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Analysis of Motility Characteristic of Pesisir Bulls Sexed Semen with Different Pre-Freezing Method Based on Computer Assisted Sperm Analyzer (CASA)

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ABSTRACT

This study aimed to find the best combination between pre-freezing method and layer of post sexing on semen quality of Pesisir Bulls. This study used 2 Pesisir Bulls. The semen was evaluated using Computer Assisted Sperm Analyzer (CASA). This study used factorial randomized block design with 2 factors. Factor A was sexed semen layer of Bovine Serum Albumin (BSA) column with 2 level, whereas Factor B was pre-freezing method with 3 level. According to the analysis of variance, there is no interaction of the combination of two factors on each paramters. Pre-freezing method showed highly statistically significant effect (P<0.01) on the quality of motility on upper layer and bottom layer which was about (52.86-72.93%; 52.32-65.50%) and about (40.17-56.99%; 40.33-53.18%) on progressive motility. The value of Distance Curve Line (DCL) was about (39.15-43.24 μm; 49.25-53.86 μm), Distance Average Path (DAP) was about (22.38-24.60 μm; 26.52-29.94 μm) and Distance Straight Line (DSL) was about (17.32-19.07 μm; 20.93-24.25 μm), Velocity Curve Line (VCL) was about (88.67- 97.50 μm/s; 109.85-117.90 μm/s), Velocity Average Path (VAP) was about (50.98- 55.63 μm/s; 59.32- 64.69μm/s), Velocity Straight Line (VSL) was about (34.45-42.37 μm/s; 46.80-50.62 μm/s), Beat Cross Frequancy (BCF) was about (23.91-25.17 Hz; 25.63-27.35 Hz), Straightness (STR) was about (0.75-0.77; 0.77-0.80), and Wobble (WOB) was about (0.56-0.57; 0.53-0.55), highly statistically significant (P<0.01) on post sexing layers. Meanwhile, the value of Amplitude of Lateral Head (ALH) was about (4.24-4.94 μm; 4.25-4.44 μm) showed highly significant effect (P<0.01) on every factor, and value Linearity (LIN) was about (0.42-0.44; 0.43-0.44) showed not significant effect (P>0,05) on every factor According to the study, it can be concluded that the best treatment was treatment modified procedure layer by space the straw 16 cm every layers and The quality of post sexing X- and Y- spermatozoa motility effect on motility characteristic.

Keywords: Pesisir Bull, Spermatozoa sexing, Pre-freezing, CASA

Introduction

Pesisir Cattle are local breed cattle of Indonesia which distribution spread in West Sumatera Province. Pesisir Cattle have some advantages that are very productive, adaptive to unfavorable environment which lack in forage and resistant to diseases (Hendri., 2013). Quality improvement of Pesisir Cattle is necessary as well as population increase. Attempt to improve the quality of Pesisir Cattle can be carried out by utilization of artificial insemination (AI) technology. Artificial insemination technology required semen from superior bulls with excellent quality. The production of frozen semen for Pesisir Bulls are not yet produced, therefore this study aimed to attain the frozen semen of Pesisir Bull, particularly on sexed frozen semen of Pesisir Bull.

Biotechnology of spermatozoa sexing is one of the efficient and effective reproduction technology, for this technology might enable to determine the sex gender in order to reach the breeding purpose desired (Bhalakiya *et al*., 2018). Spermatozoa sexing is based on the difference of DNA content, measurement, electrical charge on sperms's surface, chromosome fluorescence, difference in weight, and motility of the sperm (Aini *et al*., 2016). There are much more chromatin content of x-chromosome bearing spermatozoa causing it tend to be bigger than y-chromosome bearing spermatozoa (Hafez and Hafez, 2000); Moreover, x-chromosome bearing spermatozoa contain more 3.8% DNA than y-chromosome bearing spermatozoa (Sureka *et al*., 2013).

Numerous methods of spermatozoa sexing have been reported, one of them is Bovine Serum Albumin (BSA) column method. BSA column method might enable spermatozoa sexing based on the difference of the BSA concentration. Spermatozoa with high motility have ability to permeate albumin medium (Susilawati, 2002). Gunawan *et al*. (2015) reported that spermatozoa separation with 5% and 10%

BSA column, yielded 85% of resemblance to the sex gender from artificial insemination result. According to Afriani *et al.* (2022) reported that the semen quality of Pesisir Bull after sexing by albumin gradient method were 58.33% motility on x-chromosome bearing spermatozoa and 60.83% motility on y-chromosome bearing spermatozoa. Sexing will decrease the quality of the spermatozoa when the cells got separated due to physical and chemical effect.

Factor that could affect the quality of semen is the production system of frozen semen (Seidel, 2009). According to Cochran *et al.* (1993) claim that pre-freezing and thawing were the factors that could affect a decline in semen quality. In general, pre-freezing is conducted in only a single step process due to the time efficiency of production. Several studies showed that modification of pre-freezing process on space of the straw resulted best percentage of motility on upper layer at 55,6% and 41,47% on bottom layer, whereas progressive motility on upper layer was 32.83% and 19.79% on bottom layer (Ondho and Udrayana, 2018). According to Susilawati (2017) increase in sexed semen quality can improved based on space of the straw. Best space for straw was about 8-10 cm above the liquid nitrogen surface.

Evaluation of spermatozoa quality can be conducted by CASA to accurately examine the motility. Motility evaluation is relevant to evaluate the spermatozoa fertility (Simmet, 2004). Evaluation using CASA is able to examine the percentage of total motility and progressive motility of spermatozoa along with the motility characteristic completely (Syarifuddin *et al.*, 2018). Motility characteristic of the spermatozoa positively correlate with fertility rate (Pelumal *et al.*, 2014). The purpose of this study was to determine the best pre-freezing procedure on sexed frozen semen of Pesisir Bulls, with utilization of Computer Assisted Sperm Analyzer (CASA).

Materials and Methods

This study was conducted experimentally at Laboratorium Semen Beku Unit Pelaksana Teknis Dinas (UPTD) Balai Pengembangan Teknologi Sumber Daya (BPTSD) Tuah Sakato Payakumbuh (frozen semen handling laboratory of Regional Executive Unit, Indonesian Regional Agency for Resource Technology Development Tuah Sakato Payakumbuh).

Research material

Materials used in this study were semen of 2 tails Pesisir Bulls, freshly collected by artificial vagina. Other materials and tools used were test tube, measuring cylinder, filter paper, erlenmeyer flask, microscope, CASA, BSA, centrifuge, 3.02 g of tris aminomethane, 1.67 gr of citric acid, 1 g of fructose, 100 mL of aquabidest and egg yolk by 4:1 ratio, penstrep-400, object glass, cover glass, micropipette, and microtube.

Research method

Semen was collected by conducting the semen collection procedure, which then the semen must be evaluated as soon as possible. Semen that qualified the criteria was then diluted into 100 million of cells per mL. After dilution, semen got sexed by putting 1 mL of diluted semen into test tube filled with 2 mL of 10% BSA solution at the bottom segment and 2 mL of 5% BSA solution at upper segment which then incubated for 45 minutes. The spermatozoa was then collected on each column which were 10% column (y-chromosome bearing spermatozoa) and 5% column (x-chromosome bearing spermatozoa). The semen sample then was centrifuged in tris egg yolk extender at 1800 rpm for 10 minutes (Kaiin *et al*., 2013). The sexed semen was then rediluted in tris egg yolk extender by 1:4 ratio (egg yolk:tris buffer), the semen sample then filled and sealed into mini straw (0.25 mL). After being sealed into mini straw, sexed semen then equilibrated at 4°C for 4 hours. Equilibrated sexed semen then got to be frozen at liquid nitrogen vapor which had been put earlier on styrofoam box according to the treatments and kept inside a container.

Sexed frozen semen was thawed using fresh water (37°C) for 30 seconds in order to evaluate the semen quality. Spermatozoa analysis with Computer Assisted Sperm Analyzer (CASA) conducted by taking 5µL of the sexed semen, then it was put on object glass (37°C) after that the object glass put on microscope at 10 x 10 magnification and phase contrast. Image capturing of the spermatozoa was carried out on 4 field of view (Ratnawati *et al.*, 2017). Evaluation parameters consisted of motility, progressive motility, Curve Linear Distance (DCL), Average Path Distance (DAP), Straight Linear Distance (DSL), velocity curve linear (VCL), velocity average pathway (VAP), velocity straight linear (VSL), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

This study used 2x3 factorial randomized block design with 4 replications. First treatment was the layer of BSA column with 2 segments consisted of A1 column = Spermatozoa on the upper layer (X) and A2 column = Spermatozoa on the bottom layer (Y), and the second treatment was pre-freezing with 3 segments that were: B1 = SOP of UPTD BPTSD Tuah Sakato, 4 cm for 14 minutes B2 = modified procedure, 8 cm for 9 minutes then lowered to 4 cm for 9 minutes. B3 = modified procedure, 16 cm for 9 minutes then lowered to 4 cm for 9 minutes.

The data was processed by using factorial analysis of variance (ANOVA). If the treatment showed significant results, then the DMRT test using SPSS 16.0 software. Data analysis results are presented in form of mean + standard deviation based on each treatment.

Results and Discussion

Motility and progressive motility

Motility and progressive motility are very important evaluation in order to evaluate the quality

of semen. According to Table 1. It was known that the motility and pregressive motility were non significant (P>0.05) on interaction between the two factors, and upper layer (x-chromosome bearing spermatozoa) and bottom layer (y-chromosome bearing spermatozoa) were non significant (P>0.05). However, it was highly significant (P<0.01) on pre-freezing treatments can be seen on Table 3. The motility of spermatozoa on treatment B3 (72.93% and 65.50%) was highly significantly higher (P<0.01) compared to treatments B1. The motility of spermatozoa on treatment B2 (63.48% and 61.27%) was significantly higher (P<0.05) compared to treatment B1 and B3. The score of progressive motility was nonsigficant (P>0.05), but it was highly significant (P<0.01) on pre-freezing treatments in Table 3. The treatment B3 showed progressive motility (56.99% and 53.18%) significantly higher compared to the other two treatments. However, treatment B1 (40.17% and 40.33) and treatment B2 (48.03% and 44.89%) were significant. Straw spacing on 16 cm and 8 cm gap after equilibrated at 4°C for 4 hours believed that it could calibrate the cellular condition of spermatozoa on extreme temperature changes gradually and minimize the risk of cold shock in freezing procedure. According to Purwoistri *et al.* (2013) the motility quality of Limousin Bulls semen after sexing was 63% on upper layer and 53.5% on the bottom layer. Ondho and Udrayana (2018) reported that pre-freezing modification can increase the motility and progressive motility on sexed semen of Ettawa goat (55.67% and 32.83%). Criteria of spermatozoa motility is defined when the percentage of spermatozoa motility has the value of VAP<25 µm/s (Contri *et al.*, 2010). Examination on motility and progressive motility of sexed semen of Pesisir Bulls, indicated that there was an effect on pre-freezing method. According to Ondho and Udrayana (2018) during the pre-freezing procedure, it could damage the spermatozoa. Modification of pre-freezing technique is conducted to reduce the damage of the spermatozoa. Modified procedure of pre-freezing method could increase the quality of spermatozoa compared to other procedure standard.

DCL, DAP, and DSL

The analysis of the distance of spermatozoa gone through were used to determine the ability of spermatozoa fertilization to the egg cell. According to the Table 1 it is known that the value of DCL, DAP, and DSL were nonsignificant (P>0.05) on interaction between the two factors, and non significant on pre-freezing method. However, it was highly significant difference (P<0.01) on post sexing layers can seen in Table 2. The value of DCL on the bottom layer (53.86 µm; 49.25 µm; 52.36 µm) was highly significant different (P<0.01) toward the upper layer (39.15 µm; 43.24 µm; 42.61 µm). The value of DAP on the bottom layer (29.94 µm; 26.52 µm; 28.66 µm) was highly significant higher compared to the upper layer (22.38 µm; 24.60 µm; 24.20 µm). The value of DSL on bottom layer (24.25 µm; 20.93 µm; 22.48 µm) was highly significant higher compared to the upper layer (17.32 µm; 19.07 µm; 18.19 µm). The result of this study showed the normal value, indicating that spermatozoa were of good quality. The displacement produced by Y-spermatozoa is higher than X-spermatozoa in each line, the distance of displacement is directly proportional to the velocity value of the spermatozoa. Spermatozoa distance movement is spermatozoa ability to move from one point to another or ability to reach egg cell at fertilization process. The ability of the spermatozoa to move is influenced by the chromosomes of the sperm.

Table 1. Combination of Interaction post sexing layers and pre-freezing method treatment

Parameter	Factor A	Factor B		
		B1	B2	B3
Motility (%)	A1	52.86+8.87	63.48+3.42	72.93+6.70
	A2	52.32+7.42	61.27+6.41	65.50+5.31
Progresive motility (%)	A1	40.17+5.32	48.03+5.58	56.99+5.27
	A2	40.33+3.78	44.89+10.4	53.18+4.69
DCL (µm)	A1	39.15+5.04	43.24+5.57	42.61+2.35
	A2	53.86+9.28	49.25+4.54	52.36+1.82
DAP (µm)	A1	22.38+2.17	24.60+3.34	24.20+1.37
	A2	29.94+4.73	26.52+2.03	28.66+0.70
DSL (µm)	A1	17.32+1.63	19.07+3.05	18.19+1.29
	A2	24.25+3.89	20.93+1.27	22.48+1.01
VCL (µm/s)	A1	88.67+9.20	95.70+10.3	97.50+4.54
	A2	117.09+16.0	109.85+11.0	117.90+3.85
VAP (µm/s)	A1	50.98+3.50	54.65+6.42	55.63+2.74
	A2	63.82+9.17	59.32+5.15	64.69+2.05
VSL (µm/s)	A1	39.45+2.61	42.37+6.08	41.94+2.65
	A2	48.70+3.44	46.80+3.36	50.62+2.65
LIN	A1	0.44+0.01	0.43+0.02	0.42+0.01
	A2	0.44+0.01	0.43+0.02	0.43+0.01
STR	A1	0.77+0.00	0.77+0.02	0.75+0.01
	A2	0.80+0.01	0.78+0.02	0.77+0.01
WOB	A1	0.57+0.02	0.56+0.02	0.56+0.01
	A2	0.55+0.01	0.53+0.02	0.54+0.01
ALH (µm)	A1	4.49+0.23	4.24+0.23	4.94+0.19
	A2	4.33+0.12	4.25+0.31	4.44+0.19
BCF (Hz)	A1	23,91+2.01	25.17+1.18	24.01+1.27
	A2	27.35+1.33	25.88+0.94	25.63+0.91

A1 (upper layers), A2 (bottom layers), B1 (4 cm spacing straw), B2 (8 cm spacing straw), B3 (16 cm spacing straw).

Table 2. Sexing semen quality of Pesisir Bulls on differences in post sexing layers

Parameters	Post sexing layers treatment	
	Upper layer	Bottom layer
Motility (%)	63.08+10.4	59.69+8.17
Progresive Motility (%)	48.60+8.51	46.13+8.39
DCL (µm)	$41.67+4.52^{A}$	$51.82+5.83^{B}$
DAP (µm)	$23.73+2.42^{A}$	$28.37+3.09^{B}$
DSL (µm)	$18.19+2.06^{A}$	$22.55+2.61^{B}$
VCL (µm/s)	$93.96+8.57^{A}$	$114.95+11.0^{B}$
VAP (µm/s)	$53.75+4.58^{A}$	$62.61+4.41^{B}$
VSL (µm/s)	$41.25+3.96^{A}$	$48.70+3.48^{B}$
LIN	0.43+0.01	0.43+0.01
STR	$0.76+0.01^{A}$	$0.78+0.02^{B}$
WOB	$0.56+0.01^{B}$	$0.54+0.01^{A}$
ALH (µm)	$4.36+0.36^{b}$	$4.34+0.22^{a}$
BCF (Hz)	$24.36+1.51^{A}$	$26.29+1.25^{B}$

$^{a.b}$ Means in same rows with different superscript differ significantly (P<0.05), $^{A.B}$ Means in same rows with different superscript differ highly significantly (P<0.01).

VCL, VAP, and VSL

According to the Table 1 it is known that the value of VCL, VAP, and VSL were non significant (P>0.05) on interaction between the two factors, and non significant on pre-freezing treatments. However, the VCL was highly significant (P<0.01) on post sexing layer can be seen in Table 2. Y spermatozoa (A2) was significantly higher (117.09 µm/s; 109,85 µm/s; 117.90 µm/s) compared with x spermatozoa (A1) (88.67 µm/s; 95.70 µm/s; 97.50 µm/s). These result were obtained because management of frozen semen production did not affect spermatozoa biology. This results in this study is higher than the report of Mustofa *et al.* (2020) whom report that the best VCL value on sexed semen of Ongole crossbreed was 55.2 µm/s. VCL is the velocity of spermatozoa on their pathway measured in µm/s. The VCL value can be used as reference to determine the spermatozoa in vitro fertility rate. Y spermatozoa had higher velocity compared to x spermatozoa on VCL, VAP, and VSL. This experiment proved the success of spermatozoa sexing.

The VAP value of Pesisir Bulls sexed semen showed non significant on pre-freezing treatments. The upper and bottom layer after sexing showed highly significant effect (P<0.01). The bottom layer/Y-spermatozoa (63.82 µm/s; 59.32 µm/s; 64.69 µm/s) was significantly higher than the upper layer/X-spermatozoa (50.98 µm/s; 54.65 µm/s; 55.63 µm/s). Mustofa *et al*. (2020) reported the VAP the best value on Ongole crossbreed sexed semen was 32.5 µm/s. VAP is the average velocity of spermatozoa by calculating the length of spermatozoa pathway divided by time measured in µm/s.

The VSL value of Pesisir Bulls sexed semen also showed the same results as well as the VCL and VAP. The pre-freezing treatment were non significant, however it were highly significant (P<0.01) on post sexing layer can be seen in Table 2. The bottom layer/Y-spermatozoa (48.70 µm/s; 46.80 µm/s; 50.62 µm/s) was significantly higher compared to the upper layer/X-spermatozoa (34.45 µm/s; 42.37 µm/s; 41.94 µm/s). According to Mustofa *et al.* (2020) reported that VSL of Ongole crossbreed sexed semen was 22.1 µm/s. According to value VCL showed progressivity of spermatozoa motility but didn't how the actual oscillation size of the trajectory. According to the Table 1 it is knowshow the pattern of spermatozoa motility. VCL parameter was correlated with the permeability of spermatozoa to enter into cervix mucus (Amal *et al.,* 2019). Whereas VAP and VSL were used to predict in vitro fertility (Singh *et al.,* 2020).

LIN, STR, WOB

The value of LIN and STR can show the movement pattern of spermatozoa in linear, whereas WOB sn that the value of LIN, STR, and WOB were nonsignificant (P>0.05) on interaction between the two factors. LIN value showed that there was nonsignificant difference on every treatments factor can be seen on Table 2 and 3.

Table 3. Sexing semen quality of Pesisir Bulls on differences in pre-freezing methods

Parameters	Pre-freezing method treatment		
	4 cm	8 cm	16 cm
Motility (%)	$52.59+7.58^{A}$	$62.36+4.90^{BC}$	$69.21+6.86^{C}$
Progresive Motility (%)	$40.25+4.27^{A}$	$46.46+7.56^{AB}$	$55.08+5.05^{C}$
DCL (µm)	46.51+10.4	46.24+5.69	47.49+5.56
DAP (µm)	26.16+5.28	25.56+2.76	26.43+2.59
DSL (µm)	20.79+4.61	20.00+2.38	20.33+2.53
VCL (µm/s)	102.88+19.4	102.77+12.4	107.70+11.5
VAP (µm/s)	57.40+7.76	56.99+5.94	60.16+5.33
VSL (µm/s)	44.07+5.69	44.58+5.13	46.28+5.23
LIN	0.44+0.01	0.43+0.02	0.42+0.01
STR	0.78+0.02	0.77+0.02	0.76+0.01
WOB	0.56+0.01	0.55+0.02	0.55+0.01
ALH (µm)	$4.41+0.19^{A}$	$4.24+0.26^{A}$	$4.69+0.35^{B}$
BCF (Hz)	25.53+2.42	25.43+1.06	24.82+1.34

$^{a.b}$ Means in same rows with different superscript differ significantly (P<0.05), $^{A.B}$ Means in same rows with different superscript differ highly significantly (P<0.01).

The LIN value on the upper layer was about 0,42-0,44 whereas the value on the bottom layer was about 0.43-0.44. According to Maulana *et al.* (2022) the LIN value of Ongole Crossbreed Bulls sexed semen on upper layer was about 0.36-0.40 and about 0.64-0.67 on the bottom layer. The value of STR and WOB showed highly significant difference (P<0.01) on post sexing layers in Table 2, however it was nonsignificant on pre-freezing treatments. The bottom layer (0.77-0.80) was highly significant higher compared to the upper layer (0.75-0.77). The value of WOB on the upper layer (0.56-0.57) was highly significant higher compared to the bottom layer (0.53-0.55). According to Maulana *et al.* (2020) that reported the WOB value sexed semen on the upper layer was 0.58 and 0.59 on the bottom layer. According to Penfold *et al.* (1998) stated that there was difference on the swimming pattern (LIN and STR) between X and Y spermatozoa. According to the Table 3 the average spermatozoa move linearly, this is due to LIN >0.35 and STR > 0.5.

ALH and BCF

Anlaysis of ALH and BCF in CASA were the parameters to showed the waves pattern of spermatozoa (Ratnawati *et al*., 2017). According to the Table 1 it is known that the value of ALH, and BCF were nonsignificant (P>0.05) on interaction between the two factors. Can be seen in Table 2 and 3 it is known that the value of ALH were highly significant results (P<0.01) on post sexing layer and pre-freezing method. The upper layer/X-spermatozoa was significantly higher (4.49 µm; 4.24 µm; 4.94 µm) compared to the bottom layer/Y-spermatozoa (4.33 µm; 4.25 µm; 4.44 µm). Treatment B3 (4.94 µm; 4.44 µm) was significantly higher compared to treatment B1 and B2. These results indicate the amplitude/distance between of the lateral head is affected by splitting and freezing. ALH is acquired by mathematical calculation of maximum excursion of track crosses and average track crosses (Ratnawati *et al*., 2017). According to Perreault (2002) ALH results of different studies might be different depending on the number of the factors particularly on the kind of CASA used.

BCF or beat cross frequency is the number of trajectories of spermatozoa through the average track per second. The BCF value showed was no have interaction between the two factors in Table 1 and nonsignificant on pre-freezing treatments, but highly significant results (P<0.01) on post sexing layer which can be seen in Table 2. The bottom layer was significantly higher (27.35 Hz; 25.88 Hz; 2.,63 Hz) compared to the upper layer (23.91 Hz; 25,17 Hz; 24.01 Hz). The frequency of progressive motility was 60 hertz, low frequency can be caused because of temperature decrease and progressiveity ending (Sarastina *et al*., 2006). According to Maulana *et al.* (2022) VCL, VAP, VSL, ALH, and BCF are motility variables that useful as indicator of fertility and high probability of pregnancy.

Conclusions

There was no interaction between post sexing layers with pre-freezing method. The motility and progressive motility quality of X- and Y-spermatozoa showed nonsignificant effect on different pre-freezing method, but has significant effect on motility characteristic such as DCL, DAP, DSL, VCL, VAP, VSL, STR, WOB and BCF. Besides that, ALH showed significant effect on post sexing layers and pre-freezing layers, and LIN showed there was nonsignificant effect on post sexing layers and pre-freezing method. The best pre-freezing method in this study was the modified procedure on every post sexing layer by space the straw 16 cm above the liquid nitrogen surface for 9 minutes then lowered to 4 cm for 9 minutes.

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