



**UNIVERSITI PUTRA MALAYSIA**

***EFFECTS OF DIFFERENT CONCENTRATION OF  
DIMETHYLSULFOXIDE AND GLYCEROL ON MOTILITY, MORTALITY  
AND MORPHOLOGY OF CRYOPRESERVED BULL SEMEN***

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GLYCEROL ON MOTILITY, MORTALITY AND MORPHOLOGY OF  
CRYOPRESERVED BULL SEMEN

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It is hereby certified that we have read this project paper entitled “Effects Of Different Concentration Of Dimethyl Sulfoxide And Glycerol On Motility, Mortality And Morphology of Cryopreserved Bull Semen”, by Afiqah Binti Zainurin and in our opinion it is satisfactory in terms of scope, quality, and presentation, as partial fulfilment of the requirement for the course VPD 4901 – Project.

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## **DEDICATION**

This thesis is dedicated  
To My Teachers and My Parents  
For their profound gratitude

And

To My Fiance  
Amir Hamzah Bin Ahmad  
For his eternal love

## ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude and appreciation to Professor Dr. Abd. Wahid Haron, my supervisor for his invaluable guidance, daily basis instruction, continuous supervision, initiation, patience, encouragement and understanding throughout my study.

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## ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901- Projek.

### KESAN PERBEZAAN KEPEKATAN DIMETHYLSULFOXIDE DAN GLISEROL TERHADAP PERGERAKAN, KEMATIAN DAN MORFOLOGI AIR MANI LEMBU YANG DIKRIOAWET

oleh

Afiqah binti Zainurin

2015

Penyelia: Prof. Dr. Abd Wahid Haron

Krioawetan gamet dan embrio secara digunakan secara rutin dan meluas dalam mamalia . Krioawet sperma, oosit dan embrio digunakan untuk pernian beradas dan pemindahan embrio dalam industri ternakan. Oleh itu, keupayaan untuk meramal kualiti sperma selepas pencairan dan kesuburan dari rutin fungsi sperma akan sangat bermanfaat bagi kejayaan krioawetan. Tujuan kajian ini adalah untuk menilai kesan berbeza kepekatan dimetilsulfoksida (DMSO) dan gliserol pada pergerakan, kematian dan morfologi air mani lembu jantan yang dikrioawet. Dua sampel air mani dari dua ekor lembu jantan telah dikumpul dan dibekukan dalam pencair Tris kuning telur

dengan DMSO dan gliserol pada kepekatan yang berbeza-beza iaitu 5%, 10% dan 20%. Data yang diperolehi dibandingkan dengan kumpulan kawalan yang mengandungi 3% gliserol. Parameter kualiti air mani diuji selepas pencairan ialah peratusan pergerakan sperma progresif, peratusan keseluruhan pergerakan, peratus hidup dan peratus sperma bermorfologi tidak normal.. Keputusan menunjukkan gliserol pada kepekatan 5% mempunyai kesan yang paling besar terhadap kemandirian sperma manakala DMSO pada kepekatan 5% memberikan purata kemandirian sperma yang lebih rendah berbanding dengan purata kemandirian sperma kumpulan kawalan kedua-dua untuk hari 1 dan hari 7 selepas pencairan.Kepekatan lain menunjukkan penurunan dalam kemandirian bagi setiap kenaikan kepekatan. Tiada perbezaan yang signifikan pada peratusan purata morfologi tidak normal untuk kedua-dua gliserol dan DMSO ( $p > 0.05$ ). Kesimpulannya, 5% gliserol dalam pencair Tris-kuning telur menunjukkan kemandirian yang baik sebagai bahan krioawetan untuk air mani lembu dikrioawet.

Kata Kunci: Gliserol, DMSO, lembu, air mani, bahan krioawet

## **ABSTRACT**

Abstract from project paper for submission to Faculty of Veterinary Medicine in fulfilment of the requirements for the subject VPD 4901- Project.

### **EFFECTS OF DIFFERENT CONCENTRATION OF DIMETHYLSULFOXIDE AND GLYCEROL ON MOTILITY, MORTALITY AND MORPHOLOGY OF CRYOPRESERVED BULL SEMEN.**

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Cryopreservation of gametes and embryos are routinely and widely applied in the mammalian. Cryopreserved sperm, oocytes and embryos are used for artificial insemination and embryo transfer in the livestock industry. Hence, the ability to predict post-thaw sperm quality and fertility from a routine sperm function assay would be greatly beneficial to the success of cryopreservation. The aim of this study was to evaluate the effects of different concentration of dimethyl sulfoxide(DMSO) and glycerol on motility, mortality and morphology of cryopreserved bull semen. Semen samples from two bulls were collected twice from each bull and frozen in egg-yolk Tris extender with variable DMSO and glycerol concentrations of 5%, 10% and 20%. The result was compare with control group which consist of 3% glycerol. Semen quality parameters assisted after thawing were sperm percentage progressive, percentage total

motile, percentage live and percentage abnormal morphology of sperms. Results indicated that 5% glycerol had the highest effect on survivability of the sperm while 5% DMSO had lower survivability compared to the control group both for day 1 and day 7 post thawing. Other concentration shows decreasing in survivability as the concentrations increased. There is no significant different on mean percentage of abnormal morphology for both glycerol and DMSO ( $p>0.05$ ). In conclusion 5% glycerol in egg-yolk Tris showed better survivability of cryopreserved bull semen.

Keywords: Glycerol, DMSO, bull, semen, cryoprotectant

## CHAPTER 1

### GENERAL INTRODUCTION

Artificial insemination is widely used to produce the good and valuable genetics. It is the first animal biotechnology advancement that has made a large contribution to the genetic improvement, particularly in dairy bulls which single ejaculate from males can be used for inseminations of many females (Büyükleblebici *et al.*, 2014). One of the most important processes prior to artificial insemination is semen cryopreservation. It has been indicated that there are many benefits resulting from the process of semen cryopreservation such as increase breeding efficiency, increase ability to access superior genetics for a fraction of the price of buying a bull and extremely important for preservation of superior genes from valuable animals (Lemma, 2011). Cryoprotectant is the term to describe as any additive which can be provided to the cells before freezing and gives a higher post-thaw survival which cannot be obtained in its absence (B.Fuller, 2004).

According to Büyükleblebici *et al.* (2014), the successful of cryopreservation is not depend only on preserving the viability of the spermatozoa but also on maintaining their metabolic functions. Cryoprotactant was added to extender to protect the sperm from damage during freezing process. The amount and type of cryoprotectant also influence the viability of the spermatozoa. Therefore, the suitable cryoprotectant and extender are important factors for successful semen cryopreservation (Dorji *et al.*,

2014). A cryoprotectant concentration of about 5% to 15% is usually all that is required to permit survival of a substantial fraction of isolated cells after freezing and thawing from liquid nitrogen temperature (Jennifer, 2007). Jennifer(2007) also mentioned that the best and most commonly used cryoprotectants are a class of cryoprotectants called penetrating cryoprotectants which are small molecules that easily penetrate cell membranes and prevent excessive dehydration of cells during the freezing process.

The property of glycerol that can reduce the mechanical damage to spermatozoa during freezing process make it as most widely used cryoprotectant for bull semen. Conversely, according to Martins-Bessa *et al.*(2006), glycerol can induce alterations in the viscosity and organization of the sperm cytoplasm and in stability and permeability of the plasma membrane through disruption of protein and phospholipid structural organization.

Dimethyl sulfoxide (DMSO) was used in first human cryopreservation protocol to ease the freezing process. According to Gurtovenko & Anwar (2007), DMSO is observed to exhibit distinct modes of action in different concentration range which at low concentrations, DMSO induces membrane thinning and increases fluidity of the membrane's hydrophobic core and at higher concentrations, DMSO induces transient water pores into the membrane.

Therefore, the objectives of this study were:

1. To determine the effects of different concentration of DMSO and glycerol on survival rate of cryopreserved bull semen

2. To evaluate the mortality, motility and morphology of cryopreserved bull semen post thawing on day 1 and day 7 DMSO and glycerol as cryoprotectants.



## **CHAPTER II**

### **LITERATURE REVIEW**

#### **2.1 Introduction**

There has been an increased use of frozen semen in the past decade in breeding industry. This is because of the many advantages of utilizing frozen-thawed bull semen. Some of those advantages include preserving genetic material for the future and the ability to utilize the bull semen to produce pregnancies even after the death of the bull.

#### **2.2 Cryopreservation**

Cryopreservation is a process which maintains cell, whole tissue or any substance that susceptible to damage for an extended period of time at subzero temperature. The aim of cryopreservation is to minimize cell membrane damage associated with exposure to low temperature, regulate cell volume during the procedure and prevent lethal intracellular ice crystal formation. This can be achieved by controlling intracellular and extracellular movement of solutes and water.

Many factors contribute to success of cryopreservation which may include interactions between cryoprotectant, type of extender, cooling rate, thawing rate and packaging as well as the individual animal variation (Lemma, 2011).

## 2.3 Cryoprotectant

Cryoprotectant (CPA) is the functional derive term which describe any additive which can provide to cell before freezing and yields a higher post-thaw survival that can be obtained in its 'absence' (B.Fuller, 2004). The general properties required for cryoprotectants are that they have low molecular weight, are nontoxic and cheap. They also need to provide high levels of cell viability post thaw, and have no other influence on the cells.

According to Lemma (2011), cryoprotectant may be divided into either penetrating or non-penetrating depending on their action. Penetrating cryoprotectant such as glycerol, dimethyl sulfoxide (DMSO) and propylene glycol are able to penetrate the plasma membrane of the spermatozoa and act intracellularly as well as extracelullarly. Non-penetrating cryoprotectant such as lactose, mannose and protein only act extracelullarly.

### 2.3.1 Glycerol

The property of glycerol that can reduces the mechanical damage to spermatozoa during freezing process make it as most widely used cryoprotectant for bull semen. Conversely, according to Martins-Bessa *et al.*( 2006), glycerol can induce alterations in the viscosity and organization of the sperm cytoplasm and in stability and permeability of the plasma membrane through distrupction of protein and phospholipid structural organization.

### **2.3.2 Dimethyl sulfoxide ( DMSO)**

Dimethyl sulfoxide (DMSO) was used in first human cryopreservation protocol to ease the freezing process. According to Gurtovenko & Anwar (2007), DMSO is observed to exhibit three distinct modes of action in different concentration range which at low concentrations, DMSO induces membrane thinning and increases fluidity of the membrane's hydrophobic core and at higher concentrations, DMSO induces transient water pores into the membrane.

According to Tavassoli (2009), DMSO has been used as a cryoprotectant for cryopreservation of many species of protozoa and the optimum concentration to support the viability of that parasite was 10%. In this study, semen sample was collected to substitute with protozoa.

According to Rosato and Iaffaldano (2013), concentration of DMSO at 5% and 10% showed the least toxic effects and the use of 10% DMSO producing the best post-thaw sperm motility and membrane integrity after conventional freezing of rabbit semen. In this study, bull semen was used as the samples.

### **2.4 Mechanism of Cryoprotectant**

According to Swelum *et al.* (2011) the cell are exposed to a hyperosmotic environment during the addition of permeating cryoprotectant to a cell suspension. As water leaves through the plasma membrane, the cell will shrink. The cell will swell as

the cryoprotectant enter the cell and water reenter to maintain chemical potential. When the cryoprotectant was removed, cell will initially swell due to an influx of water and then slowly return to isosmotic volume as cryoprotectant and water leave the cell. The repeated changes in solution osmolarity can cause significant loss in functional integrity such as sperm motility and cell death without loss of plasma integrity.

## **2.5 Sperm Morphology**

Sperm morphology reflects the condition of seminiferous tubules and epididymis (de Lucio *et al.*, 2014). Sperm structure and morphology have a major impact on successful of fertilization rates, pregnancy rate, early embryonic development in artificial insemination. According to de Lucio *et al.*, (2014) sperm abnormalities has been classified according to the morphological region ( acrosome, head and tail) and origin (primary-testicular, secondary- epididymis and tertiary-accessory gland, or post ejaculation). The major sperm defects are double head, abnormal loose head, proximal droplet, abnormal mid piece, accessory tail and strongly foled while minor defect are detached acrosome, distal droplet, abaxial implantation, coiled tail with proximal droplet and normal loose head.

## **2.6 Semen Extender**

According to Dorji *et al.* (2014), the extender were freshly prepared before the dilution in which the Tris- egg yolk extender was prepared by dissolving 3.025g Tris buffer, 1.67 g citric acid and 1.25g fructose, 500IU of Penstrep in 50ml of distilled water

by stirring after which the volume was adjusted to 73 ml. In this experiment the volume was adjusted to 60ml.

An ideal extender should have nutrients as an energy source, substances that buffer against harmful changes of pH that provides a physiological osmotic pressure and concentration of electrolytes that prevents bacterial growth and protects the cells from cold shock during the freezing and thawing processes. (Concannon et al., 1989)

## **2.7 Staining**

A thin smear of thawed semen and Eosin and Nigrosin was prepared on a microscopic slide and the viability was assessed by counting 100 spermatozoa per slide under phase contrast microscope (x1000). Sperm that take up stain was considered to be non-viable while sperm that did not take up stain was considered as viable. The viability was assessed by counting 100 cells (per slide) under a microscope and percentage live spermatozoa was estimated by counting minimum 200 spermatozoa on the slide (Gohar.A *et.al.*, 2014). In this experiment the viability and percentage live was assessed by counting 200 spermatozoa.

Nigrosin-eosin stain is commonly used because it is effective, simple and, in addition to allowing sperm to be readily visualized, it is a so-called "live-dead" stain as it allowing one to assess membrane integrity at the same time as morphology. This

technique is assessed using standard bright- field microscopy and does not require negative phase contrast optics which is difficult to obtain (Bjorndahl, 2003).

## **2.8 Cooling**

According to Gohar.A *et.al.* (2014), in order to cool the semen from 37°C to 4°C gradually, the test tubes were placed to chiller (4°C) and were kept for 4 hours to achieve equilibrium. The cooling process is to prevent the semen to undergo sudden change of temperature which can cause damage in cellular integrity.

## **2.9 Freezing**

Semen was loaded into 0.5ml straw with the help of modified 1ml syringe and sealing machine. Then they are placed in a liquid nitrogen cooling tank to vapour cool to -85°C in 10 minutes and the straws were plunged into liquid nitrogen tank and stored after the temperature reached -85°C (Dorji *et al.*, 2014).

The frozen semen is stored in liquid nitrogen that is constantly monitored and replenished which the all the tanks are inspected daily and continuously monitored. This method is proven to provide the safest means of storage since the entire system is not dependent on electricity (Healthcare.utah.edu, 2015). Regardless of where the semen is stored, it is important to maintain adequate levels of liquid nitrogen in the tank to maintain the viability of sperm (Loomis and Squires, 2005).

### 3.0 Thawing

Thawing of frozen semen straw was carried out at 37°C for 30seconds in hot water bath (Gohar.A *et.al.*, 2014). In this experiment, thawing was done on day 1 and day7 post-cryopreservation. It is important to pre-freeze the forceps involved in taking the semen out of a liquid nitrogen tank and also to work below the frost line. When the sperm are exposed to temperatures above -100°C there is a chance of damage. One of the most causes of damaging sperm is during the process of removing samples from a liquid nitrogen tank. The safest way is to work below the frost line of the tank and make sure that the goblets are full of liquid nitrogen when the sperm are brought up into the neck of the tank before the straws was taken out for thawing (Loomis and Squires, 2005). Thawing is important to evaluate the viability of the sperm after cryopreservation and to compare it with the viability of the sperm before cryopreservation.

## **CHAPTER III**

### **MATERIALS AND METHODS**

#### **3.1 Animals**

Semen from two adult Fresian bulls, estimated to be more than two years old from Taman Pertanian Universiti, University Putra Malaysia was collected. The bulls were clinically healthy and free from any general and genital problems.

#### **3.2 Experimental Design and treatment**

The experiment was conducted with Completely Randomized Block Design. There are three groups which are one control group and two treatments group. Control groups consist 3% glycerol which the concentration is currently used to cryopreserved bull semen. Three ejaculations from each bull were collected and assigned to two treatments groups. Treatments 1 and Treatment 2 consist of extending the semen with Egg Yolk Tris added with different concentrations of glycerol (5%,10%,20%) and Egg yolk Tris added with different concentrations of DMSO(5%,10%,20%), respectively.

### **3.3 Semen Collection and evaluation**

Semen was collected from each bull using electro ejaculation method. Each bull was collected three times at different days. The collection tube containing the semen is transferred to a water bath maintained at 35°C. The volume, color and consistency were recorded. Sperm concentration, gross motility, progression and sperm motion was analyzed using Computer- Assisted Semen Analyzer (CASA). Semen was stain with Eosin-Nigrosin stain to assess sperm viability and morphology abnormality (Dorji *et al.*, 2014).

### **3.4 Preparation of Extender and semen dilution**

According to Dorji *et al.*, (2014), the extender were freshly prepared before the dilution in which the Tris- egg yolk extender was prepared by dissolving 3.025g Tris buffer, 1.67 g citric acid and 1.25g fructose, 500IU of Penstrep in 50ml of distilled water by stirring after which the volume was adjusted to 60 ml (Dorji *et al.*, 2014). Each ejaculates was divided into six equal aliquots and diluted using prepared Tris-Egg yolk extender with Glycerol (5%, 10%, 20%) or DMSO (5%, 10%, 20%).

### **3.5 Semen freezing and Thawing**

The extended semen was allowed to equilibrate for 2 hours in a refrigerator set at 4°C (Martins-Bessa *et al.*, 2006). The semen was loaded into 0.5ml straw with the help of modified 1ml syringe and sealing machine. Then they are placed in a liquid nitrogen cooling tank to vapour cool to -85°C in 10 minutes and the straws were plunged into

liquid nitrogen tank and stored after the temperature reached  $-85^{\circ}\text{C}$  (Dorji *et al.*, 2014). The straws were thaw at  $37^{\circ}\text{C}$  for 30-60seconds after storage of 24hours and 7days. The post thaw motility was evaluated using CASA and stained using Eosin-Nigrosin stain to assess sperm viability and morphology post-thaw.

### **3.6 Assessment of semen quality parameters**

The parameters evaluated were sperm concentration, motility, progressive movement, percentage live and morphology.

#### **3.6.1 Analysis of microscopic sperm variables**

Computer –Assisted Semen Analyzer (CASA) was used to analyze motion characteristic of the sperms. The thawed semen was put onto a pre-warmed chamber slide, and sperm motility characteristic were determined with 10x objective at  $37^{\circ}\text{C}$  (Büyükleblebici *et al.*, 2014). The motility values that being recorded were CASA motility (%), progressive motility (%), and percentage total motile. Sperm motility assessed directly after thawing for 24 hours and after 7 days.

For the assessment of morphology, the sample was stained using Eosin-Nigrosin stain. A drop of the mixture was place on a slide and cover with coverslip and was examine under oil immersion, 1000x. The live percentage was determined by counting a total 200 spermatozoa under light microscope. Sperm that take up stain was considered as dead sperm. Out of the dead sperm, the abnormal morphology was recorded.

### **3.7 Statistical analysis**

All data were analyzed using two-way analysis of variance (ANOVA) by using computerized statistic analysis (SPSS) 20.0 version. Differences were considered significant for  $p < 0.05$ .



## CHAPTER IV

### RESULTS

Table 1 shows the mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by concentration of cryoprotectant. Different concentration of both cryoprotectants did not significantly affect the semen concentration and percentage of abnormal morphology of the sperm ( $p>0.05$ ). However, there is significantly difference in CASA progressive total motile, CASA total motile sperm and percentage live of the sperm in different concentration of both cryoprotectants ( $p<0.05$ ).

**Table 1: Mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by concentration of cryoprotectant**

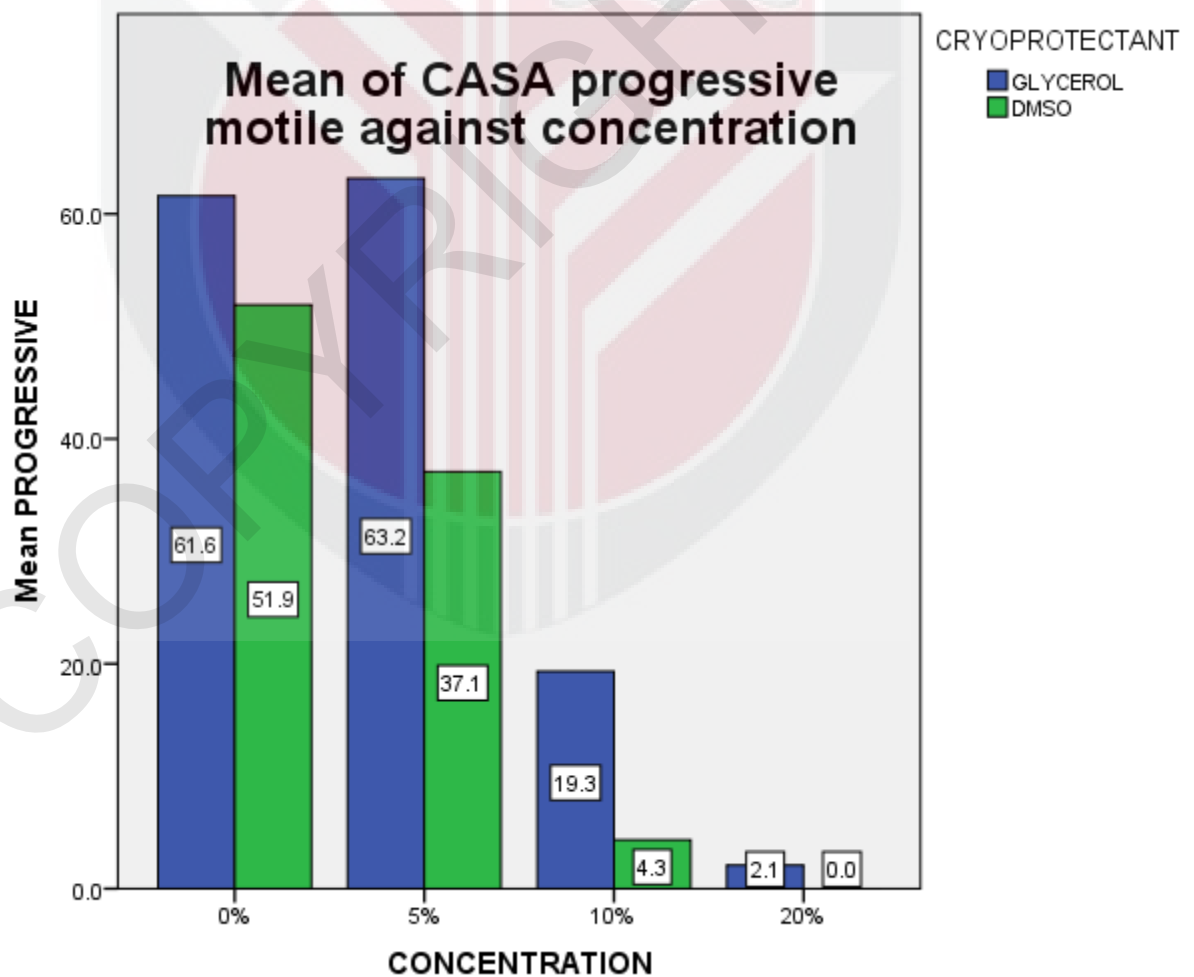
Conc. of cryoprotectant (%)	Semen conc.(%)	Progressive motile sperm (%)	Total motile (%)	% live	Abnormal morphology (%)
<b>Control</b>	109.3 $\pm$ 20.1	56.8 $\pm$ 5.2 <sup>a</sup>	42.0 $\pm$ 4.3 <sup>a</sup>	55.4 $\pm$ 3.4 <sup>a</sup>	2.9 $\pm$ 0.1
<b>5</b>	103.8 $\pm$ 18.6	50.1 $\pm$ 6.1 <sup>b</sup>	40.4 $\pm$ 5.1 <sup>b</sup>	49 $\pm$ 4.6 <sup>b</sup>	2.6 $\pm$ 0.2
<b>10</b>	69.1 $\pm$ 11.7	11.8 $\pm$ 3 <sup>c</sup>	11.8 $\pm$ 2.1 <sup>c</sup>	18.8 $\pm$ 2.3 <sup>c</sup>	2.8 $\pm$ 0.1
<b>20</b>	70.6 $\pm$ 12.6	1.0 $\pm$ 0.7 <sup>d</sup>	0.3 $\pm$ 0.2 <sup>d</sup>	4.4 $\pm$ 1.1 <sup>d</sup>	2.7 $\pm$ 0.1
<b>p</b>	NS	0.000	0.000	0.000	NS

a-d: Different superscripts within the same column demonstrate significant differences between groups ( $p<0.05$ )

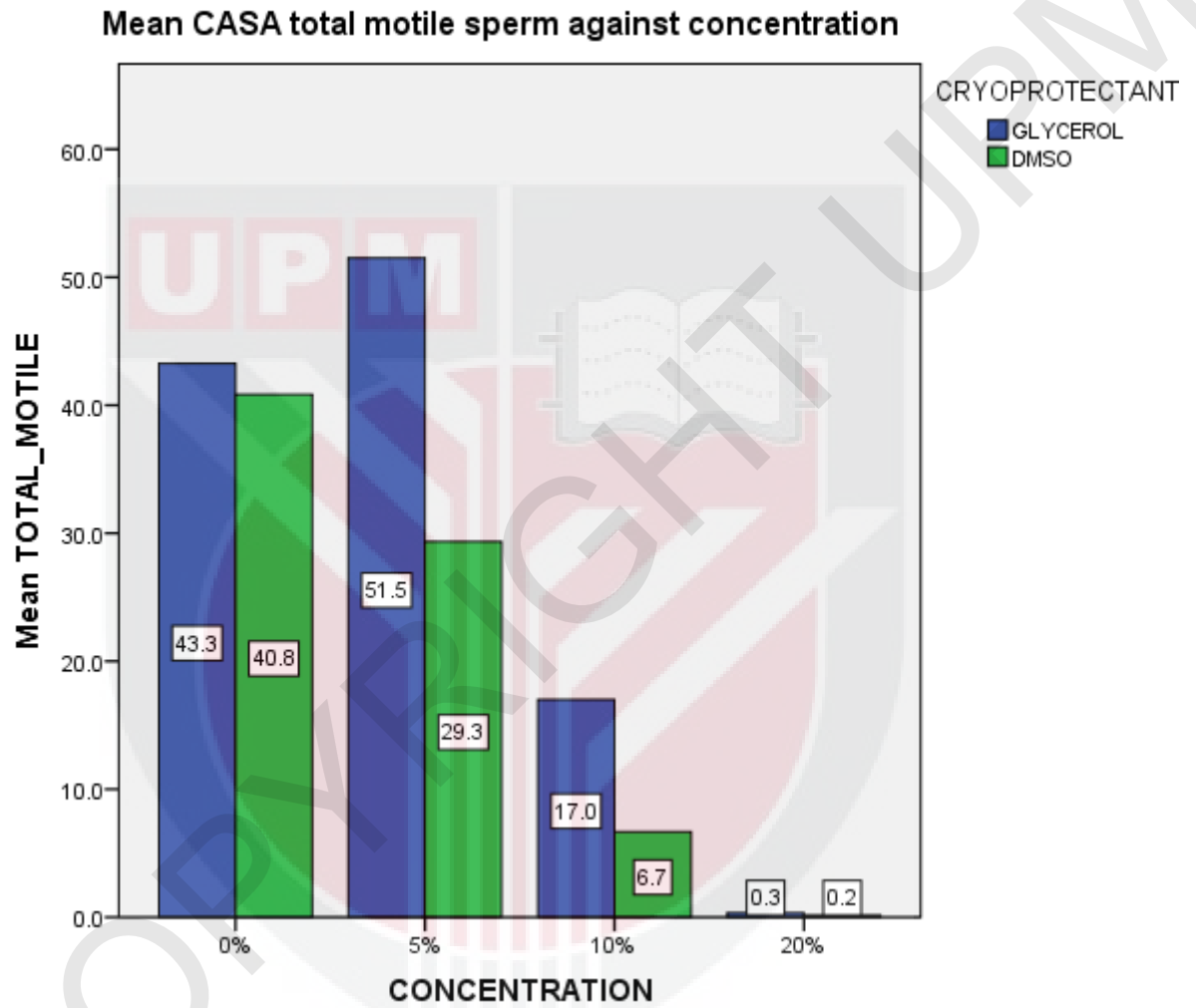
NS, no significant difference ( $p>0.05$ )

Figures 1,2 and 3 show 5% concentration of glycerol give highest mean for CASA progressive total motile, CASA total motile sperm and percentage live of the sperm as for DMSO, control group has highest mean for CASA progressive total motile, CASA total motile sperm and percentage live of the sperm. When compare between types of cryoprotectant, glycerol works better compare to DMSO in all parameters evaluated.

**Figure 1: Different Concentration of Cryoprotectants With Mean Of CASA Progressive Motile Sperm**



**Figure 2: Different Concentration of Cryoprotectants and Mean Of CASA Total Motile Sperm**



**Figure 3: Different Concentration of Cryoprotectants and Mean Of percentage live of sperm**

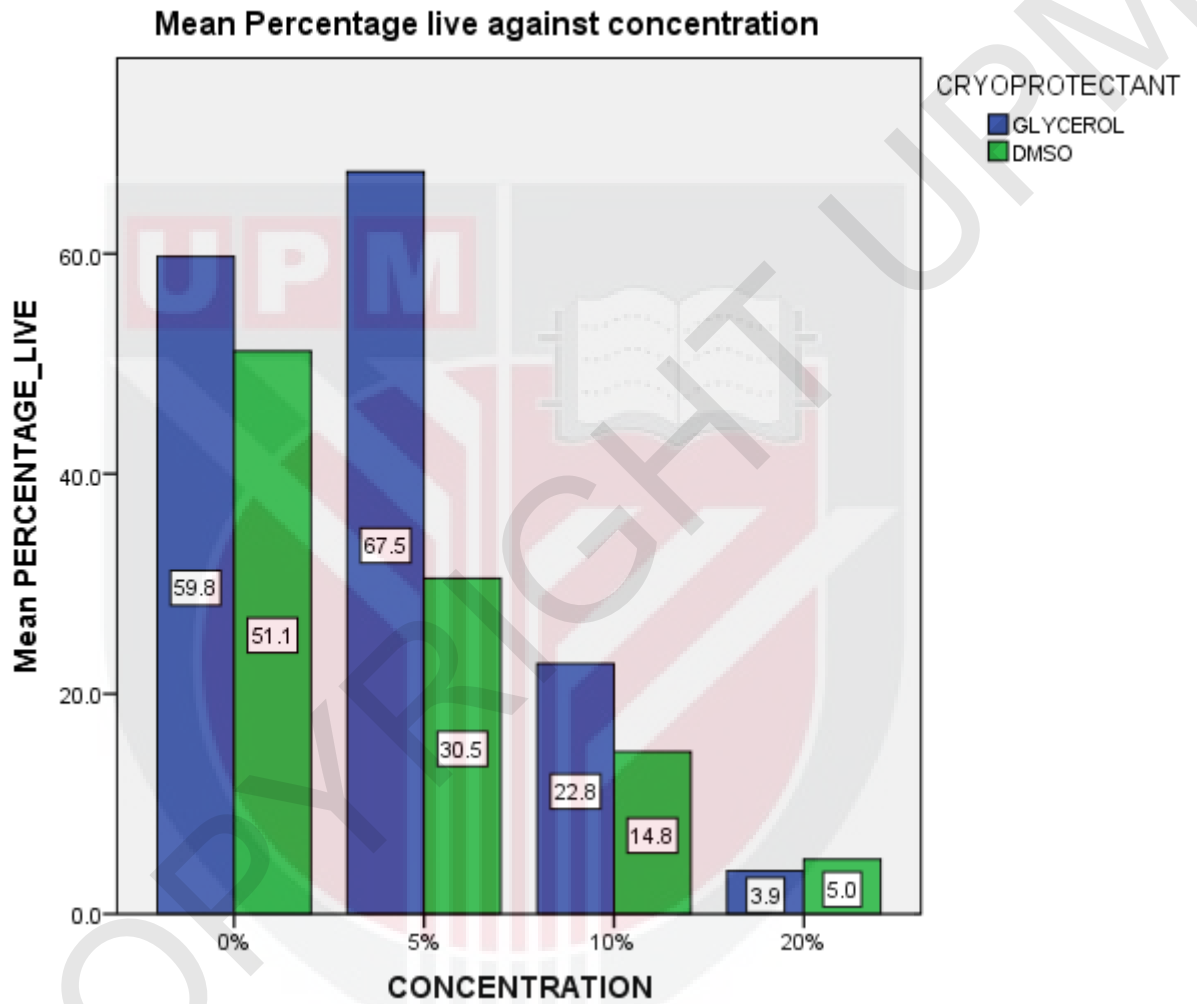


Table 2 shows the mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by types of cryoprotectant. Different types of cryoprotectants did not significantly affect the semen concentration and percentage of abnormal morphology of the sperm ( $p>0.05$ ). However, there is significant difference in CASA progressive total motile, CASA total motile sperm and percentage live of the sperm in different types of cryoprotectants ( $p<0.05$ ). Glycerol shows higher mean in CASA progressive motile sperm, CASA total motile sperm and percentage live of sperm ( $36.5 \pm 4.7$ ,  $28.0 \pm 3.6$ ,  $38.5 \pm 4.2$ ) compare to mean of DMSO in variables ( $23.3 \pm 4.3$ ,  $19.3 \pm 3.5$ ,  $25.3 \pm 3.1$ ).

**Table 2: Mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by types of cryoprotectant**

Types of Cryoprotectant	Semen conc. (%)	Progressive motile sperm (%)	Total motile sperm (%)	% live	Abnormal morphology (%)
<b>Glycerol</b>	$89.9 \pm 13.2$	$36.5 \pm 4.7^a$	$28.0 \pm 3.6^a$	$38.5 \pm 4.2^a$	$2.8 \pm 0.1$
<b>DMSO</b>	$86.6 \pm 9.9$	$23.3 \pm 4.3^b$	$19.3 \pm 3.5^b$	$25.3 \pm 3.1^b$	$2.7 \pm 0.1$
<b>p</b>	NS	0.032	0.044	0.019	NS

a-b: Different superscripts within the same column demonstrate significant differences between groups ( $p<0.05$ )

NS, no significant difference ( $p>0.05$ )

Table 3 shows the mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by day of thawing. Different day of thawing did not significantly affect the semen percentage of abnormal morphology of the sperm ( $p>0.05$ ). However, there is significantly difference in CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm and percentage live of the sperm in different types of cryoprotectants ( $p<0.05$ ). There was high mean for CASA progressive motile sperm, CASA total motile sperm and percentage live in day 1 post thawing compare to mean of all variables in day 7 post thawing.

**Table 3: Mean ( $\pm$ SEM) CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live and percentage abnormal morphology in frozen-thaw bull semen by day of thawing**

Day of thawing	Semen conc.(%)	Progressive motile sperm (%)	Total motile sperm (%)	% live	Abnormal morphology (%)
Day 1	59.7 $\pm$ 10.6 <sup>a</sup>	40.6 $\pm$ 5.3 <sup>a</sup>	31.9 $\pm$ 4.2 <sup>a</sup>	37.1 $\pm$ 3.9 <sup>a</sup>	2.7 $\pm$ 0.1
Day 7	116.7 $\pm$ 11.2 <sup>b</sup>	19.3 $\pm$ 3.1 <sup>b</sup>	15.4 $\pm$ 2.3 <sup>b</sup>	26.7 $\pm$ 3.5 <sup>b</sup>	2.8 $\pm$ 0.1
<b>p</b>	0.000	0.015	0.007	0.048	NS

a-b: Different superscripts within the same column demonstrate significant differences between groups ( $p<0.05$ )

NS, no significant difference ( $p>0.05$ )

Bivariate analysis was used in this study to identify only the most relevant variables, grouping those that may be correlated within each of the variables. All the parameters of the semen used in this experiment are shown in Table 1. The most relevant variables and correlated of coefficient within each variable are shown in Table 4. There is no significant correlation between CASA semen concentration with CASA progressive motile sperm, CASA total motile sperm and percentage live of the sperm ( $p>0.05$ ). There is no significant correlation between CASA progressive motile sperm with semen concentration however, there is significant correlation ( $p<0.05$ ) between CASA progressive total motile sperm and percentage live of sperm. The data is positively correlated ( $r=0.808, 0.656$ ). There is no significant correlation between CASA total motile sperm with semen concentration however, there is significant correlation ( $p<0.05$ ) between CASA progressive total motile sperm and percentage live of sperm. The data is positively correlated ( $r=0.808, 0.645$ ). There is no significant correlation between percentage live of sperm with semen concentration however, there is significant correlation ( $p<0.05$ ) between CASA progressive motile sperm and CASA progressive total motile sperm. The data is positively correlated ( $r=0.808, 0.645$ ).

**Table 4: Kendall's tau b correlation coefficient (r) of CASA semen concentration, CASA progressive motile sperm, CASA total motile sperm, percentage live for all samples (N=96)**

<b>Variable</b>		<b>Semen concentration (%)</b>	<b>Progressive motile sperm (%)</b>	<b>Total motile sperm (%)</b>	<b>% live</b>
<b>Semen concentration (%)</b>	Correlation coefficient	1.000	0.011	0.015	0.034
	p value	.	0.875	0.832	0.630
<b>Progressive motile sperm (%)</b>	Correlation coefficient	0.011	1.000	0.808a*	0.656a*
	p value	0.875	.	0.000	0.000
<b>Total motile sperm (%)</b>	Correlation coefficient	0.015	0.808a*	1.000	0.645a*
	p value	0.832	0.000	.	0.000
<b>% live</b>	Correlation coefficient	0.034	0.656a*	0.645a*	1.000
	p value	0.630	0.000	0.000	.

a: correlated variables ( $r > 0.05$ )

\* Correlation is significant at 0.01 (2 tailed)

## CHAPTER V

### DISCUSSION

The present study demonstrated that glycerol gives better effect on mortality of the cryopreserved bull semen compare to DMSO. At concentration of 5%, glycerol shows maximum viability of the sperm. According to Wayne (2000), bull semen may tolerate exposure to glycerol in concentrations from between 7% to 9%. The property of glycerol that increasingly high viscosity during lowering of the temperature, inhibit or retard ice crystal formation on a kinetic basis.

Tavassoli (2009) stated that the optimum concentration of DMSO to cryopreserved protozoa is approximately 10% while in this experiment the optimum concentration to cryopreserved the bull semen using DMSO is less than 5%. Although DMSO has been used to cryopreserved other types of cell, it was not an effective cryoprotectant to cryopreserve semen of bull. The concentration may vary in different types of sample used. High concentration of cryoprotectants may effects the viability of the sperms. According to Swelum, Mansour, Elsayed & Amer (2011), higher concentration of glycerol can lead to cell death.

DMSO also being reported by Shuyang & Wood (2004) can damage plasma membrane due to its high toxicity to cell. From the result in this study, glycerol shows better results compare to DMSO.

Different species of the semen sample may also affect the result as the previous study done by Rosato & Iaffaldano (2013), reported that concentration of DMSO at 5% and 10% showed the least toxic effects and the use of 10% DMSO producing the best post-thaw sperm motility and membrane integrity after conventional freezing of rabbit semen. However, in bull semen sample, concentration of 3% shows the best post-thaw motility.

In this study, two bulls were used to collect the semen sample. Result of this study might be affected because the semen was not being pooled and was evaluated separately. Different animal have different quality of semen. There are many factors that can affects semen quality such as age, environment, health status and activity of the animal itself.

Adult animal will have good quality of semen compare to young animal. This is because young animal the reproductive system may not fully develop and matured as compared to adult animal. According to Fuerst-Waltl *et al.* (2006), there are significantly differences between ages of the animal with the semen quality.

Environmental factor may include season. Seasonal effects are not only the result of various factors such as temperature, humidity, length of day, feed composition but also management (Fuerst-Waltl *et al.* 2006). Optimal ambient temperature for semen production was found to be approximately 15-20 ° C.

According to Alexendra (2013), sperm morphology is generally dependent on spermatogenesis or events that occur after spermiation. Poor handling techniques or problem during cooling and freezing could also damage the acrosome or cause reflection of the sperm tail. Disturbances during spermatogenesis in the testis or during epididymal transit that affect sperm morphology can be classified in a variety of ways, including the spermiogram, a differential count of sperm morphology (Alexendra, 2013). In this study, there is no significant difference between abnormal morphology of sperm with types of cryoprotectant, day of thawing and different concentrations of cryoprotectant. If there is significantly difference, it may due to poor handling techniques or problem during cooling and freezing process.

## CHAPTER VI

### CONCLUSION

Based upon the findings of these studies, the following conclusions could be made:

- Glycerol is better than DMSO as cryoprotectant for cryopreservation of bull semen.
- Highest viability of sperm is at 5% concentration of glycerol
- Different concentration for both cryoprotectants does not affect the morphology of the sperm
- High concentration of cryoprotectant affect the viability of the sperm

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