staining for viability using eosin nigrosin with staining method which (A) Upward slide then laid into the line of stain (B) The upward slide mixed the stain and pushed forward to create a smear.

Source: Brito (2017)

Eosin component will penetrate through the plasma membrane of the dead sperm cell while nigrosin provides background contrast to differentiate between live and dead sperm. Sperm cell that have stain purple is dead while live sperm will appears white colour.

### 3.9.3 Assessment of membrane integrity

Salvador, Yaniz, Viudes-de-Castro, Gomez, & Silvestre (2006) stated, membrane integrity was observed by using hypo-osmotic swelling test (HOST). The ingredient of HOST solution is 1.8 g fructose and 0.98 g sodium citrate were dissolved in 200 ml distilled water. A 100 µl of semen were mixed with 200 µl of HOST solution (100 mOsm/kg). Then, incubate for 1 hour at 37°C. Aliquot 10 µl of HOST solution was placed on a microscope slide covered with cover slip and evaluated using microscope under 400x. Spermatozoa with intact plasma membrane will swelled the tail while the damage sperm membrane will not show swelled tail (Zubair et al., 2013).

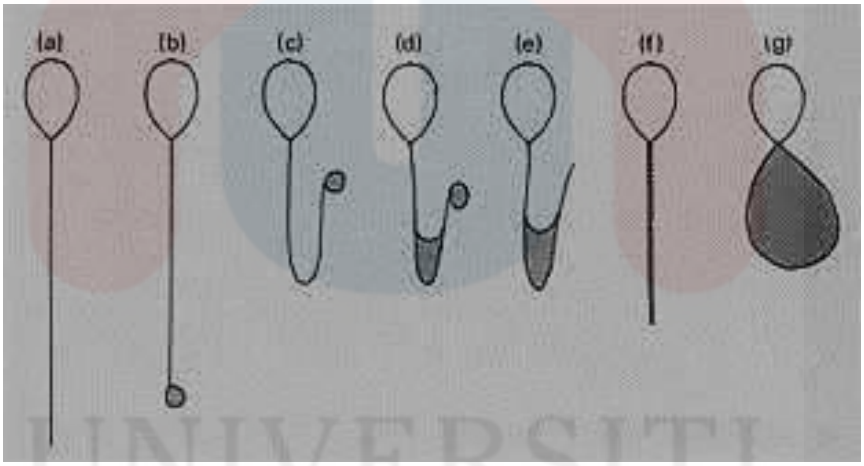


**Figure 3.6:** Schematic presentation of no reaction (a) and with mild (b) and severe (c-g) HOST reaction sperm cells.

Source: Jeyendran et al. (1984) and Fonseca et al. (2005).

### 3.10 Calculation of Percentage of Spermatozoa

Percentage of motile, HOST reactive and viable was calculated by the following formula:

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

### 3.11 Statistical Analysis

All the data in motility, membrane integrity and viability are analysed follow by ANOVA using the SAS software system to determine the differences between all the parameters (version 9.1, SAS Institute Inc, 1996, Cary, NC, USA). Tukey's post hoc test was used to compare the differences between P values ($p<0.05$) consider as statically significantly. All data are express as mean ± standard error of mean (SEM).

# CHAPTER 4

# RESULTS & DISCUSSION

Extensive research for the innovation of semen extender has been conducted for investigating the use of natural extract from plant-based for preserving animal semen (El-Sisy et al., 2016). Date palm pollen grains (DPPG) in these recent years categorized as an herbal remedy that containing high natural antioxidant which provides as a solution to problems in semen cryopreservation (Bishr et al., 2012). DPPG is also the second highest contain antioxidant among 28 fruits commonly consumed in China (Ghnimi et al., 2017).

Experiment 1 shows the effect of addition DPPG with different concentration (TCFY, TCFY + 2% DPPG, TCFY + 4% DPPG and TCFY + 6%. DPPG on sperm parameters did not show any significant different (P>0.05) after seven days of storage in chilling. The extender with 6% of DPPG had the highest mean value compared to the control group in membrane integrity (90.82 ± 2.39), viability (90.22 ± 1.22) and motility (87.37 ± 0.42). Additionally, enrichment of 6% of DPPG give beneficial to the sperm compared to the control group in chilled sperm. However, the supplementation with 2% of DPPG had no effect on all post-thawing semen parameters in semen chilled and the effect was nearly similar to control group.

**Table 4.1:** Percentage (mean ± SEM) of the quality sperm parameters on chilled semen in a Tris buffer supplemented with different concentration of DPPG.

| DPPG Enrichment % (w/v) | Sperm parameters (%) | | |
|---|---|---|---|
| | Total motility | Membrane integrity | Viability |
| Control | 70.90 ± 8.64 | 86.30 ± 2.48 | 84.31 ± 5.73 |
| DPPG 2% | 74.03 ± 2.31 | 85.98 ± 0.56 | 85.89 ± 0.13 |
| DPPG 4% | 80.70 ± 7.67 | 88.75 ± 3.59 | 89.03 ± 0.15 |
| DPPG 6% | 87.37 ± 0.42 | 90.82 ± 2.39 | 90.22 ± 1.22 |

[SEM] Standard error. (n)=12

Experiment 2, shows the effect of addition DPPG with different concentration (TCFY, TCFY + 2% DPPG, TCFY + 4% DPPG and TCFY + 6% after seven days of storage in frozen. Viability is significantly different ($P<0.05$) in 6% of DPPG (71.25 ± 1.04[b]) as compared with the control group (56.47 ± 4.69[a]). This proved that, 6% concentration of DPPG give beneficial to the sperm viability among the other treatment groups. Besides, the addition of 2% and 4% of DPPG resulted non-significant ($P>0.05$) in motility (47.58 ± 5.58; 52.08 ± 8.83) and membrane integrity (85.54 ± 4.28; 85.12 ± 3.45). Additionally, enrichment of 6% of DPPG shows the highest mean value compared to the control group in frozen. The overall result shows that, the sperm quality was dramatically reduce from chilled to frozen.

**Table 4.2**: Percentage (mean ± SEM) of the quality sperm parameters on frozen sperm in a Tris buffer supplemented with different concentration of DPPG.

| DPPG Enrichment % (w/v) | Sperm parameters (%) | | |
|---|---|---|---|
| | Total motility | Membrane integrity | Viability |
| Control | 45.32 ± 9.84 | 85.05 ± 2.56 | 56.47 ± 4.69[a] |
| DPPG 2% | 47.58 ± 5.58 | 85.54 ± 4.28 | 59.26 ± 6.15[ab] |
| DPPG 4% | 52.08 ± 8.83 | 85.12 ± 3.45 | 60.80 ± 4.38[ab] |
| DPPG 6% | 56.46 ± 9.55 | 88.14 ± 3.03 | 71.25 ± 1.04[b] |

[a,b] Mean ± SEM within each rows, mean with different alphabetic superscript were significantly different (ANOVA-post hoc test at $p<0.05$). (n=12)

### 4.1 Viability

The novel finding in this study indicated that the viability of frozen semen in 6% concentration of DPPG is significantly higher ($p<0.05$) compared to control group. This proved that the concentration at 6% of DPPG helped to improve and protect the sperm viability from damage. This results in accordance with Malik, Irwan Zakir, & Syarif Djaya (2016) whom reported was significantly difference of viability during freezing. This study showed that the sperm quality decreased after freezing for all groups. These could resulted from ice crystal formation disruption during cryopreservation that may alter membrane protein and carbohydrates composition which can lead to the damage of membrane structural and subsequently resulting in viability sperm decrease (Pedersen & Lebech, 1971; Hezavehei et al., 2018). DPPG utilization as extender helps to protect the sperm viability. Reported by Hafez and Hafez (2000), sperm viability is an important component to assess the structural and functionality activity. Mammalian sperm that exposed to physical and chemical damage resulted from transition phase during

cryopreservation. DPPG as extender provide positive effects to various sperm species on viability and motility including rabbits (Faleh & Sawad, 2006), rats (Bahmanpour et al., 2006) and humans (Rakesh et al., 2015). In contrast, sperm viability was not affected by DPPG addition as reported by El-Sisy, El-Badry, El-Sheshtawy, & El-Nattat (2016). This may be due to various factor including different breeds, locations and seasonal changes during semen collection which sperm quality could be affected (Leboeuf, Restall, & Salamon, 2000).

The result shows that DPPG was proved that can protect the viability of sperm. This may be due to the composition of DPPG which contain vitamin C permits sperm protection viability by reducing cold shock effect. Vitamin C is an essential cofactor enzymes that are free radicals scavengers (Michael, Alexopoulos, Pontiki, & Hadjipavlou-litina, 2008) which possibly improved sperm function by reducing cell damage (Gangwar et al., 2015). Higher DPPG concentration as extender gives positive results and improve sperm survivability when compared to control group which current study in agreement with El-sheshtawy et al., 2014; El-sheshtawy, El-nattat, Shalaby, Shahba, and Al-se, 2016; El-Sisy et al., (2016).

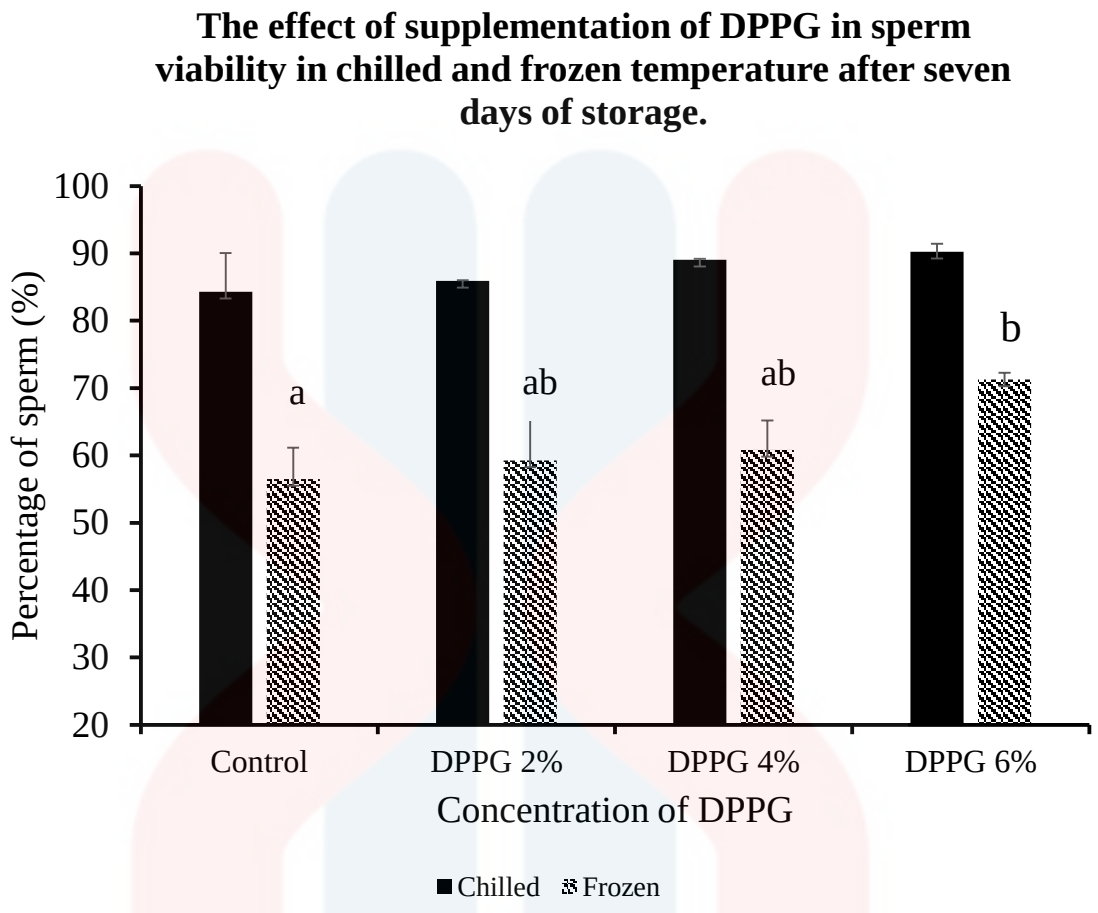


**Figure 4.1:** The comparison between chilled and frozen of sperm viability. (a) and (b) shows significant different.

Figure 4.1 shows the percentage of sperm viability with the different concentration of DPPG supplemented in extender after seven days of storage. The graphs shows, the percentage of sperm viability in chilled is slightly increase while dramatically decrease in frozen semen. SEM bars of each treatments were displayed at the top bars. At 6% of DPPG shows significant difference compared to control group. It can be prove that, at 6% concentration of DPPG give positive impact in sperm viability.

Viability and motility are the most important factor in sperm fertilization performance. They are closely related to each other for the successful of fertilisation

between sperm and ovum. Sperm can be divided into two main part which are head and tail (Pesch & Bergmann, 2006). Sperm head consists acrosome and nucleus which contain DNA and enzyme that help the sperm to break the cell membrane of an egg while sperm tail consist of mitochondria organelle which help to provide energy for tail to movement. Cold shock is the effect of rapid cooling from normal room temperature to freeze temperature (Ferrusola & Gonza, 2009) which cause the water does not have time to move outside the cell. Thus, large intracellular ice crystals formed and damage the sperm cells. Ice formation can damage either on sperm head or tail which both of these consist important organelles for the successful of fertilisation. When the ice formation interrupts the sperm head it will directly damage the plasma membrane and DNA inside the nucleus of the sperm which may result the non-viability to the sperm. The addition of DPPG give protection to sperm viability which it help to reduce the damage at the surround of sperm head.

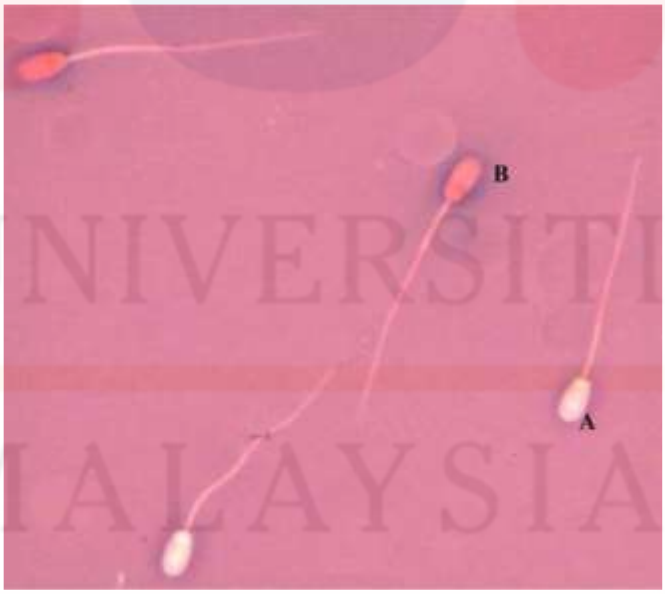


**Figure 4.2:** The comparison between live and dead sperm using eosin nigrosin solution with staining method (400x). (A) Unstained sperm cells indicate viable sperm and (B) Stained sperm indicate dead sperm with a damage cell membrane.

Source: Kondracki, Wysokińska, Kania, & Górski (2017)

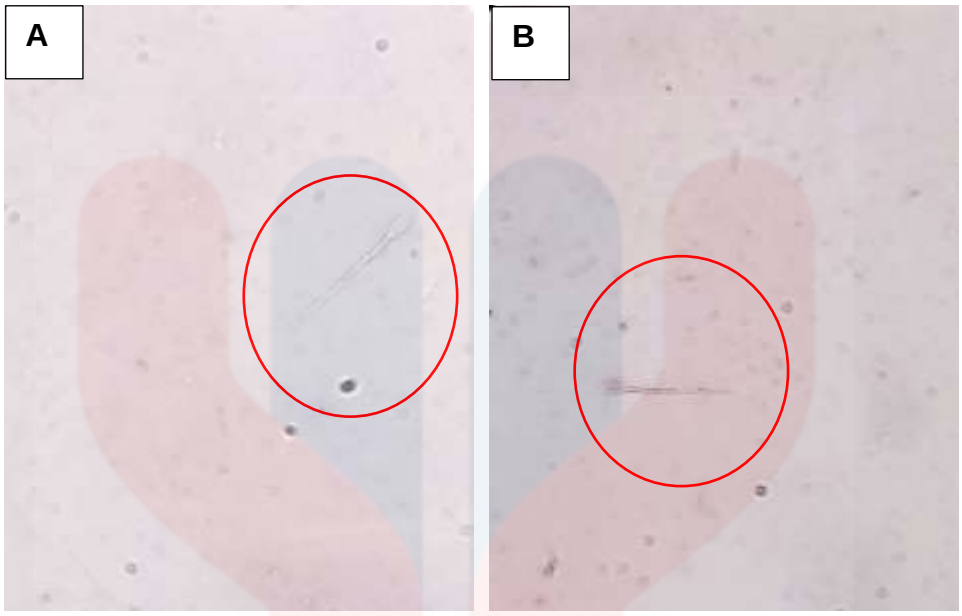


**Figure 4.3:** Sperm viability in 6% DPPG in frozen using eosin nigrosin solution with staining method (400x). A) Viable sperm; B) Non-viable sperm

## 4.2 Motility

The second finding that highlighted in this study is that the concentration of DPPG in motility of chilled and frozen semen is non-significantly different ($p>0.05$) with other treatment groups. Figure 4.4 shows the percentage of sperm motility with the supplementation of DPPG in extender after seven days of storage has no effect. The graphs shows, the percentage of sperm motility in chilled is increase gradually while dramatically decrease in frozen semen. Among the treatments, there is no significant different of the concentrations on sperm motility noticeable.

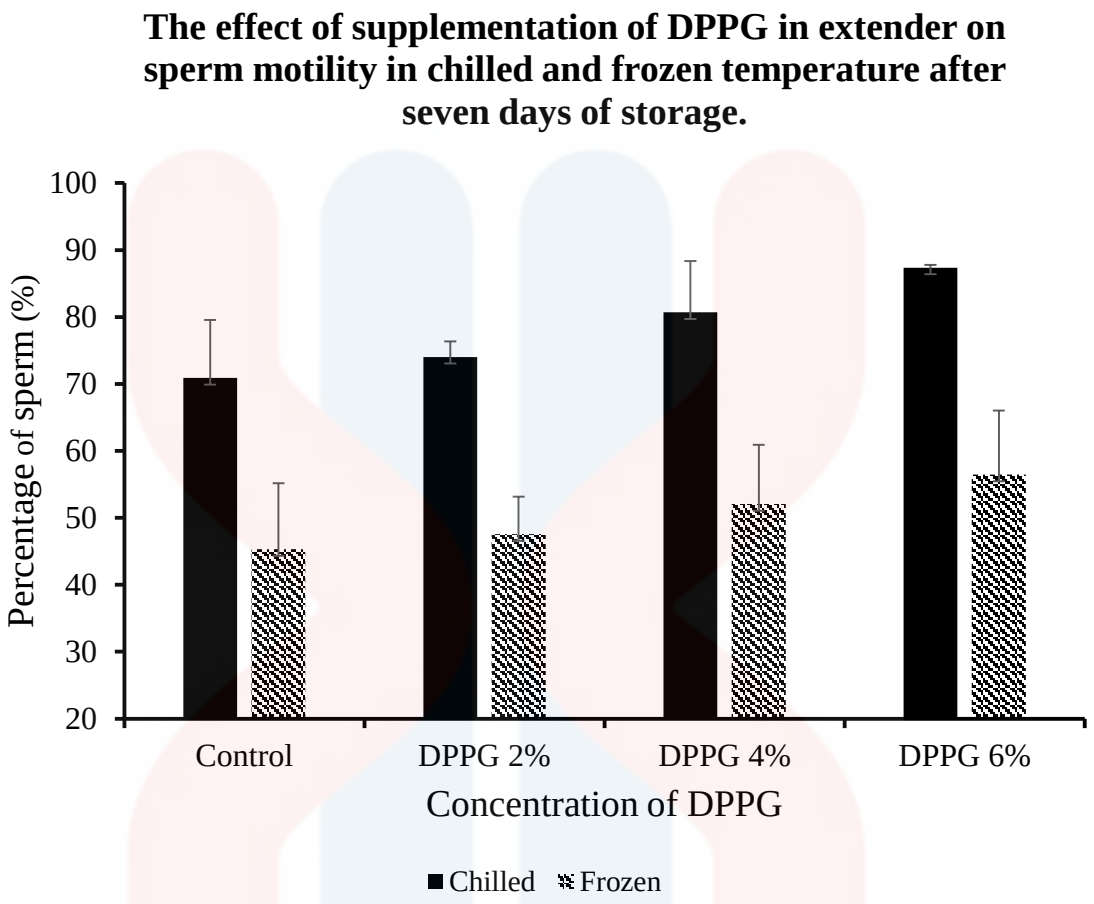


**Figure 4.4:** The comparison between chilled and frozen of sperm motility

This may due to the effect of the concentration of DPPG which is not enough effective to protect the sperm motility. From the previous study, it can be concluded that the best concentration to protect the sperm motility during cryopreservation is at 150 mg to 250 mg of DPPG. Reported by El-Sisy et al., (2016), the addition of 100 mg and 150 mg of DPPG give the significant improvement in sperm motility in chilling and freezing of Arabian stallion sperm. Next, previous study conducted by El-sheshtawy et al., (2016) also mentioned that the addition of 100 mg DPPG in chilled and 250 mg DPPG in frozen semen showed positive impacts on buffalo sperm motility. Other than that, El-sheshtawy et al., (2014) also proved that the addition 250 mg of DPPG showed the significant

improvement in bull sperm motility in chilled and frozen semen. In contrast with this study reported, the addition of 20 mg failed to give protection on the sperm motility. However, the addition of DPPG give positive impact to the sperm viability only.

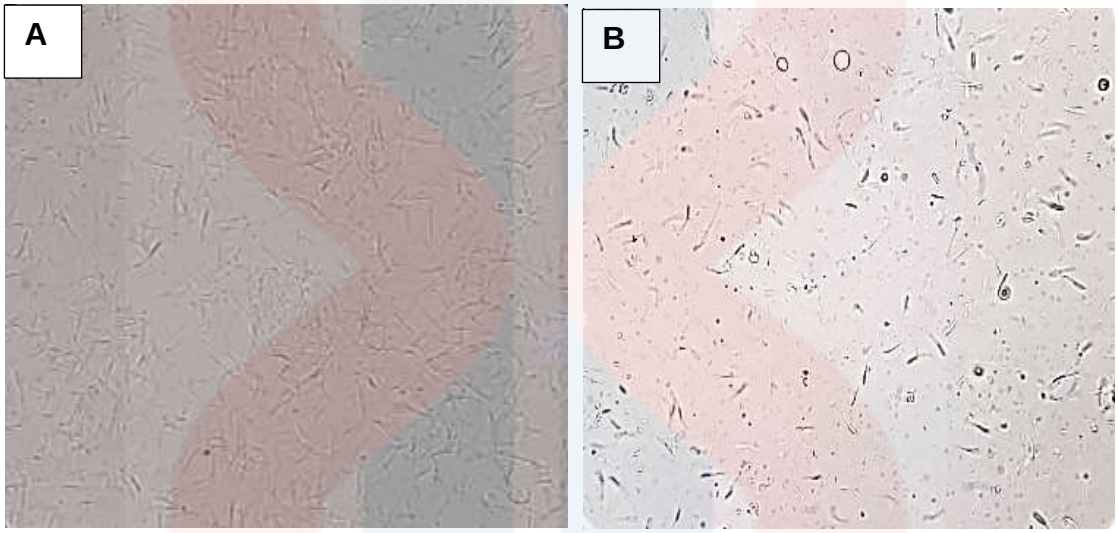


**Figure 4.5:** Sperm motility with 6% of DPPG (400x). (A) Sperm in chilled and (B) Sperm in frozen

The overall result shows that, the sperm quality was dramatically reduce from chilled to frozen. The result showed same trend with Nijs et al. (2009) which reported the percentage of sperm motility decreased from 50.6% to 30.3% after cryopreservation. Motility is an important indicator for *in vivo* fertilisation (Vijayaraghavan, 2003) because it contributes abundant of energies sources to the spermatozoa cell (Verstegen, 2002). The effect of immotile spermatozoa occurs on post thawed which may reduce the sperm motility (Ozkavukcu et al., 2008). Ice formation also happened on the sperm tail and directly disrupt the sperm mitochondrial which will reduce the percentage of motile sperm. The rapid changes in the osmolality and cooling phases will lead to the intracellular ice crystal formation in the cryopreservation process (Pedersen & Lebech, 1971; Hezavehei

et al., 2018). Cold shock give negative impact to cell such as damage spermatozoa mitochondria (Ferrusola & Gonza, 2009), permeability of sperm membrane (Meyers, 2005) and acrosomal membrane (Januskauskas, Johannisson, & Rodriguez-martinez, 2003). Cold shock also can disturb spermatozoa capacitation (Januskauskas, Lukosevici ute, & Nagy, 2005) and stability of spermatozoa chromatin structure. Thus, the fertilisation capability decreases down to 50% (Carla et al., 2008) of spermatozoa viability during cryopreservation (Defoin, Granados, & Donnay, 2008). DPPG was reported to have an effective composition that can help to prevent cold shock and improved sperm motility such as zinc, selenium, iron, cooper and cobalt which stimulates the progressive forward movement (Hassan, 2011). DPPG also contain antioxidant material including α-tocopherol which according to previous studies that it can protect sperm motility from oxidative harm.

### 4.3 Membrane Integrity

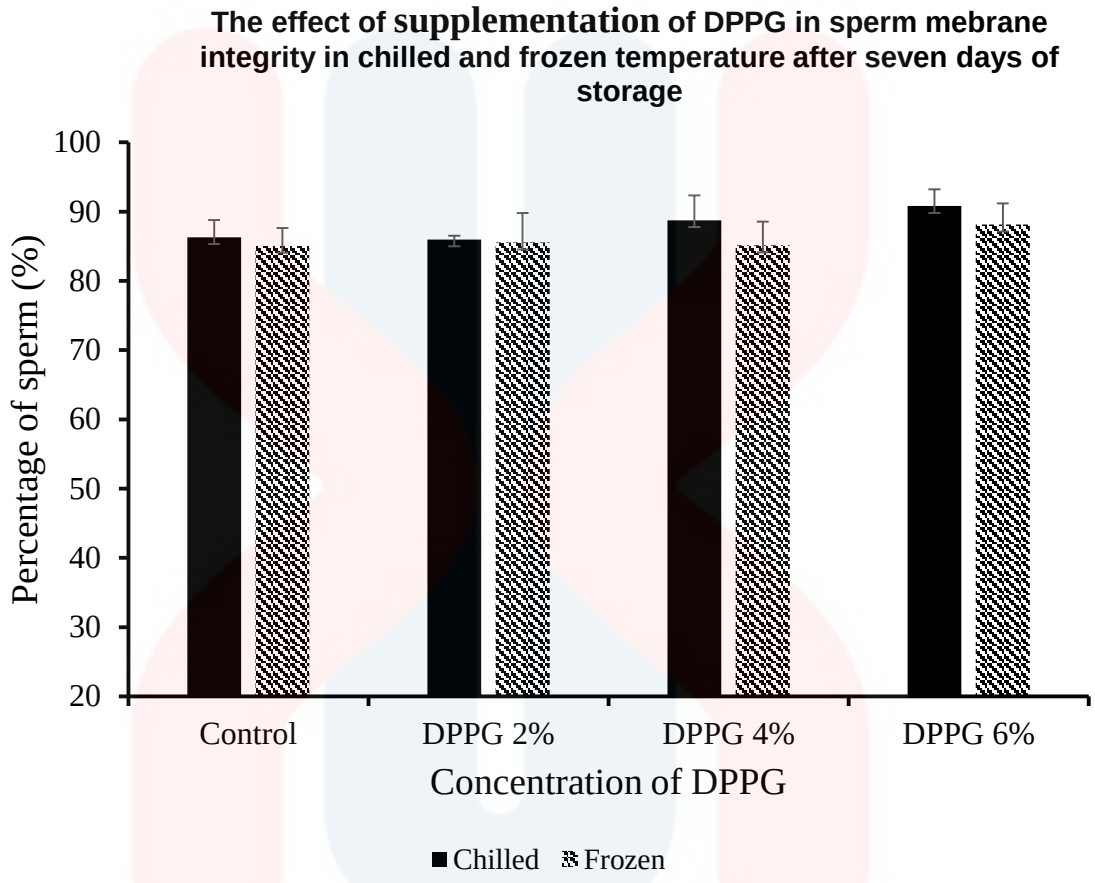


**Figure 4.6:** The comparison between chilled and frozen of sperm membrane integrity.

Next finding shows that, the addition of DPPG on extender did no shows any huge effect on the sperm membrane integrity. Figure 4.6 shows the percentage of sperm membrane integrity with the supplementation of DPPG in extender after seven days of storage shows the change barely noticeable between chilled and frozen semen. Among the treatments, there is no significant different of the concentrations on sperm membrane integrity was noticed. It can be seen on this graph all the treatments group give same improvement to the sperm integrity. However, 6% concentration of DPPG did not shows any effect to the sperm membrane integrity.

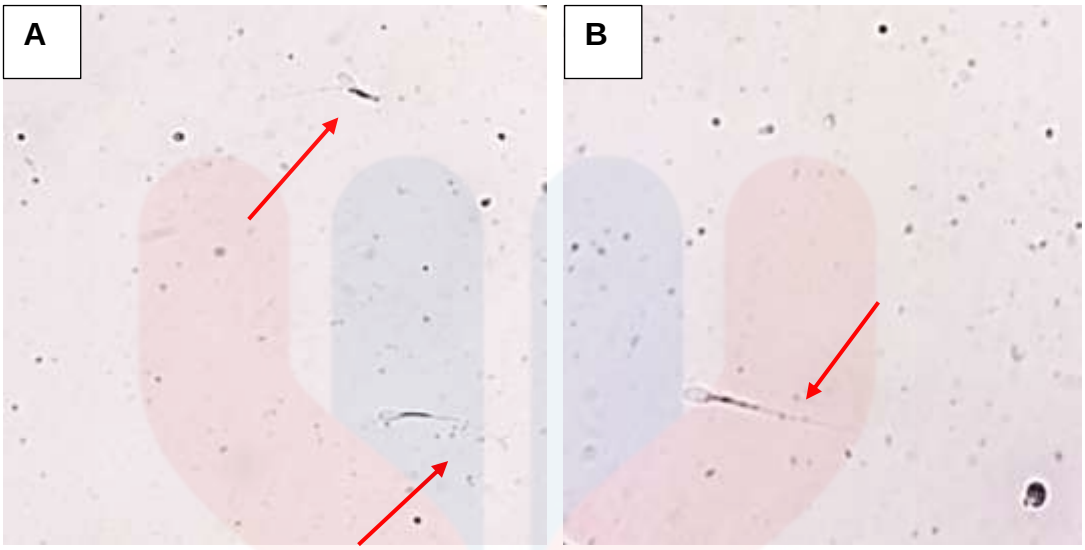


**Figure 4.7:** Sperm membrane integrity in 6% DPPG in frozen (400x). A) Viable sperm B) Non-viable sperm

The HOST induces the sperm tail to swell under hypo-osmotic conditions due to an influx of water that leads to expanded cell volume. Thus, undamaged sperm tail membrane permits the fluids to passage into the cytoplasmic space which cause swollen and the pressure generated makes tail fibres curl while the damaged tail membrane allows fluid to pass across the membrane without occur the swelling and curling of the tail.

## 4.4 General

Next finding proved that DPPG can protect the sperm from cryoinjury. Malik et al., (2016) reported that natural sugar contain in DPPG gives additional protection to the sperm during cooling, freezing and thawing (Holtz, Sohnrey, Gerland, & Driancourt, 2008). DPPG is also the ideal sugar substitute because it contain higher natural sugars and bioactive compounds (Ghnimi et al., 2017). Purdy (2006) mentioned that sugar supply energies in the extender to maintain the semen quality. During cryopreservation, sperms are subjected to tremendous chemical and physical damage caused by the phase

transition of temperature. Spermatozoa produces various types of reactive oxygen species (ROS) (Homa et al., 2015) which contains oxygen such as peroxide, superoxide, hydroxyl radical and oxygen singlet (Hayyan, Hashim, & Alnashef, 2016). The function of ROS is also important to drive tyrosine phosphorylation cascade which associated sperm capacitation. Seminal plasma contain natural antioxidant which can protect the spermatozoa from the ROS attack (Fingerova et al., 2009). However, excessive ROS production from spermatozoa unable to be effectively controlled by the antioxidant in the seminal plasma may lead to oxidative stress (OS) which further reduces the fertility of the sperm (Fingerova et al., 2009). ROS can increase dramatically during the stress environment (Devasagayam et al., 2004) especially after froze thawed (Kim, Yu, & Kim, 2010 ; Baumber, Ball, Linfor, & Meyers, 2003). The effect of ROS and OS during the semen freezing process may lead to the serious damage of sperm (Hezavehei et al., 2018).

Noted by the author Lushchak (2014) that more than 90% of ROS were produced from mitochondria. Bovine semen cryopreservation induces ROS which may attack the sperm cell due to the activities reduction from the antioxidant enzymes. Thus, limitation of ROS production of sperm during cryopreservation are possible with the addition of antioxidant which can balance the ROS level and further improved the post-thawed (Agarwal, Ong, & Durairajanayagam, 2014). Mansouri, Embarek, Kokkalou, & Kefalas (2005) reported that DPPG contain rich of phenolic compound and phytochemicals as a natural antioxidant such as *p*-coumaric, ferulic and sinapic acids, flavonoids, procyanidins and carotenoids which can help to protect the sperm against the ROS.

Besides, the investigation from this study determine that the mass motility is most the important part because it can affect the quality of the sperm during the semen

processing. In this study, the mass motility was used in score 4. The figure 4.8 shows the movement of the sperm with different score.

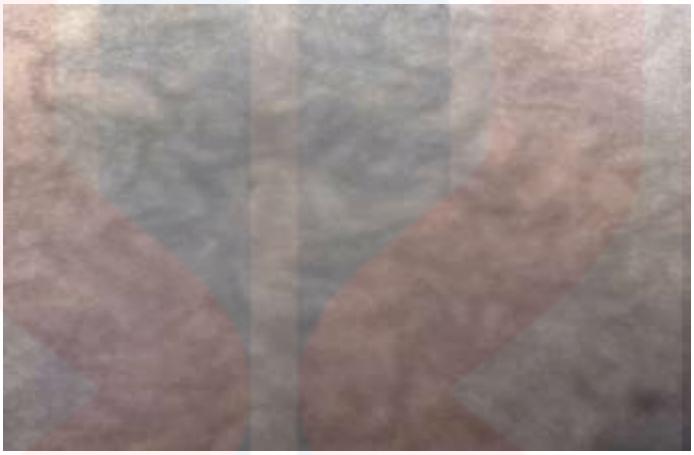

**Figure 4.8:** Bovine sperm in score 4 of mass motility (100x)

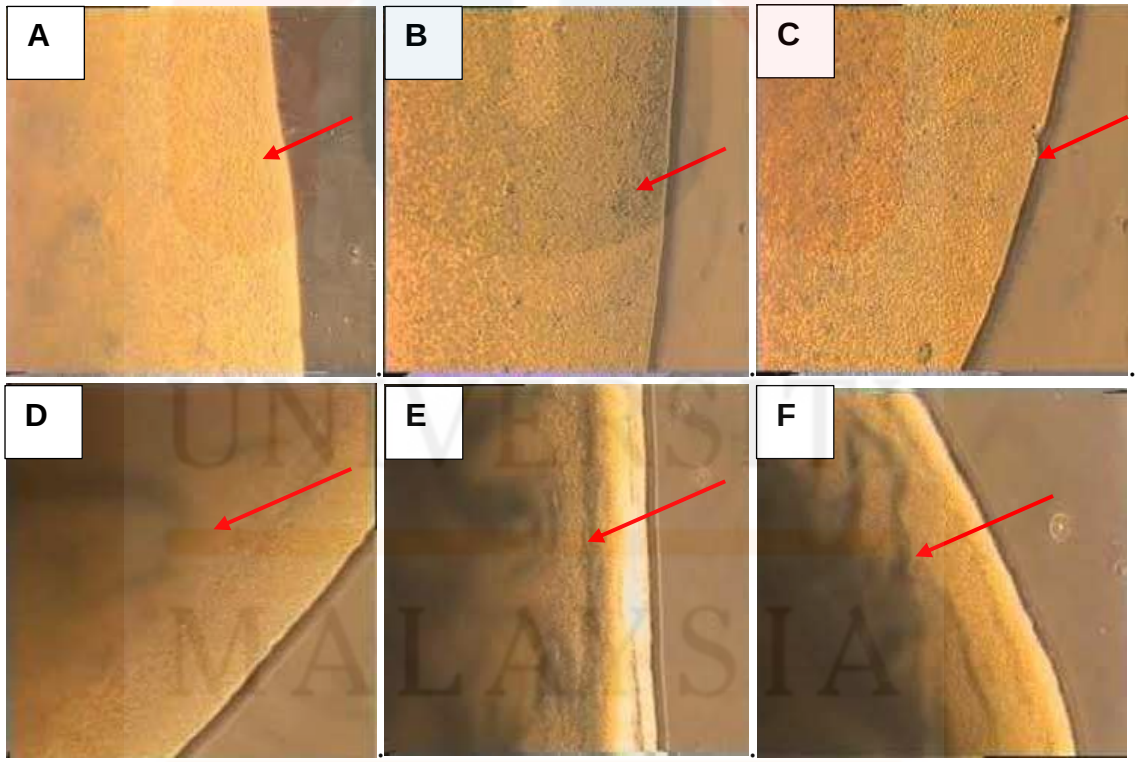


**Figure 4.9:** Mass motility rating system for ejaculated ram sperm A)Score 0; B)Score 1; C)Score 2; D)Score 3; E)Score 4 and F)Score 5. Arrow indicate the movement of the sperm

Source: David (2015)

The present of cryoprotective agents (CPA's) in extender acts as buffers and antibiotics that can help to protect the cell from osmosis properties that caused by rapid dehydration of cells during cryopreservation. Glycerol also act as protection for sperms as the temperature decreases. As observed from current study that low concentration of glycerol caused poor protection for spermatozoa against cryoinjury (Silva, A. R., Cardoso, R.C.S., Uchoa, C.Silva, 2002). The addition of egg yolk can also protect the sperm from cooling damage and help to repair the membrane cell because egg yolk contains low density of lipoprotein. Some components in DPPG can also inhibit the growth of the microbial in the semen extender and improved the semen preservability through reduction of bacteria growth. Thus, the addition 6% concentration of DPPG can give positive impact to the sperm. The result showed addition 6% DPPG concentration increased the percentage in sperm quality. In this study also proved that enriching with 6% concentration can also give beneficial to the sperm cells both in chilled and frozen semen. The result showed that 6% of DPPG was the most effective concentration of all sperm parameters compared to the control. Based from previous literature on DPPG, concentration that used in this study were lower than the concentration carried out from previous studies which is 20 mg of DPPG. However, the result still showed that the addition 6% of 20 mg of DPPG can improve sperm's survivability in chilled and frozen semen compared to the control group after seven day of chilling and frozen.

The finding of this study uses low DPPG concentration as extenders when compared to others studies. Despite using the same freezing protocol, different DPPG concentration as extender and various performance at experimental animals can produce dissimilar results when compared to previous literatures. Previous study also indicated juice date palm is an excellent materials for producing refined sugar which suitable for

extender based (Assirey, 2015). However, the current study demonstrated that the used of powdered date palm did not negatively affect sperms but instead provide other positive impact to the sperm. Powdered date palm can be easily stored and readily sent to regions where fresh DPPG were limited (Hafez & Hafez, 2000).

# CHAPTER 5

# CONCLUSION

## 5.1 Conclusion

This study conclude that the addition of DPPG can improved the sperm performance in the cryopreservation process compared without adding DPPG in the extender. The best concentration DPPG in this study is at 6% concentration. This shows that 6% concentration of DPPG had a beneficial effect on the chilled and frozen of Brahman bull semen. Even though all the sperm parameters in chilling did not show any significant different ($P>0.05$) after seven days of storage but the concentration of 6% DPPG had the highest mean value compared to the others treatment groups in chilled and frozen. Next, this study showed that the quality of sperm decreased after freezing in all groups. However, the viability of frozen semen in 6% concentration of DPPG is significantly higher ($p<0.05$) compared to control group.

The overall result shows that the addition of 20 mg in 6% concentration of DPPG in the extender give positive impact in sperm viability. In contrast with this study, the addition of 20 mg was not enough effective to protect the sperm motility. The finding of this study is differ from the previous study which is the best concentration to protect the sperm motility during cryopreservation is at 150 mg to 250 (El-Sisy et al., 2016; El-

sheshtawy et al., 2014). It can be assumed that, despite using the same protocol of freezing, different DPPG concentration as extender used in this study and variability performance at experimental animals might also generate dissimilar results when compared to previous literatures. Besides, the addition of DPPG in extender did no shows a huge effect on the sperm membrane integrity. It can be seen the concentration of DPPG in semen extender give same improvement in chilled and frozen semen. Thus, 6% concentration of DPPG can provide more beneficial impacts compared to other treatment groups in semen qualities.

## 5.2 Recommendation

To further improve the work, more parameters on the diluted semen with date palm pollen grain (DPPG) extender are required to justify which concentration gives the best result to the sperm quality such as acrosome integrity. Besides, sperm quality should be observed daily to determine the duration of time that would affect toxicity.

Next, the number of the sample in the study should be increase for a better analysis. Due to time constrain, the present study managed to obtain sufficient data for analysis.

Other than that, further study on the use of DPPG in animal extender should be done and provide the scientifically detailed information about the most effective of DPPG concentration on the sperm quality.

# REFERENCES


Abdolreza Rastegarnia, Abdolhossein Shahverdi, Tohid Rezaei Topraggaleh, Bita Ebrahimi, V. S. (2013). Effect of Different Thawing Rates on Post-Thaw Viability, Kinematic Parameters and Chromatin Structure of Buffalo (Bubalus bubalis) Spermatozoa. *Cell Journal*, *14*(4), 306–313.

Abed El-Azim, M. H. M., Yassin, F. A., Khalil, S. A., Amani, A., & El-Mesalamy, M. D. (2015). Hydrocarbons, fatty acids and biological activity of date palm pollen (phoenix dactylifera L.) growing in Egypt. *IOSR Journal of Pharmacy and Biological Sciences*, *10*(3), 46–51. https://doi.org/10.9790/3008-10314651

Aboagla, E. M., & Terada, T. (2004). Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology*, *62*, 1160–1172. https://doi.org/10.1016/j.theriogenology.2004.01.013

Agarwal, A., Ong, C., & Durairajanayagam, D. (2014). Contemporary and future insights into fertility preservation in male cancer patients. *Translational Andrology and Urology*, *3*(13), 27–40. https://doi.org/10.3978/j.issn.2223-4683.2014.02.06

Al-Daraji, H. J. (2014). Impact of extender supplementation with tomato juice on semen quality of chicken semen during liquid storage. *International Journal of Biological Science and Applications*, *1*(1), 19–23.

Anjum, F. (2012). Phytochemical characteristics of Date Palm (Phoenix dactylifera) fruit extracts. *Pakistan Journal Food science*, *22*(3), 117-127.

Aponte, P. M., Rooij, D. G. De, & Bastidas, P. (2005). Testicular development in Brahman bulls. *Theriogenology*, *64*, 1440–1455.https://doi.org/10.1016/j.theriogenology.2005.03.016

Assirey, E. A. (2015). Nutritional composition of fruit of 10 date palm (Phoenix dactylifera L.) cultivars grown in Saudi Arabia. *Journal of Taibah University for Science*, *9*(1), 75-79.

Auger, J. (2010). Assessing human sperm morphology: Top models, underdo or bio metrics? *Asian Journal of Andrology*, *12*(1), 36–46. https://doi.org/10.1038/aja.2009.8

Bahmanpour, S., Talaei-khozani, T., Vojdani, Z., & Poostpasand, A. (2006). Effect of Phoenix Dactylifera Pollen on Sperm Parameters and Reproductive system of Adult Male Rats. Iranian Journal of Medical Sciences, 31, 208-212.

Bailey, J. L., Bilodeau, J. F., & Cormier, N. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *Journal of Andrology*, *21*(1), 1,7. https://doi.org/10.1002/j.1939-4640.2000.tb03268.x

Bansal, A. K., & Bilaspuri, G. S. (2011). Review Article: Impacts of oxidative stress and antioxidants on semen functions. *Veterinary Medicine International*, 1–7. https://doi.org/10.4061/2011/686137

Baumber, J., Ball, B. A., Linfor, J. J., & Meyers, S. A. (2003). Reactive oxygen species and cryopreservation DNA fragmentaion in equine spermatozoa. *Journal of Andrology*, *24*(4), 621–628.

Baust, J. G., Gao, D., & Baust, J. M. (2009). Cryopreservation: An emerging paradigm change. *Organogenesis*, *5*(3), 90–96.

Beccaglia, M., Anastasi, P., Chigioni, S., Luvoni, G.C. (2009). Tris-lecithin extender supplemented with antioxidant catalase for chilling of canine semen. *Reprod. Domestic Anim*. 44 (sup.2), 345-349. https://doi.org/10.1111/j.1439-0531.2009.01410.x

Bishr, M., Desoukey, S. Y., & Palm, D. (2012). Comparative Study of the Nutritional Value of Four Types of Egyptian Palm Pollens, *2*, 50–56.

Bopape, M. A., Lehloenya, K. C., Chokoe, T. C., & Nedambale, T. L. (2015). Comparison of Electro Ejaculator and Artificial Vagina on Semen Collection from South African Indigenous Goat Following Assessment by Computer Aided Sperm Analysis. *Open Journal of Animal Sciences*, *5*, 210–218. https://doi.org/http://dx.doi.org/10.4236/ojas.2015.52023

Brito LFC. 2007. Evaluation of stallion sperm morphology. Clin Tech Eq Pract 6: 249–64.

Cardoso, R. D. C. S., Silva, A. R., Uchoa, D. C., & Da Silva, L. D. M. (2003). Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. *Theriogenology*, *59*(3–4), 743–751. https://doi.org/10.1016/S0093-691X(02)01151-2

Carla, E., Celeghini, C., Paes, R., Arruda, D., Andrade, C. De, Nascimento, J., Rodrigues, M. (2008). Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin, *104*, 119–131. https://doi.org/10.1016/j.anireprosci.2007.02.001

Chauhan, M. S., & Anand, S. R. (1990). Effect of egg yolk lipids on the freezing of goat semen. *Theriogenology*, *34*(5), 1003–1013. https://doi.org/10.1016/0093-691X(90)90568-E

Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Reviews of Reproduction*, *5*, 46–52. https://doi.org/DOI: 10.1530/ror.0.0050046

David, I., Kohnke, P., Lagriffoul, G., Praud, O., Plouarboué, F., Degond, P., & Druart, X.(2015). Mass sperm motility is associated with fertility in sheep. *Animal Repro duction Science*, *161*, 75–81. https://doi.org/10.1016/j.anireprosci.2015.08.006

Defoin, L., Granados, A., & Donnay, I. (2008). Analysing Motility Parameters on Fresh Bull Semen Could Help to Predict Resistance to Freezing : A Preliminary Study, *611*, 606–611. https://doi.org/10.1111/j.1439-0531.2007.00964.x

Devasagayam, T. P. A., Tilak, J., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., & Lele, R. D. (2004). Free radicals and antioxidants in human health: current status and future prospects. *J Assoc Physicians India*, *52*, 794–804. https://doi.org/10.1016/S0300-483X(03)00149-5

El-Kashlan, A. M., M. M. Noh, W. A. Hassan & S. M. Rizk. (2015). Therapeutic potential of date palm pollen for testicular dysfunction induced by thyroid disorders in male rats. *Plus One.* http://sci-hub.tw/https://doi.org/10.1371/journal.pone.0139493

El-sheshtawy, R. I., El-nattat, W. S., Sabra, H. A., & Ali, A. H. (2014). The Effect of Phoenix dactylifera Pollen Grains Tris-Infusion on Semen Preservability of Local Bull Breeds. *Global Veterinaria*, *13*(5), 728–732. https://doi.org/10.5829/idosi. wasj.2014.32.10.911

El-sheshtawy, R. I., El-sisy, G. A., & El-nattat, W. S. (2016). Effects of pomegranate juice in Tris based extender on cattle semen quality after chilling and cryopreservation. *Asian Pacific Journal of Reproduction*, *5*(4), 335–339. https://doi.org/10.1016/j.apjr.2016.06.001

El-Sisy, G. A., El-Badry, D. A., El-Sheshtawy, R. I., & El-Nattat, W. S. (2018). Effects of Phoenix dactylifera pollen grains extract supplementation on post-thaw quality of arabian stallion semen. *Bulgarian Journal of Veterinary Medicine*, 40–49. https://doi.org/10.15547/bjvm.1044

Evans, J. (2011). *Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice*. (D. K. Gardner & T. Falcone, Eds.), *The Obstetrician & Gynaecologist* (1st ed., Vol. 13). New York: Cambridge University Press. https://doi.org/10.1576/toag.13.4.278.27697

Faleh, B. H & A. A. Sawad, 2006. Effect of palm pollen grains extracts (Phoenix dactylifera L) on spermatogenic activity of male rabbits. Basrah Journal For Date Palm Research, 5, 1-10

Ferrusola, C. O., & Gonza, L. (2009). Review Article Mitochondria in Mammalian Sperm Physiology and Pathology : A Review Role of Mitochondria in Sperm Physiology Role of Mitochondria in Sperm Pathology, *349*, 345–349. https://doi.org/10.1111/j.1439-0531.2008.01211.x

Fingerova, H., Oborna, I., Novotny, J., Svobodova, M., Brezinova, J., & Radova, L. (2009). The measurement of reactive oxygen species in human neat semen and in suspended spermatozoa: A comparison. *Reproductive Biology and Endocrinology*, *7*, 1–6. https://doi.org/10.1186/1477-7827-7-118

Flesh, F., & Gadella, B. (2000.). Dynamics of the mammalian sperm membrane in the process of fertilization. *Biochim. Biophys. Acta*, *1469*, 197-235.

Foote, R. H. (2002). The history of artificial insemination: Selected notes and notables. *Journal of Animal Science*, 80(2), 1–10. https://doi.org/10.2527/animalsci2002.80E-Suppl_21a

Futino, D., Mendes, M., Matos, W., Mondadori, R., Lucci, C. (2010). Glycerol, methyl-formamide and dimethyl-formamide in canin semen cryoprservation. Reprod. Domest. Anim. 45, 214-220. https://doi.org/10.1111/j.1439-0531.2008.01208.x

Gangwar, C., S. D. Kharche, R. Ranjan, S. Kumar, A. K. Goel, S.K. Jindal, S. K. A. (2015). Effect of Vitamin C Supplementation on Freezability of Barbari Buck Semen. *Small Ruminant Research*, *129*, 104–107. https://doi.org/10.1016/j.smallrumres.2015.06.002

Ghnimi, S., Umer, S., Karim, A., & Kamal-Eldin, A. (2017). Date fruit (Phoenix dactylifera L.): An underutilized food seeking industrial valorization. *NFS Journal*, *6*, 1–10. https://doi.org/10.1016/j.nfs.2016.12.001

Gupta, K. R and Singh, K. V. (2012). Artificial Insemination : Boon for Indian Dairy Industry. *Double Helix Research*, *5*, 49–51.

Hafez, B., & Hafez, E. S. (2000). Reproduction Behavior. In *Reproduction in Farm Animals* .7 ed. New york: Lippincott Williams & Wilkens, pp: 293-306.

Hayashi, Y. (2005). Characteristics of Cryopreserved Spermatozoa from a Holstein-Friesian Bull Thawed at Different Temperature. *International Development and Cooperation*, *12*(1), 107–110.

Hayyan, M., Hashim, M. A., & Alnashef, I. M. (2016). Superoxide Ion: Generation and Chemical Implications. *Chemical Reviews*, *116*(5), 3029–3085. https://doi.org/10.1021/acs.chemrev.5b00407

Hassan, H. M. M., 2011. Chemical composition and nutritional value of palm pollen grains. Global Journal of Biotechnology and Biochemistry, 6, 1-7.

Hezavehei, M., Sharafi, M., Kouchesfahani, H. M., Henkel, R., Agarwal, A., Esmaeili, V., & Shahverdi, A. (2018). Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive BioMedicine Online*, *0*(0), 1–13. https://doi.org/10.1016/j.rbmo.2018.05.012

H. J. Al-Daraji. (2014). Impact of extender supplementation with tomato juice on semen quality of chicken semen during liquid storage. *International Journal of Biological Sciences and Applications*, *1*(1), 19-23.

Holt, W. V. (2000). Basic aspects of frozen storage of semen. *Animal Reproduction Science*, *62*(1-3), 3-22.

Holtz, W., B. Sohnrey, M. Gerland and M.A. Driancourt, 2008. Ovsynch synchronization fixed-time insemination in goats. Theriogenology, 69(7): 785-792.

Homa, S. T., Vessey, W., Perez-Miranda, A., Riyait, T., & Agarwal, A. (2015). Reactive Oxygen Species (ROS) in human semen: determination of a reference range. *Journal of Assisted Reproduction and Genetics*, *32*(5), 757–764. https://doi.org/10.1007/s10815-015-0454-x

Hoon, T., Choel, S., Hyun, J., Yoon, J., Hong, J., Seo, U., Han, J. (2017). Cryopreservation and its clinical applications. *Integrative Medicine Research*, *6*(1), 12–18. https://doi.org/10.1016/j.imr.2016.12.001

IAEA, Improving artificial breeding of cattle in Africa guidelines and recommendations (2005): VIENNA, Pp: 10-19

Januskauskas, A., Johannisson, A., & Rodriguez-martinez, H. (2003). Subtle membrane changes in cryopreserved bull semen in relation with sperm viability , chromatin structure , and field fertility, *60*, 743–758. https://doi.org/10.1016/S0093-691X(03)00050-5

Januskauskas, A., Lukoseviciute, K., & Nagy, S. (2005). Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation, *63*, 160–178. https://doi.org/10.1016/j.theriogenology.2004.04.002

Kamel, R. M. (2013). Assisted reproductive technology after the birth of Louise Brown. *Journal of Reproduction and Infertility*, *3*(3), 1–9. https://doi.org/10.4172/2161-0932.1000156

Kim, S. H., Yu, D. H., & Kim, Y. J. (2010). Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. *Theriogenology*, *73*(3), 282–292. https://doi.org/10.1016/j.theriogenology.2009.09.011

Kondracki, S., Wysokińska, A., Kania, M., & Górski, K. (2017). Application of two staining methods for sperm morphometric evaluation in domestic pigs. *J Vet Res*, *61*, 345–349. https://doi.org/10.1515/jvetres-2017-0045

Kopeika, J., Thornhill, A., & Khalaf, Y. (2014). The effect of cryopreservation on the genome of gametes and embryos : principles of cryobiology and critical appraisal of the evidence, *0*(0), 1–19. https://doi.org/10.1093/humupd/dmu063

Leboeuf, B., Restall, B., & Salamon, S. (2000). Production and storage of goat semen for artificial insemination. *Animal Reproduction Science*, *62*(1–3), 113–141. https://doi.org/10.1016/S0378-4320(00)00156-1

Len, J. A. (2008). *Effects of Centrifugation on Equine Spermatozoa Immediately and*. University of Guadalajara.

Lobo, M. G., & Yahia, E. (2014). *Biology and postharvest physiology of pineapple*. *Handbook of Pineapple Technology: Postharvest Science, Processing and Nutrition*. https://doi.org/10.1002/9781118967355.ch3

Loomis, P. (14 November, 2017). *The Major Causes of Damage to Sperm During Freezing*. Retrieved from Select Breeders Service: http://info.selectbreeders.com/blog/the-major-causes-of-damage-to-sperm-during-freezing-water-and-salts-and-ice-oh-my

Loomis, P. R. (2006). Advanced methods for handling and preparation of stallion semen. Veterinary Clinics of North America: Equine Practice, 22, 663–676. https://doi.org/10.1016/j.cveq.2006.07.002

Loomis P.R. Squires EL. (2005). Frozen semen management in equine breeding programs.*Theriogenology*,v.64,480-491.https://doi.org/10.1016/j.jevs.2009.04.184

Lushchak, V. I. (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions*, *224*(October), 164–175.

Lyashenko, A. (2015). Effect of different thawing procedures on the quality and fertility of the bull spermatozoa. *Asian Pacific Journal of Reproduction*, *4*(1), 17–21. https://doi.org/10.1016/S2305-0500(14)60051-8

Malik, A., Irwan Zakir, M., & Syarif Djaya, M. (2016). Effects of Addition of Juice Date Palm to the Extender on the Semen Qualities of Frozen Thawed in Bull Spermatozoa. *Global Veterinaria*, *16*(1), 100–104. https://doi.org/10.5829/idosi.gv.2016.16.01.10274

Meyers, S. A. (2005). Spermatozoal response to osmotic stress. Animal Reprod Sci, *89*, 57–64. https://doi.org/10.1016/j.anireprosci.2005.06.026

Michael, A. J., Alexopoulos, C., Pontiki, E. A., & Hadjipavlou-litina, D. J. (2008). Quality and reactive oxygen species of extended canine semen after vitamin C supplementation. *Theriogenology*, *70*, 827–835. https://doi.org/10.1016/j.theriogenology.2008.05.043

Mishra, B., Alam, M., Khandokar, M., Mazumder, S., & Munsi, M. (2010). Qualities of goat semen in Tris-Citrate-Glucose extender containing glutathione. *Bangladesh Veterinarian*, *27*(2), 46–55. https://doi.org/10.3329/bvet.v27i2.7554

Mohammed, A. (2018). Artificial Insemination and its Economical Significancy in Dairy Cattle : Review. *International Journal of Research Studeis in Microbiology and Biotechnology*, *4*(1), 30–43.

Mansouri, A., Embarek, G., Kokkalou, E., & Kefalas, P. (2005). Food Chemistry Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Phoenix dactylifera). *Food Chemistry*, *89*, 411–420. https://doi.org/10.1016/j.foodchem.2004.02.051

Naing, S. W., Wahid, H., Mohd Azam, K., Rosnina, Y., Zuki, A. B., Kazhal, S., San, M. M. (2010). Effect of sugars on characteristics of Boer goat semen after cryopreservation. *Animal Reproduction Science*, *122*(1–2), 23–28. https://doi.org/10.1016/j.anireprosci.2010.06.006

Nijs, M., Creemers, E., Cox, A., Janssen, M., Vanheusden, E., Castro-Sanchez, Y., Ombelet, W. (2009). Influence of freeze-thawing on hyaluronic acid binding of human spermatozoa. *Reproductive BioMedicine Online*, *19*(2), 202–206. https://doi.org/10.1016/S1472-6483(10)60073-9

Ozkavukcu, S., Erdemli, E., Isik, A., Oztuna, D., & Karahuseyinoglu, S. (2008). Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. *Journal of Assisted Reproduction and Genetics*, *25*(8), 403–411. https://doi.org/10.1007/s10815-008-9232-3

Pedersen, H., & Lebech, P. E. (1971). Ultrastructural changes in the human spermatozoon after freezing for artificial insemination. *Fertility and Sterility*, *22*(2), 125–133. https://doi.org/10.1016/S0015-0282(16)38048-7

Pesch, S., & Bergmann, M. (2006). Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron*, *37*(7), 597–612. https://doi.org/10.1016/j.micron.2006.02.006

Polge, C., Smith, A. U., & Parkes, A. S. (1949). *Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures*. *Nature* (Vol. 164). Nature Publishing Group. https://doi.org/10.1038/164666a0

Purdy, P. H. (2006). A review on goat sperm cryopreservation. *Small Ruminant Research*, *63*(3), 215-225.

Rasekh, A., H. K. Jashni, K. Rahmanian & A. S. Jahromi. (2015). Effect of Palm Pollen on Sperm Parameters of Infertile Man. *Pakistan Journal of Biological Sciences*, 18: 196-199. 10.3923/pjbs.2015.196.199

Rauch, A. (2013). Cryopreservation of bovine semen in egg yolk based extenders (Master's thesis, University of Saskatchewan Saskatoon). Retrieved from https://pdfs.semanticscholar.org/77c5/e9f88ad5e237e1c7b926f63e0e89a3d8f562.pdf

R.Jothipriya*, S.Sasikumar, EK.Madhankumar, A. P. and S. K., & Nova. (2014). A Study if Hypo Osmotic Swelling Test un Human Spermatozoa. *International Journal of Current Research and Academic Review*, *2*(12), 47–63.

Royere, D., Barthelemy, C., Hamamah, S., & Lansac, J. (1996). Cryopreservation of spermatozoa : a 1996 review, *2*(6), 553–559.

Saadat Parhizkar, S. B. (2014). Testicular morphology of male rats exposed to mscrocarpa (Mahkota dewa) aqueous extract. *Iranian Journal of Basic Medical Sciences*, *7*(5), 384-389.

Saleh, R. a, Agarwal, a, Kandirali, E., Sharma, R. K., Thomas, a J., Nada, E. A, Alvarez, J. G. (2002). Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertility and Sterility*, *78*(6), 1215–1224. https://doi.org/10.1016/S0015-0282(02)04237-1

Salvador, I., Yaniiz, J., Viudes-de-Castro, M. P., Gomez, E. A., & Silvestre, M. A. (2006). Effect of solid storage on caprine semen conservation at 5 degree. *Theriogenology*, *66*(4), 974–981. https://doi.org/10.1016/j.theriogenology.2006.02.042

Sansone, G., M. J. F. Nastri & A. Fabbrocini, 2000. Storage of buffalo (Bubalus bubalis) semen. *Animal Reproduction Science*, 62, 55-76. https://doi.org/10.1016/S0378-4320(00)00154-8

Santo, M. Di, Tarozzi, N., Nadalini, M., & Borini, A. (2012). Human Sperm Cryopreservation : Update on Techniques , Effect on DNA Integrity , and Implications for ART. *Adv Urol*, *2012*, 1–12. https://doi.org/10.1155/2012/854837

Sarıözkan, S., Numan, M., Barbaros, P., Ulutas, P. A., & Bilgen, A. (2009). Cryobiology The influence of cysteine and taurine on microscopic – oxidative stress parameters and fertilizing ability of bull semen following cryopreservation q. *Cryobiology*, *58*, 134–138. https://doi.org/10.1016/j.cryobiol.2008.11.006

Sánchez-Calabuig, M. J., Maillo, V., Beltrán-Breña, P., de la Fuente Martínez, J., Galera-Carrillo, S., Pérez-Gutiérrez, J. F., & Pérez-Cerezales, S. (2017). Cryopreservation of canine sperm using egg yolk and soy bean based extenders. *Reproductive Biology*, *17*(3), 233–238. https://doi.org/10.1016/j.repbio.2017.05.007

Schleh, C., & Leoni, A. (2013). How to optimize the benefits of computer assisted sperm analysis in experimental toxicology. *Journal of Occupational Medicien and Toxicology*, *8*(6), 6–9. https://doi.org/10.1186/1745-6673-8-6

Silva, A. R., Cardoso, R.C.S., Uchoa, C.Silva, L.D.M., (2002). Effect of Tris-Buffer , Egg yolk and glycerol on canine semen freezing. *The Veterinary Journal*, *164*, 244–246. https://doi.org/10.1053/tvjl.2002.0704

Sola Agboola, O. (2003). Nutritional Composition of the Fruit of the Nigerian Wild Date Palm, Phoenix dactylifera. *World Journal of Dairy & Food Sciences*, *2*, 196-200.

Tadesse, B. (2008). Calf Sex Ratios in Artificially Inseminated and Natural Mated Female Crossbred Dairy Herd. In: Proceedings of the 13th annual Conference of the Ethiopian Society of Animal Production. Addis Ababa, Ethiopia. Pp. 227

Tarig, A.A., Wahid, H., Rosnina, Y., Yimer, N., Goh, Y.M., Baiee, F.H., Khumran, A.M., Salman, H., Ebrahimi, M. (2017). Effect of Different Concentrations of Egg Yolk and Virgin Coconut Oil in Tris-based Extenders on Chilled and Frozen-thawed Bull Semen. *Animal Reproduction Science*, 182, 21-27. http://dx.doi.org/10.1016/j.anireprosci.2017.03.024

Temesgen T., Tibebu, M., Usman, L. (2017). Review on Status and Constraints of Artificial Insemination in Dairy Cattle in Developing Countries : The Case of Ethiopia. *Journal of Biology, Agriculture and Healthcare*, *7*(5), 79–87.

Valergakis, G. E., Arsenos, G., & Banos, G. (2007). Comparison of artificial insemination and natural service cost effectiveness in dairy cattle. *Animal*, *1*(2), 293–300. https://doi.org/10.1017/S1751731107340044

Verstegen, J. M.-O. (2002). Computer assisted semen analyzers in andrology. *Theriogenology*, *57*, 149-179

Vessey, W., Perez-Miranda, A., Macfarquhar, R., Agarwal, A., & Homa, S. (2014). Reactive oxygen species in human semen: Validation and qualification of a chemiluminescence assay. *Fertility and Sterility*, *102*(6), 1576–1583. https://doi.org/10.1016/j.fertnstert.2014.09.009

Vidal, A. H., Batista, A. M., da Silva, E. C. B., Gomes, W. A., Pelinca, M. A., Silva, S. V., & Guerra, M. M. P. (2013). Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. *Small Ruminant Research*, *109*(1), 47–51. https://doi.org/10.1016/j.smallrumres.2012.07.022

Vijayaraghavan, S., 2003. Sperm motility: patterns and regulation. In: *Introduction to Mammalian Reproduction*, ed D. Tulsiani, Kluwer Academic Publishers, Boston, USA. pp. 79-91.

Vishwanath, A., & Goldhaber, G. M. (2003). An Examination of the Factors Contributing to Adoption Decisions among Late-Diffused Technology Products. *New Media & Society*, *5*(4), 547–572. https://doi.org/10.1177/146144480354005

Walters, E. M. (2009). The history of sperm cryopreservation. *Cambridge University Press*, *1*, 1-10.

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, (60–61), 481–492.

Williams, A. C., & Ford, W. C. L. (2005). Relationship between reactive oxygen species production and lipid peroxidation in human sperm suspensions and their association with sperm function. *Fertility and Sterility*, *83*(4), 929–936. https://doi.org/10.1016/j.fertnstert.2004.11.031

Williams, M. A. (2001). Technical note : Artificial vagina vs a vaginal collection vial for collecting semen from rams. *J.Anim Sci*, 2964-2967.

Yodmingkwan, P., Guntaprom, S., Jaksamrit, J., & Lertchunhakiat, K. (2016). Effects of Extenders on Fresh and Freezing Semen of Boer Goat. *Agriculture and Agricultural Science Procedia*, *11*, 125–130. https://doi.org/10.1016/j.aaspro.2016.12.021

Zubair, M., Lodhi, L. A., & Ahmad, E. (2013). Hypo osmotic swelling test as screening for evaluation of semen of bull. *Journal of Entomology and Zoology Studies*, *1*(6), 124–128.

# APPENDIX

Media recipe

Table A.1: Control extender recipe

| Components | Amount |
|---|---|
| Tris | 3.028 g |
| Citric Acid | 1.675 g |
| Fructose | 1.250 g |
| Penomycin | 0.1 g |
| Glycerol | 0.72 ml |
| Egg Yolk | 20 ml |

Table A.2: Stock solution of DPPG

| Components | Amount |
|---|---|
| Tris | 1.514 g |
| Citric Acid | 0.8375 g |
| Fructose | 0.625 g |
| Penomycin | 0.05 g |
| Tris buffer (TCFY) | 50 ml |
| DPPG | 0.02 g |

Table A.3: HOST Solution

| Components | Amount |
|---|---|
| Fructose | 1.8 g |
| Sodium Citrate | 0.98 g |
| Distilled Water | 200ml |

Table A.4: The preparation of treatment group which Semen:Extender represent (1:12)

| Ingredients | Control | G1 | G2 | G3 |
|---|---|---|---|---|
| TCFY | 10.28 ml | 9.80 ml | 9.32 ml | 8.84 ml |
| TPG | 0 ml | 0.48 ml | 0.96 ml | 1.44 ml |
| Semen | 1 ml | 1 ml | 1 ml | 1 ml |
| Glycerol | 0.72 ml | 0.72 ml | 0.72 ml | 0.72 |
| Total extender | 12 ml | 12 ml | 12 ml | 12 ml |

Table A.5: The calculation of DPPG in percentage

| Treatment | Percentage in Extender | DPPG/Extender | Total DPPG (ml) |
|---|---|---|---|
| 2% of DPPG | $\frac{2}{100} X\ 12ml\ (Extender)$ = 0.24mg/ml | $\frac{0.24\ mg/ml}{0.5mg}$ | =0.48ml |
| 4% of DPPG | $\frac{4}{100} X\ 12ml\ (Extender)$ = 0.48mg/ml | $\frac{0.48\ mg/ml}{0.5mg}$ | =0.96ml |
| 6% of DPPG | $\frac{6}{100} X\ 12ml\ (Extender)$ = 0.72mg/ml | $\frac{0.72\ mg/ml}{0.5mg}$ | =1.44ml |

Preparation of Extender

**Figure A1:** Date palm pollen grain in powder form

**Figure A2:** Preparation of Tris buffer's ingredients

**Figure A3:** Filter the egg yolk

Semen Collection

**Figure A4:** Preparation of the semen collection's apparatus

**Figure A5:** Semen collection using electro ejaculation

**Figure A6:** Trimmed the penis feather

**Figure A7:** The collection of semen using rubber tube

Semen Processing And Freezing

**Figure A8:** The preparation of semen dilution in water bath (37°C)

**Figure A9:** The semen samples were stored in refrigerator (4°C)

**Figure A10:** The straws were placed on the liquid nitrogen vapour

**Figure A11:** The straws were stored in the liquid nitrogen tank up to seven days