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Effect of semen dilution and equilibration time on semen quality of Pesisir bull

Tinda Afriani[1]*, Zaituni Udin[1], Jaswandi[1], Adisti Rastosari[1], Fandtya Dwi Putra[1], Mangku Mundana[1], and I Made Merdana[2]

[1]Department of Livestock Production, Faculty of Animal Science, Andalas University, Padang - 25175, Indonesia
[2]Department of Veterinary Pharmacy and Pharmacology, Faculty of Veterinary Medicine, Udayana University, Denpasar - 80361, Indonesia

Corresponding Author: Tinda Afriani
*tindaafriani@ansci.unand.ac.id

ABSTRACT
The process of freezing semen, a common problem, is generally caused by cold shock to frozen cells and the formation of ice crystals. One way to overcome this is to look for diluents and optimal equilibration times to reduce damaged spermatozoa during freezing. This study aims to determine the semen quality of Pesisir Bull and the interaction between the two types of diluent and the four equilibration times. The diluent used was egg yolk tris and andromed. The equilibration times were 6, 8, 10, and 12 hours. The variables observed included macroscopic and microscopic semen evaluation. This study used a factorial randomized block design with two factors and four treatment replications. Data are reported as the mean and standard deviation analyzed using analysis of variance. The average percentage of semen motility before equilibration was 80±0.00; after equilibration, 60.00±0.00 up to 72.50±0.00. The mean percentage of viability before equilibration was 89.26±2.70 after equilibration, which was in the range of 65.44±2.72 to 78.78±2.99. The average rate of abnormalities before equilibration was 3.00±0.50 after equilibration, which was in the field of 5.81±1.29 up to 11.41±1.63. The mean percentage of IPM before equilibration was 84.18±1.18 after equilibration, which was 71.37±3.72 up to 77.85±3.53. The average rate of IAC before equilibration was 87.84±3.14 after equilibration, which was 77.69±3.65 up to 83.07±2.11. The egg yolk tris diluent treatment showed highly significant ($P<0.01$) differences with the andromed diluent treatment in motility, viability, IPM, and IAC. We concluded that the Semen of Pesisir Bull equilibrated from 6 to 12 hours using Andromed and egg yolk tris diluent is still categorized as suitable for further processing to manufacture frozen semen or for artificial insemination (AI).

Keywords: Semen Quality; Semen Dilution; Equilibration; Pesisir Bull

Introduction

Pesisir bull is local livestock native to West Sumatra which is commonly found in the Pesisir areas of Sumatra with the dominant color characteristics being brick red with variations in yellowish, brownish, to blackish colors on the head, blond eyelashes, blackish brown dorsal line, white on the legs, white and has hair on a black tail. Other characteristics are a small body shape, having a small gumba and wattle, and having a small horn shape [1]. Afriani et al. [2] stated that the main obstacle in developing Pesisir Bull is the low rate of population increase. According to Sirajuddin et al. [3], Artificial Insemination (AI) was a program aimed at increasing the production of livestock as well as the income of farmers and alternative technology being developed to improve the genetic quality and population of cattle in Indonesia. There are many problems with implementing artificial insemination in the field, which is commonly encountered in the quality of the frozen semen [4]. One of the problems in semen freezing is that a large population of sperm are incapacitated after cooling to and warming from liquid nitrogen temperatures [5].
Semen is a liquid containing spermatozoa and semen plasma secreted by the sex glands in the testes [6]. Semen includes two main elements, namely semen plasma and spermatozoa cells [7]. An equilibration time of approximately 3-4 hours before semen cryopreservation is standard practice to maintain membrane integrity and motility of male sperm. However, several studies have shown that the overnight equilibration period before freezing improves the quality of post-semen. Thawing, thus optimizing the level of pregnancy [8]. Fleisch et al. [9], stated that the rate of improvement in semen quality depended on the diluent used, and the extension of the equilibration time from 4-72 hours did not affect fertility in the field.
To maintain the shelf life, one of the conditions that must be possessed as a sperm diluent is that it contains nutrients and can protect spermatozoa [10]. The requirement for spermatozoa diluent is that it includes elements almost the same as the physical and chemical properties of semen. Various types of diluents are currently used for both homemade and commercial uses, including skimmed milk-egg yolk (SMEY), Tris–egg yolk (TEY), and Andromed® [11, 12]. The previous study, Baharun et al. [12] reported motility test on fresh, diluted, equilibrated, and after-thawed semen using Androvision®. The results showed that after thawing, the motility of sperm diluted in AndroMed® (58.64%). Salmah et al. [7] explained that the egg yolk tris diluent significantly affected the percentage of semen motility of Holstein Friesian cattle compared to the Andromed diluent after freezing. The percentage of spermatozoa motility diluted with

egg yolk tris diluent was above 40% in each group, while the percentage of spermatozoa diluted with Andromed diluent was below 40% in all groups. The results indicated that cow semen diluted with egg yolk tris diluent was still suitable for artificial insemination. According to Mukhlis et al. [13], to increase the percentage of spermatozoa motility of frozen Aceh cattle, you should only use a 20% concentration of AndroMed® diluent. As for the tris egg yolk citrate diluent, Setiono et al. [14] explained that adding a 6% glycerol dose was the best dose used in egg yolk tris citrate diluent. This study aims to determine whether the type of diluent and different equilibration times affect the semen quality of post-equilibration Pesisir bull.

Methods

Research design

This research used a randomized block design experimental method with a 2x4 factorial pattern with four treatment replications. The first treatment was a type of diluent with two levels, namely A1= Andromed; A2= Egg yolk Tris and the second treatment equilibration time with 4 levels, namely B1= 6 hours; B2= 8 hours; B3= 10 hours; B4= 12 hours. This research was conducted at the Animal Reproduction Laboratory, Faculty of Animal Husbandry, Andalas University, using Pesisir Bull semen collected from the UPT Farm Experiment, Faculty of Animal Husbandry.

Preparation of diluents

The diluents used were AndroMed® and egg yolk Tris. The composition of the egg yolk tris is 3.634 g of tris aminomethane, 2.17 g of citrate, 20 ml of egg yolk, pen strep-400 antibiotic, and 100 ml of aqua destilation. The step for making egg yolk tris diluent is to put tris amino methane crystals and citrate, which has been weighed, into an Erlenmeyer, then add 100 ml of distilled water and stir until it is homogeneous and becomes a solution buffer. Discarded 20 ml of buffer solution made before and added 20 ml of egg yolk, mixed until homogeneous. The final step is adding antibiotics and stirring until homogeneous.

Egg yolks prepared are made by removing eggshell pests by wiping them with cotton soaked in 70% alcohol, then breaking the eggs and separating the whites from the yolks using a*n egg separator*. Use filter paper to separate the egg yolks from the rest of the egg whites. The egg yolk is then pricked with a needle to remove the yolk liquid, which is covered in a vitelline membrane.

AndroMed® diluent was prepared by mixing 5 ml of AndroMed® with 20 ml of aquadest in a 100 ml measuring cup and then adding semen whose volume, concentration, and motility had been counted. The importance of diluent added to each semen is calculated by the formula:

Diluent Volume $= \frac{(Semen\ volume\ x\ sperm\ concentration\ x\ motility)}{(desired\ sperm\ concentration/ml)}$ - v. seed

Mass movement

According to Susilawati [15], mass motility was observed using a microscope without a cover glass with a magnification of 400x or 100x at a constant temperature of 37^0C. The evaluation of the mass movement is then stated based on very good (+++), good (++), not good (+), and bad (0), and scores of 0, 1, 2, 3, 4, and 5.

Spermatozoa concentration

Calculation of spermatozoa concentration using*Neubauer chamber*. Semen was dripped into the counting chamber*Neubauer chamber*, covered with *cover glass*. The counting of spermatozoa cells was carried out in five counting chambers. The number of spermatozoa cells counted in five rooms is multiplied by 10^6 cells/ml [16, 17].

Percentage of Live Spermatozoa

Evaluation of live spermatozoa using 2% eosin in physiological NaCl to prepare media *stain*. One drop of semen and one drop of eosin is placed on the *object glass* and mixed by wiping with *the cover glass* so that it is evenly distributed and left to dry. After that, 100 spermatozoa were observed under a microscope in at least five positions, and spermatozoa that were colored red were marked dead. Colorless spermatozoa were declared alive, meaning they did not absorb eosin. According to Hafez [18], The percentage of live spermatozoa is calculated using the formula:

Live spermatozoa (%) $= \frac{Number\ of\ live\ spermatozoa}{Total\ number\ of\ live\ and\ dead\ spermatozoa}$ x 100%

Spermatozoa abnormalities

Abnormality is done by making a thin smear preparation by mixing one drop of eosin dye. Furthermore, the sperm is evaluated under a microscope with a magnification of 40x10; then, the spermatozoa are counted whose morphology changes to look like a rolled tail, broken tail, and folded middle part. According to Toelihere [19], how to calculate normal and abnrmal spermatozoa by using the formula:

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$$\text{Abnormality (\%)} = \frac{Abnormal\ spermatozoa\ count}{The\ number\ of\ spermatozoa\ counted} \text{ x } 100\%$$

Intact plasma membrane (IPM)

Intact plasma membrane was observed using the HOS-Test method, with a solution of osmolarity that had been incubated for 1 hour. Spermatozoa in an osmolarity solution are placed on top *of object glass* and closed with *cover glass*, then observed under a microscope with a magnification of 400 times. Then a calculation of 100 spermatozoa was carried out with the criteria for spermatozoa with a normal IPM that would retain hypoosmotic fluid in the cell so that a circular or bent tail indicated an intact plasma membrane. In contrast, spermatozoa with straight tails indicated that the plasma membrane had been damaged. Modified to Susilawati [16], the calculation of the percentage of spermatozoa that reacted to the HOS solution was carried out by:

$$\text{IPM (\%)} = \frac{number\ of\ intact\ plasma\ membrane\ spermatozoa}{number\ of\ spermatozoa\ counted} \text{ x } 100\%$$

Intact acrosome cover (IAC)

One drop of semen is put in *a microtube* containing a solution-*formol-saline* (2.54 g potassium dihydrogen phosphate, 5.41 g sodium chloride, 6.19 g di-sodium hydrogen phosphate dehydrate, 125 mL formaldehyde solution (37%), and 875 mL aqua dest [20]. Fresh semen was put in solution-*formol-saline* with a ratio of 1:100. Leave for 1 hour and take a drop, then place it on the object glass and cover it with a cover glass. Acrosome status is calculated using the following formula:

$$\text{IAC (\%)} = \frac{Spermatozoa\ with\ TAU}{Total\ number\ of\ spermatozoa} \text{ x } 100\%$$

Data analysis

Research data were processed using analysis of variance (ANOVA) through SPSS versions 26 for windows, and if there were significant differences (P<0.05) it was continued with Duncan's test.

Results and Discussion

The average volume of semen for Pesisir Bull is 2.25±0.29 ml; the results of this study by Bhudiyadnya et al. [21], who state that the semen volume of Pesisir bull only reaches 2-5 ml of ejaculate. Thats results was categorized as below normal because, according to Arifiantini et al. [2], the semen volume of cattle in the normal category ranged from 3.20-7.30 ml, and the average semen volume of cattle was 4-8 ml. Differences in body weight can cause differences in the book of semen in cattle, the age of the bulls used, or the libido of the bulls when the shelter. Results of post-equilibration semen spermatozoa motility evaluation can be seen in Table 1.

Table 1. Post Equilibration Semen Spermatozoa Motility in Pesisir Bull

Factor A	Factor B				Average
	B1	B2	B3	B4	
A1	60	60	60	60	60±0,00^{A}
A2	72,50	72,50	72,50	72,50	72,5±0,00^{B}
Total	132,50	132,50	132,50	132,50	
Average	66,25±8,84	66,25±8,84	66,25±8,84	66,25±8,84	

Note: Superscripts with different letters towards the column indicate significantly different (P<0.05).

Table 1 shows that for the A factors, namely A1 and A2, respectively, the average is 60±0.00 and 72.50±0.00%. The egg yolk tris treatment (A2) showed higher results than andromed (A1). In factor B, namely B1, B2, B3, and B4, the average results were 66.25±8.84, 66.25±8.84, 66.25±8.84, and 66.25±8.84%.

The statistical analysis showed a significant difference (P<0.01) in the two diluent treatments. In the four equilibration treatments, the effect was not significantly different (P ≥0.05). The results of the analysis of variance showed that there was no interaction between factor A (diluent) and factor B (equilibration time), showing no significant effect (P ≥0.05) on the percentage of post-equilibration motility. The results of the analysis of diversity on factor A (diluent) showed a highly significant (P<0.01) effect on spermatozoa motility. In contrast, the egg yolk tris diluent (72.5 ± 0.00) produced a relatively higher percentage of spermatozoa motility. Higher than that of andromed diluent (60±0.00). Results of the analysis of variance on factor B (equilibration time) showed no significant difference (P ≥0.05), where factors B1, B2, B3, and B4 showed average results of 66.25±8.84, 66.25±8.84, 66.25±8.84, and 66.25±8.84%.

The results obtained in this study were higher when compared to a survey conducted by Shahverdi et al. [22], where the equilibration time of up to 16 hours in the TRIS diluent resulted in sperm motility of 54.1±10.3. It should be noted that mammalian spermatozoa can be stored at 5˚C for at least 24 hours without significantly reducing motility and fertility

[23]. The results reported in this study were that the average percentage of sperm motility after equilibration for 24 hours was 51.75% in the treatment water jacket and 59.50% in the treatment-*free water jacket*. In this study, the average percentage of motility in egg yolk tris diluent was 72.50±0.00, and Andromed was 60.00 ± 0.00) the requirements for cattle spermatozoa to be said to be alive and moving forward (motile) after dilution are 55% and after thawing at least ≥40%.

The results of the post-equilibration semen spermatozoa viability evaluation can be seen in Table 2.

Table 2. Post Equilibration of Viability Semen of Pesisir Bull

Factor A	Factor B				Average
	B1	B2	B3	B4	
A1	69,31	65,15	64,27	63,03	65,44±2,72^{A}
A2	82,29	79,87	77,65	75,31	78,78±2,99^{B}
Total	151,6	145,02	141,92	138,34	
Average	75,8±9,18^{a}	72,51±10,41^{a}	70,96±9,46^{a}	69,17±8,68^{b}	

Note: Superscripts with different letters towards the column indicate significantly different (P<0.05).

Table 2 shows that the A factors, namely A1 and A2, respectively, have an average of 65.44±2.72 and 78.78±2.99. Egg Yolk Tris treatment (A2) showed higher results than andromed (A1). In factor B, namely B1, B2, B3, and B4, the average results were 75.8±9.18, 72.51±10.41, 70.96±9.46, and 69.17±8.68. The highest average was in treatment B1 (6 hours), higher than treatment B2 (8 hours), B3 (10 hours), and B4 (12 hours). The results of the analysis of variance showed that there was no interaction between factor A (diluent) and factor B (equilibration time), showing no significant effect (P ≥0.05) on the percentage of post-equilibration viability. The results of this study were compared with similar studies by Mariani and Alimuddin [24], who reported that the average viability of semen spermatozoa of Bali cattle after equilibration for 24 hours using andromed diluent, namely 75.20±6.72, was different from the results obtained from this study, namely 65.44±2.72%.

The results of Wahjuningsih et al. [24], reported that the average percentage of sperm viability after equilibration for 24 hours using egg yolk tris diluent was 64.4, lower than the study, namely 78.78±2.99%. According to Hoesni [25], tris diluent functions as a buffer material to prevent the occurrence *could shock* due to sudden temperature drops and changes in pH due to lactic acid, as well as maintaining osmotic pressure, electrolyte balance, and energy sources.

The results of the post-equilibration semen spermatozoa abnormality evaluation can be seen in Table 3.

Table 3. Abnormalities of Semen Spermatozoa Post-Equilibration of Pesisir Bull

Factor A	Factor B				Average
	B1	B2	B3	B4	
A1	6,72	8,63	11,39	12,56	9,83±2,65^{a}
A2	4,89	7,42	8,58	10,25	7,79±2,25^{b}
Total	11,61	16,05	19,97	22,81	
Average	5,81±1,29^{Aa}	8,03±0,86^{Oops}	9,99±1,99^{Abc}	11,41±1,63^{Bc}	

Note: Superscripts with different letters towards the column indicate significantly different (P<0.05).

Table 3 shows that the A factors, namely A1 and A2, respectively, have an average of 9.83±2.65 and 7.79±2.25%. The Andromed treatment (A1) showed higher results than Egg Yolk Tris (A2). In factor B, namely B1, B2, B3, and B4, the average results were 5.81±1.29, 8.03±0.86, 9.99±1.99, and 11.41±1.63%. The highest average was in treatment B4 (12 hours), higher than treatment B1 (6 hours), B2 (8 hours), and B3 (10 hours).

The results of the statistical analysis showed that there was a significant difference (p<0.05) between the two diluent treatments, namely treatment A1 (Andromed) and A2 (egg yolk tris). In contrast, the diluent treatment, namely treatment B1 (6 hours), was not significantly different (P> 0.05) from treatment B2 (8 hours) but significantly different (P<0.05) from treatment B3 (10 hours) and very significantly different (P<0.01) with treatment B4 (12 hours). Treatment B2 (8 hours) was not significantly different (P>0.05) with treatment B3 (10 hours) but significantly different (P<0.05) with treatment B4 (12 hours). The B3 treatment (10 hours) was not significantly different (P>0.05) from the B4 treatment (12 hours). There is no interaction between the diluent and the equilibration time.

Abnormality increases with increasing equilibration time. Manehat *et al.* [26], state that spermatozoa abnormality is the degree of abnormality or physical damage to spermatozoa that occurs during the formation of spermatozoa in the seminiferous tubules or due to the process of transporting spermatozoa through the channels of the male genital organs. The average percentage of spermatozoa abnormalities with egg yolk tris diluent was lower with andromed diluent. According to Manehat et al., this is thought to be caused by lipoprotein and lecithin contained in egg yolk, which can maintain and protect the integrity of the plasma membrane sheath of spermatozoa from cold shock so that they can suppress damage to the plasma membrane in spermatozoa.

The results of post-equilibration intact plasma membrane (IPM) semen spermatozoa can be seen in Table 4.

Table 4. IPM Spermatozoa Semen Post Equilibration of Pesisir Bull

Factor A	Factor B				Average
	B1	B2	B3	B4	
A1	75,35	74,33	70,72	68,74	72,29±3,09^{A}
A2	80,34	78,93	76,82	74,00	77,52±2,76^{B}
Total	155,69	153,26	147,54	142,74	
Average	77,85±3,53^{Aa}	76,63±3,25^{Oops}	73,77±4,31^{Abc}	71,37±3,72^{Bc}	

Note: Superscripts with different letters towards the column indicate significantly different (P<0.05).

Table 4 shows that factor A, A1, and A2, each have an average of 72.29 ± 3.09 and 77.52±2.76, respectively. Egg Yolk Tris treatment (A2) showed higher results than andromed (A1). In factor B, namely B1, B2, B3, and B4, the average results were 77.845±3.53, 76.63±3.25, 73.77±4.31, and 71.37±3.72. The highest average was in treatment B1 (6 hours), higher than treatment B2 (8 hours), B3 (10 hours), and B4 (12 hours).

The results of statistical analysis showed that there was a very significant difference (P<0.01) in the treatment of the two diluents, namely A1 (andromed) and A2 (egg yolk tris). Treatment B1 (6 hours) was not significantly different (P>0.05) from treatment B2 (8 hours) but significantly different (P<0.05) from treatment B3 (10 hours) and very significantly different (P<0.01) with B4 treatment (12 hours). Treatment B2 (8 hours) was not significantly different (P>0.05) from treatment B3 (10 hours) but very significantly different (P<0.01) from treatment B4 (12 hours). The B3 treatment (10 hours) was not significantly different (P>0.05) from the B4 treatment (12 hours). There is no interaction between the diluent and the equilibration time.

The average IPM percentage is higher than the results of Arifiantini and Maheswari [27], which reported that the average IPM percentage of semen spermatozoa with egg yolk tris diluent was 34.59±3.06. The results of this study are not much different when compared to the results of the study of Indriani et al. [11], who reported that the average percentage of IPM spermatozoa in bovine semen used diluent *Cauda Epididymal Plasma* (CEP-2) was added with 10% egg yolk which had been equilibrated for 24 hours with treatment *water jacket* is 66.91% while the treatment *free water jacket* is 74.30%.

The results of the evaluation of the intact acrosome cap (IAC) of post-equilibration semen spermatozoa can be seen in Table 5.

Table 5. IAC Post Equilibration Semen Semen of Pesisir Bull

Factor A	Factor B				Average
	B1	B2	B3	B4	
A1	80,87	79,72	77,91	75,11	78,40±2,51^{A}
A2	85,22	83,99	82,79	80,27	83,07±2,11^{B}
Total	166,09	163,71	160,7	155,38	
Average	83,04±3,08^{Aa}	81,85±3,02^{Oops}	80,35±3,45^{ABb}	77,69±3,65^{Bc}	

Note: Superscripts with different letters towards the column indicate significantly different (P<0.05).

Table 5 shows that factor A, A1, and A2 each has an average of 78.40±2.51 and 83.07±2.11, respectively. Egg Yolk Tris treatment (A2) showed higher results than andromed (A1). In factor B, namely B1, B2, B3, and B4, the average results were 83.045±3.08, 81.855±3.02, 80.35±3.45, and 77.69±3.65. The highest average was in treatment B1 (6 hours), higher than treatment B2 (8 hours), B3 (10 hours), and B4 (12 hours).

The results of statistical analysis showed that there was a very significant difference (P<0.01) in the treatment of the two diluents, namely A1 (andromed) and A2 (egg yolk tris). Treatment B1 (6 hours) was not significantly different (P>0.05) from treatment B2 (8 hours) but significantly different (P<0.05) from treatment B3 (10 hours) and very significantly different (P<0.05). 01) with B4 treatment (12 hours). Treatment B2 (8 hours) was not significantly different (P>0.05) from treatment B3 (10 hours) but very significantly different (P<0.01) from treatment B4 (12 hours). The B3 treatment (10 hours) was significantly different (P<0.05) from the B4 treatment (12 hours).

There is no interaction between the diluent and the equilibration time. The results of this study, with an average IAC percentage range of 77.69±3.6 up to 83.07±2.11, were much higher than the results of a similar survey of Sitepu and Marisa [28], who reported that intact acrosomes of spermatozoa semen of Simmental cattle using egg yolk tris diluent after equilibration was 61%. Sitepu et al. [29], stated that a low percentage of intact acrosome caps is associated with low motility, viability, and intact plasma membrane.

Conclusion

There is a difference in the average semen quality of Pesisir Bull in the treatment of diluent and at equilibration. Egg yolk tris diluent had higher average motility, viability, IPM, and IAC percentages than Andromed diluent and lower abnormalities than andromed diluent. Semen of Pesisir Bull equilibrated from 6 to 12 hours using andromed and egg

yolk tris diluent is still categorized as suitable for further processing for the manufacture of frozen semen or artificial insemination (AI).

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Conflict of Interests
The authors declare that they have no competing interests.

Author Contributions
TA and MM designed the research. TA, ZU, J, AR and FDP conducted the study, and analyzed the data. TA drafted the manuscript, IMM and MM provided critical feedback and revisions, then TA finalized the manuscript.

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