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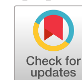

Successful Separation of X- and Y-Spermatozoa Ongole Crossbreed Using a Nano-Albumen Gradient Column

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ABSTRACT

The objective of this study was to determine the efficacy of separating X- and Y-sperm using nano-albumen (NA). The material used in this study was fresh semen of Ongole Crossbreed bulls with progressive motility of ≥ 70%. Treatments for the top and bottom fractions were T0 (BSA 5% : 10%) as a control, T1 (NA 2% : 4%), T2 (NA 4% : 6%), and T3 (NA 6% : 8%). Sexed semen was evaluated for characteristics and kinematics, and the proportions of sexed semen were validated using morphometric and polymerase chain reaction methods. The data on the characteristics and motility kinematics of X- and Y-sperm were analyzed by ANOVA using Minitab 18. The results revealed that the semen separated by the NA column generally showed the same sperm quality characteristics as the BSA column. However, the total motility of the T3y treatment in the bottom fraction was significantly lower ($p<0.05$) compared to the control. The kinematics of spermatozoa motility in the top fraction was not significantly different; however, the progressive motility value of T3y was significantly different ($p<0.05$) compared to that of T1y, and the BCF value of T2y was significantly different from that of the control in the bottom fraction. The acrosome status in the top fraction was better than in the bottom fraction, and the DNA integrity value of the top and bottom fractions showed DNA damage ranging from 2% to 5%. The present study indicated that: (1) the Nano-Albumen gradient column could be used successfully to separate X- and Y-spermatozoa, (2) characteristic values and sperm kinematics, acrosomal status, and DNA integrity were comparable to those of the BSA control, (3) combination column in treatments NA 4% (T2x) and 6% (T2y) appeared the good ability to separated X and Y-sperm.

Keywords: kinematic; nano-albumen; ongole crossbreed; sexing; sperm

INTRODUCTION

The ability to control the sex of offspring in farm animals is a topic of great interest for agricultural researchers and has commercial importance. For example, females are preferred in the dairy industry, whereas males are preferred in the meat industry. Controlling the sex ratio results in direct returns in the livestock sector, allowing for better food production management, animal welfare improvement, faster genetic selection, and reduced environmental impact (De Canio *et al.*, 2014). Separation of X- and Y-sperm for pre-selection of the desired sex is economically important in livestock production, allowing the livestock sector to produce an optimal proportion of males and females (Yang *et al.*, 2014). It can also reduce management costs by selectively managing for superior bulls or cows (Prakash *et al.*, 2014).

Various methods of sperm sexing have been developed, such as sedimentation, albumin gradient column, centrifugation, percoll density gradient, electrophoresis, filtration by Sephadex column, HY antigen, and flowcytometry sorting cells (Sharma & Sharma, 2016). However, due to various advantages and disadvantages, only a few of these are commonly used for sexing. One of the most common sexing methods, particularly in Indonesia, is bovine serum albumin in column separation (Kaiin *et al.*, 2013). However, several issues arise when separating sperm with albumin columns, including a decreased sperm quality after freezing, high cost, and limited albumin availability (Maulana *et al.*, 2019), making it difficult for commercial use. Based on these issues, it is necessary to provide an alternative low-cost and dependable local material without reducing the quality of the sexed sperm.

The use of egg white as a separation medium for sexing sperm has been investigated (Afiati, 2004), but it still has many limitations because it is not in powder form, making it unstandardized and unsuitable for long-term storage. This study aimed to investigate whether egg white nano-albumin (NA) extract could be used in albumin columns to separate X- and Y-sperm.

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MATERIALS AND METHODS

This study was carried out at the Animal Reproduction Laboratory, Research Center for Biotechnology, Indonesian Institute of Sciences, Bogor, from January to April 2021. A total of two Ongole Crossbreed (OC) bulls with live mount and teaser were used, with six ejaculates. The maintenance and use of experimental animals followed the Animal Ethics Committee of the Indonesian Institute of Sciences (No. 9879/WK/HK/XI/2015). One day old fresh chicken eggs (Lohmann Brown Layer) were kindly provided by local farmers. Egg white was separated from the yolk and stirred gently to prevent foam formation and provide a homogeneous mixture (IKA Eurostar 200). The NA was made from extracted albumin from egg white and processed for nanoparticles using the methods of Dwiastuti *et al.* (2016) with a slight modification. In this study, extracted albumin was homogenized using an Ultra-Turrax® homogenizer (model T 25, IKA-Werke GmbH & Co.KG) for 60 s, then sonicated using an ultrasonic processor (Vibracell 75043 20 kHz, Bio block Scientific, USA) for 30 min with 5-s pulses and stabilized at 20–30 °C. The NA was then stored at –80 °C before the freeze-drying process, which was carried out according to Maulana *et al.* (2019).

Sexing Sperm Using Gradient Nano-Albumen

Semen was collected using an artificial vagina. Immediately after collection, the fresh semen was taken to the laboratory for macroscopic and microscopic evaluations. The macroscopic evaluation included volume, color, consistency, and pH. The microscopic evaluation included mass movement, motility, viability, sperm concentration, and morphology (Arif *et al.*, 2020). The semen was diluted to a 200 × 10^6 sperm/mL final concentration in Tris buffer (Tris aminomethane 3.03 g citric acid 1.78 g, fructose 1.25 g, H_2O 100 mL; Maulana *et al.*, 2019). Aliquots of 1 mL were added into sexing tubes for the following treatments:
T0: Control BSA (Bovine Serum Albumin-A3608, Sigma) 5%–10% (top–bottom) gradient column; T1: Nano-albumen 2%–5% (top–bottom) gradient column; T2: Nano-albumen 4%–6% (top–bottom) gradient column; T3: Nano-albumen 6%–8% (top–bottom) gradient column. The bottom fraction with high concetration was predicted as Y sperm and top fraction as X sperm (Kaiin *et al*., 2013; Maulana *et al*., 2019).

Tubes containing semen were incubated for 40 min at room temperature (27 °C) before each sperm fraction was collected and transferred using a micropipette into 15-mL centrifuge tubes (Corning, USA) and containing Tris buffer (Tris aminomethane, Merck, Germany). The tube was then centrifuged at 312 RCF for 10 min at room temperature (Hettich® EBA 20, Germany), and the pellet was supplemented with 1 mL Tris buffer. The quality of sperm in each gradient was assessed based on Maulana *et al.* (2019).

Sexing Sperm by Kinematic Motility

Motility patterns of spermatozoa were assessed objectively using a computer-assisted semen analyzer (CASA, Spermvision 3.7; Minitube, Germany). Aliquots of 5–10 µL of semen were deposited on warmed slides at 38 °C and covered with coverslips. The microscope (Axioo-Scope A1, Carl Zeiss, Germany) was set to one phase contrast and 200× magnification. Sperm images in four fields were auto-scanned to analyze the sperm kinematic patterns. The parameters measured in this study were spermatozoa motility (motility, progressive motility, velocity curve line (VCL), velocity straight line (VSL), velocity average path (VAP), linearity (LIN), straight (STR), amplitude head displacement (ALH), and beat cross frequency (BCF).

Sperm Viability, Abnormality, and Membrane Integrity

Sperm viability (%) and abnormality (%) were assessed according to Arif *et al*. (2020), modified. Twenty microliters of semen with 40 µL of eosin–nigrosine solution were homogenized, smeared, and dried above a heated plate. Sperm that did not take up the eosin–nigrosine stain were considered alive, whereas those that did were considered dead. Sperm membrane integrity (%) was determined by calculating the percentage of sperm having an intact plasma membrane by hypo-osmotic swelling (HOS) test, performed by incubating 100 µL of sperm sample in 1 mL of 150 mOsm/L hypo-osmotic solutions for 60 min at 37 ^{o}C. Sperm with intact plasma membranes were indicated by curled or swollen tails, while straight tails marked defective or dead sperm (Padrik *et al.,* 2012). The stained slides were evaluated using a light microscope at 400× magnification and at least 200 sperm cells. The viability, abnormality, and membrane integrity values are expressed as percentages (%).

Sperm Acrosomal Status and DNA Integrity

The acrosomal status of the sperm was evaluated by using lectin peanut agglutinin (FITC-PNA, Sigma St. Luis MO) fluorescence stain. Samples of X- and Y-sperm were smeared on glass slides and fixed for 10 min in 96% ethanol at room temperature. Samples were air-dried, 30 µL (100 µg/mL) PNA added dropwise, and incubated for 30 min at 37 °C. Two hundred cells per slide were examined under a fluorescence microscope (Imager Z7, Axiovision Carl Zeiss; 380/420 nm excitation/barrier filter). Sperm with intact acrosomes exhibit a green fluorescence in the acrosome, whereas non-intact acrosome sperm exhibit dark fluorescence (Rajabi-Toustani *et al.,* 2019).

DNA integrity of sperm for sexing was evaluated by using the acridine orange staining technique. Samples of X-sperm (Tx) and Y-sperm (Ty) were smeared on glass slides, air-dried, and fixed for 2 h in Carnoy's solution. After fixation, the samples were stained with acridine orange solution (at 1000× dilution

with GL-PBS) overnight (Said *et al.*, 2015). Each slide was then washed with distilled water and sealed with synthetic resin to prevent it from drying. Slides were examined under a fluorescence microscope (Imager Z7, Axiovision Carl Zeiss; 490/530 nm excitation/barrier filter). Two hundred cells were observed in each treatment slide. Sperm with normal DNA content present a green fluorescence, whereas sperm with abnormal DNA content emit fluorescence in a spectrum varying from yellow-green to red.

Sperm Morphometrics

The sperm smear was fixed by flame on the slide glass over a Bunsen burner and washed using absolute ethanol for 4 min, and the glass was then air-dried. The glass slide was washed in chloramine 0.5% solution for 2 min, then washed with distilled water and 95% ethanol. The sperm slide was then stained by Williams staining for 8-10 min and immediately washed with flowing water and air-dried (Mansur *et al.*, 2018). The sperm was evaluated by Imager Z7 Carl Zeiss microscope with 400× magnification and then analyzed using Image Raster 3 software to measure the area, perimeter, length, and width of the head and tail of the sperm. A total of 500 sperm were measured in each fraction. X-chromosome-bearing sperm have larger head morphometry than do Y-chromosome-bearing sperm (Santolaria *et al.*, 2016).

DNA Extraction and Amplification by Polymerase Chain Reaction

According to the instruction manual of the DNA extraction kit, a total of 10 × 10^6 cells/mL of sperm were extracted by the spin column method using the gSYNC DNA Extraction Kit (GENEAID). The total sperm DNA obtained was stored at a –20 °C until the next stage.

The PCR analysis (Mastercycler® gradient (Eppendorf, Hamburg, Germany) was conducted to validate the X- or Y-sperm after separation to amplify the BRY gene 300 bp (Rattanasuk *et al*., 2011; forward primer 5′-CTCAGCAAAGCACACCAGAC-3′, reverse primer 5′ GAACTTTCAAGCAGCTGAGGC 3′) and the FOXP3 gene 204 bp (Arishima et al., 2017; forward primer 5′-TCAGATGCAGACCCCGATAC-3', reverse primer 5′-CTGAGTCAGGGCAGCATAGA-3′). The PCR product was then visualized by electrophoresis using 1% agarose gel followed by SyBr staining and captured in GBox documentation System (Syngene, Cambridge, UK).

Statistical Analysis

All data concerning semen characteristics, kinematic motility, acrosomal status, and DNA integrity of X- and Y-sperm have been presented as means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) using Minitab 18.1 (Minitab for Windows, Minitab, Inc. USA). Differences between treatments were considered statistically significant at p<0.05 by Fisher LSD analysis.

RESULTS

The characteristics of fresh OC bull semen are shown in Table 1. The sperm motility, concentration, and volume of the semen were 80.08%, 1914.83 × 10^6/mL, and 5.50 mL, respectively. According to National Standard Institute (2017), the characteristics of the OC bull semen used in this study are categorized as of good quality with motility >70%, concentration >800 x10^6/mL, and abnormal sperm <20%.

Morphometric identification of X- and Y-bearing sperm, as indicated in Table 2, shows the morphometric of X sperm in NA treatments varied from 35.67-36.09 μm^2, whereas Y sperm ranged from 27.97-28.79 μm^2. This finding was comparable to the control, X-bearing sperm (35.33 μm^2), and Y-bearing sperm (28.39 μm^2). The characteristics of sperm after separation in the top and bottom fractions can be seen in Tables 3 and 4. The Tables show that all NA treatments were not significantly different from the control group for all parameters, except T3y treatment with lower total motility in bottom fraction compared to the control group but not significantly with other NA treatments. This indicates that the NA treatments were found to

Table 1. Characteristics of fresh Ongole Crossbreed bull semen

Variables	Quality (Mean ± SD)
Volume	5.50 ± 1.92
pH	6.58 ± 0.34
Color	Cream
Mass motility	++
Consistency	Moderate
Concentration (x10^6/mL)	1914.83 ± 294.27
Motility (%)	80.08 ± 3.62
Viability (%)	84.22 ± 4.25
Abnormality (%)	14.98 ± 1.86
Membrane integrity (%)	74.35 ± 9.38

Note: ++ = Mass movement motility of sperm swirling motion.

Table 2. Morphometric and sex proportion of X- and Y- bearing sperm

Sperm characteristics variables	Treatments (Mean ± SD)			
	T0	T1	T2	T3
Top fraction sperm (μm²)	35.33 ± 1.89	35.95 ± 2.04	36.09 ± 2.69	35.67 ± 2.49
Female (X) proportion (%)	67.62 ± 7.84	72.86 ± 6.39	75.52 ± 8.94	56.94 ± 12.22
Bottom fraction sperm (μm²)	28.39 ± 1.29	28.02 ± 1.53	27.97 ± 1.46	28.79 ± 1.03
Male (Y) proportion (%)	75.84 ± 7.30	63.12 ± 3.68	72.45 ± 5.84	48.43 ± 10.75

Note: T0= (Control group, BSA 5% : 10%); T1x= (Nano-Albumen, 2% : 5%); T2= (Nano-Albumen, 4% : 6%); T3= (Nano-Albumen, 6% : 8%).

Table 3. Characteristics of sperm in the top fraction (X)

Sperm characteristics variables	Treatments (Mean ± SD)			
	T0x	T1x	T2x	T3x
Total motility (%)	86.24 ± 3.90	87.16 ± 3.14	83.77 ± 6.78	83.33 ± 4.69
Viability (%)	86.77 ± 2.32	87.13 ± 5.28	83.31 ± 11.15	84.85 ± 7.30
Abnormality (%)	6.8375 ± 0.82	9.96 ± 5.88	8.90 ± 2.76	11.48 ± 1.85
Membrane integrity (%)	83.4525 ± 2.48	83.71 ± 4.08	83.33 ± 6.93	78.05 ± 11.04
Concentration ($x10^6$)	270.8 ± 23.16	391 ± 207.52	297.4 ± 254.86	263.95 ± 134.71

Note: T= Treatment; NA= Nano-albumen; T0x= (BSA 5%); T1x= (NA2%); T2x= (NA 4%); T3x= (NA 6%).

Table 4. Characteristics of sperm in the bottom fraction (Y)

Sperm characteristics variables	Treatments (Mean ± SD)			
	T0y	T1y	T2y	T3y
Total motility (%)	87.62 ± 3.41 [a]	82.13 ± 6.4 [ab]	70.49 ± 7.04 [ab]	59.04 ± 26.96 [b]
Viability (%)	83.66 ± 4.02	82.35 ± 7.67	79.77 ± 8.72	72.52 ± 17.94
Abnormality (%)	10.55 ± 3.82	12.19 ± 3.58	12.61 ± 1.71	11.69 ± 7.73
Membrane integrity (%)	81.41 ± 7.84	72.36 ± 9.59	62.85 ± 5.99	59.09 ± 20.19
Concentration ($x10^6$)	148.3 ± 20.76	155.94 ± 80.87	116.96 ± 108.87	121.85 ± 82.12

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). T= Treatment; NA= Nano-albumen; T0y= (BSA 10%); T1y= (NA5%); T2y= (NA 6%); T3y= (NA 8%).

Table 5. Kinematic motility of sperm in the top fraction (X)

Variables	Treatments (Mean ± SD)			
	T0x	T1x	T2x	T3x
Progressive (%)	70.13 ± 6.97	74.84 ± 5.40	73.71 ± 7.48	77.07 ± 6.78
VAP (µm/s)	98.91 ± 22.42	104.68 ± 15.27	106.39 ± 11.94	99.39 ± 22.95
VCL (µm/s)	164.24 ± 36.74	180.92 ± 30.91	183.18 ± 29.05	155.12 ± 31.92
VSL (µm/s)	67.92 ± 26.71	70.45 ± 18.57	69.18 ± 14.74	69.30 ± 23.78
STR (%)	63.18 ± 13.11	64.93 ± 10.57	63.08 ± 7.34	65.73 ± 11.46
LIN (%)	37.48 ± 38.87	37.60 ± 7.16	36.42 ± 2.94	40.85 ± 9.25
WOB (%)	58.95 ± 3.47 [ab]	57.38 ± 3.38 [b]	58.15 ± 4.02 [b]	61.77 ± 5.04 [a]
ALH (µm)	4.85 ± 1.28	5.68 ± 1.95	5.56 ± 1.52	5.11 ± 1.18
BCF (Hz)	31.41 ± 5.20	30.60 ± 3.94	29.25 ± 3.02	31.02 ± 5.56

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). T= Treatment; NA= Nano-albumen; T0x= (BSA 5%); T1x= (NA2%); T2x= (NA 4%); T3x= (NA 6%). VAP= velocity average path; VCL= velocity curve line; VSL= velocity straight line; STR= straight; LIN= linearity; ALH= amplitude head displacement; BCF= beat cross frequency.

be effective in preserving motility and viability and preventing membrane damage during the sperm separation process.

The kinematic motility of sexed sperm in the top and bottom fractions can be seen in Tables 5 and 6. The results show the kinematic motility in the top fraction; there were no significant differences in progressive velocity, STR, LIN, ALH, and BCF parameters among the control group and for all NA treatments (Table 5). Likewise, in the bottom fraction (Table 6), the velocity, LIN, STR, and ALH values showed no significant differences, except in progressive motility of 8% (T3y) treatment was significantly different ($p<0.05$) compared to T1y treatment. Likewise, for BCF, the T2y treatment showed a significantly lower ($p<0.05$) compared to the control group.

The acrosome status and DNA integrity of the sexed sperm in this study are shown in Table 7. Sperm acrosome status in the top fraction was higher than in the bottom fraction, indicating that the acrosome's bottom fraction is more damaged, and the NA treatments appeared to be more effective to protect Sperm DNA than the control. Sperm with intact acrosome shows fluorescence in the acrosome area; those with non-intact acrosome look darker on the acrosome (Figure 1a, 1b). Otherwise, the value of DNA integrity showed that the NA treatments and control had a DNA fragmentation value of 2%-5% and DNA integrity is generally good (Figure 2).

The efficiency of separating X- and Y-sperm *in vitro* using NA were determined using morphometrics (Table 2). The study indicated that the percentage of X-sperm in the NA treatments ranged between 56.94% and 75.52%, while the percentage of Y-sperm varied between 48.43% and 72.45%, with the best sperm separation using NA being 4% in the top fraction and 6% in the bottom fraction. The results of the molecular validation of the PCR method using two pairs of primers for the male-specific gene (BRY) and the female-specific gene (FOXP3) are demonstrated in Figure 3. The T1y,

Table 6. Kinematic motility of sperm in the bottom fraction (X)

Variables	Treatments (Mean ± SD)			
	T0y	T1y	T2y	T3y
Progressive (%)	75.98 ± 7.53 [ab]	72.97 ± 11.49 [a]	63.83 ± 7.41 [ab]	51.38 ± 22.69 [b]
VAP (µm/s)	112.31 ± 22.00	98.19 ± 15.86	95.90 ± 20.18	99.44 ± 23.76
VCL (µm/s)	183.67± 34.32	166.26 ± 24.56	162.32 ± 34.26	171.51 ± 32.88
VSL (µm/s)	77.35 ± 25.96	66.38 ± 12.60	61.91 ± 17.59	67.38 ± 27.81
LIN (%)	64.33 ± 11.55	65.58 ± 8.98	65.35 ± 8.89	67.73 ± 13.28
STR (%)	38.67 ± 8.96	39.58 ± 4.29	39.72 ± 7.76	40.02 ± 10.00
WOB (%)	59.97 ± 4.03	60.42 ± 5.04	60.45 ± 5.10	58.48 ± 4.15
ALH (µm)	5.40 ± 1.29	4.92 ± 1.68	4.80 ± 1.75	4.54 ± 1.57
BCF (Hz)	32.68 ± 5.56 [a]	30.57 ± 2.62 [ab]	28.06 ± 1.91 [b]	29.46 ± 2.19 [ab]

Note: Means in the same row with different superscripts differ significantly (p<0.05). T= Treatment; NA= Nano-albumen; T0y= (BSA 10%); T1y= (NA5%); T2y= (NA 6%); T3y= (NA 8%). VAP= velocity average path; VCL= velocity curve line; VSL= velocity straight line; STR= straight; LIN= linearity; ALH= amplitude head displacement; BCF= beat cross frequency.

Table 7. Acrosomal status and DNA integrity of sexed sperm

Variables	Sperm	Treatments (Mean ± SD)			
		T0	T1	T2	T3
Acrosomal status	X	66.51 ± 1.76 [b]	63.26 ± 2.3 [c]	73.80 ± 2.14 [a]	37.64 ± 1.73 [d]
	Y	34.46 ± 3.23 [b]	35.33 ± 1.52 [b]	54.31 ± 1.62 [a]	34.02 ± 3.87 [b]
DNA Integrity	X	97.57 ± 1.98 [ab]	95.89 ± 2.12 [b]	97.01 ± 0.88 [ab]	98.30 ± 2.12 [a]
	Y	97.06 ± 1.49	96.13 ± 2.55	95.98 ± 0.26	95.03 ± 2.66

Note: Means in the same row with different superscripts differ significantly (p<0.05).T0= (Control group, BSA 5% : 10%); T1= (Nano-Albumen, 2% : 5%); T2= (Nano-Albumen, 4% : 6%); T3= (Nano-Albumen, 6% : 8%).

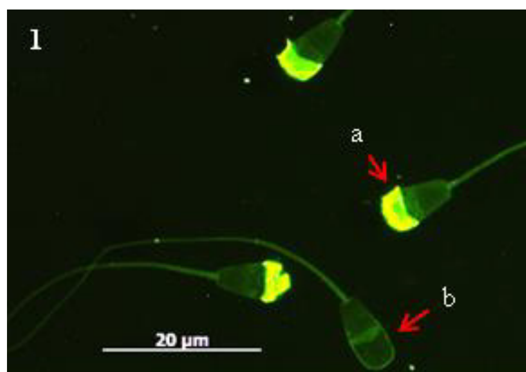

Figure 1. Acrosomal status (Magnification 60x, microscope fluorescence, Imager Z7, Carl Zeiss, Germany); a. Intact acrosome (green fluorescence in the acrosome), b. Non-intact acrosome (dark fluorescence in the acrosome).

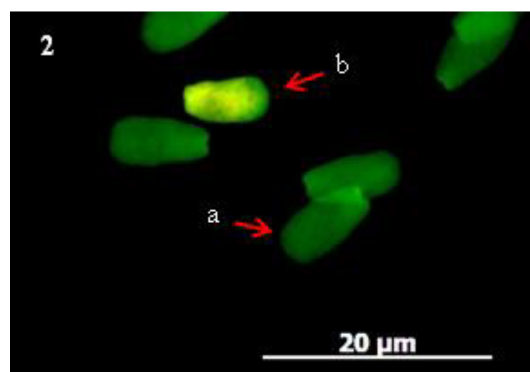

Figure 2. DNA integrity of sexed sperm (Magnification 40x, microscope fluorescence, Imager Z7, Carl Zeiss, Germany): a. Normal DNA (green fluorescence); b. Fragmented DNA (yellow-green to red fluorescence).

T2y, and T3y NA treatments using BRY primers are visible and show the same size of molecular gene weight (300 bp) as that reported by Rattanasuk *et al.* (2011). Meanwhile, all NA treatments (T1x, T2x, and T3x) that were validated using the FOXP3 primer are also visible and of the same size (203 bp) as those reported by Arishima *et al.* (2017). The use of BRY and FOXP3 genes in our study did not quantify the X- or Y-Sperm in samples after NA treatments but only evaluated the presence of X- or Y- DNA. Therefore, the accuracy of the validation method using BRY and FOXP3 gene was quite low due to the limitation of detecting the amount of “contamination” or “undesirable” sperm after NA treatment. However, the BRY and FOXP3 genes in this study provided information about thickness differentiation on PCR product bands on electrophoresis results (Figure 3) that were assumed to correlate with the amount of the X- or Y- Sperm after NA treatments.

DISCUSSION

Morphometry analysis of sex-sorted sperm by using Image Raster 3 software in this study revealed a difference between X and Y sperm. The X sperm was larger (35.67-36.09 µm^2) than the Y sperm (27.97-28.79 µm^2). This finding agreed with Susilawati *et al.* (2011), who found that the X sperm was larger than the Y sperm. The X sperm has more chromatin in its head than the Y

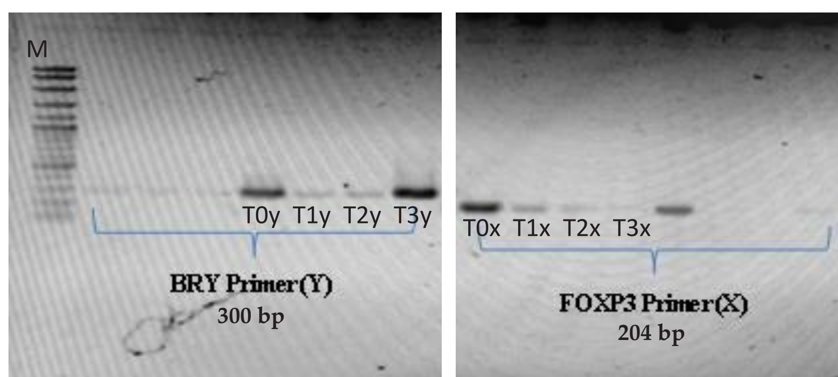

Figure 3. Results of sexing sperm by PCR; (M= 100bp DNA ladder, T0x= BSA 5%, T1x= NA 2%, T2x= NA 4%, T3x= NA 6%, T0y= BSA10%, T1y= NA 5%, T2y= NA 6%, T3y= NA 8%).

sperm, resulting in larger head size in X sperm (Hafez & Hafez, 2000). According to Garner (2006), the area of the mammal's sperm head measured by flowcytometry was 34.5 μm^2, with a 3.8% ratio of X and Y sperm DNA content.

According to this study, the separation of sperm with NA incubated for 40 min at room temperature did not affect sperm quality, and it was still feasible for the freezing procedure. The lowest motility required for sexed sperm utilizing an albumin gradient column (BSA and channalbumin) is 60% (Kaiin *et al.*, 2013; Maulana *et al.*, 2019). The low motility observed in the T3y treatment was most likely due to the high concentration and viscosity of the NA used in the bottom fraction of the column. Its condition necessitated more energy for the sperm to penetrate the column, and the lower fraction remained motile. Then, the post-separation sperm washing process also contributed to sperm motility disruption. According to Kusumawati *et al.* (2017), sperm motility decreases due to various treatments such as separation, washing, and cooling, which leads to sperm expending a great deal of energy to maintain their physiological parameters. Furthermore, eliminating seminal plasma immediately after sperm collection enhanced the sort of rate and the number of viable sperm after sorting (Burroughs *et al.*, 2013). After the sorting process, the NA treatment showed good quality and did not differ significantly from the control group BSA.

Penfold *et al*. (1998) reported no difference in spermatozoa velocity between X- and Y-sperm. The viscosity of diluents, pH, energy sources, and osmolarity are all factors that can affect spermatozoan velocity, progressive motility (PM), and velocities (VCL, VSL, and VAP) have a significant impact on fertilization (Perumal *et al.*, 2014). Another factor that reduces velocity during storage is the generation of reactive oxygen species (ROS) due to spermatozoa membrane damage. CASA provides for evaluating the fertilizing capacity of sperm based on spermatozoa velocity parameters. CASA VAP readings could be utilized to predict the fertilization capacity of frozen-thawed bull sperm (Nagy *et al.*, 2015).

According to Malik *et al.* (2011) and Blondin *et al.* (2009), sperm sexing methods such as centrifugation can reduce motility and velocity. The sperm of both sexes must adjust to changing settings, reducing motility and spermatozoa membrane integrity. In vivo bovine fertility can be predicted using PM, motility, and BCF as a standard (Oliveira *et al.*, 2013). The BCF values represent the frequency with which spermatozoa move along their average trajectory. Sarastina *et al.* (2007) observed BCF values in fresh semen of Brahman cattle at 29.39±3.85 and 28.91±6.83 in Ongole, respectively. Katiravan *et al.* (2011) found high motility will increase the BCF; frozen semen of Bos taurus had BCF values of 27.6±1.0 and Indicus 27.9±0.5. According to Oliveira *et al.* (2013), numerous sperm characteristics, such as total motility, progressive motility, and BCF, were important predictors of fertility.

The higher the ALH value, the lower the quality of the spermatozoa, which can hinder their movement (Amal *et al.*, 2019). ALH was significantly higher in high fertility (HF) bulls (reflected by high amplitude flagellar movement under microscopic evaluation) with numerically lower LIN and higher VCL values compared to low fertility (LF) bulls (Shojaei *et al.*, 2012).

The ALH and BCF parameters describe the spermatozoa wave pattern. ALH is determined by trajectory and flow rate and is obtained by calculating the maximum distance from the flow average and the maximum excursion of the track path mathematically, and BCF is the number of sperm trajectories crossing the average flow per second (Susilawati, 2011). The levels of ALH and BCF are determined by VAP, and their number varies amongst CASAs that calculate VAP differently. VCL> 150 μm/s, LIN 50%, and ALH>7 μm were the hyperactive spermatozoa group with SCA 5.0 (Susilawati, 2011). According to Kathiaravan *et al*. (2011), higher VCL (>70 μm/s) and ALH (>7 μm) levels indicate hyper-activated sperm.

In this study, sperm acrosome damage was produced by several influencing factors, including the washing of the sperm after separation and the nature of the sperm separating medium, which causes sperm capacitation. Kaiin *et al.* (2018) stated that using BSA with a bracket and oliphant (BO) medium in the sperm sexing process does not cause capacitation. The presence of an acrosome, which can activate the acrosome response and cause oocyte fusion, determines the success of fertilization. One of the most important processes in mammalian fertilization is the acrosome response (AR),

which is normally initiated when the sperm penetrates the oocyte. The AR occurs when the plasma membrane and the underlying outer acrosomal membrane fuse, allowing the acrosomal contents to be released (Costa *et al.*, 2010). Acrosomes are essential in the fertilization process; when spermatozoa bind to the zona pellucida, the AR is stimulated, resulting in the release and activation of acrosome enzymes, allowing spermatozoa to pass through the zona pellucida (Miranda *et al.*, 2009). To fertilize oocytes, spermatozoa must be acrosome-intact; if the acrosome reaction occurs before the spermatozoa reach the fertilization stage, the spermatozoa will lose their ability to fertilize the oocyte. Damage to sperm DNA does not impede oocyte fertilization or completion of early cleavage stages but may block blastocyst formation by inducing an apoptosis-like phenomenon (Insani *et al.*, 2014).

Manna *et al*. (2003) reported the use of the BRY gene in buffalo embryo selection and showed a sex-specific pattern of amplification on genomic DNA, as well as embryonic samples. In addition, Arishima *et al*. (2017) used the FOXP3 gene to study infertility in female Japanese Black cattle. In this study, the amount of "contamination" or "undesirable" sperm after treatment could not be determined by using the BRY and FOXP3 gene validation method. However, BRY and FOXP3 gene validation provided scientific evidence that the NA treatments successfully separated the X- and Y-sperm.

CONCLUSION

It is concluded that (1) a nano-albumin gradient column could be used successfully for the separation of X- and Y-spermatozoa, (2) characteristic values and sperm kinematics, acrosomal status, and DNA integrity were comparable to the BSA control, and (3) combination of the column in treatments nano-albumen 4% (T2x) and 6% (T2y) appeared the good ability to separated X- and Y-sperm.

CONFLICTS OF INTEREST

We certify no conflicts of interest concerning any financial, personal, or other relationships with people or organizations related to the material discussed in the manuscript.

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