

Original Research Paper

Kinetic Motilities of Cryopreserved Bull Spermatozoa: Owing to the Effect of *Eurycoma longifolia* Jack Aqueous Extract

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Abstract: This study was carried out to improve the kinetic motilities of frozen-thawed bull semen diluted with tris-based egg yolk diluent that was supplemented with *Eurycoma longifolia* jack aqueous extract. A total of 24 ejaculates were obtained from six cross-bred bulls using an electro-ejaculator. The extract of *Eurycoma longifolia* jack was distributed into three low doses and three high doses; cryopreserved samples were evaluated into three different times to confirm the results of kinetic motilities through different times and between groups. Path velocity (VAP $\mu\text{m/s}$), progress velocity (VSL $\mu\text{m/s}$), track speed (VCL $\mu\text{m/s}$), lateral amplitude (ALH $\mu\text{m/s}$), Beat Frequency (BCF Hz), straightness (STR %), linearity (LIN %), were evaluated three different times using Computer-assisted sperm analysis. Results revealed that the percentage of VAP, ASL and VCL were higher ($p < 0.05$) in the frozen-thawed semen group supplemented with 5 mg mL^{-1} *Eurycoma longifolia* jack extract (73.19 ± 1.91 , 58.34 ± 2.06 and 117.91 ± 2.68 in first evaluation, then 74.22 ± 2.06 , 57.45 ± 1.72 and 118.92 ± 2.55 in second evaluation and 72.95 ± 2.27 , 56.75 ± 1.30 and 119.07 ± 3.54 in third evaluation; respectively). In conclusion, *Eurycoma longifolia* Jack aqueous extract supplementation to the semen diluent at 5 mg mL^{-1} significantly improved sperm kinetic motilities of frozen-thawed bull semen.

Keywords: Bull Semen, Kinetic Motility, Cryopreservation, *Eurycoma longifolia*, Tongkat Ali

Introduction

The energy and the nutrient materials which maintain mature spermatozoa are found in spermatozoon itself or in their environment. In natural breeding, the ejaculated spermatozoa obtained their feeding and energy from the secretion of female genital tract and that fluid maintain and nurse the spermatozoa until they reach the storage site (Isthmus) or fertilization site (Ampullae) of uterine tube. However, in semen that is collected *in vitro* using one of semen collection methods, spermatozoa utilize what they found in seminal plasma and semen diluent, but, in that case, the energy and nutrient materials are usually limited, leading to decrease the ability of sperm

motility and increase the oxidative stress. Sperm motility is one of most important parameter in evaluation of the semen for natural and Artificial Insemination (AI) breeding (Correa *et al.*, 1997; Verstegen *et al.*, 2002). Clearly, the motility rate of frozen semen decreased owing to freezing shock (Peña *et al.*, 2009; Yimer *et al.*, 2015), decrease the pH in semen medium (Contri *et al.*, 2013) and the oxidative stress (Ashrafi *et al.*, 2013). Moreover, the ability of frozen semen to incubation at body temperature for long period is sharply declined as compared to fresh or chilled semen (Ahmad *et al.*, 2015).

In fact, the evolution of Computer-Assisted Sperm Analysis (CASA) improves the confidence in the precision of semen parameters evaluation. In addition,

CASA affords more parameters than the conventional method; for instance, path velocity, progress velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, elongation and area (Joshi *et al.*, 2003; Kumar *et al.*, 2010). Holt and Palomo (1996) and Farrell *et al.* (1998) found that the estimation of sperm motility value using CASA is much precise with high repeatability than when using the subjective methods. Furthermore, sperm velocity is among the most significant values of seminal quality as a result of its correlation with sperm fertility (Verstegen *et al.*, 2002). Thus, the aim of this study was to find out an organic, natural and that is rich in bioactive components material that could improve and maintain the kinetic motilities of frozen-thawed bull semen. Hence, the fertility rate of AI frozen-thawed straws will be improved.

Eurycoma longifolia Jack (EL) which is known locally in Malaysia as Tongkat Ali. Even so, there are various names of EL in South East Asia countries but Tongkat Ali becomes the global common name of this herbal medicinal plant. Traditionally it is used to treat fever, malaria, erectile dysfunction (Bhat and Karim, 2010). EL has been well described to be anti-oxidant (Varghese *et al.*, 2013), anti-tumour (Al-Salahi *et al.*, 2014; Tong *et al.*, 2015), anti-inflammatory (Han *et al.*, 2016), anti-malarial (Kuo *et al.*, 2004), anti-microbial (Khanam *et al.*, 2015), anti-parasite (Kavitha *et al.*, 2012) and anti-stress (Talbot *et al.*, 2013). It has also been reported to improve sex derive of the male (Ang and Sim, 1997; Low *et al.*, 2013; Ebrahimi *et al.*, 2016) and female (Abdulghani *et al.*, 2012).

Eurycoma longifolia Jack contains quassinoids such as 14, 15-dihydroxy-klaineaneone, eurycomanone, 13, 21-dihydro-eurycomanone, 13 α (21)-epoxy-eurycomanone, 14,15 β dihydroxy-klaineaneone, longilactone and eurycomalactone (Han *et al.*, 2015), eurycomanol, eurycomanol-2-O- β -D-glucopyranoside (Teh *et al.*, 2011), alkaloids, glycosides (Bhat and Karim, 2010), glycoproteins (Sambandan *et al.*, 2006), biophenylneolignans, triterpenes, canthine-6-one and β -carboline (Guo *et al.*, 2005).

Materials and Methods

Selection of Animals

At least four successful ejaculates were obtained from each of six bulls crossbred (Semintal-Brangus) at the farm of University Putra Malaysia (UPM); using an Electro-Ejaculator Technique (EEJ; Sarsaifi *et al.*, 2013). Bulls were selected at age 4.80 \pm 0.59 years old and weigh 573.33 \pm 39.78 kg. All bulls were fed *Brachiaria decumbens* grass and commercial palm kernel cake at a rate of 3 kg/body weight. Furthermore, mineral blocks and water were provided *ad libitum*.

Table 1. The components of tris-based egg yolk diluent

Ingredient	P ₁	P ₂
Tris*	2.24 g	2.24 g
Citric acid*	1.48 g	1.48 g
Fructose*	1 g	1 g
Penicillin-streptomycin**	50000 IU	50000 IU
Egg yolk	20%	20%
Glycerol*	-	12.8%
Distilled H ₂ O	100 mL	100 mL
<i>Eurycoma longifolia</i> ***	Dose depended	Dose depended

(Amirat-Briand *et al.*, 2010; Baiee *et al.*, 2017); *Sigma Aldrich Co. Sigma., St. Louis, MO, USA; **15140122, gibco® USA; ****Eurycoma longifolia*: (TAE009110614) was provided from L Jack (M) Sdn. Bhd, 43500 Semenyih, Selangor; Malaysia

Preparation of Semen Diluent

Tris-based egg yolk diluent was prepared according to Amirat-Briand *et al.* (2010) and modified by Baiee *et al.* (2017). Briefly, the diluent was divided into two parts, (P1 and P2) (Table 1). The second part (P2) had double amount of glycerol to make the final concentration of glycerol after mixing with P1 6.4%. Then, each part was aliquoted into seven test tubes depends on the concentration of EL extract. More over, semen diluent was prepared weekly and stored in -20°C.

Semen Collection and Evaluation

Samples of semen were collected two times a week using EEJ. Bull was prepared prior semen collection by cleaning the preputial orifice and evacuating the rectum. Later on the rectal probe was inserted with gel application on it and EEJ device was switched on and sample was collected using the collecting tube. Bulls did not treat with any sedatives or narcotic drugs. The collected ejaculates were stored at 37°C and taken to the Theriogenology and Cytogenetics lab of Faculty of Veterinary Medicine, UPM for evaluation. Only those with general motility \geq 70%, viability and morphology \geq 85% and concentration \geq 600 \times 10⁶ were included in the study (Baiee *et al.*, 2017). CASA was used for the determination of semen concentration and sperm motility rate. The sperm viability and morphology were evaluates by Eosin-Nigrosin stain (EN) as described by Felipe-Pérez *et al.* (2008). A drop of 10 μ L semen was mixed with 20 μ L of EN stain. The mixture was smeared on glass slide and dried on a slide warmer at 45°C. The slides were then viewed and evaluated under phase-contrast microscope at 400 x for viability or 1000 x for morphology. For each value 200 sperm were counted.

Experimental Design

Seven groups of tris-base egg yolk diluent have been established and EL extract was distributed in six of them at different doses; one group was without adding EL as a

control group, three groups were low doses of EL concentrations, $T_1 = 0.25 \text{ mg mL}^{-1}$, $T_2 = 0.5 \text{ mg mL}^{-1}$ and $T_3 = 1 \text{ mg mL}^{-1}$ and three other groups were high doses of EL $T_4 = 2.5 \text{ mg mL}^{-1}$, $T_5 = 5 \text{ mg mL}^{-1}$ and $T_6 = 7.5 \text{ mg mL}^{-1}$. The kinetic motilities for all samples was checked after 2, 7 and 14 days (d) of storage in liquid nitrogen using CASA technique.

Effect of EL on Kinetic Motility of Frozen Bull Semen Quality

For each frozen-thawed sample, six microscopic fields of both sides of CASA slide were evaluated using CASA. Path velocity (VAP $\mu\text{m/s}$), progress velocity (VSL $\mu\text{m/s}$), track speed (VCL $\mu\text{m/s}$; Fig. 1), lateral amplitude (ALH $\mu\text{m/s}$), beat frequency (BCF Hz; Fig. 2), straightness (STR %), linearity (LIN %), were recorded in all three different time evaluation for all groups.

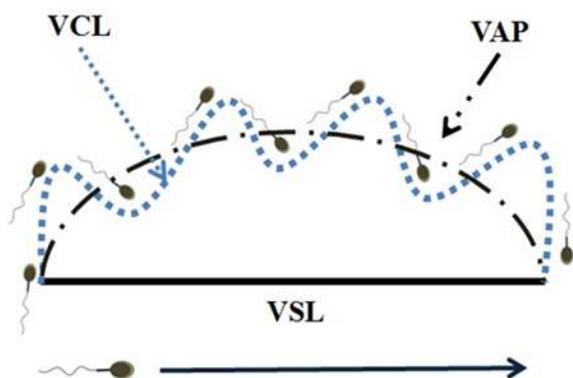


Fig. 1. Diagram shows some kinetic parameters of sperm; VCL = Curvilinear velocity; VAP = Average path velocity; VSL = Straight line velocity

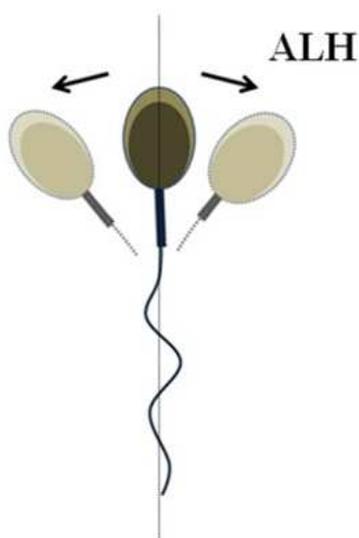


Fig. 2. Head lateral amplitude (ALH)

Statistical Analysis

SPSS program version 22 for Windows (SPSS Inc., Chicago, IL; the USA) was used for data analysis. Normality of distribution of data was checked using Shapiro-Wilk test. Differences among groups and time were determined by two-way ANOVA. Least significant Difference (LSD) test was used as a post hoc test to compare the differences among groups.

Results

The outcomes of different doses of EL extract that were supplemented into tris-base egg yolk diluent on frozen-thawed kinetic motility parameters in bull spermatozoa for 1st, 2nd and 3rd evaluation are summarized in Table 2-4 and Fig. 3, respectively. The percentage of VAP, VSL and VCL were higher ($p < 0.05$) in the frozen-thawed semen supplemented with 5 mg mL^{-1} EL extract (73.19 ± 1.91 , 58.34 ± 2.06 and 117.91 ± 2.68 , in 1st evaluation, then 74.22 ± 2.06 , 57.45 ± 1.72 and 118.92 ± 2.55 in 2nd evaluation and 72.95 ± 2.27 , 56.75 ± 1.30 and 119.07 ± 3.54 in 3rd evaluation, respectively) compared to control and other groups. While the percentages of ALH, STR and LIN were not different among groups ($p > 0.05$).

In addition, adding 5 mg mL^{-1} EL extract in the frozen semen diluent increased the percentage significantly of BCF (24.65 ± 0.99 in 1st evaluation, 25.17 ± 0.83 in 2nd evaluation and 24.56 ± 1.13 in 3rd evaluation, respectively) compared to T_3 , T_6 and control groups (Fig. 3).

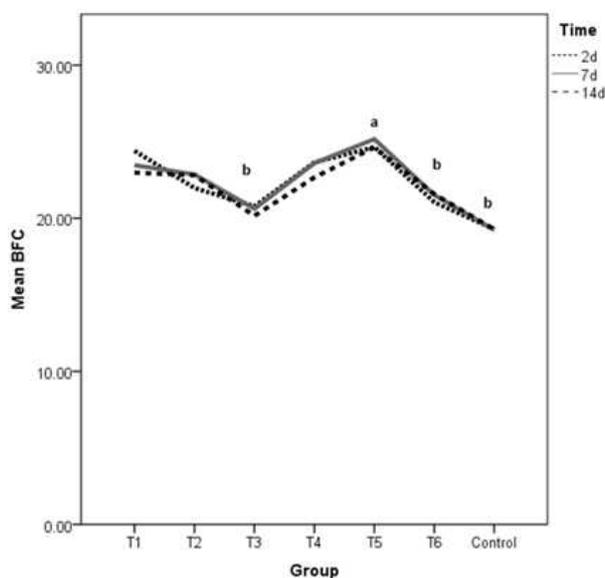


Fig. 3. Mean percentage of Beat frequency (BCF Hz) of frozen-thawed bull sperm evaluated into three different times (2, 7 and 14 d); Values with different superscript differ at ($p < 0.05$), LSD test. $T_1 = 0.25 \text{ mg mL}^{-1}$, $T_2 = 0.5 \text{ mg mL}^{-1}$, $T_3 = 1 \text{ mg mL}^{-1}$, $T_4 = 2.5 \text{ mg mL}^{-1}$, $T_5 = 5 \text{ mg mL}^{-1}$, $T_6 = 7.5 \text{ mg mL}^{-1}$ and Control = 0.0 mg/mL ; $n = 24$

Table 2. Kinetic motilities of frozen bull semen using tris-based egg yolk diluent supplemented with *Eurycoma longifolia* extract after 2 days of cryopreservation (Mean ± SEM)

1st evaluation	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	Control
VAP $\mu\text{m/s}^*$	59.03±1.31 ^b	67.88±1.76 ^c	67.85±1.39 ^c	66.77±1.80 ^c	73.19±1.91 ^a	62.30±1.72 ^b	66.67±1.43 ^c
VSL $\mu\text{m/s}$	46.55±0.87 ^b	53.71±1.03 ^c	53.80±0.76 ^c	52.84±0.90 ^c	58.34±2.06 ^a	47.88±1.29 ^b	52.34±1.22 ^c
VCL $\mu\text{m/s}$	101.21±2.31 ^c	113±3.67 ^{ab}	110.11±2.48 ^{ab}	110.82±3.58 ^{ab}	117.91±2.68 ^a	104.15±2.77 ^c	109.06±1.73 ^b
ALH $\mu\text{m/s}$	6.58±0.30	6.08±0.24	5.84±0.12	6.36±0.20	6.32±0.21	5.78±0.5	5.85±0.25
STR %	78.86±1.20	79.12±0.72 ^a	79.29±1.39 ^a	79.14±0.99 ^a	79.71±1.65 ^a	76.85±1.16 ^b	78.51±0.70
LIN %	45.99±0.79	47.53±0.68	48.86±1.13	47.68±0.96	49.47±1.60	45.97±1.20	47.99±1.11

Values within rows with different superscript differ at ($p < 0.05$), LSD test; * VAP $\mu\text{m/s}$ means Path velocity, VSL $\mu\text{m/s}$ progress velocity, VCL $\mu\text{m/s}$ track speed, ALH $\mu\text{m/s}$ lateral amplitude, BCF Hz beat frequency, STR % straightness and LIN % linearity.

T₁ = 0.25 mg mL⁻¹, T₂ = 0.5 mg mL⁻¹, T₃ = 1 mg mL⁻¹, T₄ = 2.5 mg mL⁻¹, T₅ = 5 mg mL⁻¹, T₆ 7.5 mg mL⁻¹ and Control = 0.0 mg mL⁻¹; n = 24

Table 3. Kinetic motilities of frozen bull semen using tris-based egg yolk diluent supplemented with *Eurycoma longifolia* extract after 7 days of cryopreservation (Mean ± SEM)

2nd evaluation	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	Control
VAP $\mu\text{m/s}^*$	60.44±1.69 ^b	68.58±1.99 ^c	68.56±1.36 ^c	65.84±1.89 ^c	74.22±2.06 ^a	60.29±1.51 ^b	66.32±1.40 ^c
VSL $\mu\text{m/s}$	48.46±1.16 ^b	53.61±1.02 ^c	54.92±0.91 ^{ac}	52.64±1.07 ^c	57.45±1.72 ^a	46.88±1.89 ^b	52.47±1.19 ^c
VCL $\mu\text{m/s}$	103.17±2.61 ^b	112.70±3.66 ^{ab}	110.54±2.16 ^b	110.14±3.63 ^b	118.92±2.55 ^a	103.74±2.55 ^b	108.55±1.92 ^b
ALH $\mu\text{m/s}$	6.68±0.26	6.26±0.21	5.87±0.19	6.44±0.13	6.12±0.19	6.07±0.46	5.86±0.22
STR%	80.18±1.34 ^a	78.17±0.80	80.11±1.16	79.95±0.82	77.40±1.51 ^b	77.75±1.36 ^b	79.12±0.62
LIN%	46.97±0.95	47.57±0.66	49.68±0.75	47.79±0.60	48.31±1.31	45.18±1.01	48.34±1.05

Values within rows with different superscript differ at ($p < 0.05$), LSD test; * VAP $\mu\text{m/s}$ means Path velocity, VSL $\mu\text{m/s}$ progress velocity, VCL $\mu\text{m/s}$ track speed, ALH $\mu\text{m/s}$ lateral amplitude, BCF Hz beat frequency, STR % straightness and LIN % linearity.

T₁ = 0.25 mg mL⁻¹, T₂ = 0.5 mg mL⁻¹, T₃ = 1 mg mL⁻¹, T₄ = 2.5 mg mL⁻¹, T₅ = 5 mg mL⁻¹, T₆ 7.5 mg mL⁻¹ and Control = 0.0 mg mL⁻¹; n = 24

Table 4. Kinetic motilities of frozen bull semen using tris-based egg yolk diluent supplemented with *Eurycoma longifolia* extract after 14 days of cryopreservation (Mean ± SEM)

3rd evaluation	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	Control
VAP $\mu\text{m/s}^*$	59.59±1.75 ^c	67.38±2.05 ^b	68.00±1.32 ^b	65.98±1.88 ^b	72.95±2.27 ^a	58.40±1.49 ^c	66.34±1.25 ^b
VSL $\mu\text{m/s}$	47.83±1.02 ^c	54.38±1.49 ^{ab}	54.15±0.65 ^{ab}	53.01±1.09 ^b	56.75±1.30 ^a	45.09±1.15 ^c	53.11±1.06 ^b
VCL $\mu\text{m/s}$	102.20±3.55 ^b	112.94±3.76	109.90±2.22	110.23±3.59	119.07±3.54 ^a	103.07±2.43 ^b	108.60±1.54 ^b
ALH $\mu\text{m/s}$	6.48±0.27	6.19±0.17	5.83±0.18	6.41±0.14	6.18±0.14	6.02±0.30	5.77±0.25
STR%	80.27±1.35	80.71±0.67	79.63±1.33	80.34±0.88	77.79±1.32	77.21±1.43	80.06±0.60
LIN%	46.80±1.14	48.14±0.63	49.27±1.00	48.09±0.69	47.66±1.29	43.74±1.12	48.90±0.91

Values within same rows with different superscript differ at ($p < 0.05$), LSD test; * VAP $\mu\text{m/s}$ means Path velocity, VSL $\mu\text{m/s}$ progress velocity, VCL $\mu\text{m/s}$ track speed, ALH $\mu\text{m/s}$ lateral amplitude, STR % straightness and LIN % linearity; T₁ = 0.25 mg mL⁻¹, T₂ = 0.5 mg mL⁻¹, T₃ = 1 mg mL⁻¹, T₄ = 2.5 mg mL⁻¹, T₅ = 5 mg mL⁻¹, T₆ 7.5 mg mL⁻¹ and Control = 0.0 mg mL⁻¹; n = 24

The frozen semen was evaluated into three different times after cryopreserved and stored in liquid nitrogen, so the first evaluation was done after 2 d of freezing, the second evaluation was done after 7 d of freezing and the third evaluation was done after 14 d of freezing. Based on the present findings, there were no differences in terms of sperm kinetic motilities among times of evaluation.

Discussion

The highest quality of semen is indicated by high sperm general motility, VAP and VSL movement of sperm cells (Lenz *et al.*, 2011; Ibănescu *et al.*, 2016). To the best our knowledge, this is the first study that investigated the effect of EL aqueous extract added to

tris-based egg yolk diluent on sperm kinetic motilities of frozen-thawed bull semen. Based on the study findings, VAP, VSL, VCL and BCF of frozen-thawed bull semen were significantly improved at 5 mg mL⁻¹ EL extract compared to the control group in all the different times of evaluation. Our findings are in line with several previous studies that have been conducted to investigate different ingredients for improving kinetic motilities. For instance, Gualtieri *et al.* (2014) revealed that adding 10 $\mu\text{g mL}^{-1}$ zinc chloride, 500 $\mu\text{g mL}^{-1}$ D-aspartate and 40 $\mu\text{g mL}^{-1}$ coenzyme Q10 to the bull semen after thawing improved all kinetic motilities of semen as compared to the control group. El-Raey *et al.* (2014) found that addition of 1 mM of melatonin for tris-based egg yolk diluent improved sperm motility, viability values and the

fertility rate as well compared to control group in buffalo bulls. Moreover, Daramola and Adekunle (2015) found that the progressive motility of chilled buck semen were improved in extender that was supplemented with pineapple and cucumber juices compared to control group. Authors concluded that, the anti-oxidant effect of these fruit juice improved the chilled semen quality (Daramola and Adekunle, 2015). Kaka *et al.* (2015a) found that addition of 5 ng mL⁻¹ of α -Linolenic acid to the Bioxcell® diluent improved the VAP, VSL, VCL, STR and BCF of frozen bull semen. Moreover, they found, in another study, addition of 5 or 10 ng mL⁻¹ of α -Linolenic acid to tris-based egg yolk diluent increased the general sperm motility (Kaka *et al.*, 2015b). Iqbal *et al.* (2016) found that the kinetic motilities of frozen-thawed buffalo bull semen were improved using tris-based egg yolk diluent supplemented with 2 mM of L-cysteine when compared with other groups. The improvement of kinetic motilities in these studies was attributed to the anti-oxidant activities of their additives, by improving the membrane fluidity of sperm and resistance against freezing-thawing shock (Kaka *et al.*, 2015b). In an earlier study that was established by Crespilho *et al.* (2012), it was found that lecithin based diluent did not differ much with tris-based egg yolk diluent in terms of sperm's kinetic motilities of frozen bull semen in exception of STR and LIN, the high value of these two parameters was attributed to low viscosity of lecithin based diluent as compared to other diluents such as tris-based egg yolk and skim milk diluents (Thun *et al.*, 2002).

In bulls and other animal species, no study found in the literature assessing the impact of EL extract on frozen-thawed sperm kinetic motilities. The middle piece of spermatozoon contains mitochondria that provide the energy needed for sperm motility from inner and outer ATP store. Hence, increasing ATP in semen diluent leads to an increased in the percentage of sperm motility (Marin-Guzman *et al.*, 2000). When mitochondria provide the spermatozoon with energy to promote its motility and other metabolic operations, the Reactive Oxygen Species (ROS) will increase as by-product of respiration. Likewise, sperm cell membranes are rich with poly unsaturated fatty acids which are sensitive to oxidative stress owing to freezing processes. Therefore, these sites regard to be the main sites of ROS production in frozen semen. EL has anti-oxidant activity (Panjaitan *et al.*, 2013; Varghese *et al.*, 2013; Khanam *et al.*, 2015) and Superoxide Dismutase (SOD) activity (Tambi and Imran, 2010) that can reduce the amount of ROS and protect cell membranes from the deleterious effect of ROS in frozen-thawed semen. Moreover, EL has about 70 bioactive components (Kuo *et al.*, 2004; Bhat and Karim, 2010; Meng *et al.*,

2014) and also EL is well known as energy provider (Bhat and Karim, 2010; George and Henkel, 2014) which may contribute to protect and promote sperm survival and motility by providing energy. On the other hand, we evaluated the kinetic motilities of frozen-thawed semen into three different times: results showed that time of evaluation did not differ between periods of evaluation i.e., the storage semen in frozen form at (-196°C) using liquid nitrogen will not change the kinetic values of sperm even stored for long period. This is in contrast with semen stored in chilled form even in different storage conditions (Bayemi *et al.*, 2010).

Conclusion

In summary, the collected data implied that supplementation of tris-based egg yolk diluent with *Eurycoma longifolia* Jack (Tongkat Ali) aqueous extract at 5 mg mL⁻¹ improved the kinetic motilities of frozen-thawed bull semen.

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Author's Contributions

Falah Baiee, the 1st author, is the person who did the field and laboratory work and wrote the manuscript. Falah Baiee, Prof. Dr. Abd Wahid, Prof. Dr. Rosnina, Prof. Dr. Mohammed Ariff and Dr. NurHusein Yimer, participated in study conception and design, Falah Baiee, Dr. Zaid, Salman, Tarig, Fitri and Dr. Umer acquisition of data, Falah Baiee and Prof. Dr. Ariff Analysis and interpretation of data. All authors critically revised the manuscript for important intellectual content and approved the final draft to be submitted.

Ethics

The experiment was accepted by Institutional Animal Care and Use Committee. Faculty of Veterinary Medicine, UPM, (RO73/2015).

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