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Submission date: 20-Jan-2020 01:57PM (UTC+0700)

Submission ID: 1243964747

File name: In_Press_202,_SJS.pdf (637.3K)

Word count: 7213

Character count: 37079



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Journal:	<i>Songklanakarín Journal of Science and Technology</i>
Manuscript ID	SJST-2018-0022.R3
Manuscript Type:	Original Article
Date Submitted by the Author:	29-Oct-2018
Complete List of Authors:	Yendraliza, Yendraliza; Faculty of Agriculture and Animal Science, UIN Suska Riau; Faculty of Agriculture and Animal Science, UIN Suska Riau
Keyword:	Motility, Plasma Membrane Integrity, Non Return Rate, Recovery Rate, Pregnancy Rate

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Original Article

**Quality of Bali Bull Sperm Cryopreserved Using Different
Extenders and Equilibration Times on Pregnancy Rate of Bali
Cows**

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Abstract

This study aimed to investigate effects of three different extenders (TEY, AND[®], and TSY) and three equilibration periods (2, 4 and 6 hours) on the storage of Bali bull spermatozoa. This research used a completely randomized block design with two factors (extenders and equilibration time) and 12 replications. The measured variables were fresh semen quality of Bali cattle, Motility, Viability, Abnormality, Plasma Membrane Integrity (PMI), Recovery Rate (RR), Pregnancy Rate (PR), and Non Return Rate (NRR). The result showed that the spermatozoa of the extended semen with TEY and AND[®] within 4 hours equilibration period was able to show better Motility, Viability, PMI and

RR than the spermatozoa of the extended semen with TSY within 2 hours and 6 hours equilibration period. The conclusion of this research is that the TEY, AND®, and TSY extenders on Bali bulls semen with 4 hours equilibration time yield 90% PR and 100% NRR.

Keywords: Motility, Plasma Membrane Integrity, Non Return Rate, Recovery Rate, Pregnancy Rate.

1. Introduction

Artificial Insemination (AI) is one of technologies to increase local breed population. One of the critical success factors of AI is the quality of the frozen semen used. The quality of the frozen semen is affected by the quality of the diluent and the freezing method used. The type of extender required is preferably locally available, fast, cheap and able to maintain the motility and the survival of spermatozoa. The time required in the process of preserving semen at low temperatures (Equilibration) will determine the quality of spermatozoa. It is because at that time the spermatozoa were preparing to enter a cold shock. The effects of that cold shock could damage the plasma membrane of the cell and result in spermatozoa death. According to Tsutsui et al. (2003), the egg yolk serves to protect the spermatozoa from that cold shock during storage. Moreover, the contents of lipoprotein and lecithin in egg yolk also serve to maintain and protect the intensity of the envelope and lipoprotein of spermatozoa from cold shock and stabilize the plasma membrane (White, 1993). Some extenders have been widely used to increase the survival of spermatozoa after freezing such as tris-egg yolk, skim milk-egg yolk, lactose-egg yolk. Some of commercial extenders are Andromed®, Bioexcell®, Triladyl®, Biladyl®, Biochips plus (Gil et al., 2003; Rothe, 2003; Minitub Germany,

2005). Andromed[®] is a commercial extender composed of phospholipids, tris-(hydroxymethyl)-aminomethane, citric acid, fructose, glycerol, tylosin tartrate, gentamicin sulfate, spectinomycin, and lincomycin. The use of tris egg yolk and skim milk tris has been widely reported by previous researchers (Afriantini & Purwantara, 2010) in Friesian Holstein cow (Wiratri, Susilawati, & Wahjuningsih (2014) in limousin bulls study, and buffalo (Sukhato, Thongsodseang, Utha, & Songsasen, 2001).

The Andromed[®] extender is a commercial diluent already used in the Simental bull limousine and buffalo semen extender (Yendraliza, 2014), yet it has not been used on Bali cattle. Therefore, a quality test of Bali cattle was conducted to obtain the quality of Bali cattle semen in several extenders with the best equilibration time. Watson (1995) argues that the success of cryopreservation sperm process is influenced by species-specific selection, freezing method, thawing rates and diluents. Further, Paulenz et al. (2002) state that this type of cement diluent varies greatly in providing sperm value. Align with this idea, Kulaksiz et al. (2010) confirm that a suitable diluent is a basic necessity for the successful preservation of spermatozoa and for obtaining higher conception rates in field trials using diluted semen.

2. Materials and Methods

Research was performed in two stages; diluting and semen freezing. Semen was collected using artificial vagina (IVM, France), (at 42°C) from three Bali bulls with similar age (4-5 years) for six week periods. Only ejaculates with a concentration of greater than 800×10^6 sperm/mL were having >70% motility sperm, and >80% of the sperm with normal morphology were selected. The total of 6 ejaculates was individually processed for preservation. The next stage was AI on 60 heads Bali cows. The female breed used has given birth and has healthy reproductive organs. All selected Bali cows

were synchronized by using GnRH (Fertagyl) on the first day with a dose of 3 ml and PGF2 α (2.5 ml, Lutyase) on the 7th day after GnRH injection. The cows showed an estrus after the injection of PGF2 α in AI with frozen semen of first stage research. Pregnancy rate was determined on two tests during sixty days following the application of AI. The first pregnancy test was conducted on day 21 following insemination which indicated non return rate. Based on this test, all cows have been observed whether or not the cows returned to estrus. Whereas the second test of the pregnant test, it was conducted on day 60s following AI. The pregnancy was determined using rectal palpation methods instead of estrus detection (Hafez & Hafez, 2016).

Preparation of extender

Tris-citric egg yolk (TEY) extender was prepared by using 3.0 g tris-(hydroxymethyl-aminomethane) and 1.56 g citric acid, fructose 0.2% w/v, glycerol 7.0 ml (Merck, Germany), and egg yolk 20% in 74 ml distilled water. All chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibiotics benzylpenicillin (1000 IU/ml, Pharmacia & Upjohn, Belgium) streptomycin sulphate (1000 μ g/ml, Pharmacia & Upjohn, Belgium) were added to TEY extender. Tris-skim milk-egg yolk (TSY) extender was prepared by using 3.0 g tris-(hydroxymethyl-aminomethane) and 1.56 g skim milk, fructose 0.2% w/v, glycerol 7.0 ml (Merck, Germany), and egg yolk 20% in 74 ml distilled water. Andromed[®] (AND[®]) extender was prepared according to manufacturer's instructions (IVM, France).

Semen Evaluation and Processing

Sperm progressive motility was determined microscopically ($\times 400$, Olympus BX20, Tokyo, Japan) and sperm concentration was determined using a digital photometer (IVM, France). Each pooled sample was split into two aliquots and diluted with AND[®]

extender or TEY or TSY at 37°C, added in a single step for a final concentration of 25×10^6 sperm/ml. After dilution, semen was maintained in a water bath for 10 minutes at 35°C for stabilization; thereafter, it was cooled from 37 to 25°C in approximately one hour at room temperature. Straws designated for the same duration of equilibration time transfer used liquid nitrogen (freezing rates -20°C/min, from 5 to -120°C, duration: 10 minutes), varying only for the equilibration time at 5°C: 2 hours, 4 hours, and 6 hours for a total of two factors, three treatments. French straws (IVM, France) with a suction pump at 4°C in a cold cabinet unit (IVM, France) were placed in liquid nitrogen vapors, 5 cm above the level of liquid nitrogen. Straws were then plunged and stored under liquid nitrogen (-196°C). After 72 hours, four frozen straws from each group were thawed individually at 37°C for 30 seconds in a water bath for evaluation.

Parameters measured in the first stage of the study were viability, motility, abnormalities and integrity of the plasma membrane fresh semen quality of Bali cattle and quality of Bali cattle's sperm after thawing. The second stage of research measured recovery rate, pregnancy rate, and non-return rate.

Viability

The fresh and treated semen is dripped on the object glass then Eosin-nigrosin are dripped with the other ose and mixed. The semen mixtured with Eosin-nigrosin is made smeared with the other end of the glass object until it is obtained spread along the surface of the object's glass and then dired to dry. Then the sample was observed with a light microscope with 400x magnification.

Motility

The motile sperm was evaluated by mixing the semen gently and placing a 10 μ L drop of diluted semen on a warm slide and covered with a glass coverslip (18x18mm) from five selected representative fields. The mean of the five estimations was recorded as final motility score.

Abnormality

The percentage of abnormal sperm (detached heads, tailless, acrosomal aberrations, abnormal mid-pieces, or tail defects) was recorded by counting a total of 200 spermatozoa under the phase of contrast microscopy ($\times 1000$ magnifications; oil immersion). Abnormal sperm was examined by using Eosin-nigrosin stain. Placing 10 μ L drop of diluted semen on a slide and added it with 40 μ L drop of Eosin-nigrosin, and smeared it on a slide and dried it quickly in heating stage (37°C). Microscopes were selected randomly from ten fields, with the total of 200 cells (Ax et al., 2000).

Plasma Membrane Integrity (PMI)

Sperm plasma membrane integrity was determined using a hypo-osmotic swelling (HOS) assay (Jayendra et al., 1984). HOS solution consisted of 0.73 g sodium citrate and 1.35 g fructose dissolved in 100 ml distilled water (osmotic pressure: -190 mOsmol/Kg). To assess the sperm tail plasma membrane integrity, semen (50 μ L) was mixed with HOS solution (500 μ L) and incubated for 30 minutes at 37°C before examination with a phase contrast microscope ($\times 400$, Olympus BX20, Tokyo, Japan).

Recovery rate

Percentage of successful spermatozoa recovered from a total freezing process by comparing the percentage motile spermatozoa to fresh semen and after thawing (Hafez & Hafez, 2016).

Pregnancy rate

The number of pregnant cattle was divided by the number of cattle in the AI and multiplied by 100% (Hafez & Hafez, 2016).

Non Return Rate (NRR)

NRR is the percentage of cattle that does not estrus between 30-60 days after being mated and it will be divided by total cattle that have been mated.

Data Analysis

A completely randomized block design in two factors with 3 extenders \times 3 equilibration times, with 12 replications per experimental unit was used. Results were presented as mean \pm standard deviation. Effects of extender and equilibration time were evaluated by ANOVA, with means compared by Duncan's test at a 5% level. All the statistical analyses were performed using the SAS software (version 9.0, SAS Institute Inc., USA). The differences were considered significant at $p < 0.05$ level.

3. Results and Discussion

Fresh semen quality of Bali cattle

The average evaluation of fresh semen of Bali cattle can be seen in Table 1. The average of volume obtained during the study was 9 ml. The color of semen Bali bull was cream, smell was normal, distinctive fishy smell. The consistency of semen in this study was viscous, with concentrations of 1000 million to 2000 million or more cells per ml. The pH of fresh semen was 6.8 and it has good mass motion (++).

The volume semen of Bali bull were higher (9 ml) than Aceh bull (2.80 to 4.50 ml) (Zulyzaini, Dasrul, Wahyuni, Akmal, & Abdullah, 2016); and Bali bull was 4.5 ± 2.3 ml (Ratnawati, Affandhy, Pratiwi, & Prihandini, 2008) aged 3.5 years. But it was lower than

Bali bull in Udayana ($12 \pm 1,269$ ml) (Setyani, Sarini, & Lanang Oka, 2017). These differences may be due to the differences on species, age, body weight of cattle and interval of shelter (Hafez & Hafez, 2013). The pH semen (6.8) of Bali bull was relatively similar to Aceh bull (6.8) (Zulyzaini et al., 2016), and FH bull (6.5 to 7.0) (Arifiantini et al., 2005).

Bali bull sperm concentration (1600×10^6 /ml) was higher than concentration of Aceh bull ($1194.00 \pm 52.25 \times 10^6$ /ml) (Zulyzaini et al., 2016), Limousin ($1153.64 \pm 127.50 \times 10^6$ /ml) (Wiratri et al., 2014), and Simmental ($1129.75 \pm 180.99 \times 10^6$ sperm /ml) (Sukmawati, Arifiantini, & Purwantara, 2014). The differences in spermatozoa concentrations among bull were due to genetic quality in bull (Hafez & Hafez, 2016).

Sperm motility (80%) was higher than Bali Bull in Indonesia ($74.50 \pm 3.69\%$) (Dewi, Ondho, & Kurnianto, 2012), and Limousin bull ($67.56 \pm 1.46\%$) (Lestari, Saleh, & Maidaswar, 2013). It was due to differences in feed, shelter frequency, technique and maintenance management (Hafez & Hafez, 2016).

The percentage of live sperm Bali bull (90%) was higher than Aceh bull (70%) (Zulyzaini et al., 2016; and Bali bull in the Station research of Semarang ($88,03 \pm 3,07\%$) (Ratnawati et al., 2008). On the other hand, it was relatively similar to the percentage of live sperm Limousine bull (94,08%) (Sukmawati et al., 2014). The percentage of abnormal sperm (3.00) was relatively similar to Bali bull that is kept in Indonesia ($6,56 \pm 3.05\%$) (Ratnawati et al., 2008) and the Aceh bull (Zulyzaini et al., 2016).

Quality of Bali cattle's sperm after thawing

The post-thaw sperm quality of Bali bull in 3 types of extenders and 3 different equilibration times were very significant ($p < 0.01$) in motility values, plasma integrity membrane, and recovery rate values, while the values of abnormality sperm were not

significant. The results showed similar to on Limousin bull (Wiratri et al., 2014), but different from FH bull (Afriantini & Purwantara, 2010). This difference is likely due to the chemical content of several different types of extenders. However, these could be happened due to differences in extender density, viscosity or even the presence of large particles (Anzar, Kroetsch, & Boswall, 2011). Celeghini et al. (2008) reported that during prolonged equilibration, sensitive sperm undergoes membrane and axonemal changes that lose their ability to move in a straight line, which results in a decrease in some kinematic parameters such as linearity, and straightness during freezing and thawing processes.

Percentage of Viability

The average viability rate of Bali bull sperm was higher in the use of TEY (76.67%), AND® (74.4%) compared to TSY extender (71.34%) with 4 hours equilibration time (Figure 1). This is probably due to the TEY and TSY extender having a high lactose content that accelerates the metabolism of spermatozoa and the build up of lactic acid due to sperm metabolism causes dead spermatozoa, besides fat in skim milk also inhibits sperm motion (Suharyati & Hartono, 2011). The results of this study agree with the results of research conducted by Shah, Andrabi, and Qureshi (2016) on Nili-Ravi Buffalo semen with 4 hours equilibration time. However it is different from Arifantini, Yusuf, and Graha (2005), where triladyl extender is the best quality for sperm FH bull.

Motility

Different types of extenders and equilibration times have a significant effect on Bali bull sperm motility after thawing, where type extender interacts with equilibration time in giving post-thaw sperm motility values (Figure 2). The TEY extender with 4 hours equilibration time gave higher motility value (66.66%) compared to the AND® (63.67%)

and TSY (60.66%). This is probably caused by TEY having lecithin and lipoproteins that are capable of maintaining motility. The structure of lipoprotein in TEY was similar to a structure of plasma membrane and it could protect the spermatozoa (Gotham & Mayes, 2009). The TEY was able to protect spermatozoa from cold shock (Alves et al., 2013). The motility of spermatozoa depends on the energy source of the mitochondrial metabolism derived from fructose in the diluent. At low temperatures, metabolism spermatozoa will run slowly so as to save the use of energy sources. The fructose present in the extender can be a source of energy for sperm (Schorin et al., 2012) and become the main source of energy for motility sperm (Stefanov, Anev, & Abadjieva, 2015).

Sperm motility of Bali bull is higher than Limousine bull (36-55%) (Wiratri et al., 2014) and lower than FH bull (73%) (Afriantini & Purwantara, 2010). This difference is likely due to the chemical content of several different types of extenders.

Abnormality of Spermatozoa

The average of Bali bull sperm's abnormality is not significantly different in the three types of extender but significantly different at the time of equilibration 4 and 6 hours (Figure 3). The percentage of abnormality is still within the normal range. Ax et al. (2000) pointed out that the requirement of spermatozoa value of abnormality as frozen cattle is 10-20%. The percentage of abnormalities was lower than Holstein Bull of Iraq (15.94 % up to 20.91%) used to TEY extender (Hussain, Shahad, & Al-Badry, 2016). However, if it is more than 25%, it will affect the decrease in fertility (Parera, Prihatini, Souhoka, & Rizal, 2009). Gordon (2017) suggested that the longer the storage time, the higher the abnormality will be.

Plasma Membrane Integrity (PMI)

Different types of extenders and equilibration times have a significant effect on Bali bull sperm of PMI after thawing, where type extender interacts with equilibration time in giving post-thaw sperm PMI value (Figure 4). The AND® and TEY extender with 4 hours equilibration time gave higher PMI value (72.33%, 73.33%) compared to the TSY (69.33%). This difference is caused AND® and TEY contained by active constituent of egg yolk, low-density lipoprotein (LDL) fraction, is responsible for protecting sperm cells from cold shock (Manjunath, 2012). As LDL adheres to the cell membrane during the cryopreservation it helps in restoring the loss of phospholipids and apparently induces a temporary change in its composition consequently preventing rupture of the plasma membrane (Wahjuningsih, Hermanto, Nuryadi, & Bhontoro, 2012).

The value of Bali bull PMI is different from the results obtained by Febretrisiana et al. (2016) in Boer goat, that was 79% which preserved at 5°C with Triladyl® extender and Rizal (2009) in Bali bull with 51.60%. The differences in PMI values are likely due to the TEY requiring sufficient time to enter the cell membrane and keep the fluid balance of the cells stable. So it did not damage the spermatozoa and the substrate and electrolyte in the cell smoothly (Ariantje, Yusuf, Sajuthi, & Arifiantiny, 2014). The egg yolk will protect and maintain the integrity of the spermatozoa sheath due to lecithin and lipoprotein (Manjunath, 2012). Furthermore, Hayati (2011) added the high value of membrane integrity obtained in Bali bull semen plasma which is due to its ability to protect the plasma membrane better, so that only a few phospholipids of plasma membranes are peroxidation.

Recovery Rate (RR)

Different types of extenders and equilibration times have a significant effect on Bali bull sperm RR after thawing, where type extender interacts with equilibration time

in giving post-thaw sperm RR value (Figure 5). The TEY and AND[®] extender with 4 hours equilibration time gave higher RR value (82.50%, 78.75%) compared to the TSY (75.00%). This is probably due to the basic ingredients of TEY in accordance with Bali bull sperm which is thought to be better able to protect the sperm from the bad effect of freezing because the base material and the buffers, sugars are used differently (Arifiantini et al., 2005). Cell damage during clotting and thawing is due to lipid peroxidation of spermatozoa in order to decrease survival (Alvarez & Storey, 1982). The first damage to the spermatozoa cell membrane occurs in freezing and thawing between temperatures of -15 to -60 °C, but it does not occur during dispersal in liquid nitrogen (Park & Graham, 1993). The average value of Bali bull RR sperm with TSY was lower than that of Bali bull RR sperm that was extended with TEY and AND[®]. This may be caused by damage to plasma membranes of spermatozoa due to lipid peroxides. In accordance with Maxwell and Watson (1996) that spermatozoa membranes contain a large amount of unsaturated fat that is particularly susceptible to lipid peroxidation reactions.

Pregnancy Rate (PR) based on Non-Return Rate (NRR) value.

The percentage of Bali-cows pregnancy is influenced by many things such as the lascivious quality of the AI acceptor and the quality of the sperm used. However, the observation on day 21 indicated that 90%, 100 %, 100% inseminated cows with semen use AND[®], TEY and TSY extender did not show estrus sign on day 21 after insemination (Table 2). High NRR values are caused by all acceptor cows which are in luteal phase, so the synchronization hormone can work well. Other than that, Bali cattles have been reported to be superior to other breeds in fertility and conception rate (Toelihere, 2002).

The pregnancy test was also performed on day 60 following insemination using rectal palpation methods instead of visual estrus detection method. The result showed that

88.88%, 90%, 70% of inseminated cows were pregnant (Table 2). There was a decrease in number of cows that did not show sign of estrus on day 21 following insemination with number of cows that pregnant using rectal palpation. Jainudeen, Wahid, & Hafez (2000) described the main cause of pregnancy failure in cattle was embryonic death followed by placentation, male factor, fetal, lethal gen and ovum transport.

This PR is higher than the sheep in Bangladesh (Rekha, Zohara, Bari, & Alam, 2016), the buffalo in Pati (Rizal & Riyadhi, 2016). This difference may be caused by different values of PMI, RR and Motility of semen used. The PR is also determined by the type and age of the livestock (Hafez & Hafez, 2016). High PR in this research probably was due to implementation of timely insemination, because the sign of estrus in both treatments were very clear, so the inseminator can be the appropriate time at which to inseminate.

4. Conclusion

The TEY, AND[®] and TSY extender, with 4 hours equilibration time produces sperm motility respectively: 66.66%, 63.67%, 60.66%, Abnormality: 6.33%, 6.33%, 7.33%, PMI: 73%, 72%, 69%, RR: 82%, 78%, 75%, PR: 90%, 88.8%, 70%, and NRR: 100 %, 90%, 100%.

Acknowledgments

The authors would like to thank Balitbang Bengkalis for financial support and the provision of research location. The authors also thank to Team of BIBD Tenayan Raya especially for Mr. Fauzun Rais, Dedy Junianto, Berliana and Sanif Tandi for the kind assistance throughout the experiment. There is no conflict of interest in this study.

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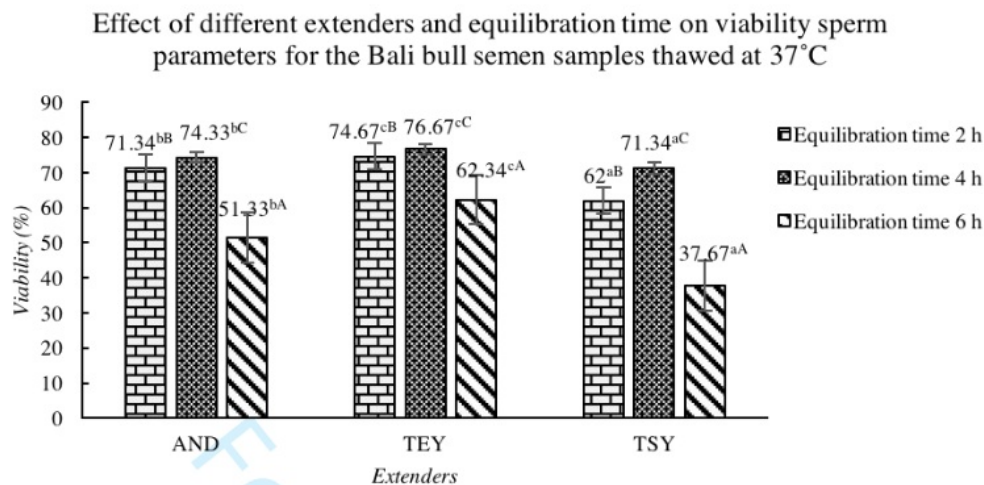


Figure 1 Effect of different extenders and equilibration time on viability sperm parameters for the Bali bull semen samples thawed at 37°C. Data are means \pm SD. ^{a, b, c} ; Values in the same row with different superscript different significantly ($p < 0.05$) and ^{A,B,C} Values in the same column with different superscript different significantly ($p < 0.05$). AND: Andromed, TEY: Tris Egg Yolk, TSY : Tris skim Yolk.

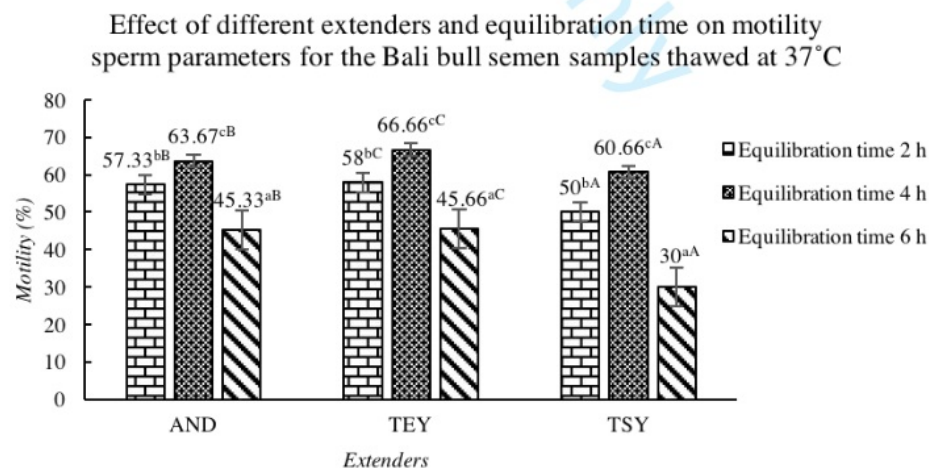


Figure 2 Effect of different extenders and equilibration time on motility sperm parameters for the Bali bull semen samples thawed at 37°C. Data are means \pm SD. ^{a, b, c} ; Values in the same row with different superscript different significantly ($p < 0.05$) and ^{A,B,C} Values in the same column with different superscript different significantly ($p < 0.05$). AND: Andromed, TEY: Tris Egg Yolk, TSY : Tris skim Yolk.

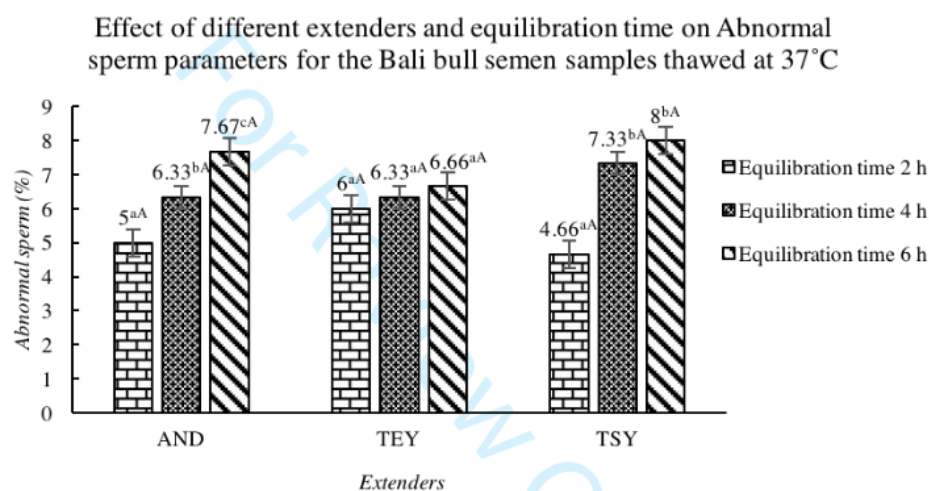


Figure 3 Effect of different extenders and equilibration time on abnormal sperm parameters for the Bali bull semen samples thawed at 37°C. Data are means \pm SD. ^{a, b, c} ; Values in the same row with different superscript different significantly ($p < 0.05$) and ^{A,B,C} Values in the same column with different superscript different significantly ($p < 0.05$). AND: Andromed, TEY: Tris Egg Yolk, TSY : Tris skim Yolk.

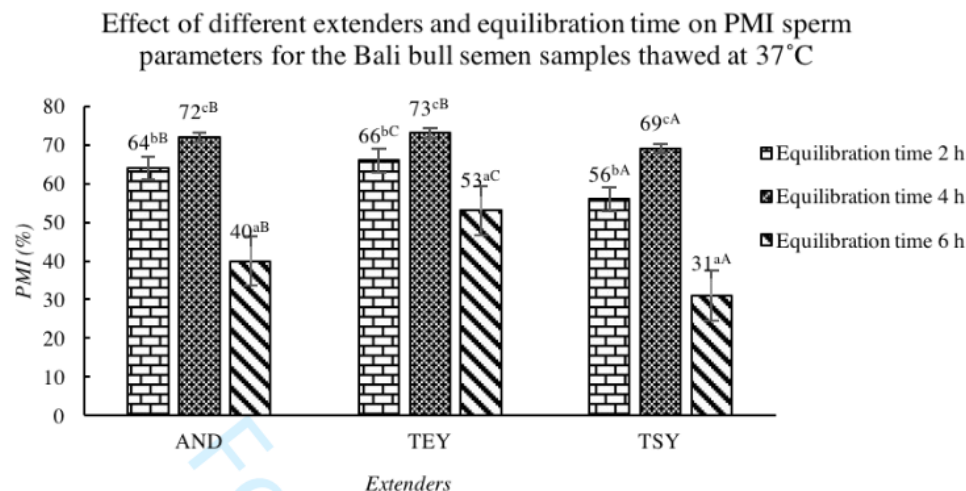


Figure 4 Effect of different extenders and equilibration time on Plasma Membrane Integrity (PMI) sperm parameters for the Bali bull semen samples thawed at 37°C. Data are means \pm SD. a, b, c ; Values in the same row with different superscript different significantly ($p < 0.05$) and A,B,C Values in the same column with different superscript different significantly ($p < 0.05$). AND: Andromed, TEY: Tris Egg Yolk, TSY : Tris skim Yolk.

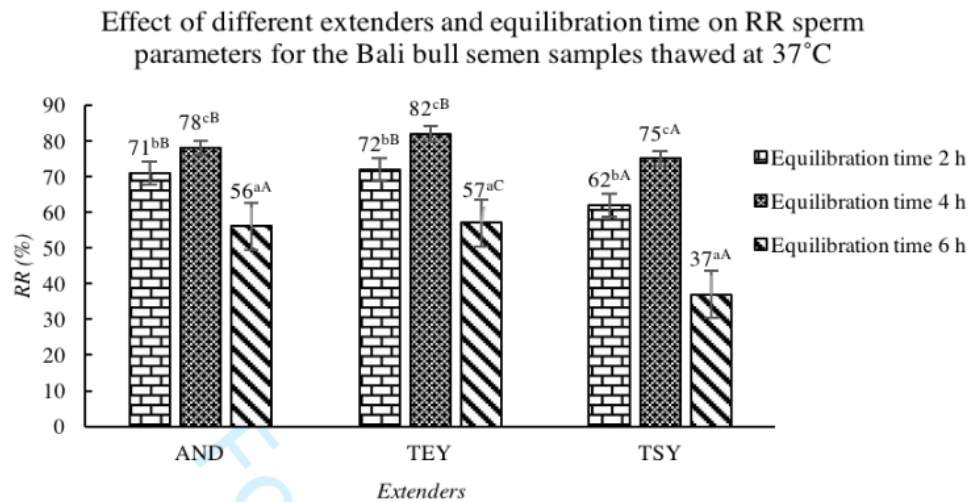


Figure 5 Effect of different extenders and equilibration time on Recovery Rate

(RR) sperm parameters for the Bali bull semen samples thawed at 37°C. Data are means \pm SD. ^{a, b, c} ; Values in the same row with different superscript different significantly ($p < 0.05$) and ^{A, B, C} Values in the same column with different superscript different significantly ($p < 0.05$).

AND: Andromed, TEY: Tris Egg Yolk, TSY : Tris skim Yolk.

Table 1. The average evaluation of fresh semen of Bali Bull in Pekanbaru

Characteristics	Average
Macroscopic Evaluation of Cement	
Volume	9 ± 2.5 ml
pH	6.8 ± 1.2
Colour	Cream
Consistency	Viscous
Microscopic Evaluation of Spermatozoa	
Concentration	1600 ± 1.5
Mass motion	++
Individual motion	2
Motility (%)	$80,00 \pm 1.5$
Viability (%)	$90,00 \pm 2.7$
Abnormality (%)	$3,00 \pm 1.5$
PMI (%)	$81,00 \pm 2.5$

Table 2. Effect different extender with 4 h equilibration time on NRR and pregnancy rate for 60 Bali-cow

Extenders <i>(equilibration 4 h)</i>	Variables	
	NRR	Pregnancy Rate
AND (20 n)	90 % (18/20)	88.88 % (16/18)
TEY (20 n)	100 %	90 % (18/20)

TSY (20 n)	100 %	70 % (14/20)
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