UNIVERSITI PUTRA MALAYSIA

CHARACTERISTICS OF FROZEN-THAWED SEMEN OF DIFFERENT CATTLE BREEDS, STORAGE TIME, PACKAGING AND SOURCE OF PRODUCTION

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CHARACTERISTICS OF FROZEN-THAWED SEMEN OF DIFFERENT CATTLE BREEDS, STORAGE TIME, PACKAGING AND SOURCE OF PRODUCTION

NOR LIYANA BINTI MOHD DZIN

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CERTIFICATION

It is hereby I certified that we have read this paper project entitled "Characteristics of frozen-thawed sperm of different breeds, storage time, packaging and source of production", by Nor Liyana binti Mohd Dzin and our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 49999 – Project.

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DEDICATIONS

In the name of Allah, the Beneficient, and the Merciful.

This thesis is dedicated especially to my parents

and my family.

Thank you for your endless love and support.

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ABSTRAK

Abstrak kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD4999- Projek

CIRI SPERMA SEJUK BEKU-CAIR DARIPADA BAKA LEMBU, MASA DAN BEKAS PENYIMPANAN, DAN SUMBER PENGHASILAN YANG BERBEZA

Nor Liyana binti Mohd Dzin

2016

Penyelia: Assoc. Prof. Dr. Rosnina Yusoff

Penyelia bersama: Prof. Dr. Mohamed Ariff Omar

Tujuan kajian ini dijalankan adalah untuk membandingkan kemotilan umum dan progresif, kebolehidupan, dan ciri keabnormalan sperma sejuk beku-cair dari baka lembu, masa dan bekas penyimpanan, dan sumber penghasilan yang berbeza. Enam puluh enam sampel semen sejuk beku daripada sebelas baka lembu, disimpan di dalam ampul kaca atau straw yang dihasilkan dari dalam atau luar negara dari tahun 1975

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sehingga 2010 telah diuji. Keputusan analisis menunjukkan masa dan bekas penyimpanan, dan baka lembu memberi kesan yang bermakna terhadap kemotilan umum dan progresif semen sejuk beku-cair. Manakala, sumber penghasilan memberi kesan yang bermakna terhadap kemotilan progresif, kebolehidupan semen sejuk beku-cair tetapi tidak terhadap motiliti umum. Masa penyimpanan, baka lembu dan sumber penghasilan yang berbeza memberi kesan yng bermakna terhadap kebolehidupan dan ciri keabnormalan semen sejuk beku-cair. Kesimpulannya, lembu baka Holstein mempunyai kualiti semen yang terbaik di dalam kajian ini. Semen sejuk beku-cair yang dihasilkan pada tahun 1999 adalah yang terbaik berbanding tahun yang lain. Semen sejuk beku-cair yang disimpan di dalam straw mempunyai kadar motiliti umum lebih tinggi berbanding ampul kaca. Manakala, semen sejuk beku-cair yang disimpan di dalam ampul kaca mempunyai kadar motiliti lebih tinggi berbanding straw. Semen sejuk beku tempatan telah diuji lebih berkualiti berbanding semen yang diimport.

Kata Kunci : *kemotilan, kebolehidupan, morfologi, sejuk beku-cair, mengkrioawet*

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of requirement for the course VPD 4999 – Project

CHARACTERISTICS OF FROZEN-THAWED SPERM OF DIFFERENT CATTLE BREEDS, STORAGE TIME, PACKAGING AND SOURCE OF PRODUCTION

Nor Liyana binti Mohd Dzin

2016

Supervisor: Assoc. Prof. Dr. Rosnina Yusoff

Co-supervisor: Prof. Dr. Mohamed Ariff Omar

The aim of this study is to compare the general and progressive motility, viability, and abnormality characteristics of frozen-thawed semen of different cattle breeds, duration of storage, packaging, and source of production. Sixty six semen samples from eleven breeds packed either in glass ampules or straws that were produced locally or were imported between 1975 and 2010 were evaluated for the above characteristics. Results showed that general motility was significantly different in the different storage duration, breeds and packaging, but not significantly different

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in the source of production. Progressive motility of thawed sperm was also significantly different in different storage duration, breeds, packaging, and source of production. Viability and abnormality of thawed sperm were significantly different in different storage duration, breeds, and source of production but not significantly different in packaging. Based on the results, it can be concluded that Holstein frozen-thawed semen was the best quality among other breeds. Frozen-thawed semen produced in 1999 was the best quality among the other years. Between packaging, straws have higher general motility compared to glass ampules. While, glass ampules have higher progressive motility compared to straws. Locally produced frozen-thawed semen was examined to be better compared with imported semen

Keywords: *motility, viability, morphology, frozen-thawed, cryopreserved*

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1.0 INTRODUCTION

Recent local beef and dairy production can only fulfil 25.67% and 12.93% of country self-sufficiency (DVS, 2013). Beef and dairy sectors of the livestock industry, however, are still 84.43% and 87.7% in shortage, although the total population of ruminant has increased over the last decade. By 2020, Malaysian government has targeted to raise the self-sufficiency level of beef to 32.7% (MOA, 2015), which can be translated as slaughter of more than 450,000 heads of cattle anually. Moreover, the demand for livestock products as a source of high quality protein is expected to continue to rise with the increase in population and per capita income, consistent with the overall rapid development of the country (Talukder, 2002).

An approach to the development of cattle industry in Malaysia is by increasing the population and productivity of cattle livestock. An artificial insemination (AI) is one good tool. AI can improve the genetics of dairy cattle in the minimum possible time (Malik, 2015). It was the first assisted reproductive technology to be applied commercially for the genetic improvement of animal in the mid-1900s until now (Peter, 2007). Artificial insemination (AI) with cryopreserved semen is the predominant method used in cattle reproduction around the world. According to Mustafa (1974), in Malaysia the first usage of AI using deep frozen semen from ampoules was in 1963. The initial purpose of using AI was the obtain crossbreds which can produce higher milk and beef production, the animal also less susceptible to disease as compared to the imported pure breeds (Raymond, 2010).

The success of AI program depends on several factors. The factors include high quality of semen, genetics, physiology, nutrition and management of cows

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(Walsh *et al.,* 2011). Among the aforementioned, factors for semen quality is considered most critical especially frozen thawed semen mainly biophysical and biochemical characteristics of sperm (Medeiros *et al.,* 2002). Furthermore, Hayashi and Ishobe (2005) reporteded that frozen thawed semen parameters such as viability, motility, and abnormality are crucial factors for AI to be successful. High viability, motility and abnormality of frozen-thawed spermatozoa are significant factor because the relationship between the post-thawing sperm viability and the subsequent conception rate has been reported (Correa JR, 1997).

Semen cryopreservation halts metabolic processes of spermatozoa and allowing indefinite storage without a significant loss of fertility (Lemma, 2011). It is important that spermatozoa are cryopreserved for a longer period without damaging their fertilizing ability. Frozen semen can be stored indefinitely to facilitate their use at any time, depending on the oestrous cycle of females. However, damage of sperm during freezing and thawing can lead to low sperm motility and consequently, fertility.

To maximally utilize the genetics of desired sires on a commercial basis, attempts are made to package a minimal number of spermatozoa per insemination unit without compromising fertility (Foote and Parks, 1993; Shannon and Vishwanath, 1995). Later, the introduction of plastic straws has resulted in the use of smaller volumes (0.25ml and 0.5ml) of frozen semen so that more females can be inseminated from a single ejaculate.

Nowadays, straws are generally popular as they are very convenient and easy to use in artificial inseminator. As different methods of packing have been used, the question is whether the means of packing has any effect on the success rate of

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cryopreservation. In this regard, different authors have compared spermatozoa stored in different packages (Heitland et al, 1996; Kneissl 1993; Park et al, 1995). However, there are some discrepancies in the study and was not fully explained.

There are about twelve breeds of beef and dairy sires cryopreserved in Theriogenology and Cytogenetics Unit, Faculty of Veterinary Medicine, UPM. The semen quality among these breeds in terms of sperm survivability in frozen and after thawing are unknown as there are no study done. Frozen thawed semen quality information is crucial for AI to be successfull.

The application of frozen-thawed semen technology is currently increasing worldwide including Malaysia. Theriogenology and Cytogenetics Unit, Faculty of Veterinary Medicine, UPM is one of the centre that having expertise in the production of high quality cryopreserved semen. The centre has been producing good quality of cryopreserved sperm for many years. Numbers of studies and developments have been done regarding the cryopreservation technique and the effects of sperm quality and fertility. The quality of cryopreserve semen is thought to be as good as imported. However, the quality to compare the cryopreserve semen between local and imported production has not been tested.

Thus, this project was conducted to depict the effects of storage duration and packaging materials on sperm parameters such as motility, viability and abnormality. In addition, seminal characteristics of frozen-thawed semen from different breeds of sires and source of production are also investigated.

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2.0 LITERATURE REVIEW

Factors affecting the success of semen cryopreservation

The combination of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control are the key factors that affect the lifespan of spermatozoa (Yoshida, 2000).

The success of cryopreservation depends upon many other factors, including interactions between cryoprotectant, type of extender, cooling rate, thawing rate and packaging, as well as the individual animal variation (Andrabi, 2007; Clulow *et al*., 2008; Cooter *et al.*, 2005).

Potentially damaging stressors

Cryopreservation induces the formation of intracellular ice crystals, osmotic and chilling injury that causes sperm cell damage, cytoplasm fracture or even effects on the cytoskeleton or genome related structures (Isachenko, 2003).

The cryopreservation protocol causes several damages to sperm by the influence of several factors, namely the dramatic changes in temperatures, submission to osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment (Medeiros *et al.*, 2002). Thus, cryopreservation is directly lethal to a significant proportion of spermatozoa in atypical semen sample and consequently the number of spermatozoa required for successful insemination has to be increased above that needed for fresh spermatozoa (Holt, 2000).

Sperm abnormalities among breeds

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Freezing-thawing increases the maturation of sperm membranes and capacitated acrosome reacted spermatozoa. These alterations may not affect motility but reduces lifespan, ability to interact with the female reproductive tract and sperm fertility (Medeiros et al. 2002).

A study (Menon, 2011) of associations between sperm abnormalities, breed, age, and scrotal circumference in beef bulls was conducted with the following significant findings. The most common defects were detached head (4.86% ± 5.71%), distal midpiece reflex (6.19% ± 9.13%), and bent tail (1.01% ± 1.54%). Breed, age, and scrotal circumference did not significantly affect the prevalence of head or midpiece defects, morphologically normal or abnormal sperm; however, tail defects were more prevalent in Angus and Hereford bulls compared with other breeds. Overall, based on sperm morphology, 1363 (83.0%) bulls were classified as satisfactory potential breeders and the remainder 279 (17.0%) as unsatisfactory (> 30% abnormal sperm, > 20% defective heads, or both). Although not significantly different, the breed with the highest percentage of satisfactory potential breeders was Limousin (90.6%) and the lowest was Hereford (78.8%). That 17% of bulls subjected to breeding soundness evaluation were designated as unsatisfactory solely on the basis of sperm morphology highlights its importance.

Different storage duration

A study by Spalekova (2015) of post-thawed semen characteristics of Pinzgau bull between long-term and short-term storage showed there was no difference in the

effect of storage time on the bull spermatozoa characteristics and observed a high inter-male variability in the susceptibility of bull sperm to cryoinduced damage.

Another study (Malik, 2015) on the effects of long term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed Frisian Holstein bull spermatozoa shows the concentration of sperm during one year storage in liquid nitrogen resulted in similar concentration storage as long as six years. However, the viability and motility sperm thawed storage in liquid nitrogen during six years was lower than storage on the one and two years.

Different types of semen packaging

Different authors have compared spermatozoa stored in different packages (Heitland et al, 1996; Kneissl 1993; Park et al, 1995). Their results showed an effect on spermatozoa quality manifested through reduced motility and conception rate. The reports further stressed the roles of different extenders used, interaction between extender, and means of packaging. However, the reasons for these discrepancies were not fully explained, and it was also not clear in all work how the dimensions of the straws change with volume, in addition to which different extenders and concentration of spermatozoa were used.

Classification of sperm abnormalities

Primary defect is one that originates within the testis during spermatogenesis. Whilst a secondary defects is one that originates within the epididymis. Although the primary and secondary abnormalities are widely accepted, this system of classification often means different things to different practitioners. All head defects such as

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knobbed acrosome, pyriform head, microcephalic sperm, and nuclear vacuoles are considered as primary defects. Dag defect which results from abnormal development of the axoneme and mitochondrial sheath expresses itself as a shattered midpiece within the epididymis and has therefore been called secondary abnormalities by some investigator. However, by definition of origin, the dag defect would be a primary defect (Albert, 2007).

Tolarence level of sperm abnormalities

The tolerable level that have been confirmed by more recent work sperm nuclear (head) defects is in the range of 15% to 20%, whereas acrosomal and tail defect is up to 25% of sperm may be tolerated. At least 70% of spermatozoa should be normal (Raymond, 2007).

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3.0 MATERIALS AND METHOD

3.1 Semen sample

Sixty six frozen semen samples from eleven cattle breeds packed either in glass ampules or straws. The frozen semen were produced locally or were imported between 1975 and 2010. These cryopreserved semen are currently stored in three cryogenic tanks containing liquid nitrogen at the Theriogenology and Cytogenetics Unit, Faculty of Veterinary Medicine, UPM. Details on the semen samples are provided in Table 1.

Table 1 : Frozen semen samples

ID	Bull	Prod. year	Breed	Source	Package
1	Chief	1975	Brahman	Imported	Ampule
2	UPM-IVM	1979	Friesian Cross	Local	Straw
3	Grove	1983	Jersey	Imported	Straw
4	Campolino	1992	Mafriwal	Local	Straw
5	Deloraine	1996	Sahiwal	Imported	Straw
6	J Royal	1996	Hereford	Imported	Ampule
7	Ultimate	1999	Holstein	Imported	Straw
8	Boto	2008	Nelore	Imported	Straw
9	Tickset	2008	Brangus	Imported	Straw
10	Justine	2008	Droughtmaster	Imported	Straw

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11	Mike	2010	Kedah Kelantan	Local	Straw

3.2 Thawing and handling of semen

Selected straws were removed from the liquid nitrogen tank one at a time using a pair of long forceps and shook gently to remove the liquid nitrogen retained in the lower end of the straw. Then, the straws were thawed in a water bath at 37°C for 30-60 seconds. Then, the straws were wiped dry before it was cut. The semen was then transferred into a pre-warmed test tube for further procedure.

3.3 Semen evaluation

Four parameters were chosen to evaluate the semen quality in cryopreserved semen samples which are general motility, progressive motility, sperm viability, and sperm abnormalities.

3.4 General and progressive motility

General motility and progressive motility observed under Nikon Eclipse E200 light microscope. Immediately after thawing, a drop semen sample were thawed, a drop of semen sample was pipetted on a pre-warmed glass slide and covered with a pre-warmed cover slip. Motility was immediately assessed under light microscope at X10 objective lens. Then, individual motility was assessed under X40 magnification. Three replicates were prepared to present motility of each straw and the mean of was

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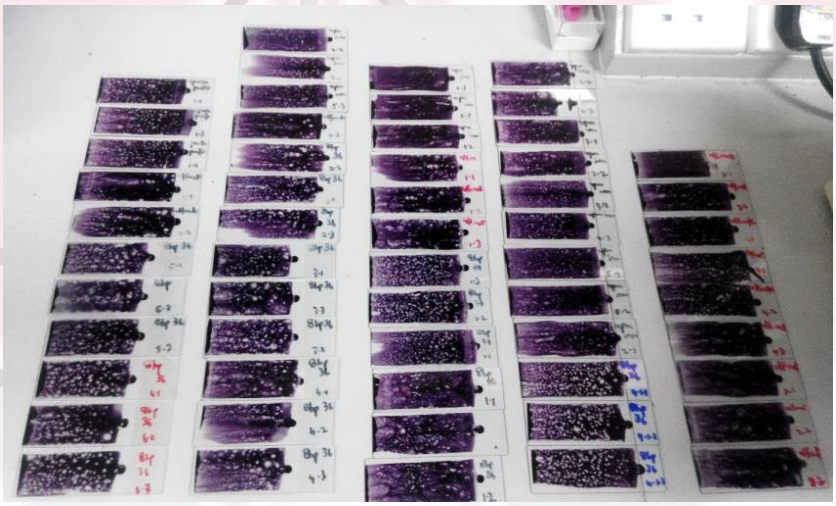
recorded as final motility score. For motility analysis, each semen sample was observed less than 15 minutes.

3.5 Sperm viability

Sperm viability was performed by staining sperm with eosin-nigrosin stain. Two drops of the stain was pipetted on a glass slide, mixed with one drop of semen and left at room temperature for 3 minutes. Then, by placing 8µ droplet of mixture on a glass slide, thin smear was done and allowed to air dry. One hundred spermatozoa were counted in at least five different fields using a cell counter. Three replicates were prepared and the mean calculated as the final viability score.

Figure 1 : Eosin nigrosin stain smear



3.6 Sperm Abnormalities

Assessment of abnormal spermatozoa was performed under a light microscope at X100 magnification using the slides from sperm viability. Abnormalities of sperm were classified morphological abnormalities such as distal midpiece reflex, dag defect, decapitation, microcephaly, cytoplasmic droplet, bent tail and others.

3.7 Statistical analysis

Statistical analysis of Independent T-Test and Mann-Whittney test were performed to compare two means of packaging and source of production. While One-Way ANNOVA (Tukey test) and Kruskal-Wallis H test were used to compare mean values of different storage duration and breeds. Differences at $P < 0.05$ were considered as statistically significant. Statistical software IBM SPSS Statistics 20 was used. Results are reported as mean $\pm$SEM.

4.0 RESULTS

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General motility, progressive motility, viability and abnormality of different breeds, storage duration, packaging and source of production were expressed as mean ± SE are presented in Table 2, 3, 4, and 5. Common spermatozoa abnormalities such as distal midpiece reflex, dag defect, decapitation, microcephaly, cytoplasmic droplet, bent tail and others are presented in Table 6.

4.1 Quality of frozen-thawed semen among breeds of bulls

The highest general motility of frozen-thawed semen was observed in Holstein (87.22±0.70%) and the significantly lowest was in Kedah Kelantan (37.22±1.02%). The significantly highest progressive motility was found in Mafriwal (95.00±1.49%) and the significantly lowest was in Droughmaster (70.83±1.20%). The significantly highest viability of frozen-thawed semen was from in Mafriwal (76.12±2.28%) and the significantly lowest was in Kedah Kelantan (31.95±0.80%). The significantly highest abnormality of frozen-thawed semen was found in Brangus (16.77±1.08%) and the lowest was found in Kedah Kelantan (1.72±0.34%).

Table 2 : General motility, progressive motility, viability and abnormality of frozen-thawed semen among cattle breeds (Mean±SE)

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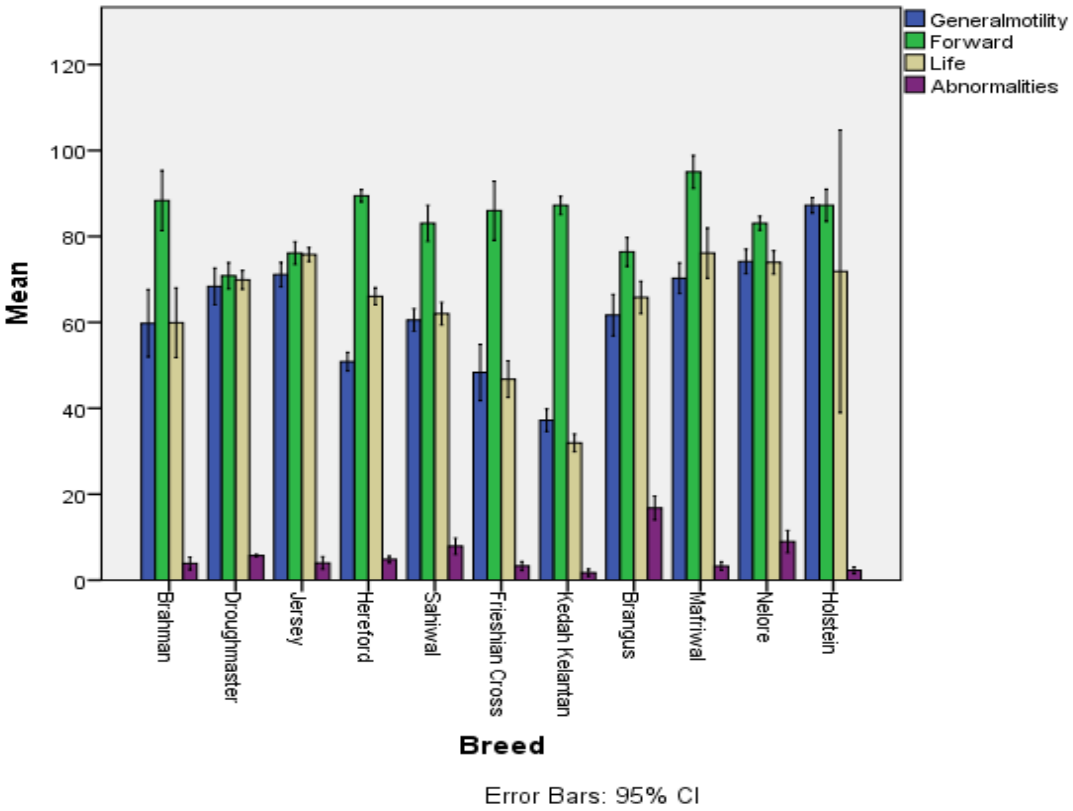
Breeds	General motility (%)	Progressive (%)	Viability (%)	Abnormality (%)
Brahman	59.78±3.06^{c}	88.33±2.72^{de}	59.89±3.13^{bc}	3.89±0.58^{abc}
Droughmaster	68.34±1.66^{de}	70.83±1.20^{a}	69.88±0.86^{c}	5.72±0.10^{cd}
Jersey	71.16±1.11^{e}	76.11±1.03^{ab}	75.77±0.64^{c}	4.00±0.57^{abc}
Hereford	50.83±0.83^{b}	89.44±0.55^{de}	66.06±0.77^{bc}	4.83±0.31^{bc}
Sahiwal	60.55±1.02^{cd}	83.06±1.63^{cd}	62.00±1.02^{bc}	7.89±0.74^{de}
Friesian cross	48.33±2.5^{b}	85.97±2.68^{d}	46.78±1.65^{ab}	3.29±0.40^{abc}
Kedah Kelantan	37.22±1.02^{a}	87.22±0.82^{d}	31.95±0.80^{a}	1.72±0.34^{a}
Brangus	61.66±1.88^{cd}	76.39±1.32^{ab}	65.78±1.47^{bc}	16.77±1.08^{f}
Mafriwal	70.27±1.389^{e}	95.00±1.49^{e}	76.12±2.28^{c}	3.28±0.39^{abc}
Nelore	74.17±1.12^{e}	83.06±0.63^{cd}	73.94±1.06^{c}	8.95±1.01^{e}
Holstein	87.22±0.70^{f}	87.22±1.46^{d}	71.83±12.80^{c}	2.28±0.31^{ab}

^{a,b,c,d,e,f} Values in the same row with different superscripts indicate significant different ($P<0.05$)

Figure 2 : Graph of general motility, progressive motility, viability and abnormality of frozen-thawed semen of different cattle breeds (Mean±SE)



4.2 Quality of semen of different storage duration

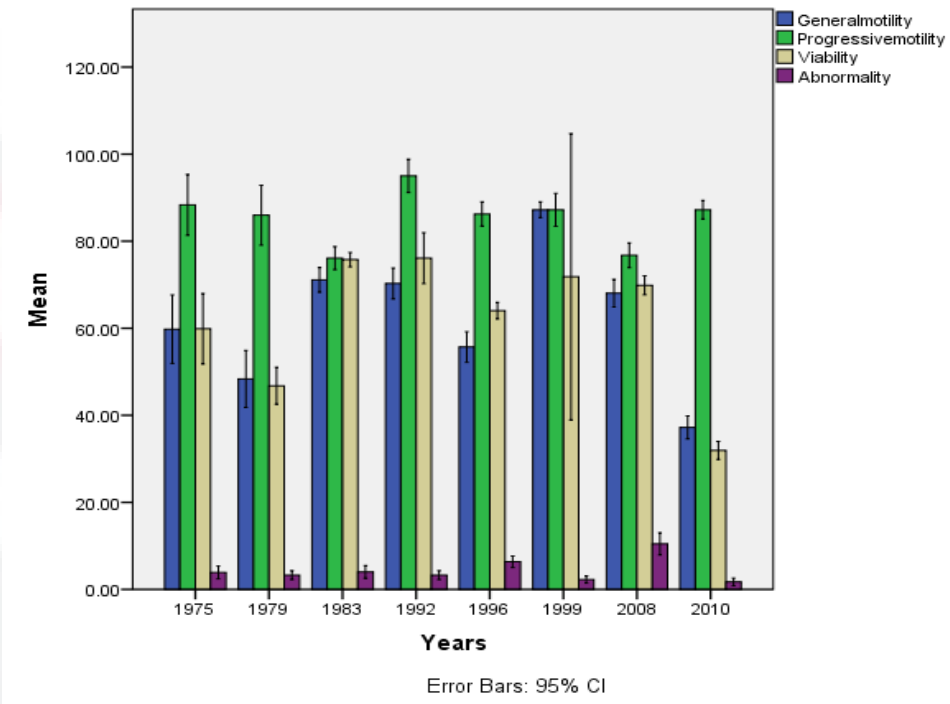
As regards to storage duration, all parameters were significantly different. The general motility of frozen-thawed semen produced in 1999 (87.23±0.70%), was significantly highest whereas frozen-thawed semen produced in 2010 (37.22±1.10%) was significantly lowest. The progressive motility of frozen-thawed semen produced in 1992 (95.00±1.49%) was significantly highest, whereas frozen-thawed semen produced in 1983 (76.11±1.03%) was significantly lowest. The viability of frozen-thawed semen produced in 1992 (76.11±2.28%) was significantly highest, whereas frozen-thawed semen produced in 2010 (31.95±0.80%) was significantly lowest. The abnormality of frozen-thawed semen produced in 2008 (10.48±1.22%) was significantly highest, whereas frozen-thawed semen produced in 2010 (1.72±0.34%) was significantly lowest.

Table 3 : General motility, progressive motility, viability and abnormalities in different years of production (Mean±SE)

Years	General motility (%)	Progressive (%)	Viability (%)	Abnormal (%)
1975	58.79 ± 3.06^{cd}	88.33 ± 2.72^{bc}	59.80 ± 3.15^{bc}	3.89 ± 0.57^{a}
2008	68.06 ± 1.51^{de}	76.56 ± 1.35^{a}	69.87 ± 1.02^{c}	10.48 ± 1.22^{c}
1983	71.11 ± 1.11^{e}	76.11 ± 1.03^{a}	75.77 ± 0.64^{c}	4.00 ± 0.57^{a}
1996	55.69 ± 1.59^{bc}	86.25 ± 1.27^{b}	64.03 ± 0.86^{c}	6.36 ± 0.50^{bc}
1979	48.33 ± 2.54^{b}	85.97 ± 2.68^{b}	46.78 ± 1.65^{ab}	3.29 ± 0.40^{a}
2010	37.22 ± 1.1^{a}	87.22 ± 0.82^{bc}	31.95 ± 0.80^{a}	1.72 ± 0.34^{a}
1992	70.27±1.38e	95.00 ± 1.49^{c}	76.11 ± 2.28^{c}	3.28 ± 0.39^{a}
1999	87.23 ± 0.70^{f}	87.20 ± 1.47^{bc}	71.83 ± 12.80^{c}	2.28 ± 0.35^{a}

^{a,b,c,d,e,f}Values in the same row with different superscripts indicate significant different ($P<0.05$)

Figure 3 : General motility, progressive motility, viability and abnormalities in different years of production (Mean±SE)



4.3 Semen quality between glass ampules and straws

There were significant differences in general motility and progressive motility between types of packaging. General motility of post-thaw semen in straw was significantly higher (64.32±1.96%) compared with glass ampules (55.31±2.13%). Progressive motility of post-thawed semen was significantly higher (88.89±1.33%) in glass ampules compared with straw (82.76±1.05%). The viability and abnormalities were not significantly different.

Table 4 : General motility, progressive motility, viability and abnormalities in different types of packaging (Mean±SE)

Packaging	General motility (%)	Progressive (%)	Viability (%)	Abnormal (%)
Glass ampule	55.31±2.13	88.89±1.33	62.97±1.81	4.36±1.34
Straw	64.32±1.96	82.76±1.05	63.78±2.38	5.99±0.64

4.4 Semen quality between imported and local production

There were significant differences in progressive motility and abnormality between sources of production. The progressive motility of frozen-thawed semen produced locally (88.56±1.11%) was significantly higher than imported (81.03±1.12%). The abnormality of imported frozen-thawed semen was significantly higher (7.44±0.70%) than locally producted (2.64±0.22%).

Table 5 : General motility, progressive motility, viability and abnormality in different source of production (Mean±SE)

Source of Production	General motility (%)	Progressive (%)	Viability (%)	Abnormality (%)
Imported	63.78±1.29	81.03±1.12	67.61±1.00	7.44±0.70
Local	60.76±4.10	88.56±1.11	56.67±4.87	2.64±0.22

4.5 Spermatozoa abnormalities among breeds

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Among breeds, there were significant differences in distal midpiece reflex, dag defect, bent tail and others while decapitation, microcephaly, and cytoplasmic droplets were not significantly different. Others consist of less common spermatozoa abnormalities such as terratoid, macrocephaly, pyriform head, double head, tail stump, diadem head, and knobbed acrosome. The distal midpiece reflex was significantly highest in Brangus. Sahiwal and Nelore were not significantly different but significant to all the other breeds.

Table 6**:** Distal midpiece reflex abnormality, dag defect, decapitation, microcephaly, cytoplasmic droplet, bent tail and others (Mean±SE)

Breeds	Distal midpiece reflex	Dag defect	Decapita-tion	Micro-cephaly	Cyto-plasmic droplet	Bent tail	Others
Brahman	3.80± 0.86 [a]	1.67± 0.67	5.80± 1.60[b]	1.00± 0.69	1.00± 1.29	2.00± 1.00[ab]	2.40± 0.45
Drough-master	6.17± 0.48[a]	1.20± 0.20	4.00± 0.26[ab]	3.0± 0.52	1.00± 1.29	2.83± 0.80 [ab]	-
Jersey	2.50± 0.50 [a]	2.80± 0.66	4.67± 1.10[ab]	2.0± 0.58	2.00± 1.29	1.67± 0.82[ab]	2.00± 0.59
Hereford	4.67± 0.61 [a]	1.75± 0.75	3.50± 0.62[ab]	2.00± 0.56	2.00± 0.74	2.00± 0.63[ab]	1.67± 0.41
Sahiwal	16.33± 2.14[b]	1.00± 1.06	4.00± 0.71[ab]	2.0± 0.49	1.67± 0.67	4.00± 1.00 [ab]	1.00± 0.51

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Friesian cross	2.20± 0.58 [a]	1.60± 0.24	2.50± 0.43[ab]	3.0± 1.0	1.00± 1.29	1.25± 0.71[a]	2.20± 0.45
Kedah Kelantan	3.17± 0.40 [a]	1.00± 1.836	1.80± 0.20[a]	-	-	1.00± 0.82 [a]	-
Brangus	25.83±0.98[c]	12.17± 1.30	1.8± 0.58[a]	1.00± 0.688	4.50± 0.76	5.17± 0.58 [b]	1.66± 0.59
Mafri-wal	4.40±2.50 [a]	1.75± 0.48	2.20± 0.58[ab]	-	1.50± 0.65	1.50± 0.71[a]	-
Nelore	16.83±0.63[b]	4.20± 1.28	2.2± 0.58[ab]	1.00± 0.973	1.00± 0.91	3.50± 0.58 [a]	1.00± 0.59
Holstein	2.25 ±1.6[a]	2.33± 0.88	1.75± 0.48[a]	1.00± 0.49	2.00± 1.00	1.20± 0.63 [a]	1.20± 0.45

[a,b,c,] Values in the same row with different superscripts indicate significant different ($P<0.05$)

A Motic image analyser was used to capture spermatozoa with abnormalities. Some of the abnormalities observed were diadem, dag defect, coiled tail, distal reflex, decapitation, cytoplasmic droplet, terratoid, and pyriform head.

Figure 4: Live and dead sperm

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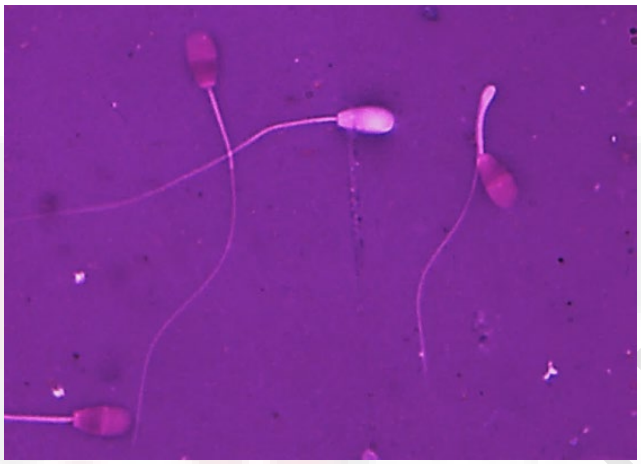
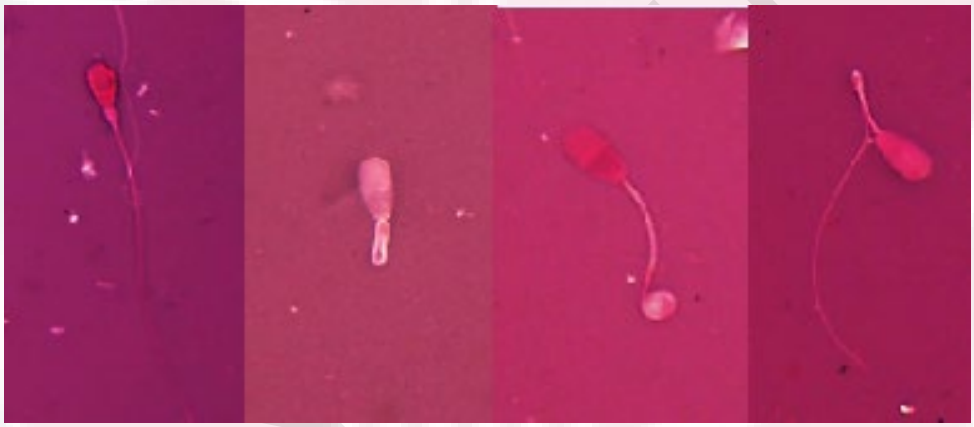
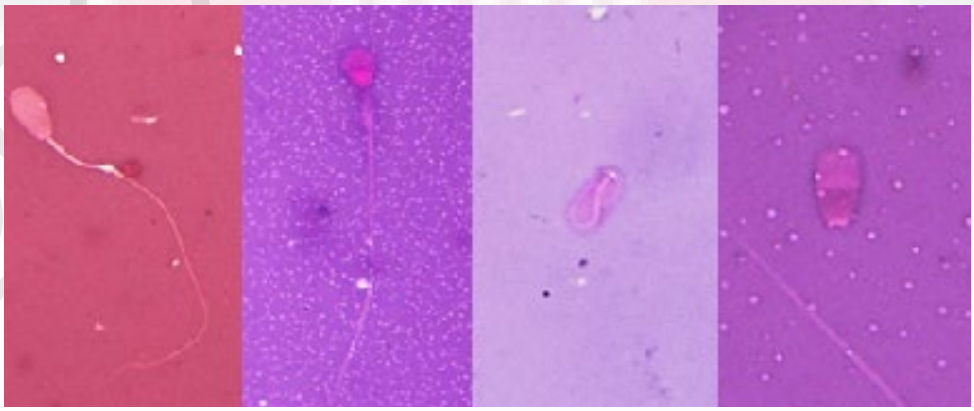


Figure 5: Spermatozoa abnormalities.



Diadem Dag defect Coiled tail Distal midpiecce reflex

Figure 6: Spermatozoa abnormalities



Cytoplasmic droplet Pyriform head Terratoid Decapitation

5.0 DISCUSSION

Quality of semen among breeds of bulls

There were significant differences among breeds for all parameters. Frozen-thawed semen of Holstein recorded 87.22% for general motility while Mafriwal recorded 95% for progressive motility. Average mass motility of fresh semen reported was 63.3% and the range was from 50-80% (Bratton *et al*., 1954) which was lower compared to the present study. Mafriwal frozen-thawed semen was significantly highest in viability which was 76.12% while Kedah Kelantan was significantly lowest 31.95%. These findings are almost consistent with the results of Check *et al*. (1991) who reported that sperm viability was decreased from 70.0 to 33.7% post-thaw possibly due to membrane damage. In fresh sperm, low percentage viability could be due to younger age of bulls, and breed difference and lower adaptability to the environmental conditions.

Spermatozoa abnormalities were highest in Brangus. This could be due to genetic, nutritional deficiency, or toxic stresses (Raymond, 2007). Dag defect, bowed midpiece, proximal droplet, and bent principle piece were suggested primarily of genetic origin and bulls with these abnormalities might be expected to not recover from this problem. In addition, Nogueira *et al.* (1999) investigated that morphological changes may occur due to by freezing and thawing on human testicular spermatozoa and they concluded that cryopreservation of human testicular spermatozoa causes similar damage to membrane and to the acrosome as seen in ejaculated cattle spermatozoa. Sperm cryopreservation induces the formation of intracellular ice crystals and the osmotic and chilling injury that gives rise to several sperm damages, namely cytoplasmic fracture, effects on the cytoskeleton and genome related

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structures (Isachenko, 2003). A sperm cell may be motile but damage and thus will reduce its fertility (Medeiros *et al*., 2002). Besides that, glycerol or dimethyl sulfoxide can induce osmotic stress and toxic effects on spermatozoa, but the extent of the damage varies according to the species and depends on its concentration in the extender solution (Purdy, 2006).

Among breeds studied, Holstein frozen-thawed semen gave the best quality. Mafriwal and Holstein were not significantly different for progressive motility, viability and abnormality. However, Holstein was significantly higher in general motility compared to Mafriwal. A study from Atiqur (2014) stated that Holstein cross bulls ranked top and superior among other bulls under his investigation. A reference from Rao and Rao (1979) observed that the average viability of fresh sperm percentage for Holstein bull was 83.5%, with range from 70%-90%. This lwere almost similar to the average live sperm percentage in this study. However, Medeiros (2002) stated that, under the best cryopreservation protocol, about one-half of the initially motile population did not survive thawing. This could be due to the method of experimentation and recording, which is not standardized in many reproductive researches. The variation of semen parameters among breeds could be due to the fact that individuals can often be classified as ‘‘good freezers’’ or ‘‘bad freezers’’. This implies that certain characteristics of membrane structure may be genetically determined, predispose towards survival under cryopreservation stress (Shamsuddin and Larsson 1993; Watson, 2000). Individual differences have been recorded for bull semen and allowances have been made for so-called poor freezers by packaging straws with more spermatozoa or by adjusting freezing protocols for individual bulls

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(Parkinson and Whitfield, 1987). However, even though preliminary semen evaluation was done, Curry (2000) stated that the concept of poor freezers suggests that 'cryosurvival' is not necessarily related to the observed quality of the semen sample such that, for certain individuals with apparently good pre-freeze sperm parameters, post-thaw survival is consistently poor.

It was recognized that cryopreserved spermatozoa had shorter lifespan within the female reproductive tract compared with fresh semen. This finding has subsequently been confirmed in a number of studies and for a range of species (Mattner *et al*., 1969; Hawk 1983). The proportion of sperm, which survive after thawing, is determined by their sensitivity towards osmotic stress during cryopreservation as well as cooling and rewarming. While there may be species differences in overall sperm sensitivity to cryopreservation, the ejaculate is heterogeneous with a variable resistance to osmotic stress amongst the cells. Those that succumb to lethal membrane damage however, are not determined stochastically. In all species, differences among individuals seems to be of genetic origin as differences in specific DNA sequences have been identified among boars in which thawed semen quality was classified as poor or good (Thurston *et al*., 2001).

Quality of semen of different storage duration

Liquid nitrogen is used widely for the cryopreservation and long term storage of human and animal semen (Malik, 2015). In this study, there is no relationship

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between storage duration for general motility, progressive motility, viability and abnormality. All semen parameters did not show any gradual increase or decrease pattern with increase in storage duration. Semen produced in 2010 recorded the lowest general motility (37.22±1.1%) and viability (31.95±0.80%). In fact, semen produced in 1975 recorded high percentage in progressive motility (58.79%) and viability (59.80%). Overall, frozen-thawed semen produced in 1999 produced the best quality among the storage years.

Varied in the result could be due to semen sample from different breeds, packaging, semen extenders used or individual differences. In the future, it is suggested to use only one type of breed, with the same semen extender, and same packaging as these may become confounding factors that might affect semen characteristics. A study (Spalekova, 2015) on semen quality between long-term and short-term storage on a single breed, semen extender and packaging showed that there were no significant findings in total motility between long-term and short-term storage. In contrast, a more recent study by Malik (2015) who also examine semen quality from a single breed, packaging and semen extender showed that viability and motility of thawed sperm in liquid nitrogen were gradually decreased over the years.

The success of cryopreservation depends upon many factors, including interactions between cryoprotectant, type of extender, cooling rate, thawing rate and packaging, as well as individual variation (Andrabi, 2007; Clulow et al, 2008; Cooter et al, 2005). Some loss in spermatozoa viability is unavoidable due to processing prior to freezing as well as during the freezing process. In this study, the method of cryopreservation, cryoprotectant and types of semen extender used were unknown and

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using different methods of cryopreservation may produce different results. (Barbas, 2009). There are two methods that can be used for gamete cryopreservation which are slow freezing and vitrification. Slow freezing uses low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method that decreases cold shock, but usually it is not performed because heat transfer in sperm cells is too slow to permit vitrification, without the risks of "solution effects" or crystallization (Arav *et al*.,2002).

Semen quality between glass ampules and straws

The general motility of post-thawed semen in straws was significantly higher (64.32±1.96%) compared to glass ampules (55.31±2.13%). Glass ampules took longer time to freeze and to thaw. This may cause sperm injury and reduce motility. Knutson and Stoner (1988) studied that glass ampules are difficult in achieving high rates of cooling or warming because of the glass thickness and package geometry. Over rapid freezing may cause lethal intracellular ice formation. The optimal cooling rate should be slow enough to prevent intracellular ice formation, but fast enough to minimize the harmful effect ("solution effects") of the prolonged exposure to high salt concentrations of cryoprotectant (Holt, 2000). Fast thawing is required to avoid recrystallisation of any intracellular ice present in the spermatozoa (Barbas, 2009). Fisher *et al.* (1987) studied that spermatozoa thawed at a faster rate are exposed less time to the concentrated solute and cryoprotectant, and the restoration of the intra and extracellular equilibrium is more rapid than with slower thawing.

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The progressive motility of post-thawed semen was significantly higher in glass ampules (88.89±1.33%) compared to straw (82.76±1.05%). This could be due to semen with lowest quality belonging to Kedah Kelantan produced in 2010 was packed in straws. Therefore, the mean score from straws recorded lower than glass ampule. It is recommended in the future to use a constant breed as well as produced from the same year to test the effects of frozen semen in different types of packaging. Types of semen extenders used and interaction between cryoprotectant might also affect the quality of frozen-thawed semen (Lemma, 2011) between glass ampules and straws.

Semen quality between imported and local production

Locally produced frozen-thawed semen was examined to be better compared with imported semen. This could be affected by different method of cryopreservation and semen extender used between local and imported frozen semen as stated previously as an interactions between cryoprotectant, type of semen extender used, cooling rate, thawing rate and packaging, as well as individual animal variation. Semen is extended for protecting spermatozoa during cooling, freezing and thawing. Commonly, sperm cryopreservation extenders include a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol,ethylene glycol, or dimethyl sulfoxide), a buffer (Trisor Test), one or more sugars (glucose, lactose, raffinose, saccharose, or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Evans and Maxwell, 1987). The extenders used for semen preservation must have adequate pH and buffering capacity, suitable osmolality and should protect sperm cells from cryogenic injury (Salamon and

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Maxwell, 2000). Besides that, semen freezing-thawing survival is greatly influenced by semen dilution rate (Barbas, 2009) which was also unknown in this study.

In cattle, good fertility rates are obtained with sperm doses containing approximately 20 million spermatozoa, making AI commercially viable (Watson, 2000). Fertility with frozen-thawed semen is similar to fresh semen though it is necessary to use higher sperm concentrations (Vishwanath *et al*., 1996). In the mammalian species, losses of fertility due to cryopreservation are compensated by insemination doses containing larger number of spermatozoa in all domestic species (Watson, 2000). In my study, semen concentration test in each semen sample was not done. In the future, it is recommended to test for semen concentration as some semen with moderate viability, motility with several abnormalities were selected to be cryopreserved considering the bull was from high quality pure breed.

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6.0 CONCLUSION

Among breeds studied, Holstein frozen-thawed semen produced the best quality. The quality of frozen-thawed semen produced in 1999 was the best among the

years studied. Between packaging, straws have higher general motility compared to glass ampules. While, glass ampules have higher progressive motility compared to straws. Frozen-thawed semen produced locally was better compared to those imported.

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7.0 RECOMMENDATIONS

It is recommended in the future to analyse spermatozoa motility by using a Computer Automated Semen Analysis (CASA) for faster evaluation of semen motility. Further studies should comprise of larger and equal sample size of storage duration, packaging and source of production to obtain better estimation of mean. It is recommended to use the same semen extender in the study of frozen-thawed semen. Or if different semen extender is to be used, information about the semen extender should be known.

In the future, semen quality information during preliminary semen evaluation should be recorded before being cryopreserved. Hence, the semen survivability and freezing ability can be determined.

In Malaysia, there is a lack of study regarding the frozen-thawed semen quality produced in Malaysia especially from Kedah Kelantan. In this study, Kedah Kelantan recorded the lowest quality. Therefore, more studies are needed to identify the problem of low frozen-thawed semen quality.

It is perceived that distal midpiece reflex was significantly highest in Brangus compared with other breeds. This abnormality should be investigated further as the percentage mean was more than 20% which was over the baseline of tolerated abnormality of cryopreserved semen.

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