NUSANTARA BIOSCIENCE
Vol. 9, No. 2, pp. 175-180
May 2017

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n090212

Morphometry and abnormality evaluation of sex-sorted sperm of spotted buffalo (*Tedong bonga*)

EKAYANTI MULYAWATI KAIIN♥, MUHAMMAD GUNAWAN, TULUS MAULANA
Research Center for Biotechnology - Indonesian Institute of Sciences. Jl. Raya Jakarta-Bogor Km 46 Cibinong, Bogor 16911, West Java, Indonesia. Tel. +62-21-8754627, Fax.: +62-21-8754588, ♥email: ekayantikaiin@yahoo.com.

Manuscript received: 31 August 2016. Revision accepted: 3 April 2017.

Abstract. *Kaiin EM, Gunawan M, Maulana T. 2017. Morphometry and abnormality evaluation of sex-sorted sperm of spotted buffalo (*Tedong bonga*). Nusantara Bioscience 9: 175-180.* This research aimed to determine the morphometry and the abnormality of the spotted buffallo (*Tedong bonga*) sperm after the separation of X and Y-chromosome-bearing sperms. Semen was collected from the spotted buffalo bull by using an artificial vagina.The semen quality was evaluated macro- and microscopically. Semen that met the required standard were subjected to separation by using Bovine Serum Albumin (BSA) 5% and 10% column method. The quality of sex-sorted sperm was evaluated microscopically, including motility, concentration, viability, abnormality and intact plasma membrane. Smear preparation using Eosin-Nigrosin dyes was made for sperm morphometry and abnormality evaluation.The evaluation of sperm morphometry and abnormality was conducted on 200 sperm cells for each observation and repeated in triplicates of observation, by using Axiovision Imager Z microscope at objective and ocular magnifications 40x and 10x, respectively.The morphological parameters observed were including the length and the width of head sperm and the head area of sperm X or Y. The abnormality parameter consisted of primary and secondary abnormalities. The data were analyzed descriptively. The results showed that the head size of the Y sperm of spotted buffalo (27.16 µm^2) was smaller than that of the X sperm (29.86 µm^2) with the abnormality of the Y sperm (7.85%) was also lower than that of the X sperm (9.7%).

Keywords: Abnormality, morphometry, sexing, sperm, spotted buffalo

INTRODUCTION

Spotted buffalo (*Tedong bonga*) belong to swamp buffalo group (*Bubalus bubalis*) found only in Tana Toraja, South Sulawesi Province. Currently, its breeding becomes increasingly difficult since the superior spotted buffalo bulls are offered for Toraja traditional ritual, thus, the bull is exclusively kept, isolated and separated from the female buffalo. Hence, the spotted buffalo bull has a very high economic value. The challenging breeding of spotted buffalo is feared to potentially reduce population and quality of spotted buffalo in Tana Toraja. Buffalo has a low breeding efficiency (35-40%). It is due to delay in puberty both in male and female, difficulty in detecting estrus, and low pregnancy rate (Tappa 2006). Utilization of breeding biotechnology can be done to increase the reproductive efficiency in spotted buffalo. The application of sex-sorted sperm is expected to generate more spotted buffalo bulls as a supply for the traditional ritual in Tana Toraja.

The sex of offspring in mammals, including in buffalo, is determined by the sex chromosome contained in the sperm that fertilizes the egg. As a result of reductive cell division during spermatogenesis, the sperm will contain only half of the DNA content of the somatic cell from the same species and generate either one of the sperm type, X sperm or Y sperm. Fertilizing an oocyte, an X sperm will form a female embryo, while a Y sperm will form a male embryo. The Y sperm possesses an SRY (sex-determining region Y) gene that functions in controlling the formation testes in male animals and the gene is not found in X sperm (Susilawati 2011). Macroscopic analysis indicated that the semen of spotted buffalo has a volume ranging between 0.3-3.8 mL; white and creamy in color with a motility of 68-79% (Battosamma 1985; Tappa 2006). Morphometry is a method to determine sperm size in farm animals and to identify X and Y sperm based on the size of sperm heads. In addition, morphometric analysis of sperm heads can be used as an indicator of *in vitro* fertility and in the determination of sperm fertility index (Padrik and Jaakma 2002, Roy et al. 2008, Thompson et al. 1994). Sperm morphology evaluation is undertaken to determine normality and abnormality of sperm cells. The size and shape of spermatozoa are species-specific and different among animals such as cattle (Boersma 2001, Belleti et al. 2005) and sheep (Gravance et al. 1998, Sancho et al. 1998) and such differences are controlled genetically. Maree et al. (2010) suggested that sperm morphology can be used to estimate sperm fertility. A normal sperm morphology can be used as an indicator for male fertility potency (Esteso et al. 2006). Carvalho et al. (2013) suggested that the measurement of sperm head shape and size in nanoscale can be developed into a sperm sexing method.

Sperm abnormality is aberrations or defects occurred on sperms which can fall in either of two categories, primary or secondary abnormality. The primary abnormality occurs during spermatogenesis inside testis. Meanwhile, secondary abnormality occurs after spermiation (the release of sperms into the lumen of seminiferous tubules) (Perry

and Patterson 2011). Sperm abnormalities are consistently associated with male infertility and suggested to be transmitted genetically (Chenoweth 2005). According to Rehman et al. (2013), the increase in sperm abnormality in cattle can occur as a result of the conventional semen freezing process.

The sperm sorting method by using 5% and 10% Bovine Serum Albumin (BSA) column have been widely applied to both dairy and beef cattle (Cain et al. 2013). Application of BSA-column-sorted sperms on buffalo has never been done before. To increase the yield of artificial insemination using sex-sorted sperm in spotted buffalo bull, microscopic evaluations are required in advance. This study aimed to evaluate morphometry and abnormalities of the sex-sorted sperms of spotted buffalo bulls.

MATERIALS AND METHODS

Semen samples were collected from two spotted buffalo bulls in the Regional Artificial Insemination Center of (Balai Inseminasi Buatan Daerah, BIBD) Puca, South Sulawesi. Semen collection was done by using an artificial vagina. The collected semen was then immediately brought to the laboratory to be analyzed according to Indonesian National Standard (SNI) procedure for the production of frozen buffalo bull semen. Evaluation of sperm quality was analyzed macro- and microscopically in BIBD Puca laboratory. Collected semen that meets the standard criteria for fresh semen then underwent sexing by using 5% and 10% BSA column method. Semen containing 300 million cells was incubated for one hour in 5% and 10% BSA column at 37,5ºC. Thereafter, each sperm fraction was collected and transferred into 15 mL centrifuge tube and added with Brackett-Oliphant (BO) medium. The tube was then centrifuged at 1800 rpm for 10 minutes, at 26-27ºC. The 5% column fraction was predicted as X sperm fraction and 10% column fraction as Y sperm (Kaiin et al. 2005, 2013).

The quality of both X and Y-sorted sperm was then observed, in term of its motility, concentration, viability, abnormality and its plasma membrane integrity. Sperm specimen preparation for morphometry and abnormality observations was done by using Eosin-Nigrosin staining (Barth and Oko 1989). 25 µl semen was pipetted onto a slide glass and then added with 50 µl staining solution. The sperm smear was then fixed by flaming the slide glass on a Bunsen burner and after that, the glass was air-dried. The analysis of plasma membrane integrity was done by adding 100 µl of sperm sample into 1 mL of 150 mOsm/kg hypoosmotic solution (0.735 g sodium citrate and 1.351 g fructose in 1 in 100 mL double-distilled water). The sperm was then incubated for 60 minutes at 37ºC. Sperm cells with an intact plasma membrane are indicated with curled tail, whilst the defect or dead sperm are marked by a straight tail.

Microscopic observation was carried out under Olympus CX23 microscope with objective and ocular magnifications of 40x and 10x, respectively. The number of samples for every observation replicate was 200 sperm cells, for both X and Y sperms. Observations were done in 3 replicates. Observation of sperm morphometry and abnormality were performed in the Laboratory of Animal Reproduction, Breeding, and Cell Culture, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, West Java, Indonesia. Observation of sperm specimen was carried out under fluorescent microscope Imager Z with an objective and ocular magnification of 40x and 10x, respectively. Sperm head dimension analysis, including measurement of sperm head length, width, and the area was done using Axiovision Rel. 4.8 application (Maulana et al. 2014), meanwhile sperm abnormality analysis includes sperm head, stem, and tail abnormalities observations. Sperm head abnormalities consist of a pear-shaped head, microcephalus, and detached sperm head. Sperm body abnormalities include abaxial, proximal droplet, and sperm body defect. Tail abnormalities include folded tail and a distal droplet (Sousa et al. 2013). The observation was done in triplicate of 200 sperm cells. Data of sperm quality evaluation was expressed as mean values and analyzed descriptively.

RESULTS AND DISCUSSION

Macro- and microscopic evaluation results of the spotted buffalo bull semen were shown in Table 1. According to the evaluation result in Table 1, it was known that the quality of spotted buffalo semen in BIBD Puca met the standard for sperm sexing. This result was in agreement with the result obtained by Battosamma (1985) which stated that the amount of semen from spotted buffalo bulls ranged between 0.3-3.8 mL with an average volume of 1.7±0.8 mL, creamy and white in color which indicate the semen was in normal and healthy conditions; a sperm concentration of 1.2 x 10^9cell/mL, and an average motility rate of 74%. Tappa et al. (2006) have reported a similar result, in which the semen volume ranged between 0.5-2 mL with an average volume of 1.71x10^9 cells/mL, an average motility rate of 74.5%, and a mass movement of +2 to +3.

The microscopic evaluation result of the sex-sorted sperm (X and Y sperm) of the spotted buffalo bull was shown in Table 2. The result indicated that X and Y sperm possess a similar motility of about 50%. The reduced motility of fresh sperm probably occurred during sperm sexing, in which the sperm undergo a more time-consuming process, starting from the separation in BSA columns up to the sperm washing, compared with those without sorting procedure. This result was similar to that of cattle sperm. Despite the reduced motility, it is still suitable for further freezing/frozen sperm straw production (Kaiin et al. 2013). The X sperm concentration was 1.29 x 10^9 cells/mL, far higher than the Y sperm concentration of 0.81x10^9 cells/mL. This difference occurred because the amount of Y sperm went through the 10% BSA column for a certain period was less than the amount of X sperm penetrating the 5% BSA column. The smaller and lighter Y sperm head made the Y sperm movement much faster than that of X sperm (Susilawati 2011). The viability of the X

sperm was 79.6%, and that of the Y sperm was 82.66 %. Meanwhile, the abnormality of the X and Y sperm were 9.70% and 7.85%, respectively. The sperm abnormality below 15-20% meant that the bull from which the sperm was collected and sorted had a good fertility. The increase in the viability and plasma membrane integrity of the sex-sorted sperm compared with that of the collected sperm (Table 1), is suspected to be caused by the filtering of dead and non-motile sperm allowing only motile sperm to pass through the BSA column.

Battosamma (1985) reported the sperm quality of spotted buffalo from Tana Toraja, South Sulawesi, in which, the sperm concentration ranged from $0.6x10^9$ to $1.0x10^9$ cells/mL with an average of 800 million sperm cells/mL and a percentage of viable sperm of 48-80%. In this study, we found that the sperm motility was at 75%, the sperm concentration was $1.79x10^9$ cells/mL with a percentage of abnormality of 17.0% (Table 2). These results were similar to the quality of fresh sperm collected from spotted buffalo bull from Tana Toraja, South Sulawesi using an artificial vagina conducted previously by Battosamma (1985), in which the sperm had a motility rate of 74%; a concentration ranging from $0.2x10^9$ to $2.5x10^9$ cells/mL; and an abnormality of 10-20% with an average of 15.06%. The X sperm with an intact plasma membrane was as much as 77.29%, while the Y-sperm was 76.30%, indicating that the plasma membrane integrity of the sperm of spotted buffalo was fairly high. Plasma membrane integrity of sperm is correlated with the motility and viability of the sperm. According to Susilawati (2011), if a sperm with an intact membrane is placed on a hypo-osmotic medium, it will increase the water content in its cytoplasm to keep the balance of the solutions inside and outside the cell, thereby causing the sperm tail to curl, hence, indicating a motile sperm.

Table 1. The quality of spotted buffalo's semen in BIBD Puca, South Sulawesi, Indonesia

Parameter	Result
Volume (mL)	4
Color	Cream
pH	6-7
Consistency	Viscous
Mass movement	+2
Motility (%)	75
Concentration (cells/mL)	$1.79x10^9$
Viability (%)	79.4
Intact plasma membrane (%)	70.9
Abnormality (%)	17.0

Table 2. The quality of sex-sorted sperm of spotted buffalo in BIBD Puca, South Sulawesi, obtained by BSA column

Parameter	Sperm quality	
	X sperm	Y sperm
Motility (%)	50	50
Concentration (cell/mL)	$1.29x10^9$	$0.81x10^9$
Viability (%)	79.6	82.66
Abnormality (%)	9.7	7.85
Intact plasma membrane (%)	77.29	76.3

Morphometry observation of sex-sorted sperm of the spotted buffalo bull showed a difference between X and Y sperm. The X sperm had a bigger size (29.86 μm^2) than that of the Y sperm (27.16 μm^2) (Table 3). This result was in agreement with Susilawati et al. (2011) which stated that the X sperm was bigger than the Y sperm. In cattle, the average length of the sperm head was 8.75 µm. Meanwhile. its average width was 4.12 µm with a head area of 32.75 µm (Susilawati et al 1999). X sperm contains more chromatin in its head than that of Y sperm, leading to a bigger head size in X sperm (Hafez and Hafez 2000).

The result of the morphometry analysis of the sex-sorted sperm of buffalo bull reported in this research was similar to the result of previous studies. Maulana et al. (2014) showed that the sperm of spotted buffalo bull obtained without semen sexing had a head length of 7.89 µm; a head width of 4.45 µm; a head area of 29.57 μm^2; and a tail length of 42.61 µm. A study by Roy (2014) reported that the sperm of Murrah buffalo and its crossing line, respectively, showed a head size of 7.59 µm and 9.18 µm; a head width of 4.45 µm and 5.11 µm; and a head area of 24.41 μm^2 and 30.76 μm^2. Chantaraprateep and Bodhipaksha (1975) found that, in buffalo, the sperm head length and width were 9.0 µm and 6.3 µm, respectively; while in buffalo, the head length and width were 12.0 µm and 6.0 µm, respectively. According to Tappa et al. (2008), different animal exhibited different sperm head size. Tappa et al. (2008) found that the buffalo sperm had a head length of 8.33 µm and a head width of 4.32 µm; the cattle sperm had a head length of 7.01 µm and a head width of 4.32 µm; meanwhile the sheep had a head length of 8.94 µm and a head width of 4.59 µm. The morphometry analysis done by Tappa et al (2008) using a micrometer was different from the analysis done in this study which used Axiovision Rel 4.8. application. Using a micrometer, Arifiantini et al (2011) obtained sperm morphometry measurement of the swamp buffalo with a head length of 6.24 µm and a head width of 3.31 µm.Varied observation results were probably due to the differences in measurement methods used. In addition, sperm size is also affected by the season at which the sperm was collected. In the summer, the length of the buffalo sperm head was 9.45 µm in dry condition and 8.52 µm in humid air. In the winter, spring and autumn, sperm had a shorter head length ranging from 7.11 to 8.01 µm (Javed et al 1997). Other sperm head measurement conducted by Hameed et al (2016) on Nili-Ravi buffalo from Pakistan also gave rise to different measurements in different seasons and breeding locations.

Garner (2006) stated that the area of the mammal's sperm head measured using flow cytometer was 34.5 µm with a ratio of X and Y sperm DNA content of 3.8%. Tapaloaga and Tapaloaga (2015) stated that the average area of Buffalo bull sperm head was 24.35 μm^2 and there was a size difference between sperm collected from the young and old bull. Thurston et al (2001) suggested there were differences in sperm shape and dimension among species as well as among individuals. Furthermore, the difference in measurement results can be due to the difference in the staining method applied that can alter the morphometry dimensions (Maree et al 2010). Staining

applied in a particular species may not be applicable in other species (Banaszewska et al 2015).

The abnormality of sex-sorted sperm of the spotted buffalo bull was shown in Table 4. From this result, it was shown that the abnormality of the Y sperm was lower than that of the X sperm. Pear shaped sperm abnormality in Y sperm (2.85%) was lower than the that of X sperm (4.28%). Similarly, in the Y sperm, other form of abnormalities such as macrocephalus (0.71%), abnormal shape (3.21%), folded tail (7.85%), and body defect (1.78%) were lower than those in the X sperm, 0.77%, 3.5%, 9.72%, and 2.72%, respectively. This was probably because the Y sperm has undergone a longer sorting process than the X sperm, consequently, more of the abnormal sperm cannot go through 10% BSA column. The most commonly occurring abnormality was the pear-shaped head, a form of primary abnormality (Table 4) (Chenoweth 2005).

The other most common found abnormality was a folded tail, which is a type of secondary abnormality, with a percentage 9.72% in the X sperm and 7.85% in the Y sperm. Arifiantini et al. (2011) discovered that the total abnormality found in the sperm of swamp buffalo was 31.86%; while, the primary abnormality constitutes 9.93%

Table 3. Morphometry of the X and Y sperm of spotted buffalo bull from BIBD Puca, South Sulawesi

Parameter	Morphometry	
	X sperm	Y sperm
Head length (µm)	8.65±0.33	7.78±0.51
Head width (µm)	4.31±0.28	4.14±0.19
Area (µm^2)	29.86±1.56	27.16±1.57

Table 4. Primary and secondary abnormalities found in the sex-sorted sperm of the spotted buffalo bull in BIBD Puca, South Sulawesi, Indonesia

Parameter	Sperm abnormality (%)	
	X chromosome	Y chromosome
Pear-shape	4.28	2.85
Macrocephalus	0.77	0.71
Abnormal shape	3.5	3.21
Detached head	2.72	2.85
Abaxial	3.5	3.9
Folded tail	9.72	7.85
Sperm body deformity	2.72	1.78
Proximal and distal droplet	1.94	2.1

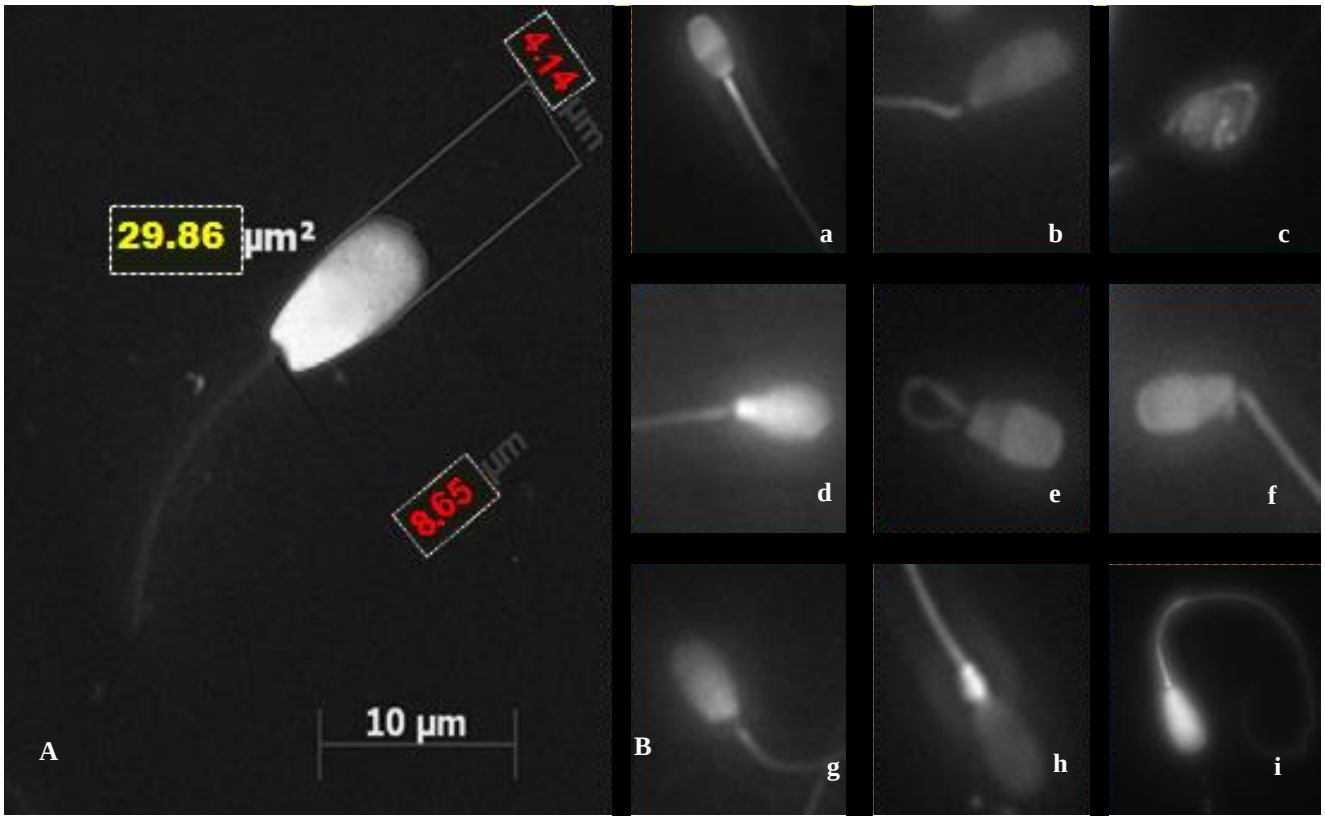

Figure 1. Abnormality morphology of sex-sorted sperm of the spotted buffalo bull. (A) Measurement of sorted spotted buffalo bull (B) Sperm morphology. a = normal sperm, b = macrocephalus, c = abnormally-shaped, d = pear-shaped, e = curled-tail, f = folded-neck, g = abaxial, h = proximal droplet, i = shrunk head.

of the total sperm observed. Susilawati (2011) suggested that the cattle sperm abnormality of 20% could reduce sperm fertility. Similarly, in sheep, there was a positive correlation between the morphology and the motility of the ejaculated semen, indicating that sperm abnormality affected male fertility (Susilawati 2011). Arifiantini et al (2011) found that a pear-shaped head abnormality on Simmental cattle was as high as 2.9% and of Bali cattle was 0.9%; meanwhile, the macrocephalus abnormality was 0.2%; and the abaxial defect was 0.1%. Sperm abnormality can be categorized into primary and secondary abnormalities. Primary abnormalities include pear-shaped head, macrocephalus, and a proximal droplet. Primary abnormalities were found in 10-15% of abnormal sperm population and these abnormalities confirmed to cause genetic infertility when found in every semen collection (Chenoweth 2005).

Factors contributing to sperm abnormalities such as the frequency of weekly semen collection and the interval between each collection. In addition, inadequate feeding as well as stresses experienced by the animal caused by both environmental factors and pathogens, are among the factors that pose detrimental effects on spermatogenesis to spermiation (Chenoweth 2005). Susilawati (2011) found that the high-temperature stress exerts a damaging impact on the sperm the most. A prolonged high temperature and humidity exposure for more than 6 weeks can cause male sterility because of the high occurrence of abnormal sperms high even during the recovery period. During this study, the research location at BIBD Puca undergone a dry season and the animal experienced inadequate intake of green fodder. These conditions were expected to be the cause of the higher abnormality observed in this study compared with that obtained by Arifiantini et al (2011) on Simmental and Bali cattle.

Based on the results of this research, it can be concluded that the morphometry of X and Y sperms from the spotted buffalo bull exhibits differences in the length, the width and the area of the sperm head. The Y sperm was smaller in size with a lower abnormality level compared with the X sperm.

ACKNOWLEDGEMENTS

The authors wish to thank the head and staff of BIBD Puca, South Sulawesi, which has facilitated the sampling in this research. This research activity was part of DIPA PN Meatpro 2014, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, West Java, Indonesia.

REFERENCES

Arifiantini RI, Purwantara B, Riyadhi M. 2011. Occurrence of sperm abnormality of beef cattle at several artificial insemination center in Indonesia. Anim Prod 12 (1): 44-49. DOI: 10.20884/1.anprod.2010.12.1.278.

Barth AD, Oko RJ. 1989. Abnormal morphology of bovine spermatozoa. 1st Edition. Iowa State University Press, Iowa.

Batosamma JT. 1985. Application of Artificial Insemination Technology for Preservation of Kerbau Belang Livestock Resources. [Dissertation]. Institut Pertanian Bogor, Bogor. [Indonesian]

Banaszewska D, Andraszek K, Czubaszek M, Biesiada-Drzazga B. 2015. The effect of selected staining techniques on bull sperm morphometry. Anim Reprod Sci 150: 17-24.

Belleti ME, Costa LF, Viana MP. 2005. A comparison of morphometric characteristics of sperma from fertile Bos taurus and Bos indicus bulls in Brazil. Anim. Reprod. Sci. 85: 105-116.

Boersma A, Rasshofer R, Stolla R. 2001. Influence of sample preparation, staining procedure and analysis conditions on bull sperm head morphometry using morphology analyser integrated visual optical system. Reprod. Dome Anim. 36 (5): 222-229.

Chantaraprateep P, Bodhipaksa P. 1975. Studies on semen of Thai swamp buffalo for artificial insemination. The Thai Vet Med Assoc 26 (3): 37-62.

Chenoweth PJ. 2005. Genetic sperm defects. Theriogenology 64: 457-468.

Carvalho JO, Silva LP, Sastori R, Dode MAN.2013. Nanoscale differences in the shape and and size of chromosome-bearing bovine sperm heads assessed by atomic force microscopy. PlosONE 8 (3) 1:7. DOI: 10.1371/journal.pone.0059387

Esteso MC, Soler AJ, Fernandez-Santos MR, Quintero-Moreno AA, Garde JJ. 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (Cervus elaphus hispanius) epididymal sperm samples. J. Andro 27 (5): 662-670.

Garner DL. 2006. Flowcytometric sexing of mammalian sperm. Theriogenology 65: 943-957.

Gravance C, Liu I, Davis R, Hughes J, Casey P. 1996. Quantification of normal head morphometry of stallion spermatozoa. J Reprod Fert 108: 41-46.

Hafez ESE, Hafez B. 2000. Reproduction in farm animals. 7th Edition. Lippincott Williams & Wilkins, Baltimore.

Hameed S, Masood S, Zaneb H, Khan MS, Younis M, Avais M, Khan MUR. 2016. Dimensional characteristic of spermatozoa of Nili-Ravi buffalo bulls: seasonal and climatic influences. Turk J Vet Anim Sci 40: 207-213.

Javed MT, Inayat A, Khan A, Asi MN. 1997. Effect of season on sperm morphometry and dead sperm percentage in different age groups of *Bubalis bubalis.* Pakistan Vet J 7 (2): 85-87.

Kaiin EM, Ginting SS, Djuarsawidjaja M et al. 2005. The quality of frozen sperm separated using dynamic and static shelving. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner, Puslitbangnak. Bogor, 12-13 September 2005. [Indonesian]

Kaiin EM, Gunawan M, Said S et al. 2013. Production of frozen sexing sperm separated with BSA column method with standardized on artificial insemination center. Proceedings International Conference on Biotechnology. RC Biotechnology LIPI, Bogor, 13-14 November 2012.

Maree L, du Plessis SS, Meckveld R, van der Horst G. 2010. Morphometric dimensions of the human sperm head depend on the staining method used. Human Reprod. 25 (6): 1369-1382.

Maulana T, Gunawan M, Kaiin EM. 2014. Abnormality and morphometry of buffalo spermatozoa collected in the UPT-IB Puca South Sulawesi and the BIBD Banjarbaru South Kalimantan. Prosiding Seminar Nasional: "Peran Bioteknologi dalam Peningkatan Populasi danMutu Genetik Ternak dalam Mendukung Kemandirian Daging dan Susu Nasional. Bioresources LIPI Expo, Bogor, 24 September 2014. [Indonesian]

Padrik P, Jaakma U. 2002. Sperm morphology in Estonian Holstein dairy bulls, factor affecting it and relation to fertility. Agraateadus 13: 243-256.

Perry G, Patterson D. 2011. Determining reproductive fertility in herds bull. Agricultural MU Guide: 1-4.

Rehman FU,Qureshi MS, Rehman SU, Khan I, Majid HA, Aftab M. 2013. Conventional semen processing technique and breeding soundness of various cattle breed bulls used in sub tropical conditioned. Sci Lett 1 (1): 9-12.

Roy B, Nagpaul PK, Pankaj PK, Mohanty TK, Raina VS, Mishra A. 2008. Studies on the biometry of sperm of riverine buffalo bulls (*Bubalus bubalis*). Buffalo Bulltein 27 (2): 192-195.

Roy B. 2014. A comparative study on sperm morphometry of cross breed and Murrah buffalo bulls. Intl J Agric SciVet Med 2 (3): 149-155.

Sancho M, Perez-Sanchez F, Tablado L, De Montserrat JJ, Soler C. 1998. Computer assisted morphometric analysis of ram sperm heads: Evaluation of different fixative techniques. Theriogenology 50: 27-37.

Sousa PC, Santos EAA, Souza ALP et al. 2013. Sperm morphological and morphometric evaluation in captive collared peccaries (*Pecaritajacu*). Pesq Vet Bras 33 (7): 924-930.

Susilawati T, Sumitro SB, Harjopranjoto S et al. 1999. Patterns capacitation of cattle caspermatozoa X and Y separated using a sephadex filtration and density gradient centrifugation percoll. Jurnal Penelitian Ilmu-ilmu Hayati 11: 29-40. [Indonesian]

Susilawati T. 2011. Spermatology. UB Press, Malang.

Tapaloaga D, Tapaloaga P-R. 2015. Study regarding age related morphometric features of Buffalo sperm. Agricult Agric Sci Proc 6: 272 -276.

Tappa B, Said S, Kaiin EM. 2006. Buffalo (*Bubalus bubalis*) breed outside their natural habitat in Tana Toraja. Proceeding International Seminar on "The Artificial Reproductive Biotechnologies for Buffaloes". Bogor, 28 Agustus-1 September 2006. [Indonesian]

Tappa B, Afiati F, Said S. 2008. Identification of spermatozoa head morphometry of buffalo, cattle, and sheep. J Protein 15 (2): 105-111. [Indonesian]

Thompson LA, Brook PF, Warren MA, Barrat CLR, Cooke ID. 1994. A morphometric comparison of the nuclear morphology of fresh and frozen thawed human zona-bound and unbound sperm. J Androl 15: 337-342.

Thurston LM, Watson PF, Milehan AJ, Holt WV.2001. Morphologically distinct sperm sub population defined by fourier shape descriptor in fresh ejaculates correlated with variation in boar semen quality following cryopreservation. J Androl 22: 282-394.