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Short communication



Comparative Study of Three Different Extenders on Fresh, Chilled and Cryopreserved Mafriwal Bull's Sperm

Nur Amalina N[1], Asmad K[1]* and Zawawi I[2]

[1]*School of Animal Science, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, Malaysia.*

[2]*National Institute of Veterinary Biodiversity (NIVB), Institut Biodiversiti Veterinar Kebangsaan (IBVK) Bukit Dinding, Pahang, 27000 Jerantut, Pahang, Malaysia.*

**Corresponding author E-mail: asmad@unisza.edu.my*

Abstract

The aim of this study is to study the effects of different types of extender and to determine the effects of cryopreservation process (before chilling, after chilling, after cryopreservation) on sperm characteristics and sperm morphology of Mafriwal bulls. Three natural based extender (*Aloe vera* based extender (AVBX), honey based extender (HBX), egg yolk based extender (EYBX)) and a control (Bioxcell®) were used as extender and compared for different semen quality parameters that can be assessed under routine laboratory condition. Semen samples were collected from three sexually matured Mafriwal bulls by using artificial vagina (AV). Samples collected were subjected to four different treatments and was evaluated after dilution with extenders. After diluted, the samples were chilled for 2 hours in 4°C. The semen samples were then packed into 0.25 ml straw for further cryopreservation process. After 24 hours, the cryopreserved semen samples were evaluated. Eosin nigrosin smearing were done to evaluate sperm morphology. The EYBX resulted in the highest percentage of almost all parameters tested in the study followed by Bioxcell®, AVBX and HBX. Semen treated with EYBX and Bioxcell® shows no significant different ($P>0.05$). All treatment shows decrease of sperm characteristics after chilling and frozen-thawed but AVBX and HBX shows rapid depletion of sperm characteristics ($P<0.05$) after chilling process. This study suggests that EYBX is the most suitable extender for cryopreservation Mafriwal bull's semen. However, further study is warranted to investigate the optimum concentration of other raw materials (i.e. *Aloe vera* and honey) in extender for semen cryopreservation.

Keywords: *Aloe vera, honey, egg yolk, Mafriwal bulls, semen cryopreservation, semen extender.*

1. Introduction

Artificial insemination (AI) is a biotechnological advancement that has greatly contribute in genetic improvement whereby a single ejaculate from male are able to inseminate into multiple females. However, AI is not possible without freezing process of male's semen. Whilst, the process of freezing-thawing leads to the generation of reactive oxygen species (ROS) that impair sperm motility, membrane integrity, and fertilizing potential (Hu et al., 2010). To reduce the freezing effect on sperm, semen extender which contains protective ingredient that enable the sperm to survive outside reproductive tract is added. Extender that is added for cryopreservation process helps to protect the sperm from mechanical damage caused by extreme cold temperature and to prevent the formation of ice crystal. There are also several substances such as egg yolk, *Aloe vera* and honey have been increasingly used as cryoprotectant in extender to reduce the damage caused by cryopreservation.

Egg yolk is the most common substance used in extender as cryoprotectant agent as it gives beneficial effect on sperm cryopreservation and act as cryoprotectant for plasma membrane and acrosome [1]. Though egg yolk are known from having a good cryopreservative ability, recent studies shows that there are several disadvantages related to adding egg yolk into extenders such as risk of microbial contamination, with the subsequent production of endotoxins capable of damaging the capabilities of spermatozoa fertilization [1, 2, 3]. Egg yolk could also reduce acrosome integrity after thawing and the viability of sperm in species [4]. The use of egg yolk produce an increase in the viscosity of medium that affect the motility by reducing the speed of spermatozoa movement [2].

Therefore, many researchers have focused on finding an alternative medium as cryoprotectant or at least supplementation that could address the constraint. As *Aloe vera* contains some substances that can act as conventional cryoprotectant, it appear to be a good alternative to egg yolk as *Aloe vera* is a vegetative material [5]. According to Souza et al. [6], *Aloe vera* is an efficient cryoprotectant for peccaries semen chilling and freezing as it provide similar values for sperm morphology, viability, osmotic response, membrane integrity, sperm motility, amplitude of lateral head (ALH), beat cross frequency (BCF), rapid, low and static subpopulation to semen treated with egg yolk based extender. Therefore, *Aloe vera* presents a good potential as an alternatives to egg yolk based extender.

Sugar is one of the essential component of most semen extenders and honey is known to consist primarily sugars such as monosaccharides, disaccharides, oligosaccharides and polysaccharides [7] that act as an energy sources to support spermatozoa survivability during cryopreservation. Honey are naturally a highly concentrated product and has the potential hy-perosmotic extracellular environment around sperm cell that en-hances efflux of intracellular fluid to minimized intracellular ice crystal formation which causes sperm damage during cryopreservation [8, 9]. This mechanism of

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protection gives honey the property of a non-permeable cryoprotectant. Honey is also known to be a potent antioxidant in protecting cells of the various organ in the body from damage due to oxidative stress [10].

There are number of studies conducted to compare each of Tris based extender; EYBX, HBX, AVBX, to commercial based extender but no study is yet done to compare all Tris based extender to a commercial based extender. Therefore, the results obtained from this study may help local farmers and small scale AI centre to gain an alternative source to commercial extender with comparable results for semen cryopreservation. Some content in commercial based extender may be harmful to spermatozoa as stated by Aurich and Spergser [11] whereby a commonly used antibiotic in commercial semen extender, Gentamicin was shown to give adverse impact to sperm motility and viability if the amount of Gentamicin is more than 1g per millilitre [12]. Therefore, by using natural based extender, the amount of antibiotic added in the natural based extender can be controlled accordingly. This study is conducted to investigate the effects of different types of extender on cryopreservation of bull's sperm and to compare the effective extender to be used in the cryopreservation of bull's semen.

The evaluation of semen can be done by using many parameter including semen volume, sperm concentration, sperm motility, proportion of life sperm, proportion of abnormal sperm and several biochemical measurements and functional tests [13]. In certain study, the initial motility, concentration and volume, post thaw motility, percent intact acrosomes, and percent primary and secondary abnormalities are included in semen quality analysis [14].

The aim of this study are to study the effectiveness of different types of extender (egg yolk-based extender (EYBX), honey-based extender (HBX) and *Aloe vera*-based extender (AVBX) and Bioxcell® extender) and to determine the effects of cryopreservation process (before chilling, after chilling, after cryopreservation) on sperm characteristics of Mafriwal bulls.

2. Materials and Methods

This study was designed to evaluate the effectiveness of four extenders on Mafriwal bull's semen cryopreservation. This study was conducted at National Institute of Veterinary Biodiversity, Jerantut, Pahang.

2.1. Experimental Animals

Three sexually matured Mafriwal with varying age and originating from one AI centre were used for this study. They were placed individually in pens at National Institute of Veterinary Biodiversity, Jerantut, Pahang. The body condition score (BCS) of these bulls were in the range of 3 to 4.

2.2. Preparation of Semen Extender

In this study, four different extenders:

i. Egg yolk-based extender
ii. *Aloe vera*-based extender
iii. Honey-based extender
iv. Bioxcell® (control)

Tris stabilizer was prepared first, followed by the addition of either egg yolk (EYBX), *Aloe vera* (AVBX) or honey (HBX). Concentration of each raw material was fixed.

2.3. Preparation of Tris Stabilizer

Tris stabilizer was prepared according to ingredients and formulation listed below [15]:

3.80 g Tris (hydroxymethyl)
2.10 g Citric acid monohydrate
0.05 ml Streptomycin solution
0.03 ml Penicillin solution

The entire ingredient above were diluted with distilled water up to total volume of 100 ml and pH of solution should be adjusted to 6.75 with 10% citric acid. Fresh semen was distributed into four tubes and diluted with different types of extender (Bioxcell®, 20% of EYBX, 30% of AVBX and 2.5% HBX) after evaluated. Bioxcell® was prepared according to manufacturer's instructions (IMV, France).

2.4. Preparation of Egg Yolk- Based Extender (EYBX)

In this experiment, Tris Citric Acid Yolk Extender (TCAYE) was prepared. Commercial chicken eggs are used as the yolk source for TCAYE. The collected pure yolk was then added with Tris stabilizer for about 1:5 ratio to produce 20 % EYBX. The mixture was centrifuged at 2000 rpm for about 15 minutes [16] to remove seminal plasma. The supernatants were collected and 1 % of fructose was added to form a complete and ready to be used TCAYE.

2.5. Preparation of *Aloe vera*-Based Extender (AVBX)

The steps for preparation of AVBX extender were similar with the preparation of TCAYE but with substitution of egg yolk to *Aloe vera* [16].

2.6. Preparation of Honey-Based Extender (HBX)

As for HBX also similar with steps TCAYE except egg yolk was replaced with local commercial honey. The honey was mixed with Tris stabilizer in a ratio of 1:40 to produce 2.5 % HBX [17].

2.7. Semen Collection and Semen Dilution

Semen samples were collected twice a week for two consecutive days by using artificial vagina (AV) where female cattle or steer was used as a teaser. The bull's preputial hair was clipped in preparation for using the AV to reduce microbial contamination of collected semen. During semen collection, the teaser was positioned in front of the bull for mounting. The semen collector should be ready with AV for semen collection. Thermal and mechanical stimulation will be used in AV method to stimulate ejaculation. The liner of AV was filled with water at 40 °C to 45 °C and the inner surface of AV was lubricated with K-Y Jelly lubricant as a precaution steps to avoid the penis from being injured. Sample collected from three Mafriwal bulls were further divided into four test tube. Each test tube containing four different extenders in this study, EYBX, AVBX, HBX and a commercial extender, Bioxcell® as a control. The semen samples were diluted with the ratio of 1:9 (semen:extender).

2.8. Semen Cryopreservation and Evaluation

For semen cryopreservation, the prepared extenders was added into the semen collected from the bulls and were extended to adjust the concentrations of sperm to 20 x 10^6 spermatozoa in 0.25 ml mini straw and it was slowly chilled for 3 hours period in 4 °C. After 3 hours period, the diluted semen was filled in 0.25 ml straw at 4°C working environment and it was kept at the same temperature for another 4 hours to equilibrate. The packed straws filled with diluted semen were placed on racks and for 10 minutes, placed the straw horizontally 4 cm above the surface of liquid nitrogen vapour. Before plunging them into liquid nitrogen, the rack were left to float with the straws on liquid nitrogen for 3 minutes.

The parameters were evaluated for fresh, diluted, after chilled and frozen-thawed semen. For fresh sample, the semen were evaluated immediately after semen collection. A drop of semen were placed on a warm glass slide and covered with cover slide. The sample

will then analysed by using Computer Assisted Sperm Analyzer (CASA). CASA evaluate the semen based on,

i. Sperm motility
ii. Progressive motility
iii. Mean velocities. An average speed for all sperm in the field of view
iv. Pathway velocity
v. Amplitude of lateral head displacement. The average distance that the sperm head "wiggles" back and forth while moving

The morphology of sperm was observed by using eosin-nigrosin (EN) stain. A total of 100 spermatozoa were examined for defect associated with sperm head (detached, tapered, giant and macro head), mid-piece (cytoplasmic droplets, bent and irregular shape) and tail region (broken, bent, coiled, looped tail). Based on the examination, unstained membrane was referred as intact membrane sperm (viable) and the stained membrane referred as damaged membrane sperm.

2.9. Statistical Analysis

Data collected from the study, which is the percentage values for fresh, post chilled and frozen thawed semen were analysed using one-way analysis of variance (ANOVA) with Duncan's multiple comparison test using Statistical Analysis Systems (SPSS) software package to compare the result between all treatments. The result was presented as means with SEM. Statistical significance were considered at $P<0.05$.

3. Results

3.1.1. The Effect of Extenders (Bioxcell®, EYBX, AVBX, HBX) on Fresh Sperm.

The result of the effect of different types of extenders on fresh bull's semen are presented in Table 1. The data shows the result of diluting fresh semen on three different extenders which were Bioxcell® as a control treatment, EYBX, AVBX and HBX. Generally, the data from Table 4.2 shows that Bioxcell® as a control gave the highest percentage of general motility of sperm compared to other treatment. EYBX on the other hand have the highest percentage of progressive motility sperm. Both AVBX and HBX shows lower reading on most of parameters.

Table 1: Mean and standard error of mean (SEM) of fresh semen quality parameters using four different extenders.

Parameter	Extender type			
	Bioxcell®	Egg yolk-based extender (EYBX)	*Aloe vera*-based extender (AVBX)	Honey-based extender (HBX)
General motility (%)	85.4 ± 1.3[a]	79.3 ± 2.2[a]	80.2 ± 2.4[a]	71.7 ± 2.5[b]
Progressive motility (%)	59.2 ± 1.4[ab]	62.1 ± 1.5[a]	56.7 ± 2.6[ab]	53.7 ± 2.6[b]
Velocity distribution (%)				
• Rapid	76.7 ± 1.6[a]	74.9 ± 2.2[a]	72.2 ± 2.7[a]	65.1 ± 3.0[b]
• Moderate	8.7 ± 0.8[a]	4.4 ± 0.5[b]	8.1 ± 0.9[a]	6.3 ± 1.4[ab]
• Slow	10.1 ± 0.9b	10.4 ± 1.3[b]	14.3 ± 1.4[b]	19.4 ± 2.0[a]
• Static	4.5 ± 1.0[c]	10.4 ± 2.0[a]	5.4 ± 1.3[bc]	9.6 ± 1.8[ab]
Pathway Velocity (μm/s)	104.5 ± 5.3b	129.6 ± 4.2[a]	110.0 ± 2.5[b]	100.1 ± 2.5[b]
Lateral amplitude (μm)	6.1 ± 0.3[ab]	6.8 ± 0.3[a]	6.6 ± 0.3[ab]	5.8 ± 0.2[b]
Normal morphology (%)	90.1 ± 1.2	91.6 ± 0.8	90.6 ± 1.4	89.8 ± 1.1

[a,b,c]Values with different superscripts across rows indicate significant differences ($P<0.05$).

3.1.1. General Motility

Bioxcell® as a control treatment demonstrated the highest percentage of general motility compared other treatment. Among all Tris-based extender, AVBX was at peak followed by EYBX and HBX. However, there were no significant different between Bioxcell®, EYBX and AVBX with P value more than 0.05. HBX on the other hand was significantly different ($P<0.05$) to the other treatment.

3.1.2. Progressive Motility

EYBX shows higher percentage of progressive motility sperm compared to other three treatment. HBX was significantly differed to EYBX ($P<0.05$). However, both treatment of Bioxcell® and AVBX were insignificantly differ ($P>0.05$) to both EYBX and HBX.

3.1.3. Velocity Distribution

EYBX shows slightly higher percentage of rapid velocity distribution compared to Bioxcell® as control treatment but both treatment including AVBX were insignificantly different ($P>0.05$). Semen treated with HBX have the least percentage of rapid velocity distribution sperm with a significant different from other treatment. In medium velocity distribution graph, Bioxcell® as a control treatment, have the highest velocity distribution compared to other treatment followed by AVBX. However, Bioxcell® and AVBX were insignificant different ($P>0.05$) but both treatments were significantly differ ($P<0.05$) to semen treated with EYBX. HBX on the other hand have no significant different from the other treatment with P value more than 0.05. For slow velocity distribution, three treatment which include the control treatment, EYBX and AVBX were insignificantly differ from each other ($P>0.05$) eventhough AVBX shows higher percentage of slow velocity distribution sperm. HBX shows the highest percentage of slow velocity distribution and are significantly different ($P<0.05$) with other treatment. In static velocity distribution graph, EYBX shows the highest percentage of non-moving sperm followed by HBX. Both treatments were insignificantly different with each other ($P>0.05$). Semen treated with Bioxcell® and AVBX have a significant different ($P<0.05$) with EYBX.

3.1.4. Pathway Velocity

EYBX have the highest pathway velocity compared to other treatment. Semen treated with EYBX was significantly different ($P<0.05$) to those treated with Bioxcell®, AVBX and HBX. All treatment except EYBX were insignificantly different from each other with P value more than 0.05.

3.1.5. Amplitude of Lateral Head Displacement (ALH)

Semen treated with EYBX had the most amplitude of lateral head displacement compared to control treatment, AVBX and HBX. However, between EYBX, control and AVBX, there were actually no significant different ($P>0.05$) among all treatments. Semen treated with HBX was significantly different ($P<0.05$) with EYBX but insignificantly different with both control and AVBX ($P>0.05$).

3.1.6. Normal Morphology

All treatments used in the study were insignificantly different (P>0.05) eventhough EYBX demonstrated a slightly higher percentage of normal sperm morphology followed by AVBX, Bioxcell® and HBX.

3.2. The Effect of Extenders (Bioxcell®, EYBX, AVBX, HBX) on Chilled Sperm.

The result of the effect of different types of extenders on chilled bull's semen are presented in Table 2.

Table 2: Mean and standard error of mean (SEM) of chilled semen quality parameters using four different extenders.

Parameter	Extender type			
	Bioxcell®	Egg yolk-based extender (EYBX)	*Aloe vera*-based extender (AVBX)	Honey-based extender (HBX)
General motility (%)	85.4 ± 1.3[a]	79.3 ± 2.2[a]	80.2 ± 2.4[a]	71.1± 2.5[b]
Progressive motility (%)	59.2 ± 1.4[ab]	62.1 ± 1.5[a]	56.7 ± 2.6[ab]	53.7 ± 2.6[b]
Velocity distribution (%)				
• Rapid	76.7 ± 1.6[a]	75.0 ± 2.2[a]	72.2 ± 2.7[a]	65.1 ± 3.0[b]
• Medium	8.7 ± 0.8[a]	4.4 ± 0.5[b]	8.1 ± 0.9[a]	6.3 ± 1.4[ab]
• Slow	10.1 ± 0.9[b]	10.4 ± 1.3[b]	14.3 ± 1.4[b]	19.4 ± 2.0[a]
• Static	4.5 ± 1.0[c]	10.4 ± 2.0[a]	5.4 ± 1.3[bc]	9.6 ± 1.8[ab]
Pathway velocity (μm/s)	104.5 ± 5.3[b]	129.6 ± 4.2[a]	110.0 ± 2.5[b]	100.1 ± 2.5[b]
Lateral amplitude (μm)	6.1 ± 0.3[ab]	6.8 ± 0.3[a]	6.6 ± 0.3[ab]	5.8 ± 0.2[b]
Normal morphology (%)	92.1 ± 0.8[a]	90.8 ± 1.1[ab]	89.7 ± 1.1[ab]	88.3 ± 1.0[b]

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

In general, EYBX resulted in the best post-chilled semen quality compared to Bioxcell® and other Tris-based extenders use for the study with higher percentage of most of parameters tested in this study.

3.2.1. General Motility

Generally, there was a decrease in percentage of motile sperm after chilling process (4℃). Bioxcell® demonstrated a higher percentage of motile sperm compared to other treatments in this study (EYBX, AVBX, HBX). However, both EYBX and AVBX treatments were not significantly different (P>0.05) with semen treated with Bioxcell®. HBX on the other hand, was significantly different with other treatments used in this study (P<0.05).

3.2.2. Progressive Motility

Semen treated with EYBX shows a slightly higher percentage of progressive motile sperm compared to other treatment. Both control treatment and AVBX were insignificantly differ from semen treated with EYBX with P value more than 0.05. HBX show the least percentage of progressive motile sperm but semen treated with control and AVBX are insignificantly different (P>0.05) with semen treated with HBX.

3.2.3. Velocity Distribution

In rapid velocity distribution, there were no significant different between control treatment, EYBX and AVBX (P>0.05). However, there was a significant different between HBX and other treatments used in this study. In medium velocity distribution, there was no significant different between Bioxcell® as a control treatment and AVBX. Both EYBX and HBX were insignificantly different (P>0.05) with each other. In slow velocity distribution, HBX demonstrated the highest percentage (P<0.05) of slow velocity distribution sperm compared to Bioxcell®, EYBX and AVBX. There was no significant different (P>0.05) between all treatments except HBX. Semen treated with EYBX shows the highest percentage of static sperm compared to other treatments. However, there was no significant different (P>0.05) between semen treated with HBX and EYBX. Bioxcell® have the least percentage of static sperm compare to other Tris-based extender.

3.2.4. Pathway Velocity

Semen treated with EYBX shows the highest (P<0.05) pathway velocity of sperm compared to Bioxcell®, AVBX and HBX. There was no significant different between the pathway velocities of sperm treated with Bioxcell®, AVBX and HBX with P value more than 0.05.

3.2.5. Amplitude of Lateral Head (ALH)

Semen treated with EYBX had the highest value of the amplitude of lateral head displacement compared to other treatment. However, semen treated with control and AVBX were insignificantly different with semen treated with EYBX (P>0.05). Semen treated with HBX had least amplitude of lateral head displacement but was not significantly different from control and AVBX (P>0.05).

3.2.6. Normal Morphology

Bioxcell® as a control treatment had the highest percentage of normal sperm morphology compared to other treatment. However, both EYBX and AVBX were not significantly different (P>0.05) from control treatment. HBX show the least percentage of normal sperm morphology but both EYBX and AVBX were not significantly different (P>0.05) from HBX.

3.3. The Effect of Extenders (Bioxcell®, EYBX, AVBX, HBX) on Cryopreserved Sperm

The result of the effect of different types of extenders on crypreserved bull's semen are presented in Table 3. Generally, EYBX resulted in the best post-freezed semen quality compared to Bioxcell® and other Tris-based extenders use for the study with higher percentage of most of parameters tested in this study.

3.3.1. General Motility

The EYBX shows significantly highest (P<0.05) percentage of motile sperm compared to other treatments in this study. Semen treated with AVBX shows the least percentage of motile sperm. However, AVBX was insignificantly different from HBX with P value more than 0.05.

3.3.2. Progressive Motility

EYBX shows significantly highest (P<0.05) percentage of progressive motile sperm followed by Bioxcell® as a control treatment. Semen treated with AVBX shows the least percentage of progressive motile sperm. However, AVBX was insignificantly different from HBX with P value more than 0.05.

Table 3: Mean and standard error of mean (SEM) of cryopreserved semen quality parameters using four different extenders.

Parameter	Extender type			
	Bioxcell®	Egg yolk-based extender (EYBX)	*Aloe vera*-based extender (AVBX)	Honey-based extender (HBX)
General motility (%)				
Progressive motility (%)	8.0 ± 2.7[b]	17.6 ± 2.7[a]	0.7 ± 0.2[c]	0.3 ± 0.1[c]
Velocity distribution (%)				
• Rapid	10.4 ± 3.6[b]	20.4 ± 3.3[a]	0.9 ± 0.3[c]	0.4 ± 0.2[c]
• Medium	4.9 ± 1.3[ab]	5.6 ± 1.1[a]	1.4 ± 0.9[b]	2.3 ± 1.7[ab]
• Slow	20.4 ± 4.4[b]	34.6 ± 3.7[a]	18.6 ± 4.2[b]	28.3 ± 5.6[ab]
• Static	64.3 ± 7.6[a]	40.0 ± 5.2[b]	79.1 ± 4.3[a]	63.6 ± 6.7[a]
Pathway velocity (μm/s)	62.9 ± 2.7[b]	80.6 ± 6.4[a]	56.2 ± 6.5[b]	45.4 ± 7.9[b]
Lateral amplitude (μm)	5.2 ± 0.3	5.6 ± 0.3	5.7 ± 0.7	4.8 ± 0.8
Normal morphology (%)	90.2 ± 1.1[ab]	91.3 ± 0.7[a]	89.3 ± 1.1[ab]	88.3 ± 0.9[b]

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

3.3.3. Velocity Distribution

In rapid velocity distribution graph, semen treated with EYBX shows highest percentage (P<0.05) of rapidly progressing sperm after cryopreservation process followed by control treatment. Both AVBX and HBX, however have the least percentage of rapidly moving sperm after cryopreservation process. In medium and slow velocity distribution, EYBX also have the highest percentage of progressing sperm compared to other treatment. In medium velocity distribution graph, there was no significant different (P>0.05) between semen treated with EYBX, control and HBX. Semen treated with EYBX had the least (P<0.05) static sperm compared to AVBX, HBX and control treatments.

3.3.4. Pathway Velocity

EYBX shows a highest (P<0.05) pathway velocity compared to other treatment. Control treatment, AVBX and HBX were insignificantly differ (P>0.05) from each other.

3.3.5. Amplitude of Lateral Head Displacement (ALH)

There were no significant different (P>0.05) on the amplitude of lateral head displacement between all treatments.

3.3.6. Normal Morphology

EYBX had the highest percentage of normal sperm morphology compared to other treatment. However, there was no significant difference (P>0.05) on the percentage of normal sperm morphology of semen treated with control and AVBX. HBX had the least percentage of normal sperm morphology and are significantly different (P<0.05) with EYBX.

3.4. The Effect of Different Type of Extenders on Cryopreservation Process of Bull's Sperm

3.4.1. Bull's sperm treated with Bioxcell® (control)

The result of the effect of Bioxcell® as a control on cryopreservation process of bull's sperm are presented in Table 4.

Table 4: Mean and standard error of mean (SEM) of Bioxcell® as a control on cryopreservation process of bull's sperm.

Parameter	Cryopreservation Phase		
	Diluted semen	Post chilled (4°C)	Frozen-thawed
General motility (%)	85.4 ± 1.3[a]	81.6 ± 2.1[a]	15.3 ± 4.6[b]
Progressive motility (%)	59.2 ± 1.4[a]	46.9 ± 2.2[b]	8.0 ± 2.7[c]
Velocity distribution (%)			
• Rapid	76.7 ± 1.6[a]	72.4 ± 2.7[a]	10.4 ± 3.6[b]
• Medium	8.7 ± 0.8[a]	9.2 ± 1.0[a]	4.9 ± 1.3[b]
• Slow	10.1 ± 0.9[b]	12.4 ± 1.3[b]	20.4 ± 4.4[a]
• Static	4.5±1.0[b]	6.0 ± 1.1[b]	64.3 ± 7.6[a]
Path velocity (μm/s)	104.5 ± 5.3[a]	109.8 ± 3.8[a]	62.9 ± 2.7[b]
Lateral amplitude (μm)	6.1 ± 0.3[b]	7.7 ± 0.2[a]	5.2 ± 0.3[c]
Normal morphology (%)	92.1 ± 0.8	90.1 ± 1.2	90.2 ± 1.1

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

There was a general decrease in post-chilled sperm quality parameters compared to diluted sperm. The motility of sperm in sperm treated with Bioxcell® were slightly decreasing after chilling procedure were done. However, there were no significant different between general motility of sperm in sperm diluted with Bioxcell® before and after chilling (4 °C) procedure with P value more than 0.05. The sperm general motility depleted (P<0.05) rapidly after frozen-thawed procedure.

The progressive motility of sperm treated with Bioxcell® was also decreasing as the cryopreservation process goes on. When the semen was freshly diluted, the progressive motility of sperm was higher (P<0.05). As the sperm went through chilling and frozen-thawed procedure, the progressive motility of sperm depleted significantly (P<0.05) with freshly diluted sperm.

Rapid and medium velocity distribution of sperm treated with Bioxcell® as a control were insignificantly different (P>0.05). However, for both parameters, the velocity distribution of sperm after frozen-thawed procedure decreased significantly with P value less than 0.05. Slow and static velocity distribution of sperm treated with control treatment (Bioxcell®) had no significant different (P>0.05) with each other. For both parameters, the velocity distribution of sperm after frozen-thawed procedure increased significantly with P value less than 0.05.

The pathway velocity of sperm treated with Bioxcell® were insignificantly different (P>0.05) before and after chilling process eventhough the value of pathway velocity after chilled was higher than in diluted sperm. However, the pathway velocity of sperm treated with Bioxcell® after frozen-thawed process were rapidly decreasing (P<0.05). The amplitude of lateral head displacement was significantly higher (P<0.05) after chilled procedure compared to freshly diluted and frozen-thawed sperm. There was no significant difference (P>0.05) in the normal sperm morphology along the phase of cryopreservation processes.

3.4.2. Bull's sperm treated with Egg Yolk-based Extender (EYBX)

The result of the treatment using EYBX on cryopreservation process of bull's sperm are presented in Table 5.

Table 5: Mean and standard error of mean (SEM) of EYBX on cryopreservation process of bull's sperm.

Parameter	Cryopreservation Phase		
	Diluted sperm	Post chilled (4°C)	Frozen-thawed
General motility (%)	79.3 ± 2.2[a]	77.0 ± 3.0[a]	25.6 ± 3.8[b]
Progressive motility (%)	62.1 ± 1.5[a]	48.2 ± 2.2[b]	17.6 ± 2.7[c]
Velocity distribution (%)			
• Rapid	74.9 ± 2.2[a]	67.7 ± 4.1[a]	20.4 ± 3.3[b]
• Medium	4.4 ± 0.5[b]	9.3 ± 1.8[a]	5.6 ± 1.1[b]
• Slow	10.4 ± 1.3[b]	14.2 ± 1.8[b]	34.6 ± 3.6[a]
• Static	10.4 ± 2.0[b]	8.9 ± 1.8[b]	34.6 ± 3.7[a]
Path velocity (µm/s)	129.6 ± 4.2[a]	115.1 ± 7.3[a]	80.6 ± 6.4[b]
Lateral amplitude (µm)	6.8 ± 0.3[a]	7.3 ± 0.4[a]	5.6 ± 0.3[b]
Normal morphology (%)	90.8 ± 1.1	91.6 ± 0.8	91.3 ± 0.7

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

There was a general decrease in post-chilled sperm quality parameters compared to diluted sperm. The motility of sperm treated with EYBX were slightly decreasing after chilling procedure were done. However, there was no significant different between general motility of sperm diluted with EYBX before and after chilling (4 °C) procedure with P value more than 0.05. The sperm general motility depleted (P<0.05) rapidly after frozen-thawed procedure.
The progressive motility of sperm treated with EYBX was also decreasing as the cryopreservation process goes on. When the semen was freshly diluted, the progressive motility of sperm were higher (P<0.05). As the semen went through chilling and frozen-thawed procedure, the progressive motility of sperm depleted significantly (P<0.05) with freshly diluted semen.
Rapid velocity distribution of sperm treated with EYBX after frozen-thawed was significantly different (P<0.05) with freshly diluted and post-chilled sperm. For medium velocity distribution, post-chilled sperm shows a higher percentage (P<0.05) then freshly diluted and frozen-thawed sperm. Slow and static velocity distribution of sperm treated with EYBX had no significant different (P>0.05) with each other. For both parameters, the velocity distribution of sperm after frozen-thawed procedure increased significantly with P value less than 0.05.
The pathway velocity of sperm treated with EYBX was insignificantly different (P>0.05) before and after chilling process eventhough the value of pathway velocity after chilled was higher than in diluted sperm. However, the pathway velocity of sperm treated with EYBX after frozen-thawed process were rapidly decreasing (P<0.05). The amplitude of lateral head displacement was significantly lower (P<0.05) after frozen-thawed procedure compared to freshly diluted and post-chilled sperm. There was no significant difference (P>0.05) in the normal sperm morphology along the phase of cryopreservation processes.

3.4.3. Bull's sperm treated with Aloe vera-based Extender (AVBX)

The result of the treatment using AVBX on cryopreservation process of bull's sperm are presented in Table 6.

Table 6: Mean and standard error of mean (SEM) of AVBX on cryopreservation process of bull's sperm.

Parameter	Cryopreservation Phase		
	Diluted sperm	Post chilled (4°C)	Frozen-thawed
General motility (%)	80.2 ± 0.4[a]	28.6 ± 5.5[b]	2.4 ± 1.0[c]
Progressive motility (%)	56.7 ± 2.6[a]	15.0 ± 3.1[b]	0.7 ± 0.2[c]
Velocity distribution (%)			
• Rapid	72.2 ± 2.7[a]	22.3 ± 4.8[b]	0.9 ± 0.3[c]
• Medium	8.1 ± 0.9[a]	6.4 ± 1.7[a]	1.4 ± 0.9[b]
• Slow	14.3 ± 1.4	17.8 ± 2.6	18.6 ± 4.2
• Static	5.4 ± 1.3[c]	53.6 ± 6.8[b]	79.1 ± 4.3[a]
Path velocity (µm/s)	110.0 ± 2.5[a]	85.6 ± 6.4[b]	56.2 ± 6.5[c]
Lateral amplitude (µm)	6.6 ± 0.3	6.9 ± 0.4	5.7 ± 0.7
Normal morphology (%)	89.7 ± 1.1	90.6 ± 1.4	89.3 ± 1.1

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

There are a rapid decreased in most of parameters tested in this study. In general motility and progressive motility parameter, there is a significant difference (P<0.05) between general motility of sperm during the early stage of cryopreservation process which is freshly diluted, post-chilled and frozen-thawed process. For rapid velocity distribution, there was a decrease in percentage (P<0.05) of rapidly distributed sperm as the sperm went through cryopreservation processes. There were no significant different (P>0.05) between freshly diluted semen and post chilled semen for medium velocity distribution graph. However, there was a rapid decreased (P<0.05) in percentage of medium velocity distribution of sperm after frozen-thawed process. There was no significant difference (P>0.05) on the percentage of slow velocity distribution along the cryopreservation phases. The pathway velocity of sperm treated with AVBX was depleted (P<0.05) after chilling and frozen thawed process. In amplitude of lateral head displacement and normal sperm morphology, both parameters shows no significant different (P>0.05) between the value of sperm along cryopreservation phases.

3.4.4. Bull's sperm treated with honey-based Extender (HBX)

The result of the treatment using HBX on cryopreservation process of bull's sperm are presented in Table 7.

Table 7: The effect of HBX on cryopreservation process of bull's sperm.

Parameter	Cryopreservation Phase		
	Diluted sperm	Post chilled (4°C)	Frozen-thawed
General motility (%)	71.1 ± 2.5[a]	35.7±6.1[b]	2.9 ± 1.8[c]
Progressive motility (%)	53.7 ± 2.6[a]	25.0 ± 4.7[b]	0.3 ± 0.1[c]
Velocity distribution (%)			
• Rapid	65.1 ± 3.0[a]	29.6 ± 5.4[b]	0.4 ± 0.1[c]
• Medium	6.3 ± 1.4	6.1 ± 1.6	2.3 ± 1.7
• Slow	19.4 ± 2.0	18.8 ± 3.5	28.3 ± 5.6
• Static	9.6 ± 1.8[c]	45.7 ± 7.5[b]	63.6 ± 67[a]
Pathway velocity (µm/s)	100.1 ± 2.5[a]	82.9 ± 7.5[a]	45.4 ± 8.0[b]
Lateral amplitude (µm)	5.8 ± 0.2	5.8 ± 0.4	4.8 ± 0.8
Normal morphology (%)	88.3 ± 1.0	89.8 ± 1.1	88.6 ± 0.6

[a,b,c]Values with different superscripts across rows indicate significant differences (P<0.05).

In general, there was rapid decreased in parameters tested in the study. In the percentage of general and progressive motility of sperm, post-chilled sperm percentage have a significant different ($P<0.05$) with diluted and frozen-thawed sperm. For velocity distribution parameter, both moderate and slow velocity distribution shows no significant different between the percentage of cryopreservation phases; freshly diluted semen, post-chilled and frozen thawed semen. For pathway velocity, there were no significant difference between freshly diluted semen and post-chilled semen ($P>0.05$) but frozen-thawed semen are significantly different to both post-chilled and frozen thawed semen. In amplitude of lateral head displacement and normal sperm morphology, both parameters shows no significant different ($P>0.05$) between the value of sperm along cryopreservation phases.

4. Discussion

During cryopreservation processes, sperm cells expose to many physical and chemical stresses. Therefore, it is unavoidable to have decreased in the sperm quality after cryopreservation processed [18, 19]. Besides, other factors such as type of extender used, will also affect the semen quality deterioration. From this study, EYBX shows a better result of sperm quality compared to other treatment including Bioxcell® a commercial extender that commonly used in cryopreserving bull's sperm. The positive effect of egg yolk to reduce cryo-shock effect have been recognized on chilling and freezing of bull semen and other domestic animals. Egg yolk has been regularly used in semen extenders for cryopreservation of sperm. The protective component of egg yolk, specifically the phospholipid portion has been well known for the cryoprotective ability. It was reported that in bovine semen protection was provided by egg phosphatidylcholines liposomes [20]. A study conducted by De Leeuw et al. [21] stated that the exclusion of egg yolk from extender lead to a pronounced drop in the amount of intact cell.

Aloe vera presents a good potential as an alternatives to egg yolk based extender. However, based on the results obtained from this study, AVBX did not performed as well as EYBX and Bioxcell® does. Previous study done by Yong et al. [22] stated that the addition of 10 % AVBX resulted in best sperm viability in the chilling of red tilapia (*Oreochromis niloticus*) and the addition of 30 % AVBX offered the best result in sperm motility score. It also stated that higher *Aloe vera* concentration yield better result. However, current study shows that the addition of 30% AVBX into semen are not comparable to EYBX and Bioxcell® with P value less than 0.05. The differences in result from previous study are because of the study conducted by Yong et al. [22] are on aquatic animal whereas this study focuses more on bull's semen cryopreservation. Since *Aloe vera* have the ability to preserve red tilapia semen, there are probabilities that it may able to preserve semen from other species of animals. However, *Aloe vera* contain phenolics and aloins that disrupt membranes by weakening hydrophobic interaction between hydrocarbon chain in phospholipid bilayers [5] which may results in poor sperm viability. Though, *Aloe vera* contain various polysaccharide [23] which serve as an energy sources for sperm. Thus, there is a need to investigate further the potential of using *Aloe vera* in extender by manipulating the concentration to determine the optimum value of it for bull's or ruminant's sperm cryopreservation.

Honey are naturally a highly concentrated product and has the potential hyperosmotic extracellular environment around sperm cell that enhances efflux of intracellular fluid to minimized intracellular ice crystal formation which causes sperm damage during cryopreservation [8, 9]. However, the advantageous properties in honey listed from previous studies did not shown in the results in this study. From this study, honey shows the least ability to protect sperm from cryo-injury based on parameter tested. A study conducted by Yimer et al. [17] stated that Tris+2.5 honey was not significantly different from Bioxcell® in all of the parameters. This study however, stated otherwise. The diversity of data from previous study might be due to the formulation of Tris from Yimer et al. [17] are different than Nor-Ashikin [15]. Yimer et al. [17] also add 2.5 % honey as a supplementation in their egg yolk-based extender.

In present day, farmers and semen processing laboratories are using commercial extender for semen cryopreservation as it has longer shelf life and give good result for semen cryopreservation eventhough it is a little bit expensive in compare to using natural ingredient as an alternative. Natural ingredient such as *Aloe vera* and honey are easily accessible but it will take time to prepare the extenders. Moreover, the natural-based extenders did not have long shelf compared to commercial extender through the observation during pesent study. The colour and physical structure of all three extenders changes after 2 weeks. Some farmers did not cryopreserve the semen of their animals since they did AI as soon as semen collection was done.

During semen cryopreservation process, the decrease in temperature will cause oxidative stress on sperm membranes. Thus, resulted in damages to sperm organelles and changes in enzymatic activities, associated with reduction in sperm motility, functional membrane integrity and fertilizing ability [24, 25]. After semen collection, freshly collected semen will not be affected by the environmental temperature. However, the quality of sperm will decreases by time. Therefore, it is important to speed up the process of semen cryopreservation to minimize the risk of reducing the semen quality. Chilling process is important for the sperm cell to prevent cold shock or intracellular damage to the cells. During freeze-thawing process, the intracellular antioxidant capacity of sperm decreases with time [25]. Therefore, semen extenders containing cryoprotectant are added to protect sperm from damages during freezing process and to reduce oxidative damage during freeze thawing of bull spermatozoa.

In semen cryopreservation research, motility and membrane integrity are two parameters frequently use to asses sperm survival. However, sperm movement strongly dependant on the condition used during determination and plasma integrity does not guarantee functional integrity of the surviving spermatozoa [21]. The morphology of sperm also play an important roles especially in fertilizing the ova. An abnormal sperm is incapable of fertilizing the ova.

Although the addition of egg yolk into Tris stabilizer gives several disadvantages to spermatozoa, it is proven from present study that it is beneficial as well. Based on similar study done by Büyükleblebici et al. [26]; 20 % (v/v) of egg yolk are added in the Tris based extender as the based for all extender.While this study are using a basic Tris stabilizer with honey without any addition of egg yolks. Therefore, to determine whether addition of honey could improve the quality of chilled or cryopreserved sperm the additional investigation on such combination of honey and egg yolk, and optimum concentration of either honey or *Aloe vera* in extender for bull's or ruminant's sperm preservation is warranted. The use of only honey or *Aloe vera* as in this study might give strong evidence on the beneficial effect of the ingredients itself toward spermatozoa. However, the addition of honey and *Aloe vera* into the Tris stabilizer may alter the pH value of the extender. It is well known that sperm cell can only survive in pH 6.8 to 7 (neutral). The early preparation of semen extender may be one of the causes that the sperm does not survive even after chilling procedure. Therefore, it is advisable to check the pH value of the extender before processing the semen as a precautious step.

5. Conclusion

This study suggests that EYBX is the most suitable extender for cryopreservation Mafriwal bull's sperm. However, further study is warranted to investigate the optimum concentration of *Aloe vera* and honey in extender for semen cryopreservation.

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