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CHARACTER OF LIQUID SEMEN MOTILITY IN VARIOUS DILUENTS ON BALINESE CATTLE DURING COLD STORAGE

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Abstract- Balinese cattle have been spread and domesticated in Indonesia especially in Bali Island. Artificial insemination using liquid semen is a solution for applicated in areas where there is no liquid Nitrogen. The objective of this study was to observe the motility of Balinese Cattle spermatozoa by using liquid semen in Tris Aminomethane, CEP-2 and skim milk diluents. The materials used were two Balinese cattle. The research was located on Beef Cattle Research Station of Grati-Pasuruan. Semen was collected by using artificial vagina then continued by fresh semen analysis and liquid semen processing. Liquid semen quality was observed up to day 5 after chilled. The parameters measured were: sperm motility, sperm progressive motility, VCL, VSL, VAP, LIN, STR, WOB, H, ALH and BCF. Data were analyzed by minitab 17 and SPSS 16. The results showed that during cold storage, the sperm motility of Balinese cattle which were diluted with Tris aminomethane and CEP egg yolk was better than skim milk. Velocity linear curve (VCL), velocity average pathway (VAP), straightness (STR), and wobble (WOB) were decreased during cold storage. CEP-2 egg yolk can maintain the sperm motility better that other diluent.

INTRODUCTION

Artificial insemination by using frozen semen requires liquid nitrogen to maintain the sperm life. freezing process can damage the spermatozoa membrane which can decrease the sperm motility, this is one of the causes the AI success with frozen semen are still low. Liquid semen which stored at refrigerator (temperature 2-4 ^{o}C) is an alternative AI for locations there is no available liquid nitrogen around. Some diluents have been developed to sustain the survival of spermatozoa and increase the volume of semen. Verberckmoes *et al*., (2004), (2005) developed diluents that resemble caudal epididymal plasma liquid (CEP) which can maintain the spermatozoa life at least for 6 days. Ducha *et al*., (2012) mentioned that CEP-2 plus 20% egg yolks can maintain the motility of Limousin cattle spermatozoa up to 8 days. Furthermore, Da Costa *et al*., (2016a) revealed that Tris aminomethane + 20% egg yolk diluent is better than the basic diluent of CEP-2+10% egg yolk in maintaining of Ongole crossbred sperm motility, viability and abnormality for 7 days during the cooling process. The study of Da Costa *et al*., (2016b) showed that liquid semen which stored in 1 and 5 days after dilution with tris aminomethane + 20% egg yolks diluent resulted the conception rate (CR) is 86.67% and 83.33%, while service per conception (S/C) is 1. 31 and 1.44. Susilawati *et al*., (2016) have been studying the replacement of BSA in CEP-2 diluent by using blood serum, blood plasma, and egg white, the result showing that the best replacement is egg white which can maintain the sperm motility to 40% motility for 2 days. Continuing by Proceeds from the Nisa'us Sholikah, *et al*., (2016) showed that BSA on CEP diluents can be replaced with egg whites and can maintain the sperm life to 8 days.

Computer- Assisted Sperm Analysis (CASA)

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systems have evolved over approximately 40 years. through advances to capture the image from the microscope, huge increases in computational power concurrent with the amazing reduction in a size of computers, new computer language, and updated/expanded software algorithms. Remarkably, basic concepts for identifying sperm and their motion patterns are little changed. Older and slower systems remain used. Most major spermatology laboratories and semen processing facilities have a CASA system, but the extent of reliance thereon ranges widely. This review describes capabilities and limitations of present CASA technology used with boar, bull and stallion sperm, Followed by possible future development (Amann and Waberski, 2014).

CASA Analysis equipment can be used to evaluate the bull sperm. in Cornell University extender and egg yolk-glycerol-tris extender (following cooling and storage in the latter two extenders) (Tardif *et al*., 1997). The purposes of this research were to evaluate the decreases of sperm motility by using CASA analysis equipment on Balinese cattle during a colling storage in three kinds of diluent including Tris Aminomethane egg yolk, CEP-2 egg yolk and Skim Milk.

MATERIALS AND METHODS

Location and Time of Study

The research was conducted Animal Reproduction Laboratory and animal housing of Beef Cattle Research Institute, Grati Pasuruan. The research was conducted within 3 months starting from November 2016 to January 2017.

Research Methods

The material used were 2 Balinese cattle with 2-years old, which are collected regularly two times a week by using artificial vagina. The semen used were those with progressive motility > 70% and mass motility 2+. In this study, the three kinds of diluents were used including tris aminomethane + egg yolk, CEP + egg yolk and skim milk with 10 times repetition.

a) CEP + Egg yolk diluent preparation

Dilution materials were prepared by several steps as follow: (1) weight 15 mmol/L NaCl, 42.9 mmol/L citric acid, 4 mmol/L MgCl2 (H_2O). 11.9 mmol/L $NaHCO_3$, 8 mmol/L NaH_2PO_4, 20 mmol/L KH_2PO_4, 55mmol/L fructose, 1 g/L sorbitol, 2 g/L Tris, 0.05 NaH_2PO_4, 20 mmol/L KH_2PO_4, 55 mL mmol/L fructose , 1 g/L sorbitol, 133.7 mmol/L BSA, 2 g/L Tris, 0.05 g/L gentamicin Verberckmous *et al*., (2004). Furthermore, 4% albumin and 10% with egg yolk were added to CEP-2 dilutions.

b) Trisamino methane+egg yolk diluent preparation

The materials consisting of Trisamino methane (1,363 g), citric acid (0.762 g), lactose (1,500 g) and fructose (0,500 g) were added to Erlenmeyer, then 80 mL aquadest were poured to Erlenmeyer. The diluents were homogenized by using magnetic stirrers for 10-15 min. After homogenized, the diluents was sterilized by heated it for 15 minutes. Then, the temperature of diluents was lowered 100 ^oC to 37 ^oC. The antibiotic including penicillin (0.1 g) and streptomycin (0.1) were added to diluents and homogenized for and for 10-15 min. The diluents were stored at the refrigerator for three days. Then the supernatant was separated from the sediment of diluents. The supernatant can be used as diluents for sperm while the sediment was discarded (Susilawati, 2013).

c) Skim milk preparation

The materials consisting 10 g of skimmed milk powder and 0.9 g of glucose in 100 mL of sterile distilled water were homogenized for 10-15 minutes. The diluents were sterilized or heated at 92-95 ^oC for 10 minutes, the after the diluents reached to room temperature, the antibiotic were added including streptomycin 100 mg (Meiji, Japan) and penicillin 100 000 IU (Meiji, Japan). Then the egg yolk with the concentration of 10% was added to a diluent solution (Kulaksiz *et al.*, 2012).

Semen Dilution

The tubes were filled with diluent according to the treatment consisting of tris aminomethane + egg yolk, CEP + egg yolk and Skim milk + egg yolk, then semen were added to the tube with to with diluent was 1:1 ratio. After that, the diluents were added to the tube until the sperm concentration to 100 million/mL. Furthermore, the semen and diluent were stored in the refrigerator at the temperature between 3-5 ^oC.the sperm motility test was performed on day 0 (the start of sperm diluted) until day 5 after diluted by using CASA (Sperm

Class Analyzer version 5.0.).

Examination of spermatozoa motility with CASA

Liquid semen as much as 3 µL was dropped on to the glass object that has warmed at temperature 37 °C, then covered it with the cover glass. The contrast phase was adjusted at pH position 1, then continued with the observation of sperm motility with 10x10 magnification. the reflector mirror was coated with the green filter (Anonymous, 2016). the diaphragm and the intensity of the light up were adjusted to the color standard specified by the microscope.e was taken in 5 fields of view. Furthermore The resulted of the analysis of each image and the average of 5 fields of view images were presented in the form of excel file.

Parameter of Research

Fresh Semen parameter were; pH, the assessment by using indicator pH; Color, the color assessment of semen volume was scored based on scaled tube directly (Susilawati, 2011) (Ax *et al.*, 2008); Consistency, the semen was moved slowly at the tube to observed the viscosity (Susilawati, 2013); Concentration, the calculation of concentration by using haemocytometer; mass motility, the subjective assessment of spermatozoa on object glass, then examined by using a light microscope with the magnification was 100 X, followed by observation of individual motility with observations with 400X magnification (Hafez, 2008; Ax *et al.*, 2008); sperm viability; the observation of living and dead sperm by using eosin and nigrosin stained which was observed by a light microscope with 100X magnification, f the living sperm was not absorbed the medium color, while the dead sperm will absorb it; sperm abnormality, the abnormality of sperm was counted from 100 of (Susilawati, 2013).

Semen diluted was observed by using SCA which were consisting of 11 parameters including: motility, progressive motility, VCL (velocity straight linear) = (µm / sec.), VAP (VSL / VAP), WOB (wobble) = (VAP / VCL), H (hyperactivity), (VSL / VCL), W (wobble) = (vapocity average pathway) = (µm / sec.), LIN (linearity), ALH (lateral head amplitude) = (µm) and BCF (beat cross frequency) = (Hz) the experimental research was performed by using completely randomized design (CRD), while the data analysis was performed by using Minitab 17 and SSPSS 16.

RESULTS AND DISCUSSION

Semen Quality

The semen quality was important to `determine whether the semen can be further processed into both the liquid semen and frozen semen. The previous study mentioned that fresh semen is feasible to be processed into frozen semen with the reaches to 70% (Susilawati, 2013). In this research, the semen quality which is processed into liquid semen were presented in Table 1.

Tabel 1. The quality of Balinese cattle semen

No.	Parameter	Number
1.	Volume (mL)	3.4 ± 0.7
2.	pH	6.6+ 2.2
3.	Consistency mid	
4.	Mass motility	2+
5.	Concentration (million/mL)	1228.0 ± 265.2
6.	Viability (%)	83.6 ± 4.3
7.	progressive motility (%)	71.0 ± 2.2
8.	Abnormality(%)	3.4 ±1.9

The volume of Balinese semen obtained was 3.4 ± 0.7 mL which were lower than Ismail *et al*., (2010) who mentioned that on cows that were maintained in oil palm plantations have a volume of 6.59 ± 0.35 mL. The sperm concentration in this study was 1.228.0 ± 265.2 million/mL, according to Ax *et al*., (2008) that the concentration of cattle spermatozoa is around 200-1800 million/mL (Ax *et al*., 2008). While in Bali cattle, based on Ismail *et al*., (2010) is 685.57 ± 61.37 x 106 sperm /and Haryana *et al*., (2016) was 803.6 ± 109.2x106 cells per ml.

The progressive motility in this study was 71.0 ±2.2%, based on Ax *et al*., (2008) the normal progressive motility in cattle is ranging from 70 - 90%, while in Bali cattle 86.7% (Haryani *et al*., 2016). The viability of semen was 83.6 ± 4.3%, according to Ax *et al*., (2008) the value of cow spermatozoa viability can reach to 80%, while in Bali cattle is 74.7%+1.5 (Haryani *et al*., 2016). The abnormal resulted was 3.4+1.9, this resulted was lower that the minimum requirement of normal sperm, therefore the semen was in normal category (Ax *et al*., 2008).

The motility of liquid semen during cooling storage

The motility test of liquid semen by using CASA showed that the percentage of motility and the

Table 2. The motility of balinese cattle sperm on different diluents.

No.	Parameters	Days	Tris+EY	CEP+EY	Skim Milk+EY
1.	Motility (%)	0	95.8±35^{ab}	96.5±1.6^{a}	96.0±1.3^{a}
		5	84.6±6.9^{a}	83.8±8.2^{a}	73.2±9.7^{ab}
2.	ProgressifMotility (%)	0	79.3±4.31^{bc}	83.7±4.8^{ab}	72.9±4.9^{d}
		5	68.0±4.5^{a}	61.4±10.6^{ab}	39.5±5.8^{c}
3.	VCL (*velocity curve linear*) (μm/sec.)	0	51.3±3.4^{bcd}	55.0±2.3^{ab}	48.0±1.6^{cde}
		5	50.9±2.7^{a}	42.0±6.0^{bc}	34.5±2.7^{c}
4.	VSL (*velocity straight linear*) (μm/sec.)	0	27.2±3.2	33.1±4.6^{a}	25.5±2.8^{cd}
		5	18.6±3.5^{abc}	20.6±3.2^{ab}	14.7±2.6^{c}
5.	VAP (*velocity average pathway*) (μm/sec.)	0	36.3±3.0c	42.7±3.5^{a}	34.3±2.7^{c}
		5	25.7±3.1^{a}	25.7±4.0^{a}	20.1+2.4^{b}
6.	LIN (*linearity*)= (VSL/VCL) (μm/sec.)	0	53.4±6.2^{a}	59.6± 6.6^{a}	52.9±5.0^{a}
		5	36.3±6.1^{c}	49.1±3.2^{ab}	42.6±6.2^{abc}
7.	STR (*straighness*) (VSL/VAP) (μm/sec.)	0	75.1±4.7^{abc}	76.8±4.4^{abc}	73.9±4.8^{c}
		5	71.6±6.1^{bcd}	80.3±2.7^{ab}	73.0±6.1^{abcd}
8.	WOB (*wobble*)= (VAP/VCL) (μm/sec.)	0	70.8±5.0^{ab}	77.4±4.6^{a}	71.5±3.9^{ab}
		5	50.6+4.7c	61.2±3.4^{ab}	58.2±4.4^{abc}
9.	Hyperactivity	0	5.2±2.0^{b}	5.2±2.0^{b}	6.7+2.8^{ab}
		5	14.2±7.1^{a}	8.4±3.9^{b}	5.7±2.2^{bc}
10.	ALH (*amplitude lateral head*) (μm)	0	1.9±0.2^{ab}	1.9±0.1^{ab}	1.8±0.1^{abc}
		5	2.9±0,1^{a}	2.1±0.2^{cde}	2.2±0.2^{bcd}
11.	BCF (*beat cross frequency*) (Hz)	0	11.5±0.9^{abcd}	10.6±1.3^{cd}	10.9±1.2^{cd}
		5	7.4±2.0^{d}	11.4±1.2^{abc}	8.8±1.7^{cd}

percentage of progressive motility in Balinese cattle during cold storage as shown in Table 2.

Spermatozoa motility value is the sum of spermatozoa fast progressive category, slowly progressive and motile non-progressive. While progressive motility value is the sum of fast progressive and slow progressive values. Massanyi et al (2008) mentioned that motile spermatozoa is a spermatozoa that moves at a rate of> 5 μm / s. While progressive motile spermatozoa is a spermatozoa that moves with speed> 20 μm / s.

The percentage of motility after diluted were not a significant difference (P> 0.05) between 3 diluents, as well as after 5 days of storage, although the motility on skim milk diluent was lower. The percentage of progressive motility in the diluent of CEP modification and Tris aminomethane was not significantly different (P> 0.05) but the skim milk diluents were significantly lower (P <0.05) as well as during storage for 5 days. Motility and progressive motility were the motility parameter level 1 in motility analysis using CASA analysis. The basis of motility classification of spermatozoa at CASA refers to human standards according to WHO (World Health Organization), because standards for animals were not available yet. There are four broad categories of spermatozoa motility, including: fast progressive (VAP> 80 μm / s), slow progressive (80 μm / s> VAP> 25 μm / s), non-progressive motile (VAP <25 μm / s) and immotile (VAP=0) (Contri et al., 2010). Based on that category, the results showed that after diluents addition the sperm motility was in the slow progressive category, while after diluted and stored on 5 days the sperm motility on skim milk diluents was in the category of motile non-progressive.

Velocity curve linear (VCL) semen diluted with CEP showed the best VCL value, although when it was compared to tris aminomethane diluent was not significantly different (P> 0.05), while it was significantly different significantly when compared to skim milk diluent (P <0.05). After storage for 5 days, the VCL value in tris aminomethan diluents was better in CEP and skim milk diluent (P <0,05). There were three parameters of spermatozoa velocity that can be measured by SCA 5.0, including VCL, VSL, and VAP. Each parameter indicates the specific spermatozoa velocity between the VCL (velocity curve linear) parameters, VSL (velocity straight linear) and VAP (velocity average pathway). Based on Perreault, (2002) opinions, that VCL is the rate of

spermatozoa along its course but does not provide speed change information during the observation period. VCL is a good indicator for "vigor" movement of spermatozoa. The VCL does not describe the direction of the spermatozoa movement nor the progression of spermatozoa. The VSL and VCL values are the complementary information.

The highest Velocity average pathway (VAP) was in CEP modified diluent, followed by the tris aminomethane and skim milk diluents significantly (P <0.05). after the fifth day of storage, the VAP in skim milk diluent was significantly lower (P <0.05) than tris aminoethane and CEP diluent. The linearity (LIN), which was the divided of VSL and VCL were showed the highest at 0 hours after dilution in CEP diluent, while the other diluents there were significantly different. After the fifth day of storage, the linearity on tris aminomethane was the lowest, while on CEP and skim milk diluents were highest than skim milk significantly (P> 0.05). Straightness (STR) is the results divided between VSL and VAP on the 0 days. STR value was lowest in skim milk diluent (P <0.05), while after storage for 5 days, did not show the difference (P> 0.05). The percentage value of spermatozoa Motile has a high correlation with VCL and low correlation with VSL. The motility of spermatozoa is influenced by the length storage and the diluent used. The motility of spermatozoa in all diluents showed the decreasing by the length of storage. This was related to the variation in oxygen uptake and metabolic activity in each diluent. Furthermore, the dilution shock when the dilution processing should be considered because it can decrease the motility value and increases the circular motility.

The correlation of motile sperm with other spermatozoa movement measures (ALH, STR and LIN) was not significant (Contri *et al*., 2010). Based on Table 2 that the values of VCL, VSL and VAP decreased gradually during cold storage. The decrease of velocity spermatozoa was associated with the availability of energy sources that were decreasing during cold storage. VSL is a simple speed parameter expressed in units of μm / s. VSL values describe the velocity of spermatozoa in one direction and are used as a leading indicator of progression of spermatozoa. VSL only informs the speed in one direction but does not provide a picture of sperm motion patterns. Sperm that moved slowly on a straight path has the same VSL value as a fast move sperm with a twisted or rotating trajectory pattern (Perreault, 2002). VAP is calculated by determining the average length of the flow and dividing it by the elapsed time to obtain VAP in μm/s. VAP depends on the average number of centroids and algorithms used. VAP value is below the VCL value and above the VSL value. Sperm with relatively straight movement in its path and regular has the VAP values more like VSL than VCL (Perreault, 2002). Tardif *et al*., (1997) mentioned that media has a contribute primarily to variations in the quality of semen in cold storage. The velocity parameter (VAP) increases after the spermatozoa were cooled, then fell slowly during the cooling process.

Wobble (WOB) is the divided between VAP / VCL, the WOB value shortly after dilution was not significantly different (P> 0.05) among all diluents, whereas after storage on five days after dilution, the WOB value on tris-aminomethane was significantly lower (P <0.05) than other diluents. LIN, STR, and WOB values were motility parameters derived from other motility parameter values, namely VSL, VCL, and VAP. STR value is VSL divided to VAP multiplied by 100; While LIN is VSL divided to VCL multiplied by 100 and WOB is VAP divided to VCL multiplied by 100. LIN is a curve linear curve alignment, STR is the mean straightness of spatial path and WOB is the measurement of actual trajectory oscillation (Udayana, 2009). Progressive spermatozoa have STR and LIN higher than circular swimming pattern sperm. STR and LIN scores were used as an attempt to identify spermatozoa movement patterns, including hyperactive sperm (strong movement) but not progressive.

The hyperactivity value at the time after the diluent was given was not a significant difference (P>0.05), whereas in the fifth day of storage showed the highest hyperactivity was tris aminomethane diluent (P> 0.05) and the lowest was skim milk diluent. Based on the Table 2 above, the percentage of hyperactive spermatozoa during cold storage were fluctuating. Balinese cattle showed the hyperactivity value that tends to increase during cold storage. The observations resulted during cold storage did not indicate an increase in spermatozoa hyperactivity. This was supported by the decreasing VCL value, stable LIN, and low ALH. While the requirements for spermatozoa are called to be hyperactive were: increased VCL, decreased LIN and increased ALH. Tardiff *et al*., (1997) have been reported that the initial values for motile

sperm of Bull in modified tyrode's solution, cornell University extender and egg yolk glycerol- tris extender were 87, 79 and 66%; Litle change Following cooling and storage at 5ºC in the latter two extenders. Also, there was a small but significant decline in sperm velocity during 3 days of storage. Hyperactive sperm increased slightly during storage.

Amplitude lateral head (ALH) and BCF (beat cross frequency) (Hz) value were not significantly different (P> 0.05) in both conditions, shortly after dilution and after storage until fifth days. Hyperactivity assessment using CASA was a level 2 motility assessment. Some other level 2 motility parameters were a linear, linear, and non-linear curve (Sarastina, 2006). However, the CASA analysis used in this study can only measure the hyperactive parameters only. The hyperactive spermatozoa category with SCA 5.0 was the VCL> 150 μm/s; LIN <50% and ALH>7 μm. However, some previous studies have a slightly different standard of hyperactive spermatozoa. Shibahara et al (2003) stated that hyperactive standards are VCL> 100 μm / s, LIN> 60% and ALH> 5 μm, While Ripp *et al*., (2003) is VCL> 100 μm / s, LIN> 60% and ALH> 7.5 μm.

In the normal conditions, fresh semen has relatively low hyperactive spermatozoa, but it will increase during capacitation. Capacitation are accompanied by changes in spermatozoa membrane structure followed by increased Ca2 + which are triggering hyperactivity and acrosomal reactions (Schmidt and Kamp, 2004). The ALH and BCF parameters illustrate the wave pattern of spermatozoa. ALH is determined by trajectory and flow rate. ALH is obtained by calculating mathematically the maximum distance from the flow average and the maximum excursion of the track path. BCF is the number of sperm trajectories across the average flow per second. The values of ALH and BCF depend on VAP, its number is variated between CASAs that calculate VAP differently (Perreault, 2002). Based on the graph above, it is known that the LIN value is relatively stable during the shelf life. The WOB value showed a downward trend in the Balinese cattle sperm during the cold storage. However, the value of STR was fluctuated.

Many factors influence the CASA outcomes value. Although the CASA system is in principle the same, each tool has different optical, hardware characteristics, algorithms, and path reconstruction. The set of tools and expertise of analysts in different CASA operations yielded the different results (Contri *et al*., 2010). Massanyi et al (2008) stated that several factors that influence the results of CASA are: sample collection, dilution, chamber type, the surface adhesivity of chamber, while Tardif *et al*., (1997) mentioned that the validity of the CASA results are depending on the sample preparation and setting of the CASA tool.

CONCLUSION

The Tris aminomethane and CEP egg yolk can maintain the spermatozoa life better than skim milk. Linear curve velocity curve (VCL), velocity average pathway (VAP), straightness (STR), and wobble (WOB) decreased, while among the 3 best diluents were CEP2 egg yolkMotilitas velocity curve linear (VCL), velocity straight linear (VSL), velocity average pathway(VAP) , straightness (STR), dan wobble (WOB) were decreased during cold storage, while the CEP 2 egg yolk were best among the three diluents.

REFERENCES

Anonim, 2016. SCA evolution-Veterinary edition. http://www.micropticsl.com/documents-and-support/sca-tutorial/. Access date 16 September 2016.

Amann, R.P. and Waberski, D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology*. 81: 5-17.

Ax, R.L., Dally, M.R., Didion, B.A., Lenz, R.W., Love, C.C., Varner, D.D., Hafez, B. and Bellin, M.E. 2008. Semen Evaluation. Reproductive in Farm Animals. 8th Edition. Edited by Hafez and Hafez. *Lea and Febiger*. 365-375.

Contri, A., Valorz, C., Faustini, M., Wegher, L. and Carluccio, A. 2010. Effect of semen preparation on casa motility results in cryo preserved bull spermatozoa. *Theriogenology*. 74 : 424-435. www.theriojournal.com.

Da Costa, N., Susilawati, T., Isnaini, N. and Ihsan, M.N. 2016a. Effect of different dilution materials usage on Indonesian Peranakan Ongole bull sperm quality during cooling process. *Indo American Journal of Pharmaceutical Sciences* (*IAJPS*). 3 (4) : 379-385 ISSN 2349-7750Available online at: http://www.iajps.com

Da Costa, N., Susilawati, T., Isnaini, N. and Ihsan, M.N. 2016b. The difference of artificial insemination successful rate of Ongole filial cattle using cold semen with different sturage time with Tris aminomethan egg dilution agent. *ISOR Journal of Pharmacy*. 6 (6) : 13-19. ISSN : 2319-4219. www.

isorphr.org.

Ducha, N., Susilawati, T. and Wahyuningsih, S. 2012. Ultrastructure and fertilizing ability of Limousin Bull sperm after storage in CEP-2 extender with and without egg yolk. *Pakistan Jornal of Biological Sciences.* 15 : 979-985.

Hafez, E.S.E. 2008. Preservation and Cryopreservation of Gametes and Embryos. *Reproductive in Farm Animals,* 8th Edition. Edited by Hafez and Hafez. Lea and Febiger. Philadelphia. 431-441.

Haryani, R., Toleng, A.L., Sonjaya, H. and Yusuf, M. 2016. Characteristic of Bali bulls sperms assessed using computerized assisted semen analysis (CASA). *International Journal of Sciences:Basic and Applied Research* (*IJSBAR*). 28 (2) : 161 - 168

Holt, W. V., O'Brien, J. and Abaigar, T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reproduction, Fertility and Development*. 19 : 709-718.

Ismail, M.I., Jaffar, F.F., Zaenaabidin, F.A., Mail, M.H., Hajarian, H., Ismail, Z., Karim, A.A.A., Jaffar, F.H.F., Nang, C.F., Hassan, N., Muhammad, S.F.S., Ibrahim, S.F., Osman, K. and Othman, A.M. 2010. Semen analysis of Bali cattle (*Bos javanicus*) bulls ranched in oil palm plantation in Malaysia. reproductive biotechnology. *Asian Reproductive Biotechnology Society*. 7 (1) : 46.

Massanyi, P., Chrenek, P., Lukac, N., Makarevich, A.V., Ostro, A., Zivcak, J. and Bulla, J. 2008. Comparison of different evaluation chambers for analysis of Rabbit Spermatozoa motility parameters using CASA syatem. *Slovak J. Anim. Sci*. 41 (2): 60-66.

Perreault, S.D. 2002. Smart use of computer-aided sperm analysis (casa) to characterize sperm motion. Reproductive Toxicology Division. U.S. Environmental Protection Agency National Health and Environmental Effects Research Laboratory.

Sarastina, Susilawati, T. and dan Ciptadi, G. 2006. Analisa Beberapa Parameter Motilitas Spermatozoa Pada Berbagai Bangsa Sapi Menggunakan Computer Assisted Semen Analysis (CASA). *J. Ternak Tropika* 6 (2) : 1-12. (Indonesia).

Tardiff, A.L., Farrel, P.B., Trouern-Trend and Foote, R.H. 1996. Computer-assisted sperm analysis for assesing initial semen quality and changes during storage at 5C. *J. Dairy Sci.* 80 : 1606-1612.

Schmidt, H. and Kamp, G. 2004. Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. *Reproduction.* 128 : 171-179.

Shibahara, H., Obara, H., Kikuchi, K., Yamanaka, S., Hirano, Y., Suzuki, Y., Takamizawa, S. and Suzuki, M. 2003. Prediction of human sperm fertlizing ability by hyperactivated pattern. *J. of Mamm Ova Res*. (20): 29-33.

Sholikah, N., Isnaini, N., Yekti, A.P.A. and Susilawati, T. 2016. Pengaruh Penggantian Bovine Serum Albumin (BSA) dengan Putih Telur pada Pengencer CEP-2 Terhadap Kualitas semen sapi Peranakan Ongole pada Suhu Penyimpanan 3-5 °C. *Jurnal Ilmu-Imu Peternakan*. (26) : 7- 15 ISSN:0852-3681 http://jiip.ub.ac.id (Indonesia).

Susilawati, T. 2011. *Spermatology*. First Print. Universitas Brawijaya Press. Malang. ISBN978-602-8960-04-5 (Indonesia).

Susilawati, T. 2013. *Padoman Inseminasi Buatan*. Universitas Brawijaya Press. Malang. ISBN 978-602-203-458-2 (Indonesia).

Susilawati, T., Wahyudi, F.E., Anggraeni, I., Isnaini, N. and dan M.N. Ihsan, 2016. Penggantian bovine serum albumin pada Pengencer CEP -2 dengan serum darah dan Putih Telur Terhadap Kualitas semen cair Sapi Limousin Selama Pendinginan. *Jurnal Kedokteran Hewan*. 10 (2) : 98 -102.

Tardif, A.L., Farrel, P.B., Trovem-Trend, V. and Foote, R.H. 1997. Computer-assisted sperm analysis for assessing initial semen quality and changes during storage at 5 °C. *Journal of Dairy Science.* 8 (8) : 1606 -1612.

Verberckmoes, S., Van Soom, A., Dewulf, J. and de Kruif, A. 2005. Comparison of three diluents for three diluents for the storage of fresh bovine semen. *Theriogenology*. 63 : 912 -922.

Verberckmoes, S., A. Van Soom, J. Dewulf and A. de Kruif, 2004. Storage of fresh bovine semen in a diluent based on the ionic composition of cauda epididimal plasma. *J. Reprod. Domestic Anim*. 39 : 410-416.