Hasanuddin J. Anim. Sci.Vol. 4, No. 1: 48-57
pISSN 2621-9182 eISSN 2621-9190
DOI:10.20956/hajas.v4i1.19439

The Quality of Bali Bull Sexed Sperm Using Soybean Extract Extender

Nur Eni Nur[a], Abdul Latief Toleng[b], Muhammad Yusuf[b*]

[a]Animal Technology Postgraduate Student, Faculty of Animal Science, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea, Makassar 90245, South Sulawesi, Indonesia
[b]Laboratory of Animal Reproduction, Faculty of Animal Science, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

*Corresponding author: E-mail: myusuf@unhas.ac.id

ARTICLE INFO

Article history:
Submission: December 31, 2021
Accepted: July 18, 2022
Published: July 25, 2022

ABSTRACT

The process of sexing spermatozoa requires a medium that is able to protect and provide an optimal environment. One of them is extender media that is used to extend the volume of semen. The extender that is commonly used is commercially but some limitation such as price and availability. Therefore, it is necessary to have an alternative extender such as soybean. The aim of this study was to know the quality of sexed sperms diluted using soybean extender. Semen of Bali bull was collected for five times and then subjected to three different extender treatments after sexing; T1 (Soybean), T2 (Tris), T3 (Tris-soybean), and T0 (Fresh semen-tris) was used as control before sexing. The parameters observed were the quality of fresh semen and after sexing. The results of the study showed that the characteristic of Bali bull fresh semen macroscopically in this study was 4.75 mL in volume, cream color, distinctive odor, pH 6.6, moderate consistency, and microscopically motility, viability, abnormality, and concentration were 94.22%, 96.06% 4.89, and 1596×10^6, respectively. Motility and viability after sexing were significantly ($P<0.05$) decrease in comparison to the fresh semen (T0) at each treatment both top and bottom layers. In conclusion, the smallest decreased of the sperms motility and viability were still greater than 50%. Different extenders as one of the treatments in the present study did not showing different motility and viability of the sperms. This suggests that the use of tris and soybean or their combination can be used as semen extender for Bali bull sexed semen.
Keywords: Soy extender, Bali bull, sexing, motility, viability.

INTRODUCTION

Efforts to increase livestock productivity in the application of artificial insemination (AI) program in cattle have developed through the use of sexed semen [1]. Furthermore, the desire sex calves production using AI program can be determined through the spermatozoa sexing method with the process of separating X and Y spermatozoa. The separation process can be carried out by distinguishing the nature, movement, and physical size of the spermatozoa [1].

In the process sexing sperms, it is necessary to use a medium that is able to protect and provide an optimal environment for spermatozoa in order to maintain the quality of spermatozoa. One of important things in maintaining sperms quality is the addition of extender media. The function of the extender media is to provide nutrients for spermatozoa during storage, allow sperm to move progressively, become a buffer for sperm, and can protect against cold shock for both frozen semen and liquid semen [2].

Generally, commercially extender is commonly used to extend the volume of semen such as Andromed. This extender contains lecithin derived from soybean extract which plays an important role in the liquid semen process [3]. However, the use of Andromed requires relatively expensive costs due to that this extender is imported, so an alternative extenderis needed.

In several studies [4]; [5]; [6] that have been carried out it was found that soybean can be used as an extender in semen. Soybean extract extender has the advantage of being more affordable and more economical. The nutritional content of soybeans is that it has several nutrients needed in spermatozoa, including lipoproteins and lecithin which can protect spermatozoa from cold stress or cold shock [7]. To our knowledge, the use of soybean as an extender is still lacking. Therefore, this study was intended to know the quality of sexed semen using soybean extract as an extender in Bali bull.

MATERIALS AND METHODS

This study was carried out in February – March 2021 at the Samata integrated farming system, Samata Village, Somba Opu District, Gowa Regency and the Animal Reproduction Laboratory of the Semen Processing Unit, Faculty of Animal Science, Hasanuddin University, Makassar.

Semen Collection and Preparation of Extender

Semen collection of a mature Bali bull about four years old was conducted using an artificial vagina (Minitube, Germany) in the morning during five times every week. After collection, the semen was then subjected to macroscopic and microscopic examination in the laboratory. That semen that passed both the examination criteria was then processed according to the treatment.

Tris crystals (Hydroximethylaminomethane) of 3.634 g, 0.50 g of glucose crystals, 1.99 g of citric acid, penicillin and streptomycin of 0.1 g were mixed inaquabidest up to 100 ml as a tris solution. Soybean extender was made from 37.5 g of peeled soybeans, 0.1 g of penicillin and streptomycin, and 5 g of sodium citrate in aquabidest of 250 ml. These two solutions were subsequently modified to be three different extender solutions; solution I = soybean extender (T1), solution II = tris extender (T2), and solution III =soybean-tris extender with a ratio of 50:50 (T3). T0 = fresh semen-tris was used as control before sexing.

Sexing the Sperms

The process of separating X and Y sperms in this study was performed using egg white media following [1]. The collected semen was diluted with tris extender with a ratio of 1:1 between semen and extender. Meanwhile, preparation of tubes for sexing the sperms with different concentration of fractions (top and bottom) was undertaking in advance. Egg whites with a concentration of 10% and 30% in the tris media were prepared at different tube. Into the tubes (10 mL in size) was first pipetting 1 mL of 30% egg white in the tris media followed by 1 mL of 10% in the top. Next, make three mediums by adding 2 ml of egg white, 30% on the bottom layer and 10% on the top layer in a test tube to form a gradient. After the top and bottom gradients were arranged, 1 ml of diluted semen was added into a test tube containing a 30% and 10% egg white gradient and then incubated for 30 minutes. Spermatozoa in the upper layer (expected as X sperms) were taken as much as 1 ml, the middle and lower layers (expected as Y sperms) were 2 ml each and put into different test tubes. Each layer was centrifuged at 2500 rpm for 5 minutes. The results of supernatant centrifugation were discarded and the density portion was then added with an extender to a scale of 2 ml and observations were made to measure motility and viability.

Evaluation of Semen

The quality of semen was evaluated both macroscopically and microscopically. Macroscopic evaluation of semen consisted of volume, pH, colour, odor and consistency, while microscopic evaluation consisted of motility, concentration, and viability.

Macroscopic Evaluation of Semen

Volume of collected semen was noted by looking the scale of the reservoir tube. Colour was determined by visually observation from milky white to cream. Consistency was evaluated by tilting the collection tube and then straightening it again and judged by the speed at which the semen returns to the bottom of the tube [8]. The assessment was based on three categories; watery (semen quickly returns to the bottom of the tube), medium (semen slowly returns to the bottom of the tube and leaves some on the tube wall) and thick (semen very slowly returns to the bottom of the tube and leaves some on the tube wall). pH was measured using pH indicator paper in the pH range of 6.0 – 8.0.

Microscopic Evaluation of Semen

Motility

Spermatozoa motility assessment was observed by making preparations or one drop of semen was dripped on a glass object and covered with a *cover glass*, then observed under a microscope with a magnification of 400× [8].

Sperms Concentration

Sperms concentrations were evaluated using a photometer SDM 6 (Minitube, Germany). The cuvette containing 3 ml of physiological NaCl solution is inserted into the device with the line facing forward and then the zero buttons is pressed. The cuvette was removed and then replaced with a cuvette containing a physiological NaCl solution to which 30 µl of fresh semen was added and then the result button was pressed, the concentration of spermatozoa would be obtained in the amount per ml [8].

Viability

Viability examination was carried out by making thin smear preparations using eosin-negrosin staining which was then observed with a microscope with a magnification of 40x10 Formula % live sperm (Viability). Sperm, in which absorbed the red colour of eosin-negrosin was considered death, otherwise was considered a live.

$$\text{Percentage of live spermatozoa} = \frac{Living\ \ spermatozoa\ \ \ percentages}{Count\ \ of\ \ spermatozoa} \times 100\%$$

Data Analysis

The quality and proportion of X and Y sperms after sexing were analyzed by One Way Analysis of Variance (ANOVA) and continued with the Least Significant Difference (LSD) test if there were treatments that showed differences. Furthermore, the proportion of spermatozoa X and Y using the Descriptive Statistical test. All calculation of data obtained were performed using Statistical package of SPSS V.16 of Windows.

RESULTS AND DISCUSSION

The Quality of Bali Bull Fresh Semen

The quality of Bali bull fresh semen was evaluated in two stages; macroscopic and microscopic. The evaluations of fresh semen soon after collection were intended to determine for further processing. The semen that met the quality required for further processing was then subjected to the treatment. Macroscopic and microscopic evaluations of fresh semen of Bali bull are shown in Table 1. The average of volume Bali bull fresh semen during collection was 4.75± 0.43 mL, ranged from 4.00 to 5.55 ml. These volumes were quite in normal range in accordance with the opinion of [2] which stated that the normal range of bovine semen volume ranges from 3.2 to 7.3 ml. However, Garner and Hafez [9] stated that the normal range of bovine semen is between 5 to 8 mL per ejaculate. This difference may due to that different breed of bulls. Furthermore, the volume of semen per ejaculation varies; this can be caused by age, temperature, nation, food and frequency of semen collection [10].

The color produced in this study was a cream colour, this indicated that the semen was in normal condition. This is in accordance with the opinion of Mukminat *et al*., [11] that normal semen is milky or cream-colored, whitish and cloudy. Suyadi *et al.* [12] explained that the color,

consistency and concentration of spermatozoa are interrelated with one another. It means that high or low concentration of sperms in the semen is affecting the color of semen.

The smell, pH, and consistency of Bali bull semen in the present study were in normal (Table 1). Inonie *et al.* [13] stated that normal semen generally has a distinctive fishy odor accompanied by the smell of the animal itself. The normal smell of semen indicates no contamination with the other substrate. In this study, the average of semen pH was 6.6±0.5. Aisah *et al.* [14] stated that the semen acidity degree for further processing ranging between 6.28 and 7.00. Normal pH is needed by spermatozoa to maintain stability and metabolic balance in the body.

Table 1. Macroscopic and Microscopic Evaluation of Bali Bull Fresh Semen

Evaluation	Parameter	Average
Macroscopic	Volume (ml) (±SD)	4.75±0.50
	Color	Creamy
	Smell	Typical
	pH (±SD)	6.6±0.5
	Consistency	Milky-moderate
Microscopic	Individual motility (%)(±SD)	94.22±2.37
	Viability (%)(±SD)	96.06±1.09
	Abnormality (%)(±SD)	4.89±1.12
	Concentration (10^6/ml) (±SD)	1596±1472

SD = Standard Deviation

Consistency of semen collected in this study was milky-moderate with an average concentration of 1596 x 10^6 ± 1472 per ml. This indicated that the semen quality is good. This is in line the statement of Garner and Hafez [15] that the concentration of bovine semen varies from 1,000 to 1,800 million per ml. Ismaya [16] also stated that the consistency of bovine semen is from medium to thick or creamy in color, indicated that the concentration of spermatozoa between 1,000 and 2,000 million spermatozoa per ml.

Individual motility and viability of the sperms obtained in the present study were 94.22% ± 2.37 and 96.06% ± 1.09, respectively. These indicated that the semen were suitable for further processing. Toliehere [17] stated that fertile males have 50-80% motile spermatozoa. Similarly, Susilawati [18] also stated that the motility of fresh bovine semen ranged from 70% to 90%. Likewise, the individual viability of the sperms in this study was consistent with the study of [19] that 50% of live and motile spermatozoa are required for use in AI. Spermatozoa that have a high percentage of life indicate that the plasma membrane is still physically intact, so that the spermatozoa cell organelles will be protected, the need for nutrients and ions for metabolic processes

Motility of Bali bull Sexed Sperms at Different Extenders

The motility of spermatozoa is the ability that needed to fertilize ovum. Fresh semen generally contains motile sperms that are able to fertilize the ovum especially in natural

breeding. Semen that is subjected to treatment before using to fertilize the ovum such as sexing is usually mostly suffered from reducing the motility. In the present study the motility of sexed spermatozoa using Bali bull semen and extending using different extenders was conducted. The motility of Bali bull sexed sperms at different extenders is shown in Table 2.

Analysis of variance showed that there was a significant difference (P<0.05) between T0 and each treatment on the top and bottom layers. In Table 2, it can be seen that the motility of T0 is higher than that of the top and bottom layers of T1, T2 and T3. The motility of the top layer of T1 and T2 was lower than that of the lower layer, while the motility of the lower layer in T3 was lower than that of the upper layer. The average motility of spermatozoa decreased after sexing compared to spermatozoa that had not been sexed. This may due to that the spermatozoa have moved out through many processes that require a lot of energy. This is in line with the study of Susilawati [1] stated that the decrease in the motility is reasonable, because the spermatozoa have undergone treatment starting from the process of separation and washing in which requires a lot of energy to maintain physiological conditions.

Table 2. Motility of Bali Bull Sperms before and after Sexing Using Different Extenders

Sperms motility	**Treatment**			
(%)(±SD)	**T0**	**T1**	**T2**	**T3**
Before sexing	89.39±5.84[a]	-	-	-
After sexing				
Top Layer (X)		66.08±11.53[b]	60.37±23.39[b]	66.55±12.10[b]
Bottom Layer (Y)		73.54±9.91[ab]	60.92±11.03[b]	62.23±17.59[b]

[a,b]Different superscripts indicate significant different (P<0.05)

T0 = Fresh semen-tris (control)

T1 = Soybean

T2 = Tris

T3 = Tris-soybean

The motility of X sperms was higher than motility of Y sperms (Table 2), this probably cause by Y sperms have a smaller head size that can move fester which can penetrate to the bottom of the tube. Hafez [20] and Afiati [21] stated that the Y sperms will move downwards while the X sperms remain in the upper layer. Sperms carrying the Y chromosome have higher motility than the spermatozoa carrying the X chromosome.

The sperms motility that was extended using soybean extender was higher than that of tris extender and tris-soybean extender. This is probably due to the ability of soybeans to suppress oxidative stress. The sperms motility in each treatment in the present study was ranging from 60 to 73%. This indicated that the sperms motility obtained in this study was suitable for further using to inseminate the cows (greater than 40%). The result of this study is higher than the previous study [22] which also using soybean as extender, which was about 52.41%.

Viability of Bali bull Sexed Sperms at Different Extenders

Sperms viability examination aims to determine the number of viable sperms. In this study, the viability of Bali bull sperms after sexing was examined and can be seen in Table 3.The average viability of Bali bull sperms before sexing was 91.13% and it was decreased significantly ($P<0.05$) after sexing using different treatments both X (top layer) and Y (bottom layer) sperms. Top layer sperms in T1, T2 and T3 were 67.75%, 61.99%, and 68.69%, respectively. While the bottom layer sperms for similar treatments were 76.77%, 65.28%, and 54.31%, respectively.

The decrease of separated sperms viability compared to the sperms viability before the separation might be occurred due to the reduced energy of the spermatozoa during the sexing process. In addition, most probably it also influenced by environmental temperature, and the components contained in the medium which resulted in structural damage to the spermatozoa membrane. This is in line with the study of [23] stated that during the process of separating spermatozoa (sexing) causes friction between the spermatozoa with the medium and other spermatozoa resulting in damage to the cell membrane. Solihati *et al.* [24] also mentioned that the decrease in spermatozoa viability was caused by an increase in the number of damaged and dead spermatozoa due to lack of energy.

Table 3. Viability of Bali Bull Sperms before and after Sexing Using Different Extenders

Sperms viability	**Treatment**			
(%)(±SD)	**T0**	**T1**	**T2**	**T3**
Before sexing	91.13±5.78^{a}	-	-	-
After sexing				
Top Layer (X)	-	67.75±11.48^{b}	61.99±23.41^{b}	68.69±12.71^{b}
Bottom Layer (Y)	-	76.77±9.42^{ab}	65.28±12.59^{bc}	54.31±24.43^{c}

^{a,b}Different superscripts indicate significant different ($P<0.05$)

T0 = Fresh semen-tris (control)
T1 = Soybean
T2 = Tris
T3 = Tris-soybean

The viability of Y sperms tended to be higher than that the X sperms. This indicates that in the top layer, immotile sperms were filtered and cannot penetrate to the bottom layer. This is supported by the study of Kaiin *et al.,* [25] stated that the high viability of Y sperms compared to the X sperms is thought to be caused by immotile sperms which allows only motile sperm to penetrate to the bottom layer.

In the Table 3 also shows that the highest sperms viability after sexing was in the T1 treatment (76.77%) using soybean extender. The viable spermatozoa are strongly influenced by the integrity of the plasma membrane, due to the presence of lipoprotein and phospholipid content in soy extender. Sugiarto *et al*. [26] stated that the survival percentage of spermatozoa depends on the integrity of the spermatozoa membrane. Damage in the spermatozoa membrane will cause disruption of the intracellular metabolic process which will cause the spermatozoa to weaken and eventually die. Ihsan [27] explained that in addition to membrane

damage, the availability of energy also greatly affects the mortality rate of spermatozoa cells. According to Hardijanto *et al.* [28] the presence of an energy source in the extender can minimize the death of spermatozoa.

CONCLUSIONS

Motility and viability of Bali bull sperms were decreased significantly ($P<0.05$) after sexing. However, the smallest decreased of the sperms motility and viability were still greater than 50%. Different extenders as one of the treatments in the present study did not showing different motility and viability of the sperms. This suggests that the use of tris and soybean or their combination can be used as semen extender for Bali bull sexed semen.

REFERENCES

[1] T. Susilawati, "Sexing Spermatozoa", Brawijaya Press University, Malang, 2014

[2] D. Hartanti, E. T. Setiati, and Sutopo, "Comparison of The Use of Extenders of Egg Yolk Citrate and Egg Yolk Tris on The Percentage of Viability of Spermatozoa in Brebes Java Bull", Animal Agriculture Journal, Vol. 1, no. 1, pp: 33-42, 2012.

[3] M. Ervandi, T. Susilawati, and S. Wahyuningsi, "The Effect of Different Extenders on Spermatozoa Quality of Cows Sexing with Albumin Gradient (Egg White)", JITV. Vol.18, no. 3, pp:177-184, 2013.

[4] F. U. Rehman, M.S. Qureshi, and R.U. Khan,"Effect of Soybean Based Extenders on Sperm Parameters of Holstein Friesian Bull During Liquid Strorage at 4^{o}C", Pakistan J. Zool. Vol. 46, no. 1, pp. 185-189, 2014.

[5] S. Chelucci, V. Pasciu, S. Succu, D. Addis, G.G. Leoni, M.E. Manca, S. Naitana, and F. Berliguer, "Soybean Lecithin-Based Extender Preserves Spermatozoa Membrane Integrity and Fertilizing Potential During Goat Semen Cryopreservation", Theriogenology, Vol. 83, no. 6, pp. 1064-1074, 2015.

[6] W. Soltan, E. El-Shenawy, L. Abd. El-Razek, M. El-Sharawy, K. Kubota, N. Yamauchi, and I. El-Shamaa,"Soybean Milk-Based Extender for Cryopreservation of Buck Spermatozoa", J. Fac. Agr. Kyushu Univ,Vol. 62, no. 2, pp. 361-366, 2017.

[7] I. Kmenta, C. Strohmayer, F. Muller-Schlosser, and S. Schafer-Somi,"Effects of a Lecithin and Catalase Containing Semen Extender and a Second Dilution with Different Enhancing Buffer on The Quality of Cold-Stored Canine Spermatozoa", Theriogenology, Vol. 75, no. 6, pp. 1095-1103, 2011.

[8] Sahiruddin, Widjiati, S.P. Madyawati, A.L. Toleng, M. Yusuf. Masturi, A. Ako, and M. F. Amrullah, "The Quality of Bali Bull Sexed Sperms at Different Incubation Time Using Egg White Sedimentation Method", IOP Conf. Series: Earth Environ. Sci., Vol. 788, 012142, pp. 1-5, 2021.

[9] D. L. Garner, and E. S. E. Hafez, "Spermatozoa and Seminal Plasma", In: E. S. E. Hafez (Ed.). Reproduction in Farm Animals, 7th Ed. Lea and Febiger, Philadelphia, 2000.

[10] J. Blegur, W. M. Nalle, and T. M. Hine, "Influence Addition Virgin Coconut Oil in Tris Egg Yolk on The Quality of Bali Bull Spermatozoa During Preservation", Jurnal Nucleus Peternakan. Vol. 7, no. 2, pp.130-138, 2020.

[11] A. Mukminat, S. Suharyati, and Siswanto,"The Effect of Various Sources Carbohydrate Supplementation in Skim Egg Yolk Extender towards The Frozen Semen Quality of Bali Bull", Jurnal Ilmiah Peternakan Terpadu, Vol. 2, no. 2, pp. 87-92, 2014.
[12] A. Suyadi, Rachmawati, and N. Iswanto,"Effect of α-TocoperolinTris-Aminomethane-Egg Yolk on The Semen Quality During Cold Storage in Boer Goats", J. Ilmu-Ilmu Peternakan, Vol. 22, no. 3, pp. 1-8, 2012.
[13] R. L. Inonie, L. O. Baa, and T. Saili, "Kualitas Spermatozoa Kambing Boerawa dan Kambing Kacang pada Penggunaan Tris-KuningTelur yang Berbeda", JITRO, Vol. 31, no. 1, pp. 52-64, 2016.
[14] S. Aisah, N. Isnaini, and S. Wahyuningsih,"Kualitas Semen Segar dan Recovery Rate Sapi Bali pada Musim yang Berbeda", Jurnal Ilmu-Ilmu Peternakan", Vol. 27, no. 1, pp. 63-79, 2017.
[15] D. L. Garner, and E. S. E Hafez,"Spermatozoa and Plasma Semen", In Reproduction in Farm Animals. E. S. E. Hafez and B. Hafez (Eds.). 7th ed. Lippincott & Williams, Baltimore, Maryland, USA, pp. 82-95, 2008.
[16] Ismaya, "Artificial Insemination Biotechnology in Cows and Buffaloes", Yogyakarta, Gadjah Mada University Press, ISBN: 979-420-848-5, 2014.
[17] M. R. Toelihere, "Fisiologi Reproduksi pada Ternak", Angkasa, Bandung, 1985.
[18] S. Susilawati,"The Effect of Centrifugation on Quality of Motility, Viability, and Acrosome Cup of Goat Sperm", Veterinaria Medika, Vol. 3, no. 1, pp. 61-64, 2010.
[19] D. Hidayatin, "Comparative Assessment of The Quality of Frozen Semen from BIB Lembang and Singosari Products in Each Distribution Channel", [Thesis] Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, 2002.
[20] E. S. E. Hafez, "Semen Evaluation in Reproduction In Farm Animals", 7 th edition, Lippincott Williams and Wilkins, Maryland, 2000.
[21] F. Afiati, "Proporsi dan Karakteristik Spermatozoa X dan Y Hasil Separasi Kolom Albumin", Media Peternakan, Vol. 27, no. 1, pp. 16-20, 2004.
[22] O. S. Ariantie, "Cryopreservation of Etawah Crossbreed (PE) Goat Semen Using Tris-Egg Yolk and Tris-Soya Extenders with Modified Carbohydrates and Different Cryoprotectants", [Thesis] Bogor Agricultural University, Bogor, 2013.
[23] A. Mahfud, N.Isnaini, A. P. A.Yekti, Kuswati, and T. Susilawati, "The Quality of Spermatozoa Post Thawing Sperm Y Frozen Semen Results from Sexing in Limousine", J. Ternak Tropika, Vol. 20, no. 1, pp.1-7, 2019.
[24] N. Solihati, R. Idi, S. D. Rasad, M. Rizal, and M. Fitriati, "Quality of Cauda Epididymal Spermatozoa of Ongole Cross Bred Bull in Egg Yolk Skim Milk, Citrate Extenders Stored at 4-5^{o}C", Animal Production, Vol. 11, no. 1, pp. 22-29, 2008.
[25] E. M. Kaiin, M.Gunawan, and T. Maulana, "Morphometry and Abnormality Evaluation of Sex-Sorted Sperm of Spotted Buffalo (Tedong Bonga)", Nus.Biosci.Vol. 9, no. 2, pp. 175-180, 2017
[26] N. Sugiarto, T. Sisilawati, and S. Wahyuningsih, 2013. "Kualitas Semen Cair Sapi Limousin selama Pendinginan Menggunakan Pengencer CEP-2 dengan Penambahan Berbegai Konsentrasi Sari Kedelai", J. Ternak Tropika, Vol. 15, no. 1, pp. 51-57, 2013.

[27] M. N. Ihsan,“Goat Semen Quality with Vitrification Freezing Use Different Level of Glycerol”, J. Ternak Tropika, Vol. 14, no. 2, pp. 38-45, 2013.
[28] S. S. Hardijanto, T. Hernawati, T. Sarjito, and T. W. Suprayogi, “Buku Ajar Inseminasi Buatan”, Fakultas Kedokteran Hewan, Universitas Airlangga, Surabaya, 2010.