UNIVERSITI PUTRA MALAYSIA

EFFECT OF EDIBLE-BIRD'S NEST IN TRIS AND BIOXCELL EXTENDERS ON BULL SEMEN CRYOPRESERVATION

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DAYANG RAKHMIOKTALEAWATTY YUSOP

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It is hereby certified that I have read this project paper entitled "Effect of edible-bird's nest in Tris and Bioxcell extenders on bull semen cryopreservation", by Dayang Rakhmioktaleawatty Yusop 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 – Project.

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DEDICATION

This thesis is dedicated to

My parents, my teachers, and my friends

For their profound gratitude

And

School of Chemical Sciences and Food Technology, UKM

(Prof Salam and Ms. Etty)

For their valuable hydrolysed edible-bird nest (EBN).

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek

KESAN SARANG BURUNG BOLEH MAKAN DALAM PENGEKAL TRIS AND BIOXCELL TERHADAP PENGKRIOAWETAN SEMEN LEMBU

oleh

Dayang Rakhmioktaleawatty Yusop

2016

Penyelia: Dr. Nurhusien Yimer

Penyelia Bersama: Prof. Madya Dr. Rosnina Yusoff dan Prof. Dr. Abd Wahid Haron

Tujuan kajian ini adalah untuk menilai kesan penambahan sarang burung boleh makan (EBN) dalam pengekal Tris (Tr) dan Bioxcell (Bx) terhadap kualiti semen lembu selepas pengkrioawetan. Sejumlah 12 sampel semen lembu diambil menggunakan kaedah elektroejakulasi. Sampel dinilai kualitinya berasaskan % kemotilan umum dan progresif, kebolehidupan, dan morfologi abnormal, dalam keadaan segar dan selepas pengkrioawetan. Sampel segar dilarut dan dikekalkan mengguna pengekal yang mengandungi 0 (kawalan), 0.03, 0.06, dan 0.12% EBN, disejukkan kepada 4°C selama 3 jam sebelum diisi ke dalam straw 0.25 mL dan di sejuk beku dalam nitrogen cecair kepada -196°C selama 48 jam. Hasil ujikaji

menunjukkan tiada perbezaan (P>0.05) tererti dalam parameter di kalangan pengekal dan pengekal dengan kawalan. Namun, pengekal yang ditambah 0.12% EBN menunjukkan % keabnormalan yang paling rendah yang menghampiri sampel semen segar. Tambahan pula, Bx menunjukkan % keabnormalan yang lebih rendah daripada Tr untuk penambahan EBN yang sama, menunjukkan perlindungan yang lebih telah berlakunya terhadap semen dalam pengkrioawetan. Kesimpulannya, EBN pada kepekatan yang diguna dalam kajian ini tidak meningkatkan secara tererti kualiti sperma selepas penyejukbekuan.

Kata kunci: pengkrioawetan semen, sarang burung boleh makan, lembu, pengekal Tris dan Bioxcell

ABSTRACT

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

EFFECT OF EDIBLE-BIRD'S NEST IN TRIS AND BIOXCELL EXTENDERS ON BULL SEMEN CRYOPRESERVATION

By

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2016

Supervisor: Dr. Nurhusien Yimer

Co-supervisor: Assoc. Prof. Dr. Rosnina Yusoff and Prof. Dr. Abd Wahid Haron

The aim of this study was to evaluate the effect of supplementing Tris (Tr) and Bioxcell (Bx) extenders with edible-bird's nest (EBN) on the quality of bull sperm upon cryopreservation. A total of 12 semen samples were collected from mature bulls by electroejaculation. A total of 12 semen samples were collected from mature bulls by electroejaculation. The semen samples were evaluated both freshly and after cryopreservation for quality based on % sperm general and progressive motility (under a microscope), viability and abnormal morphology (using eosin-nigrosin stain). The fresh samples were then diluted and extended using the two extenders containing 0%

(control), 0.03%, 0.06%, and 0.12% of EBN. Chilled at 4°C for 3 hours before packaged into 0.25 mL straws and frozen into liquid nitrogen (-196°C) for 48 hours. Results for both extenders revealed insignificant differences (P>0.05) in all parameters between the different EBN treatment groups and control. Although not significant, 0.12% EBN in both extenders showed the lowest % abnormality, close to the fresh sample reading. Moreover, Bx tended to show reduced % abnormality than Tr for the same EBN treatments, implying more protective role played. In conclusion, EBN concentrations used in this study do not significantly improve sperm quality after freezing. Further research should be carried out with increased sample size and dosage of EBN in order to clarify the minimal improvement observed for parameters such as sperm abnormality.

Keywords: Semen cryopreservation, edible-bird nest, bulls, Tris and Bioxcell extender

1.0 INTRODUCTION

Semen cryopreservation is a biotechnology to preserve and store sperm for a short or long time for many purposes such as in assisted reproduction technologies (ART), species or breed conservation and clinical medicine (Barbas and Mascarenhas, 2009). However, the quality and life-span of frozen-thawed semen reduces from its fresh quality due to the effect of storage temperature, cooling rate, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition, hygienic control and chemical composition of the extender (Barbas and Mascarenhas, 2009). Extender is a medium for semen cryopreservation which basically contain nutrient, energy sources such as glucose and fructose, buffer solution, antibiotic and cryoprotectants such as glycerol in order to maintain the metabolic activity of the sperm as well as their motility, viability and morphology (Bearden *et al.*, 2004). According to Karunakaran *et al.* (2012), antioxidants are endogenously present in the seminal plasma of the bull. However, when the production of ROS exceeds the antioxidant capacity of the seminal plasma during cryopreservation, it leads to oxidative stress which is harmful to sperm that results in loss of sperm motility and viability (Rahman, 2013).

Edible-bird's nest (EBN) is a dried glutinous secretion from salivary glands of several different swiflet species (mainly from *Aerodramus fuciphagus*) and composed mainly of glycoproteins (Deraman, 2012). EBN contains 35.80% of protein and

46.47% of carbohydrate (9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fructose) (Lee *et al.*, 2015). In addition, EBN also contains aspartic acid (6.12%) and serine (4.54%) as major amino acids (Lee *et al.*, 2015) and sialic acid that can act as an antioxidant (Deraman, 2012). Another study by Movahedian *et al.* (2006) reported that serine also has antioxidant activity. Thus, being rich in essential nutrients and antioxidant activity, EBN is believed to have a positive effect in maintaining sperm quality through a possible mechanism of reduction of spermatozoa oxidative stress as well as acting as additional source of energy during cryopreservation.

1.1 Objectives

1. To determine the effect of EBN in Tris and Bioxcell extenders on post-thaw quality of bull sperm after cryopreservation.
2. To compare between Tris and Bioxcell extenders treated with similar concentrations of EBN on post-thaw semen quality of bull sperm.

1.2 Hypothesis

Treatment of semen extenders (Tris and Bioxcell) with EBN used for bull semen cryopreservation produces a significant improvement in post-thaw quality compared to untreated controls.

2.0 Literature Review

2.1 Semen collection method

There are several methods for semen collection in bull such as collecting the semen from vagina of naturally mated cow, transrectal massage of the vesicular glands and ampullae of bull, semen collection with the artificial vagina (AV) and electroejaculator (Bearden *et al.,* 2004). Electroejaculation method for dairy bulls usually preferred under certain condition such as in older bull and have low sexual activity but in beef bulls that to be used in natural mating, it is extensively used for semen quality evaluation (Youngquist and Threlfall, 2007). The feces are removed before inserting the lubricated probes into the rectum in which the electrodes immediately positioned over the accessory glands. The stimulation is start with low voltage and then gradually increased with a few volts at a time with 4 seconds rest in which the voltage is returned to 0, and that pattern is continued until the bull is stimulated to ejaculate. The voltage ranges are from 0 to 30 (Bearden *et al.,* 2004). According to Youngquist and Threlfall (2007), the amount of electrical stimulation should be gauged at all times by the response of the bull. Since the elecroejaculator is function by stimulating the pelvic parasympathetic and sympathetic nerve, the nerves of the rear legs also involve and caused the stiffening of the hindlimb (Bearden *et al.,* 2004), thus the bull should be restraint on non-slippery floor. There are several factors of difficulties in obtaining the semen samples such as insufficient electrical stimulus due to presence of an excessive amount of air or feces in the rectum,

inadequate probe size, and weak batteries in the electroejaculator (Youngquist and Threlfall, 2007).

2.2 Semen quality evaluation

There are many parameters that can be evaluated to determine the semen quality of the animals especially in bulls such as semen density and volume, gross motility, individual motility, viability and sperm morphology (Youngquist and Threlfall, 2007). The volume of semen that can be ejaculate by a bull normally ranges from 2mL to 10mL and the appearance is creamy white in colour. However, low concentration of semen may appear watery (Bearden *et al.,* (2004). The sperm motility includes the gross or general and individual motility which are very important in semen quality evaluation because the fertility of the bull is highly correlated to the motile sperm inseminated (Bearden *et al.*, (2004). Individual motility such as progressive motility (forward movement), rotating, vibrating and backward movements are normally seen but in order to get good semen quality, there should be 70-90% of motile spermatozoa (progressive motility). The motility of the spermatozoa can be evaluate using light microscope which is very subjective or using Computer Automated Semen Analyzer (CASA) which has potential to increase objectivity of analysis (Baracaldo *et al.*, 2007).

Sperm morphology is evaluated using eosin-nigrosin stain and observation under light microscope (x100 magnification). Abnormal sperm that exceeds 20% to 25% may affect the fertility thus good semen quality normally have 5% abnormalities (Bearden *et al.*, (2004). According to Youngquist and Threlfall (2007), eosin-nigrosin is usually very hypotonic and might cause hypotonic shock to live spermatozoa, thus the stain should be dry faster by using warm slide. At the same time of observing for abnormal morphology, the live and dead sperm can also be counted. Sperm cells that stained pink were counted as dead, whereas sperm cells with white colour head as alive because the membrane integrity of live sperm still intact thus it will not take the stain (Youngquist and Threlfall, 2007).

2.3 Semen cryopreservation

2.3.1 Semen extender

Yolk-phosphate, yolk-citrate, yolk-tris, whole homogenized milk and skim milk semen extenders are available to be used for semen cryopreservation. In addition, Bioxcell extender is the commercial extender that can be used by diluting the extender using 1:5 dilution ratios with distilled water (IMV Technologies France). Tris extender which also been called yolk-tris extender are using fresh egg yolk which mixed into the formulation of the Tris extender (Bearden *et al.*, 2004). There are some criteria for good quality of semen extender such as has osmotic pressure and electrolyte balance, energy sources from glucose, fructose and lactose, able to give protection against cold shock like lipoprotein

in egg-yolk, antibiotics to get bacteria free semen, cryoprotectant such as glycerol, and lastly buffering capacity to maintain the pH of diluted semen (Chaudhari and Mshelia, 2002). The pH of the extender might change due to the product of metabolism in stored sperm thus buffer is important in semen extender as well as cryoprotectant to protect the sperm during freezing and thawing (Bearden *et al.*, 2004).

2.3.2 Chilling, packaging, and freezing

Chilling of the semen is done after extending procedure in which the fresh semen samples are diluted into semen extenders at 37°C. Chilling is one of the cryopreservation processes to reduce the environmental temperature of the semen from 37°C to 4°C to prevent cold shock of the sperm before freezing (Asr *et al.*, 2011). The chilled semen is then packaged either in ampules or straws (0.5mL or 0.25mL straws). However, recent studies mostly packaged in straws for artificial insemination (AI) purpose. Moreover, according to Bearden *et al.* (2004), the straws give increased sperm survival and slight increase in conception rate compared to ampule.

The packaging process can be done in room temperature but most preferable one is in cold cabinet to maintain the semen equilibration temperature. The straws are then sealed using seal powder before freezing. Freezing of the straws is done by placing the straws horizontally on a tray or rack above the liquid nitrogen about 4cm – 5cm. According to Bearden *et al.* (2004), the cold nitrogen vapours will freeze the semen at

about the desired rate and some research reported the semen can be frozen both too fast or too slowly and rates between 126°C per minute down to 7°C per minute will give good results. However, according to Lemma (2011), rapid cooling rate may cause large intracellular ice crystal formation causing physical damage to the spermatozoa cell membranes, thus optimum cooling rate is needed. The frozen straws are then plunged into liquid nitrogen and stored in liquid nitrogen tank.

2.4 Edible bird's nest (EBN)

Edible bird's nest (EBN) is made from a high-protein glutinous secretion of the swiflets' salivary glands that mostly from white-nest swiflet (*Aerodramus fuciphagus*) and the black-nest swiflet (*Aerodramus maximus*) (Babji *et al.*, 2015). The swiflets' nests are being consumed by the people in Chinese and Southeast Asia for a very long time and it also had been used in traditional Chinese medicine (Saengkrajang *et al.*, 2013). Besides, it is known for their health enhancing effects in human such as antiaging, growth promoting and immunoenhancing properties (Deraman, 2012). According to Lee *et al.* (2015), EBN contain 35.80% of protein and 46.47% of carbohydrate which are 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fructose. A study by Ma *et al.* (2012), EBN give significant effect to testosterone level which improve sexual function of castrated rats. Besides, Deraman (2012) reported that EBN has potential source of natural antioxidants and sialic acid is believed as an endogenous antioxidant factor.

3.0 Materials and Methods

3.1 Animals

Seven sexually matured bulls from Ladang 16, Taman Pertanian Universiti (TPU), Universiti Putra Malaysia were used for semen collection. The breeds of the bulls include Kedah-Kelantan cross, Friesien-Jersey, and Brangus with the range age of 2 to 7 years old. Twelve semen samples were collected from the bulls within three weeks period of time with minimal rest of four days for each bull that used for the next collection. On the day of collection, the bulls were transferred from their paddock into the crash in beef unit area for physical restraint.

3.2 Experimental design and treatment groups

Twelve semen samples were collected and each of the fresh semen sample was evaluated and then diluted in Tris and Bioxcell extenders that contain 0.00%, 0.03%, 0.06%, and 0.12% of edible-bird nest (EBN); of which both extenders that contain 0.00% of EBN were used as controls. Thus, there were a total of eight treatment groups for each sample including the control. The diluted semen was then processed and stored in liquid nitrogen (-196°C). After 48 hours of storage, the semen was thawed and evaluated for their quality (general motility, progressive motility, viability, and abnormal morphology of the sperm).

3.3 Semen collection procedure

Semen was collected using automatic electroejaculator (ElectroJac6, by IDEAL® Instruments) every morning. A small container contain a tube rack was provided before semen collection was done which filled with water with temperature of 39°C to 40°C to keep the semen later. The preputial hairs was trimmed short to prevent contamination from urine and feces that might have in the hair and then the prepuce and penis of the bull were cleaned by using diluted chlorhexidine and warm water, and finally dried with paper towel. After that, the bull's feces were removed by rectal palpation using rectal glove to increase the contact of the electroejaculator electrode in the rectum during semen collection. The lubricated electrode was then inserted into the rectum. Minimum of three people was involved in this collection; one person was collected the semen using sterile graduated collection vessel covered with paper towel, one person was handling the electrode, and another person was controlling the voltage of the electroejaculator. Low voltage was used for the start of stimulation which increased gradually until the erection of penile occurs and the ejaculation occurred. After the semen was collected, the vessel contain the semen was kept in a container that had been prepared earlier. The temperature should be maintained with range of 35°C to 38°C to prevent temperature shock for the semen.

3.4 Semen evaluation

Semen evaluation was done in Theriogenology and Cytogenetic laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Fresh and post-thawed semen quality was evaluated. For fresh semen, the volume and colour of the semen was recorded directly after semen collection was done. The colour include watery, thin milky, milky, and creamy. After that, a drop of fresh semen was put on the warm glass slide and was observed under light microscope using 40 x magnifications to give score of the wave pattern from 0 to 5; 0 is no wave pattern observed, 1 is slow motion, 2 is moderate motion, 3 is fast motion, 4 is dense (dark) and fast motion, 5 is dense (dark) and swiping motion (Laboratory Manual Technique in Theriogenology and Cytogenetics, Faculty of Veterinary Medicine UPM)

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Concentration of the semen, general and progressive motility of the spermatozoa were evaluated by diluting the semen with normal saline with 1:40 dilution ratios. The concentration of semen was evaluated by using Computer Automated Semen Analyzer (CASA) in which 10µl of diluted semen was placed on each chamber of a 37°C heated glass slide and covered with rectangle cover slip, and then inserted into CASA. The semen sample with concentration lower than 200 million per mL (M/mL) were rejected. After that, another 10µl from diluted semen was placed on another warm glass slide without cover slip to evaluate the general motility of the sperm using 100 x magnifications of light microscope. Another 10µl was placed on warm glass slide and covered with cover slip to

evaluate the individual motility (progressive motility) using 400 x magnification under light microscope.

For viability evaluation, the live spermatozoa percentage was calculated. A 10µl of fresh semen was mixed with 30µl of eosin-nigrosin stain on warm glass slide and left for 3 minutes. After 3 minutes, 10µl from the mixture was placed on a cleaned glass slide and then thin smear was done and it was air dried for at least 10 minutes. A total of 200 spermatozoa were examined under oil magnification of light microscope. Sperm cells that stained pink were counted as dead, whereas sperm cells with white colour head as alive (Youngquist and Threlfall, 2007). At the same time, the sperm morphology also been evaluated. The abnormal morphology that been evaluated were bent tail, coil tail or dag defect, tailless, decapitated, and head abnormalities include tapered, narrowed, and micro head. The value of general motility, progressive motility, viability and abnormal morphology were expressed in percentages (%).

3.5 Semen cryopreservation

3.5.1 Semen extender preparation

Tris buffer solution had been made by mixing 10.08 g of tris (hydroxymethyl) aminomethane, 5.75 g of citric acid monohydrate, and 4.17 g of fructose with sufficient distilled water to make 250 mL of buffer and put in the fridge. Yolk-Tris extender was then prepared a day before semen collection by adding 20% fresh egg yolk into 30mL of

tris buffer solution, and 1.5mL of Pen strep as antibiotic (10,000 Units/mL Penicillin and 10,000 µg/mL Streptomycin) with sufficient distilled water to make 50mL of extender (mixture A). Another similar extender was made but 7% glycerol was added (mixture B). Each mixture was then divided into four cleaned graduated collection vessel with 10mL each, and the remaining mixture was discarded. 0.03%, 0.06% and 0.12% of edible-bird nest was then added into each vessel and was thoroughly mixed.

For Bioxcell extender, it was also prepared a day before semen collection was done. The stock solution of Bioxcell was diluted to 1:5 ratios using distilled water and was divided into four cleaned graduated collection vessels. Similar to Tris extender, 0.03%, 0.06% and 0.12% of edible-bird nest was added into each vessel and was thoroughly mixed.

3.5.2 Extending procedure

The semen was extended after semen evaluation was done. The collected fresh semen and prepared Bioxcell and Tris extender that contain no glycerol were placed into 37°C water bath. The required diluted semen per extender was calculated using the following formula:

$$\text{Number of straws} = \frac{\text{Semen concentration (sperm/mL) x Volume of fresh semen needed (mL)}}{\text{Concentration of semen to be packed in a straw (sperm/straw)}}$$

$$\text{Volume of semen extender required} = \text{Number of straws calculated x volume of straw used (0.25 mL)}$$

Concentration of semen to be packed in a straw was decided based on the quality of the fresh semen. If the general motility of the sperm is more than 80%, the packed semen is 30 x 10^6 sperm/straw and if the general motility of the sperm is 60% to 79%, the packed semen is 40 x 10^6 sperm/straw.

Eight cleaned 10mL glass test tubes were placed in 37°C water bath and the required volume of Bioxcell extender and fresh semen was then transferred using micropipette into four of the glass test tubes and the mixture were gently mixed. For the Tris extender (contain no glycerol), only half of the calculated volume semen extender was mixed with the required semen volume into another four glass test tubes and the mixture were gently mixed.

3.5.3 Chilling procedure

All eight glass test tubes that contain diluted semen with Tris and Bioxcell extender were placed in a beaker contain 37°C of water bath. Then, the beaker was put in the refrigerator for 30 minutes as primary cooling to prevent cold shock to the sperm. After 30 minutes, the water in the beaker was removed and the beaker with all eight glass test tube was placed in a chiller with temperature of 4°C for 3 hours (Bearden *et al.*, 2004).

3.5.4 Packaging and freezing procedure

0.25 mL straws were prepared with labelled and placed in a chiller of 4°C before packaging. Tris extender that contains glycerol was then added half from the calculated volume semen extender into the chilled semen with Tris extender (contain no glycerol). After that, all of the chilled semen were uploaded into straws and sealed, and this procedure was done in the polystyrene box contained ice packs to maintain the temperature of 4°C. Then, another polystyrene box which contains liquid nitrogen was then prepared for freezing procedure.

All of the straws were placed on a cold rack in the polystyrene box and lowered into nitrogen vapour 4 to 5 cm above the liquid nitrogen surface for 5 minutes. Then, the level was lowered down to 1 to 2 cm above the liquid nitrogen surface for 5 minutes and finally, the straws were fully immersed in the liquid nitrogen for 3 minutes. After that, the straws were carefully transferred into a goblet that been cooled in the liquid nitrogen and then transferred into liquid nitrogen tank (-196°C) and were stored for at least 48 hours.

3.5.5 Thawing procedure

The frozen semen was thawed in 37°C water bath for 30 seconds. Then, the straw was dried using paper towel and the sealed end was cut using scissor and the straw was put into 5mL glass tube that placed in 37°C water bath. After that, another sealed was cut

to allow the semen to flow out from the straw. Three straws were randomly chosen to be evaluated for post-thawed quality which includes the general motility, progressive motility, viability, and abnormal morphology.

3.6 Statistical analysis

The semen quality parameters which were general motility, progressive motility, viability, and abnormal morphology of the fresh and post-thawed semen were analysed using one-way analysis of variance (ANOVA) of SPSS 20.0 software. The differences among means were tested for significance by Tukey test in which the value of $P < 0.05$ was considered to be having a significant statistical difference.

4.0 Results

The mean value of semen quality; general motility, progressive motility, viability, and abnormalities were determined using descriptive analysis. The mean semen volume of twelve samples was 7.88 mL while the mean concentration of semen was 1190.17 x 10^6 sperm/mL.

Table 1: Mean and standard deviation of fresh semen sample and post-thawed semen quality parameters processed using Bioxcell extender (Bx) treated with different concentration of EBN.

Parameters	Fresh Semen	Treatment Groups			
		Bx0	Bx1	Bx2	Bx3
General Motility	82.67±10.44 [a]	32.19±17.66 [b]	32.39±17.85 [b]	29.44±17.30 [b]	30.61±19.67 [b]
Progressive Motility	80.67±13.23 [a]	55.11±20.75	54.32±23.20	54.10±20.19	50.81±23.67 [b]
Viability	69.00±8.24 [a]	39.38±14.26 [b]	37.65±14.84 [b]	39.44±14.76 [b]	40.49±13.83 [b]
Abnormalities	5.67±1.83	7.93±4.58	8.32±5.91	8.24±5.32	6.89±4.06

- ab, means in the same row with different letter superscripts are significantly different at α=0.05
- Bx0: Bioxcell with 0.00% EBN (control),
  Bx1: Bioxcell with 0.03% of EBN,
  Bx2: Bioxcell with 0.06% of EBN,
  Bx3: Bioxcell with 0.12% of EBN

Table 2: Mean and standard deviation of fresh semen sample and post-thawed semen quality parameters processed using Tris extender (Tx) treated with different concentration of EBN.

Parameters	Fresh Semen	Treatment Groups			
		Tr0	Tr1	Tr2	Tr3
General Motility	82.67±10.44 [a]	32.25±12.18 [b]	32.58±15.96 [b]	32.90±16.78 [b]	30.89±17.59 [b]
Progressive Motility	80.67±13.23 [a]	58.21±21.38	50.43±24.09 [b]	54.35±20.24	55.97±15.25
Viability	69.00±8.24 [a]	43.65±12.62 [b]	43.01±15.45 [b]	45.40±15.09 [b]	41.07±17.55 [b]
Abnormalities	5.67±1.83	9.61±6.67	9.02±5.81	9.15±6.71	7.33±3.35

- ab, means in the same row with different letter superscripts are significantly different at α=0.05
- Tr0: Tris with 0.00% of EBN (control),
  Tr1: Tris with 0.03% of EBN,
  Tr2: Tris with 0.06% of EBN,
  Tr3: Tris with 0.12% of EBN

From Table 1 and 2, the evaluation data were expressed as the mean ± standard deviation of mean and comparison was done between fresh semen samples with eight different types of treatment groups including the control for Tris and Bioxcell extender. Comparison among the treatment groups was also done for all the parameters of bull semen quality. Both Table 1 and 2 show that there were decreases in semen quality from fresh semen to the post–thawed semen after cryopreservation for all the treatment groups.

For the general motility from Table 1, there was significant difference between fresh semen and all treatment groups but there was no significant difference among the treatment groups. The highest general motility mean recorded was Bioxcell extender with 0.03% of EBN (32.39±17.85), followed by Bioxcell extender with 0.00% of EBN (32.19±17.66), and then Bioxcell extender with 0.12% of EBN (30.61±19.67) and lastly Bioxcell extender with 0.06% of EBN (29.44±17.30). From Table 2, Tris extender with 0.06% of EBN had the highest mean value for general motility (32.90±16.78) followed by Tris extender with 0.03% of EBN (32.58±15.96), then Tris extender with 0.00% of EBN (32.25±12.18) and lastly Tris extender with 0.12% of EBN (30.89±17.59).

The progressive motility shows that there were significant differences between fresh semen and Bioxcell extender with 0.12% of EBN and Tris extender with 0.03% of EBN. The highest progressive motility mean showed by both control groups of Bioxcell and Tris extender. Tris extender with 0.03% of EBN showed the lowest progressive

motility mean among all treatment groups (50.43±24.09) followed by Bioxcell extender with 0.12% of EBN (50.81±23.67).

Average viability of the sperm for Bioxcell extender with 0.12% of EBN was found to be the highest among the Bioxcell treatment groups (40.49±13.83) whereas for Tris treatment groups, Tris extender with 0.06% of EBN showed the highest viability mean (45.40±15.09). There was significant difference between fresh semen and all treatment groups but like the other parameters, there was no significant difference among the treatment groups itself.

Figure 1: Comparison of semen quality parameters of fresh semen sample with post-thawed semen sample of all treatment groups

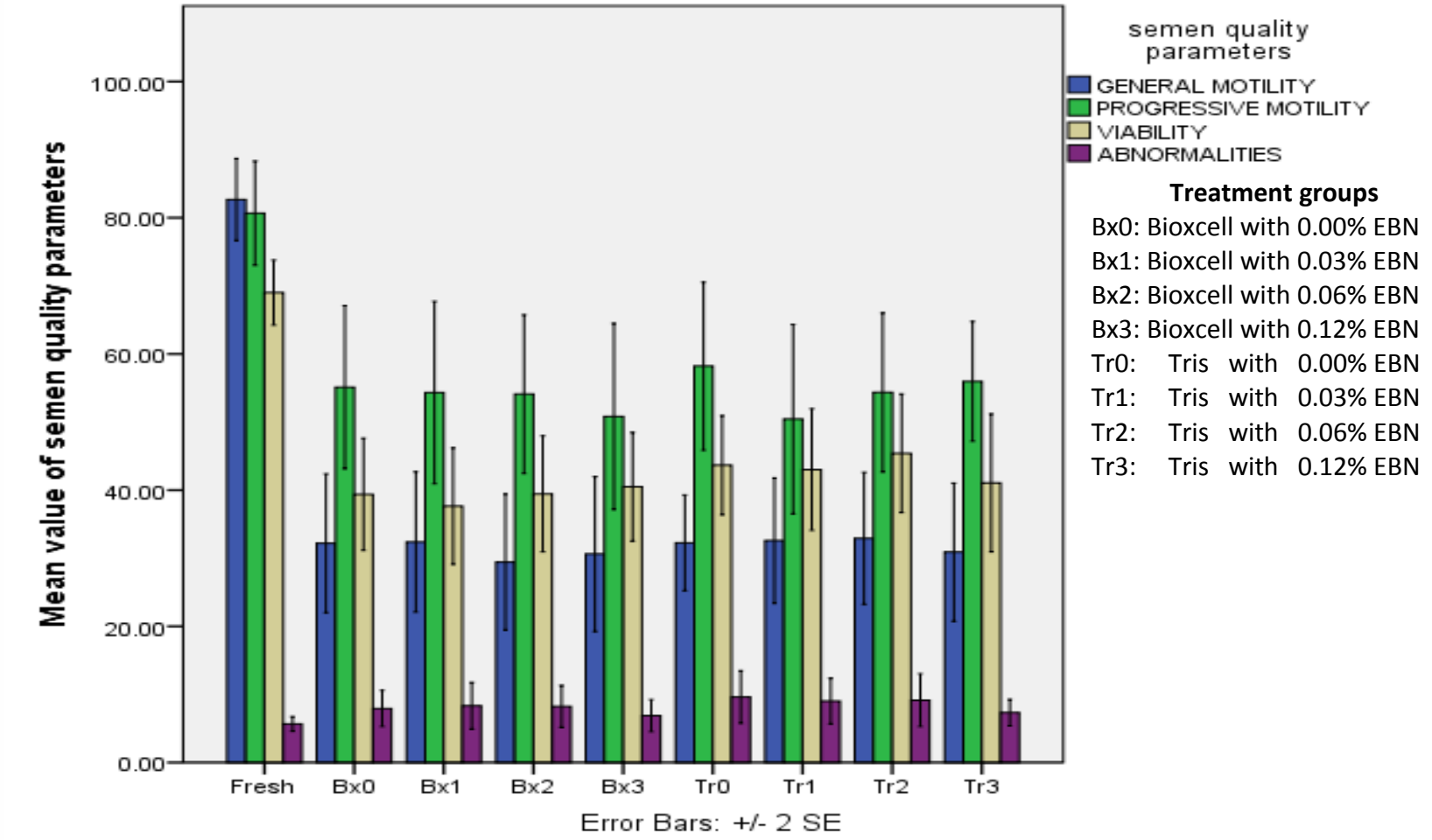
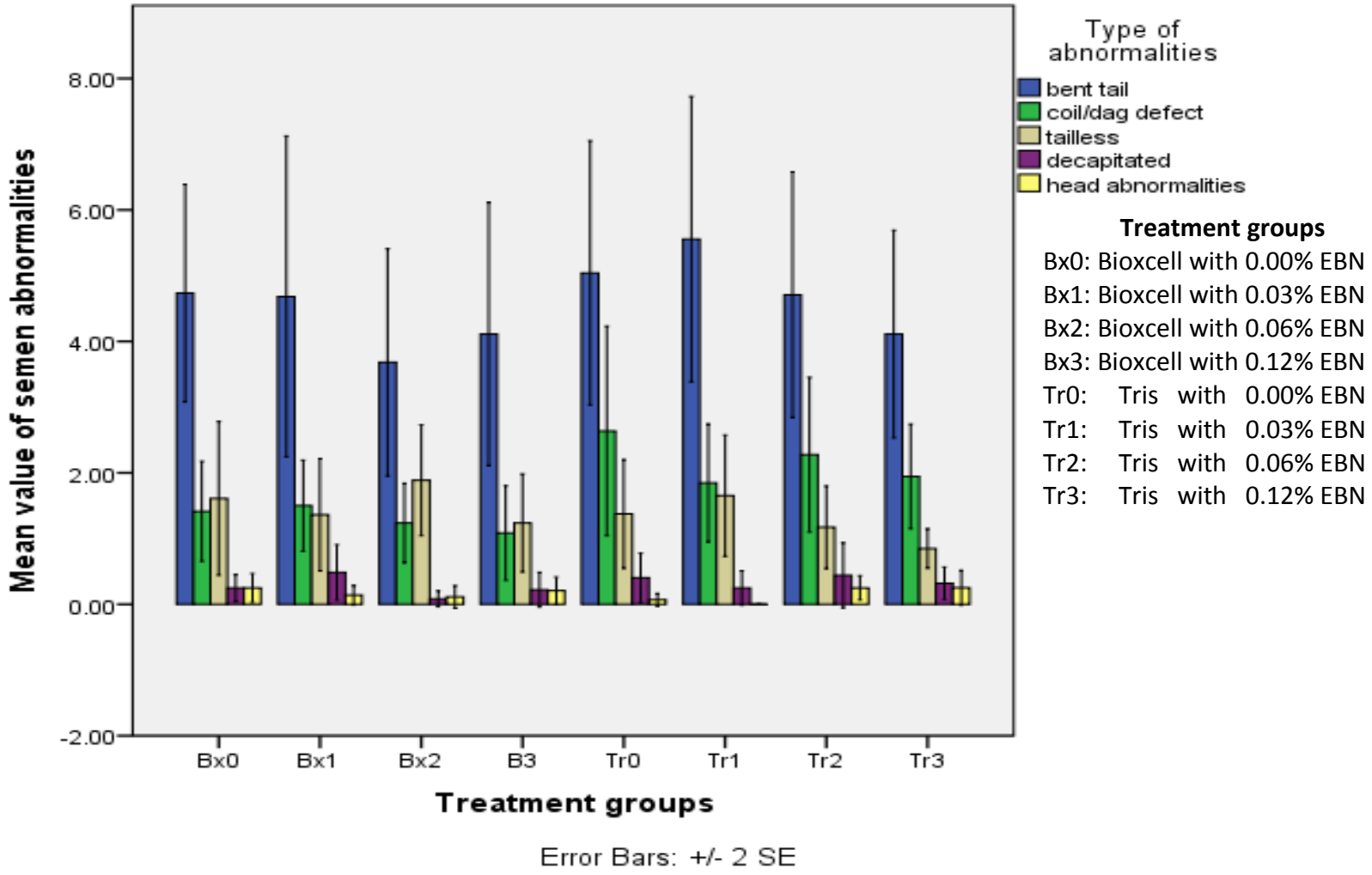


Figure 2: Comparison of abnormal morphology of fresh semen sample with post-thawed semen sample of all treatment groups



Based on Figure 1, for the general motility, progressive motility and viability, the fresh semen shows significantly higher compared to other treatment groups of the post-thawed semen sample. Besides, the abnormalities of the post-thawed treatment groups were higher than fresh semen however statistically there were no significant difference observed between them.

When comparing between control and treated groups with EBN, Bioxcell and Tris extender with 0.12% of EBN show the lowest abnormalities compared to the control group. Generally, viability for Tris extender treatment groups in this study were higher compared to Bioxcell treatment groups. However, statistically there were no significant difference observed among the control and treatment groups for all the parameters.

Based on Figure 2, there was insignificant difference on the types of sperm abnormal morphology between the treatment groups. However, Bioxcell with 0.06% of EBN shows the lowest rate for sperm bent tail and decapitated sperm compared to other treatment groups. Tris with 0.03% of EBN shows the highest rate of sperm bent tail but it shows the lowest head abnormalities. Moreover, Tris with 0.12% of EBN shows the lowest rate of tailless sperm and overall, Tris treatment groups mostly shows the highest rate of coil tail or dag defect sperm compared to Bioxcell treatment groups.

5.0 Discussion

Based on the results of this study, the post-thawed semen of the treatment groups show decrease in semen quality compared to the fresh semen sample. That is might due to temperature and environmental changes, chemical composition of extenders, freezing-thawing procedure, and oxidative stress of the sperm. According to Bansal and Bilaspuri (2011), the reactive oxygen species (ROS) will be increase during cryopreservation, thus when the sperm exposed to the cold shock and atmospheric oxygen during cryopreservation, lipid peroxidation might occur that results to the damage of sperm plasma membrane (Rahman *et al.,* 2012). Therefore it may cause loss of sperm motility, loss in membrane and morphological integrity, impaired cell functions, and induction of sperm apoptosis. Besides, the antioxidant level will be decrease during cryopreservation and thawing process (Bansal and Bilaspuri, 2011).

Comparison between the control and treatment groups revealed that there were no significant improvements on bull semen quality and this might be because the concentration of EBN used in this study not enough to give significant effect on semen quality after cryopreservation. Since there is no study regarding using of EBN to the semen extenders for cryopreservation, but there are many study of using different concentration of EBN that gave significant effect to their studies such as study by Ma *et al.* (2012), edible bird's nest with dosage of 9 mg/kg of bodyweight that used in rats intragastrically reported to result significant effect on serum hormones level. Another

study which was in vitro study by Oryza Oil & Fat Chemical Co., LTD (2013), the recommended dose for topical application of powder form EBN to give effect on wound healing was 0.1% to 0.5%.

The abnormal morphology of the sperm for fresh and post-thawed semen also show no significant difference but the abnormalities are slightly higher for post-thawed semen compared to fresh semen. One of the reasons might be because of sample handling during cryopreservation and thawing process which can accidentally injured the sperm. Besides, the temperature and osmotic effects during cryopreservation and thawing process will cause alterations in cell water volume and lead to mechanical stress on the cell membrane of the sperm (Bailey *et al.*, 2000). Although there were no significant effect in abnormalities in this study, but Tris and Bioxcell extender that contain highest concentration of EBN (0.12%) shows the lowest abnormalities among the treatment groups which nearest to the fresh semen abnormality.

6.0 CONCLUSION

Based on the results in this study, the concentrations of edible-bird nest (EBN) used might not be enough to result significant effect to the bull semen quality after cryopreservation. Although not significant in those concentrations, slight improvement in percentage abnormality at 0.12% EBN for both extenders implying possibly to indicate the importance of increasing concentration of EBN in future studies.

7.0 RECOMMENDATION

The semen sample size and dosage of edible-bird nest (EBN) should be increase in order to clarify the minimal improvement observed for semen quality parameters such as sperm abnormality. Moreover, since this study was using subjective method for semen evaluation of general and progressive motility, Computer Automated Semen Analyzer (CASA) can be used for future research to avoid differentiation in semen evaluation. Edible-bird nest also is believed to have antioxidant activity thus research on the effect of EBN to the oxidative stress level on post-thaw semen quality can be carry out in the future.

8.0 References

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