UNIVERSITI PUTRA MALAYSIA

EFFECTS OF DIFFERENT HOLDING TEMPERATURES BEFORE FREEZING ON THE QUALITY OF BULL SEMEN

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EFFECTS OF DIFFERENT HOLDING TEMPERATURES BEFORE FREEZING ON THE QUALITY OF BULL SEMEN

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It is hereby certified that I have read this project paper entitled "Effects of Different Holding Temperatures before Freezing on the Quality of Bull Semen", by Ivy Thien Shuk Yee and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This project and this project paper are dedicated to

the Almighty God,

my family and my best friends.

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KESAN SUHU PEMEGANGAN YANG BERBEZA SEBELUM PEMBEKUAN TERHADAP KUALITI SEMEN LEMBU JANTAN

oleh

Ivy Thien Shuk Yee

2017

Penyelia: Prof. Dr. Abd. Wahid Haron

Satu kajian telah dijalankan untuk menilai kesan suhu pemegangan yang berbeza sebelum pembekuan terhadap motiliti, morfologi dan kebolehidupan spermatozoa lembu jantan yang disejuk dan dibekukan. Pengumpulan semen dilakukan ke atas empat ekor lembu jantan yang matang menggunakan elektroejakulator. Selepas penilaian awal dan pencairan pertama dengan menggunakan Tris-egg yolk semen extender, empat kumpulan semen sampel yang dicair telah disimpan pada empat suhu pemegangan yang berbeza (4°C, 15°C, 25°C dan 37°C) selama 10 minit. Semua semen sampel kemudian disejukkan dalam peti sejuk pada 4°C selama sekurang-kurangnya 3 jam. Penilaian selepas penyejukan telah dijalankan selepas pencairan yang kedua dengan pengekal yang ditambahkan gliserol. Sampel semen dimasukkan ke dalam straw 0.25ml sebelum pembekuan dalam cecair nitrogen. Penilaian semen dijalankan selepas pencairan. Pencairan semen sampel beku telah dilakukan sehari selepas krioawetan dalam air yang bersuhu 37°C selama 30 saat.

Hasil kajian menunjukkan bahawa tiada perbezaan yang ketara antara empat suhu pemegangan yang berbeza bagi motiliti, morfologi dan kebolehidupan sperma selepas penyejukan dan pembekuan-pencairan sperma. Berdasarkan dapatan kajian ini, kesimpulan bahawa 10 minit masa pemegangan pada suhu yang berbeza sebelum pembekuan tidak menjejaskan kualiti spermatozoa lembu jantan yang disejuk dan dibekukan.

Kata Kunci: semen, suhu pemegangan, lembu jantan, pembekuan

ABSTRACT

An abstract of the project paper presented to Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

EFFECTS OF DIFFERENT HOLDING TEMPERATURES BEFORE FREEZING ON THE QUALITY OF BULL SEMEN

By

Ivy Thien Shuk Yee

2017

Supervisor: Prof. Dr. Abd. Wahid Haron

A study was conducted to evaluate the effect of different holding temperature before freezing on the motility, morphology and viability of chilled and frozen bull spermatozoa. Semen collection was performed on four mature bulls by electro-ejaculator. After initial evaluation and first dilution using Tris-egg yolk semen extender, four groups of diluted semen samples were kept in four different holding temperatures (4°C, 15°C, 25°C and 37°C) for 10 minutes. All semen samples were subsequently chilled in refrigerator at 4°C for at least 3 hours. Post chilling evaluation was done after second dilution with the same extender added with glycerol. The semen samples were packed into 0.25ml straws before freezing into liquid nitrogen. Thawing of frozen semen samples were done on day 1 post cryopreservation in water maintained at 37°C for 30 seconds. Semen evaluation was conducted post thawing. The results showed that there were no significant difference among the four different

holding temperatures for the post chilling and frozen-thawed sperm motility, morphology and viability. Based on the findings of this study, it can be concluded that 10 minutes holding time at different holding temperatures before freezing did not affect the quality of chilled and frozen bull spermatozoa.

Key Words: semen, holding temperature, bull, freezing

1.0 INTRODUCTION

1.1 Artificial Insemination

The use of artificial insemination (AI) in domestic animals, especially cattle has an extraordinary growth for more than one human generation (Foote, 1982). The first report on successful AI in cattle and sheep was achieved by Ivanov and colleagues in Russia in 1930 (Ball & Peters, 2007). This technology brought great improvement in efficiency of livestock production resulted from the widespread use of highly selected genetically superior sires which free from most of the known diseases (Foote, 1982). Artificial insemination also provides many advantages in terms of cost effectiveness, flexibility and ease in reproductive management of the livestock. The use of AI in dairy cattle industry reduces the cost of rearing bulls and problem arises from rearing bulls such as fighting and sexually transmitted diseases.

1.2 Cryopreservation

The advancement in cryopreservation technology is now able to store frozen bull semen for extended period of time and used for insemination. Many studies have been conducted in order to improve the techniques for collecting the maximum number of viable spermatozoa from the male through preservation of the semen quality by adding suitable media, providing appropriate cooling, freezing, storage, and thawing procedures (Foote, 1982).

1.3 Holding temperature

Correa et al. (1996) reviewed that cryopreserved spermatozoa are susceptible to the changes of osmotic conditions during freezing, thawing and processing, in which causing reduced fertility as compared with fresh semen (Watson, 2000). The cryopreservation protocol has a number of potentially damaging stresses, one of them is the change in temperature (Watson, 2000). By manipulating different holding temperature of the semen before cryopreservation may produce different effect to the spermatozoa in terms of viability, motility and morphology before and after freezing.

1.4 Objectives

The main objective of this study is to determine the effect of different holding temperatures before freezing on the quality of cryopreserved bull semen.

2.0 LITERATURE REVIEW

2.1 Semen collection by electro-ejaculation

Electro-ejaculation (EE) is one of the reliable methods to collect semen sample from male animal by the means of electrical stimulation for the purpose of evaluating breeding soundness evaluation (BSE). This technique has been a very effective method of collecting semen sample for the past half-century as it does not require mount animal and adaptable to cattle handling facilities (Palmer, 2005). A rectal probe is used to initiate electrical stimulation to stimulate ejaculation. Collection of semen was done by using a rubber cone held by a section of radiated hose.

In recent years, automated electro-ejaculator was developed in which very useful for user who is not skilled in manual technique of applying electrical stimulation. However, welfare concern is raised regarding the use of EE which is considered to be painful and inhumane. The signs of discomfort shown by bulls during EE are intense muscle contraction, struggling, vocalization and occasional recumbency (Palmer, 2005).

2.2 Semen quality and evaluation

Evaluation of fresh semen quality can be conducted macroscopically and microscopically. For macroscopic aspect, the volume and colour of fresh semen are determined. For microscopic aspect, sperm motility, concentration, viability and morphology are determined.

Computer Assisted Semen Analyzer (CASA) can be used to evaluate the sperm motility and concentration. It is also able to evaluate the sperm motility in non-biased manner (Graham & Mocé, 2005). The CASA was programmed to capture frames at the rate of 60 Hz/s at 37°C of temperature and at a video frequency of 60. The magnification factor, minimum cell size, detection contrast and cell intensity were 1.92, 5 pixel, 40, 55, respectively.

Eosin-nigrosin stain were used to determine mortality and morphology of sperm (Memon *et al*., 2011). A drop of semen sample is mixed with two drops of eosin-nigrosin stain and then a smear is made on a glass slide. After the smear is air dried, examine the stained slide under light microscope at 400x magnification. Sperms (n = 200) were counted from four microscopic fields. A normal sperm cell has the normal structure of smooth oval shaped head with clearly defined cap (acrosome) continued to the mid-piece and tail without any visible defect (Khumran *et al*., 2015). Abnormal sperm has the characteristics of large or small head sizes, abnormal mid-piece, presence of cytoplasmic droplets, broken parts and a double heads with or without tail (Nagy *et al*., 2013). Evaluation of sperm mortality can be determined based on the sperm staining characteristics by eosin-nigrosin stain. Dead spermatozoa

is stained pink-purple due to absorption of eosin-nigrosin stain while live spermatozoa remains white or colourless (Yimer *et al*., 2015).

2.3 Semen cryopreservation

Semen cryopreservation enables the storage of frozen semen for a long period of time particularly under temperature of -196°C in the liquid nitrogen tank (Lemma, 2011). This process decreases the metabolism and toxin production of the spermatozoa thus prolonging the life of spermatozoa (Bailey *et al*., 2003; Curry, 2000; Hammerstedt *et al*., 1990).

Semen cryopreservation consists of several general steps which are dilution, cryoprotection, cooling, freezing, storage and thawing (Hammerstedt *et al*., 1990). Sperms are diluted with extender under the temperature close to the body temperature (36-39°C) and then cooled to 5°C in which the addition of cryoprotectant is done (Polge *et al*., 1949). Tris-egg yolk-glycerol extender gives excellent protection for sperm either frozen or unfrozen (Davis et al., 1963; Foote, 1998). Approximately 4-8% glycerol is usually used for cryopreservation of bull spermatozoa (Holt, 2000). Glycerol is a cryoprotectant that is readily to cross the cell membrane but the concentration used should be balanced against glycerol toxicity (Curry, 2000).

Semen is usually packed into semen straws (0.25ml or 0.5ml) for freezing and storage. Straws can be frozen by putting the straws in the cool nitrogen vapour above the liquid nitrogen or in a controlled-rate freezing machine (Holt, 2000).

2.4 Sperm damage due to cryopreservation

The reasons for the reduced fertility in cryopreserved sperms are the reduced number of viable sperms and the functional defects of the remaining viable sperms (Watson, 2000). Rapidly reduction in temperature is termed as cold shock. Cold shock induces a lethal stress in sperms proportional to the rate of cooling, the temperature interval and the temperature range (Watson, 1981). The change of temperature also induces plasma membrane damage in sperms. Cryomicroscopic examination of ram sperm loaded with a marker for membrane integrity showed that the change of temperature experienced by the sperm affected the plasma membranes at the principal piece, midpiece and head (Holt and North, 1994). Sperm plasma membrane damage predisposes the sperm to morphologic defects such as abnormal acrosomes (Bailey *et al*., 2000), which is crucial for the process of fertilization.

Cryopreservation also impairs the membrane selective permeability mechanisms resulting in high amount of intracellular calcium in surviving cryopreserved sperm (Bailey and Buhr, 1994; McLaughlin and Ford, 1994). The addition and removal of cryoprotectant during the procedure of cryopreservation can induce osmotic stress to the plasma membrane of sperms as sperms are sensitive to toxic effects of cryoprotectant (Watson, 2000). Extra caution should be considered when using glycerol in spermatozoa as cryoprotectant (Katkov *et al*., 1998).

Spermatozoa can experience stress due to osmotic pressure changes during the process of freezing. When a solution is cooled to the below its freezing point, there will be ice formation from the pure water portion of the solution. The solutes are dissolved in the remaining portion of unfrozen liquid, thus increasing the osmotic pressure of the solution (Watson, 2000).

According to Drobnis *et al.* (1993), the susceptibility of the plasma membrane to undergo lipid phase transitions during cooling is inversely proportional to the content of cholesterol. Bull and ram sperm which have low cholesterol contents are known to be sensitive to cooling compared to rabbit and human sperm (Bailey et al., 2000).

During cooling to 4°C, there will be increase of reactive oxygen species (ROS) production by spermatozoa and seminal leukocytes (Wang *et al*., 1997). Disrupted balance between ROS production and detoxication by antioxidants causes oxidative stress in spermatozoa, which affect motility and oxidative metabolism in spermatozoa (Tosic, 1947). Aitken *et al.* (1998) reported ROS can cause damage to the sperm DNA.

Cryopreservation will also cause premature capacitation of spermatozoa (Bailey *et al*., 2000). Due to disrupt of membrane permeability, influx of calcium ions is more favored and resulted in abnormally high amount of intracellular calcium ions in the spermatozoa.

3.0 MATERIALS AND METHODS

3.1 Animal

Four sexually mature Brangus cross bulls from Taman Pertanian Universiti, Universiti Putra Malaysia cattle farm were used for semen collection. The age of the bulls were within the range of 3 to 7 years old with body weights between 300 to 400 kg. These bulls were kept semi-intensively, fed with palm kernel cake (PKC) and allowed to graze freely on Napier grass during the day for about 12 hours. Commercial mineral block and water were given *ad libitum* to the bulls. The sample collection was approved by Institutional Animal Care and Use Committee (IACUC) FYP.2016/FPV (13, 31).

3.2 Experimental design

Semen samples were divided into four groups. First group, the controlled group was kept in refrigerator at 4°C. Second group was kept in a cool water bath at 15°C inside an insulated thermal box. The third group was put in a water bath at 25°C inside an insulated thermal box. The last group of samples was put in a warm water bath at 37°C. All groups were maintained at its respective temperatures 10 minutes before chilling.

3.3 Semen collection and fresh semen evaluation

Two semen samples were collected from two different bulls using an automated electro-ejaculation method (Yimer *et al*., 2011). The collection was repeated three times to get a total of eight semen samples. The semen was collected into a labelled 15ml graduated centrifuged tube and kept under water bath of 37°C following the ejaculation. The semen samples were evaluated immediately based on macroscopic (volume and colour) and microscopic (spermatozoa concentration, general motility, morphology and viability) characteristics. The volume and colour of the semen samples were evaluated.

The semen sample was diluted 1 to 40 ratio with isotonic buffer (normal saline) at water bath of 37°C for initial evaluation. Evaluation of spermatozoa concentration and general motility were made by using a Computer Assisted Semen Analyzer (CASA, IVOS System, Hamilton Thorne Inc., USA).

Spermatozoa morphology and viability were evaluated using eosin-nigrosin stain technique (Yimer *et al.,* 2014). A total of 200 spermatozoa were examined under a light microscope (400 x magnifications). Dead spermatozoa were stained purple and live spermatozoa were unstained. Sperm abnormality can be determined by examining 200 spermatozoa for defects associated with sperm head (detached, tapered, giant and micro head), mid-piece (cytoplasmic droplets, bent, irregular shape) and tail (broken, bent, coiled and looped tail).

Only semen sample that achieved the standard minimum criteria can be used for the experiment. Volume of ejaculate must be more than 5ml. The colour of the ejaculate should be milky. The concentration of spermatozoa should be 500-1500 $x10^6$/ml. There should be at least more than 70% of general motility and more than 80% of live spermatozoa (viability) in the semen sample. Percentage of abnormal mid-piece, coiled tail, protoplasmic droplets and abnormal heads must be less than 4%, 5%, 4%and 18% respectively. Lastly, there should be no cells other than spermatozoa in the semen sample.

3.4 Cryopreservation procedure

3.4.1 Extender preparation

For extender preparation, 2.42g of Tris (hydroxymethyl), 1.48g of citrate, 1g of fructose (Sigma-Aldrich) and 20ml of fresh egg yolk were mixed with distilled water to produce 100ml of Tris-egg yolk extender. 2.42g of Tris (hydroxymethyl), 1.48g of citrate, 1g of fructose (Sigma-Aldrich), 20ml of fresh egg yolk and 12.8ml of glycerol were mixed with distilled water to produce 100ml of Tris-egg yolk-glycerol extender. Both of the extenders were stored under chilled temperature of 10°C.

3.4.2 Semen dilution and packing

The semen sample was pre-diluted with Tris-egg yolk extender at water bath of 37°C. Four semen samples from each bull were kept at different holding temperature of 4, 15, 25 and 37°C respectively for 10 minutes. Then, all semen samples were transferred for chilling at temperature of 4°C and kept for more than 3 hours. After 3 hours, all the semen samples were evaluated using Computer Assisted Semen Analyzer (CASA, IVOS System, Hamilton Thorne Inc., USA) and stain with eosin-nigrosin to determine the sperm general motility, progressive motility, viability and morphology (Yimer *et al.,* 2014).

The pre-diluted semen sample was then diluted with Tris-egg yolk-glycerol extender as final dilution under temperature of 4°C. The extended semen was packed into 0.25 ml labelled French straws and sealed with sealing powder under temperature of 4°C.

3.4.3 Freezing of semen

The straws were subject to a slow gradual freezing process manually before they were stored in a liquid nitrogen tank. After sealing, straws were placed horizontally on a cold rack (5°C) for 5 minutes and then lowered into nitrogen vapour (-50°C) for 5 minutes, then lowered to 3-4 cm above the surface of liquid nitrogen in a polystyrene box for another 3 minutes. When the temperature reached -100°C, the

frozen straws were transferred into goblets of appropriate size and transferred into a liquid nitrogen tank (-196°C).

3.5 Frozen semen evaluation

After 24 hours of storage, the straws were taken out from the liquid nitrogen tank and thawed in water bath with temperature of 37°C for 30 seconds. Then, the straws were cut and the semen samples were inserted into test tubes respectively in the water bath with temperature of 37°C. All the post thawed semen samples were also evaluated using Computer Assisted Semen Analyzer (CASA, IVOS System, Hamilton Thorne Inc., USA) and stain with eosin-nigrosin (Yimer *et al.,* 2014).

3.6 Statistical analysis

All the data were tested for normality by using Shapiro-Wilk Test. The result of post chilling and post thawing evaluation were analysed by Kruskal-Wallis H Test using IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). Mann Whitney U Test was used to analyse the relationship between different pairs of treatment groups. The result of fresh semen evaluation and post thawing evaluation were analysed by Wilcoxon Signed Rank Test using the software mentioned above.

4.0 RESULTS

From the test of normality, Shapiro-Wilk Test (APPENDIX II) showed that most of the data were not normally distributed, $p<0.05$. Therefore, relevant non-parametric tests were selected to analyse the data from the experiment.

The result of Kruskal-Wallis H Test for the post chilling evaluation (APPENDIX III) revealed that there were no statistically significant difference ($p>0.05$) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the different holding temperatures. The mean value of sperm viability were 66.38% for 4°C, 66.75% for 15°C, 68.88% for 25°C and 62.13% for 37°C. The mean value for sperm motility were 58.88% for 4°C, 62.38% for 15°C, 65.25% for 25°C and 62.50% for 37°C. The mean value for sperm progressive motility were 16.00% for 4°C, 15.63% for 15°C, 16.50% for 25°C and 15.63% for 37°C. The mean value for sperm normal morphology were 94.56% for 4°C, 93.56% for 15°C, 95.50% for 25°C and 96.00% for 37°C.

Besides, the result of Kruskal-Wallis H Test for the post thawing evaluation (APPENDIX IV) showed that there were no statistically significant difference in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the different holding temperatures, $p>0.05$. The mean value for sperm viability were 58.25% for 4°C, 56.63% for 15°C, 56.38% for 25°C and 55.88% for 37°C. The mean value for sperm motility were 32.13% for 4°C, 27.63% for 15°C,

31.88% for 25°C and 36.63% for 37°C. The mean value of sperm progressive motility were 6.25% for 4°C, 4.63% for 15°C, 5.13% for 25°C and 7.00% for 37°C. The mean value of sperm normal morphology were 96.00% for 4°C, 96.25% for 15°C, 95.69% for 25°C and 95.06% for 37°C.

On the other hand, the result of Wilcoxon Signed Rank Test for the fresh semen evaluation and the post thawing evaluation (APPENDIX V) showed that there were statistically significant differences ($p<0.05$) in sperm viability, sperm general motility and sperm normal morphology. However, there was no statistically significant difference ($p>0.05$) in sperm progressive motility.

From the result of Mann-Whitney U Test for the post chilling evaluation, there were no significant differences ($p>0.05$) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the holding temperature of 4°C and 15°C, 4°C and 25°C, 4°C and 37°C, 15°C and 25°C, 15°C and 37°C, 25°C and 37°C.

Furthermore, the result of Mann-Whitney U Test for the post thawing evaluation showed that there were no significant differences ($p>0.05$) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the holding temperature of 4°C and 15°C, 4°C and 25°C, 4°C and 37°C, 15°C and 25°C, 15°C and 37°C, 25°C and 37°C.

Table 1: Mean value of post chilling evaluation of different holding temperature

Temperature (°C)	Post Chilling Evaluation (% ± SEM)			
	Viability	General Motility	Progressive Motility	Normal Morphology
4	66.38 ± 4.74	58.88 ± 12.42	16.00 ± 4.23	94.56 ± 3.10
15	66.75 ± 6.45	62.38 ± 10.32	15.63 ± 3.47	93.56 ± 2.40
25	68.88 ± 5.90	65.25 ± 8.67	16.50 ± 3.15	95.50 ± 1.78
37	62.13 ± 5.17	62.50 ± 10.54	15.63 ± 3.04	96.00 ± 1.43

Table 2: Mean value of post thawing evaluation of different holding temperature

Temperature (°C)	Post Thawing Evaluation (% ± SEM)			
	Viability	General Motility	Progressive Motility	Normal Morphology
4	58.25 ± 7.29	32.13 ± 9.06	6.25 ± 2.34	96.00 ± 2.37
15	56.63 ± 4.18	27.63 ± 6.74	4.63 ± 1.48	96.25 ± 0.97
25	56.38 ± 5.05	31.88 ± 6.84	5.13 ± 1.77	95.69 ± 1.77
37	55.88 ± 5.58	36.63 ± 9.61	7.00 ± 2.26	95.06 ± 2.23

5.0 DISCUSSION

Electro-ejaculation method was used to collect fresh semen sample from the selected bulls in this study. Care should be taken when handling the ejaculates. Exposure of fresh semen sample to sunlight, faeces, dust or temperature exceed 40°C should be avoided to prevent reduction in the quality of the semen sample. The fresh semen sample was first collected in a 15ml graduated centrifuged tube covered with tissue towel and then closed by a cover tightly before kept in the ice box with 37°C water bath in upright manner. Time taken from the collection of fresh ejaculate in the farm until the evaluation of fresh semen sample in the laboratory took about 20 to 30 minutes. This duration might have affected the quality of semen sample.

In this study, Tris-egg yolk extender containing citrate and fructose as energy supplement to the spermatozoa were used to extend their life spans. Approximately 20% of egg yolk in the extender is used as standard level for most case of bull semen cryopreservation (Rahman *et al*., 2012). Before conducting fresh semen evaluation using CASA, the semen sample was diluted with normal saline in 1 to 40 dilutions. After the dilution, the diluted sample had to be evaluated immediately to prevent inaccurate result because normal saline does not serve the function as an extender. Tris-egg yolk extender was not used as diluent during fresh semen evaluation because CASA may count the egg yolk granules from the Tris-egg yolk extender as individual sperm in which will affect the outcome.

After cooling the semen sample at 4°C in the refrigerator for more than 3 hours, Tris-egg yolk-glycerol extender was used to dilute the semen before packing for cryopreservation. Tris-egg yolk-glycerol extender gives excellent protection for sperm either frozen or unfrozen (Davis et al., 1963; Foote, 1998). Approximately 4-8% glycerol is usually used for cryopreservation of bull spermatozoa (Holt, 2000). In this study, we used 6.25% glycerol for the cryopreservation of bull spermatozoa. Spermatozoa are sensitive to the changes in the osmolality of the surrounding solution, in which they will lose their motility irreversibly if the osmolality is intolerable (Gilmore et al., 1998; Willoughby et al., 1996). Therefore, we have to be aware of the dilution factor used to dilute the semen with extender for cryopreservation.

The result of this study showed that there were no statistically significant differences between the different holding temperatures before freezing to the quality of bull semen. The duration for the different holding temperatures applied in this experiment may be too short for the effect to take place on the semen quality. Besides, the use of insulated thermal box to maintain the constant holding temperature of 15°C and 25°C may not be effective. This may be one of the reasons to affect the accuracy of the outcome.

Cooling of spermatozoa to different temperatures had shown different effect of capacitation in spermatozoa. Capacitation in spermatozoa can be detected by using fluorescent antibiotic chlortetracycline staining (Curry, 2000). Changes in chlortetracycline (CTC) staining pattern have been reported for bull spermatozoa

(Cormier et al., 1997), after cooling to 4°C there is a similar increase in the capacitatated spermatozoa staining pattern. Besides, cooling of boar spermatrozoa to 5°C had a greater effect than cooling to 15°c (Maxwell & Johnson, 1997). Curry (2000) stated that if frozen thawed spermatozoa were found to have an accelerated rate of capacitation, this might lead to a shortened lifespan for these cells.

There were statistically significant differences in the quality of bull semen before and after cryopreservation. The quality of cryopreserved bull semen is lower than the quality of fresh bull semen in terms of sperm viability, sperm motility and sperm morphology. This means that the process of cooling and freezing during cryopreservation do affect the quality of bull spermatozoa in terms of sperm viability, sperm motility and sperm morphology.

Cooling and freezing of spermatozoa during process of cryopreservation causes reduction of temperature of the spermatozoa and its surrounding solution. This change in temperature induces sperm plasma membrane damage which leads to a state of sperm abnormal morphology such as abnormal acrosome (Bailey *et al*., 2000). The damaged sperm plasma membrane allows influx of calcium ions which resulted in abnormally high amount of intracellular calcium ions in the spermatozoa. This phenomenon contributes to the premature capacitation of spermatozoa (Bailey *et al*., 2000).

In this study, four sexually matured bulls of different ages and body weights from the same cattle farm. It had been reported that there is variation among

individuals in the extent to which their spermatozoa are damaged by freeze-thawing (Curry, 2000). This individual differences particularly for bull semen have been recorded and to cover this difference, freezing protocols were adjusted for individual bulls or packaging straws with more spermatozoa for "poor freezers" (Parkinson and Whitfield, 1987). The effect of different temperature to the quality of spermatozoa may be varies for different individuals from the same species. Therefore, the accuracy of result may be reduced due to this factor.

Proper handling of sample throughout the experiment is one of the crucial components. The proper way of using pipette, constant temperature of water bath machine, constant temperature of slide warmer, consistent way of making eosin-nigrosin stain can help reduce error that affects the accuracy of the result.

6.0 CONCLUSION AND RECOMMENDATION

In this study, we can conclude that different holding temperatures before freezing do not affect the quality of bull semen significantly. So, the hypothesis is rejected. However, the process of cooling and freezing during cryopreservation affects the quality of bull semen significantly.

The recommendation to improve this study is use longer duration of holding time so that the effect of temperature on the semen quality can be significantly determined. Since bull semen is more resistant towards the effect of temperature, similar study can be conducted in different species such as buck, ram and boar.

Besides, more time and numbers of suitable samples should be required to improve this study. These will allow for more tests to be conducted for different parameters and more samples can be processed. Further investigation regarding the effect of different holding temperature before freezing should be done.

REFERENCES

Aitken, R. J., Gordon, E., Harkiss, D., Twigg, J. P., Milne, P., Jennings, Z., & Irvine, D. S. (1998). Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction, 59*, 1037-1046.

Bailey, J. L., Bilodeau, J., & Cormier, N. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *Journal of Andrology, 21*(1), 1-7.

Bailey, J. L., Morrie, A., Cormier, N. (2003). Semen cryopreservation: success and persistent in farm species. *Canadian Journal of Animal Science. 83*, 393-401.

Bailey, J. L., Robertson, L., & Buhr, M. M. (1994). Relationships among in vivo fertility, computer-analysed motility and in vitro Ca 2 flux in bovine spermatozoa. *Canadian Journal of Animal Science, 74*, 53-58.

Ball, P. J., & Peters, A. R. (2004). *Reproduction in Cattle*. Oxford, UK: Blackwell Pub.

Cormier, N., Sirard, M. A., Bailey, J. L. (1997). Premature capacitation of bovine spermatozoa is intiated by cryopreservation. *Journal of Andrology,* 18, 461-468.

Correa, J. R., Rodriguez, M. C., Patterson, D. J., & Zavos, P. M. (1996). Thawing and processing of cryopreserved bovine spermatozoa at various temperatures and their effects on sperm viability, osmotic shock and sperm membrane functional integrity. *Theriogenology, 46*(3), 413-420.

Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Reviews of Reproduction, 5(1),* 46-52.

Davis, I. S., R. W. Bratton, and R. H. Foote. (1963). Livability of bovine spermatozoa at 5, -25 and -85°c in tri-buffered and citrate-buffered yolk-glycerol extenders. *Journal of Dairy Science, 46*, 333-336.

Drobnis, E. Z., Crowe, L. M., Berger, T., Anchordoguy, T. J., Overstreet, J. W., & Crowe, J. H. (1993). Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. *Journal of Experimental Zoology, 265*, 432-437.

Dziuk, P. J., Graham, E. F., & Petersen, W. E. (1954). The technique of electroejaculation and its use in dairy bulls. *Journal of Dairy Science, 37*(9), 1035-1041.

Foote, R. H. (1982). Cryopreservation of spermatozoa and artificial insemination: past, present, and future. *Journal of Andrology, 3*(2), 85-100.

Foote, R. H. (1998). *Artificial insemination to cloning: tracing 50 years of research*. Ithaca, NY: Cornell University.

Graham, J. K., & Mocé, E. (2005). Fertility evaluation of frozen/thawed semen. *Theriogenology, 64*, 492-504.

Hammerstedt, R. H., Graham, J. K., Nolan, J. P. (1990). Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of Andrology, 11*, 73-88.

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

Holt, W. V. (2000). Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology, 53*(1), 47-58.

Holt, W. V., & North, R. D. (1994). Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. *Biology of Reproduction, 51*, 414-424.

J. A. Gilmore, J. Liu, A. T. Peter, J. K. Critser. (1998). Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation, *Biology of Reproduction, 58,* 28-36.

Kaka, A., Wahid, H., Rosnina, Y., Yimer, N., Khumran, A., Sarsaifi, K., Ebrahimi, M. (2015). α-Linolenic acid supplementation in BioXcell® extender can improve the quality of post-cooling and frozen-thawed bovine sperm. *Animal Reproduction Science, 153*, 1-7.

Katkov, I.I., Katkova, N., Critser, J.K., Mazur, P. (1998). Mouse spermatozoa in high concentrations of glycerol: chemical toxicity vs. osmostic shock at normal and reduced oxygen concentrations. Cryobiology 37, 325-338.

Khumran, A., Yimer, N., Rosnina, Y., Ariff, M., Wahid, H., Kaka, A., Sarsaifi, K. (2015). Butylated hydroxytoluene can reduce oxidative stress and improve quality of frozen–thawed bull semen processed in lecithin and egg yolk based extenders. *Animal Reproduction Science, 163*, 128-134.

Lemma, A. (2011). Effect of cryopreservation on sperm quality and fertility. *Artificial Insemination in Farm Animals,* 191-216.

Maxwell, W. M. C. and Johnson, L. A. (1997). Chlortetracycline analysis of boar spermatozoa after maturation, flow cytometric sorting, cooling or cryopreservation. *Molecular Reproduction and Development, 46*, 408-418.

Mclaughlin, E. A., & Ford, W. C. (1994). Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone. *Molecular Reproduction and Development, 37*, 241-246.

Memon, A. A., Wahid, H., Rosnina, Y., Goh, Y.M., Ebrahimi, M., Nadia, F., & Audrey, G. (2011). Effect of butylated hydroxytoluene on cryopreservation of Boer goat semen in Tris egg yolk extender. *Animal Reproduction Science, 129*(1), 44-49.

Nagy, S., Johannisson, A., Wahlsten, T., Ijäs, R., Andersson, M., & Rodriguez-Martinez, H. (2013). Sperm chromatin structure and sperm morphology: Their association with fertility in AI-dairy Ayrshire sires. *Theriogenology, 79*(8), 1153-1161.

Palmer, C. W. (2005). Welfare aspects of theriogenology: Investigating alternatives to electroejaculation of bulls. *Theriogenology, 64*(3), 469-479.

Parkinson, T. J. and Whitfield, C. H. (1987). Optimisation of freezing conditions for bovine spermatozoa. *Theriogenology, 27*, 781-797.

Polge, C., Smith, A. U., & Parkes, A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature (London), 164*, 666-666.

Rahman, H. J. U., N. Ahmad, N. U. Rahman, S. Waheed, M. Ahmad, M. Younis, T. Ahmad. (2012) Effects of different levels of pigeon egg-yolk in extenders on the post-thaw semen quality of Sahiwal bulls. *Pakistan Veterinary Journal, 32*, 315-318.

Taşdemir, U., Büyükleblebici, S., Tuncer, P. B., Coşkun, E., Özgürtaş, T., Aydın, F. N., Gürcan, I. S. (2013). Effects of various cryoprotectants on bull sperm quality, DNA integrity and oxidative stress parameters. *Cryobiology, 66*(1), 38-42.

Thibault, C., Laplaud, M., and Ortavant, R. (1948). L'Electro-ejaculation chez le Taureau, Technique et Résultats. Comptes Rendus de l'Académie des Sciences. Paris, 226: 2006.

Tosic, J. (1947). Mechanism of hydrogen peroxide formation by spermatozoa and the role of amino-Acids in sperm motility. *Nature, 159*, 544-544.

Wang, A. W., Zhang, H., Ikemoto, I., Anderson, D. J., & Loughlin, K. R. (1997). Reactive oxygen species generation by seminal cells during cryopreservation. *Urology, 49*, 921-925.

Watson, P. F. (1981). The effects of cold shock on sperm cell membranes. In: Morris, G. J., Clarke, A. (Eds.), Effects of Low Temperatures on Biological Membranes. Academic Press, London, pp. 189-218.

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

Willoughby, C. E. (1996). Osmotic tolerance limits and properties of murine spermatozoa. *Biology of Reproduction, 55*(3), 715-727.

Yimer, N., Muhammad, N., Sarsaifi, K., Rosnina, Y., Wahid, H., Khumran, A., & Kaka, A. (2015). Effect of honey supplementation into Tris Extender on Cryopreservation of bull spermatozoa. *Malaysian Journal of Animal Science, 18*(2), 47-54.

Yimer, N., Noraisyah, A., Rosnina, Y., Wahid, H., Sarsaifi, K., & Hafizal, A. (2014). Comparison of cryopreservative effect of different levels of omega-3 egg-yolk in citrate extender on the quality of goat spermatozoa. *Pakistan Veterinary Journal, 34*(3), 347-350.

APPENDIX I

Standard Minimum Criteria

1. Volume (ml)	≥5 (5-10)
2. Colour	milky
3. Concentration (x10^6/ml)	500 – 1500
4. Motility (wave motion: 0 – 5)	3-5
5. Percentage motile (general motility)	≥70%
6. Percentage live (viability)	≥80%
7. Percentage abnormal mid-piece	≤4%
8. Percentage coiled tail	≤5%
9. Percentage protoplasmic droplets	≤4%
10. Percentage abnormal heads	≤18%
11. Cells other than spermatozoa	Nil

APPENDIX II

Table 3: Tests of Normality

	Shapiro-Wilk		
	Statistic	df	Sig.
Fresh Viability	.845	32	.000
Fresh Motility	.854	32	.001
Fresh Progressive Motility	.793	32	.000
Fresh Morphology	.722	32	.000
Post Chill Viability	.983	32	.871
Post Chill Motility	.785	32	.000
Post Chill Progressive Motility	.845	32	.000
Post Chill Morphology	.652	32	.000
Post Thaw Viability	.956	32	.218
Post Thaw Motility	.948	32	.127
Post Thaw Progressive Motility	.880	32	.002
Post Thaw Morphology	.673	32	.000

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

APPENDIX III

Table 4: Kruskal-Wallis H Test for post chilling evaluation of different holding temperatures

	Post Chill Viability	Post Chill Motility	Post Chill Progressive Motility	Post Chill Morphology
Chi-Square	1.595	.266	.696	1.310
Df	3	3	3	3
Asymp. Sig.	.661	.966	.874	.727

Table 5: Mean Ranks for post chilling evaluation of different holding temperatures

	Temperature	N	Mean Rank
Post Chill Viability	4	8	16.81
	15	8	17.88
	25	8	18.31
	37	8	13.00
	Total	32	
Post Chill Motility	4	8	17.69
	15	8	15.69
	25	8	16.94
	37	8	15.69
	Total	32	
Post Chill Progressive Motility	4	8	18.19
	15	8	16.06
	25	8	17.25
	37	8	14.50
	Total	32	
Post Chill Morphology	4	8	18.38
	15	8	13.38
	25	8	16.88
	37	8	17.38
	Total	32	

APENDIX IV

Table 6: Kruskal-Wallis H Test for post thawing evaluation of different holding temperatures

	Post Thaw Viability	Post Thaw Motility	Post Thaw Progressive Motility	Post Thaw Morphology
Chi-Square	.049	.517	.398	.935
Df	3	3	3	3
Asymp. Sig.	.997	.915	.941	.817

Table 7: Mean Ranks for post thawing evaluation of different holding temperatures

	Temperature	N	Mean Rank
Post Thaw Viability	4	8	16.69
	15	8	16.81
	25	8	16.63
	37	8	15.88
	Total	32	
Post Thaw Motility	4	8	16.13
	15	8	15.00
	25	8	16.56
	37	8	18.31
	Total	32	
Post Thaw Progressive Motility	4	8	17.19
	15	8	15.25
	25	8	15.75
	37	8	17.81
	Total	32	
Post Thaw Morphology	4	8	19.25
	15	8	15.31
	25	8	15.69
	37	8	15.75
	Total	32	

APENDIX V

Table 8: Wilcoxon Signed Ranks Test for fresh semen evaluation and post thawing evaluation

	Post Thaw Viability – Fresh Viability	Post Thaw Motility – Fresh Motility	Post Thaw Progressive Motility – Fresh Progressive Motility	Post Thaw Morphology – Fresh Morphology
Z	-4.601[b]	-4.283[b]	-1.826[b]	-2.340[c]
Asymp. Sig. (2-tailed)	.000	.000	.068	.019

b. Based on positive ranks.
c. Based on negative ranks.

APENDIX VI

Table 9: Mean Ranks for fresh semen evaluation and post thawing evaluation

		N	Mean Rank	Sum of Ranks
Post Thaw Viability – Fresh Viability	Negative Ranks	27[a]	18.89	510.00
	Positive Ranks	5[b]	3.60	18.00
	Ties	0[c]		
	Total	32		
Post Thaw Motility – Fresh Motility	Negative Ranks	26[d]	18.96	493.00
	Positive Ranks	6[e]	5.83	35.00
	Ties	0[f]		
	Total	32		
Post Thaw Progressive Motility – Fresh Progressive Motility	Negative Ranks	19[g]	17.95	341.00
	Positive Ranks	12[h]	12.92	155.00
	Ties	1[i]		
	Total	32		
Post Thaw Morphology – Fresh Morphology	Negative Ranks	7[j]	15.64	109.50
	Positive Ranks	22[k]	14.80	325.50
	Ties	3[l]		
	Total	32		

a. Post Thaw Viability < Fresh Viability
b. Post Thaw Viability > Fresh Viability
c. Post Thaw Viability = Fresh Viability
d. Post Thaw Motility < Fresh Motility
e. Post Thaw Motility > Fresh Motility
f. Post Thaw Motility = Fresh Motility
g. Post Thaw Progressive Motility < Fresh Progressive Motility
h. Post Thaw Progressive Motility > Fresh Progressive Motility
i. Post Thaw Progressive Motility = Fresh Progressive Motility
j. Post Thaw Morphology < Fresh Morphology
k. Post Thaw Morphology > Fresh Morphology
l. Post Thaw Morphology = Fresh Morphology