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Research article

Evaluation of quality and sex ratio of sperm in post-thaw sexed Brahman semen using magnetic-activated cell sorting conjugated with Y-scFv antibodies (M-Zlex)

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Abstract

Brahman cattle *(Bos indicus)* are the breed suitable for beef production in tropical climate rearing conditions, where the use of sexed semen helps to pass on the good genetics of the superior sire and expand the herd rapidly and efficiently. This research evaluated the motility, kinematic variables, and sex ratio of sperm in post-thaw sexed Brahman semen using magnetic-activated cell sorting conjugated with Y-scFv antibodies (M-Zlex) by computer-assisted sperm analysis (CASA) and imaging flow cytometry. Fresh semen samples from a total of 26 ejaculates were collected one per week from eight Brahman bulls (2-4 ejaculates/bull) and divided into two parts to produce conventional (CONV) semen and M-Zlex semen. CASA was used to evaluate sperm motility and kinematic variables, and imaging flow cytometry was used to assess the X-/Y-chromosome bearing sperm ratio. The results found that sperm motility and all kinematic variables of M-Zlex semen did not differ from those of CONV semen. A significant difference was observed between M-Zlex and CONV in the proportion of X-sperm ($p < 0.05$) in eight bulls. 76.10%–78.78% X-sperm was present in M-Zlex, while the range of CONV was between 50.60% and 51.35%. Therefore, M-Zlex sexing method is another alternative for producing sexed Brahman bull sperm, which has the same sperm quality as CONV but increases the number of X-chromosome-bearing sperm that tend to produce female calves.

Keywords: Beef, *Bos indicus*, Sexed sorting, Spermatozoa

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INTRODUCTION

Currently, the livestock sector is beginning to favor sexed semen (Thaworn et al., 2022). The use of sexed semen makes possible higher production, reproductivity, and accelerated genetic development in the cattle industry (Soleymani et al., 2020). Moreover, the availability of sexed semen supports the production of more heifers for herd expansion and herd replacements at a rapid rate. (Habermann et al., 2005; Chowdhury et al., 2019; Mostek et al., 2020). Also, by manipulating the gender of the calves, the possibility for increased revenue and profit can be enhanced (Crites et al., 2018). Brahman cattle *(Bos indicus)* are the breed suitable for beef production in tropical climate rearing conditions (Chaokaur et al., 2015; Bessa et al., 2021). This international breed, which was bred mostly from American Brahmans, is present in over 70 countries (Santarosa et al., 2023). Since the 1950s, Thailand has maintained a breeding program using imported American Brahman cattle that have been selected for suitability under Thai environmental circumstances, including high temperatures, high humidity, and challenges from insects and diseases. The developed breed is known as Thai Brahman, and approximately six million beef cattle are raised in Thailand, with the Brahman breed being one of the more common breeds (Waritthitham et al., 2010; Kamprasert et al., 2019). In brahman cattle, the female cow is still crucial for the production of beef, even if male calves typically need to be fattened in order to produce beef. Female cows have the potential to pass on the good genetics of their sire, which helps expand the herd rapidly and efficiently. Furthermore, the superior-genetic female cow is more valuable compared to a typical male bull. Sexed semen refers to semen that has undergone sorting and selection procedures to alter the natural ratio of X-chromosome-bearing sperm (X-sperm) and Y-chromosome-bearing sperm (Y-sperm), assisting in the production of calves with the desired gender (Jo et al., 2021; Quelhas et al., 2021; Paitoon et al., 2024). Sex separation based on assumed differences in sperm weight, density, size, motility, and surface charge was the method employed by researchers to separate sperm carrying the X- and Y-sperm. Nevertheless, none of these techniques has proven as effective as flow cytometry cell sorting, which is based on differences in DNA content between X-sperm and Y-sperm and contains approximately 3.7–4.2% depending on the breed (Quan et al., 2015; Sringarm et al., 2022). Cell sorters are advanced flow cytometers that activate a fluorescent dye attached to the sperm DNA with a laser, enabling sperm sorting with an accuracy rate of over 90% (Sang et al., 2011; Thongkham et al., 2021). This technology has been used successfully to produce sex-sorted units of bovine sperm cells for commercial use. However, compared to conventional or non-sex-sorted semen, sex-sorted semen produced using this technology had worse viability, fertility, and overall quality following cryopreservation and thawing (Habermann et al., 2005; Thomas et al., 2017, 2019). Therefore, knowledge of immunology has been applied to sexing sperm.

The fundamental idea behind immunological sexing procedures is based upon different proteins on the surface of X- and Y- sperm, as well as techniques that use the H-Y antigen, sex-specific antigens (OSSAs), and sex chromosome-specific protein (SCSP) (Soleymani et al., 2020). Nowadays, several studies have been capable of creating antibodies specific to Y-sperm,

notably monoclonal antibodies (Mab-1F9) (Thongkham et al., 2021) and WholeMom (Uhm et al., 2023) and soluble single-chain fragment variable (scFv) antibodies, which are generated from the Y-sperm of Holstein Friesian (Bos taurus) bulls (Thaworn et al., 2022). In a previous study, magnetic-activated cell sorting (MACS) was conjugated with scFv antibodies specific to Y-sperm developed by Sringarm et al. (2022), which can be used to separate bovine sperm into two fractions, including X-enriched fractions: unbound sperm with PY-microbeads, which contained a high concentration of X-sperm, and Y-enriched fractions: sperm trapped on the PY-microbeads, which contained a high concentration of Y-sperm. A schematic of magnetic-activated cell sorting conjugated with Y-scFv antibodies (M-Zlex) is shown in Figure 1. Furthermore, the M-Zlex sexing method was used to separate Y-sperm from X-sperm of Holstein Friesian bull semen *(Bos tautus)* through the Y-sperm trapped on the PY-microbeads, and the magnetic microbeads were exposed to a strong neodymium magnet. Sexed semen from this sexing technique has a high percentage of X-sperm (80.24%) and does not adversely affect sperm quality compared with conventional semen (Sringarm et al., 2022). Nevertheless, no research has been conducted regarding sexing semen by M-Zlex in *Bos indicus*, i.e., Brahman.

Thus, this research evaluated the motility, kinematic variables, and sex ratio of sperm in post-thaw sexed Brahman semen using magnetic-activated cell sorting conjugated with Y-scFv antibodies (M-Zlex) by CASA to evaluate sperm motility and kinematic variables and imaging flow cytometry to discriminate between X- and Y-sperm.

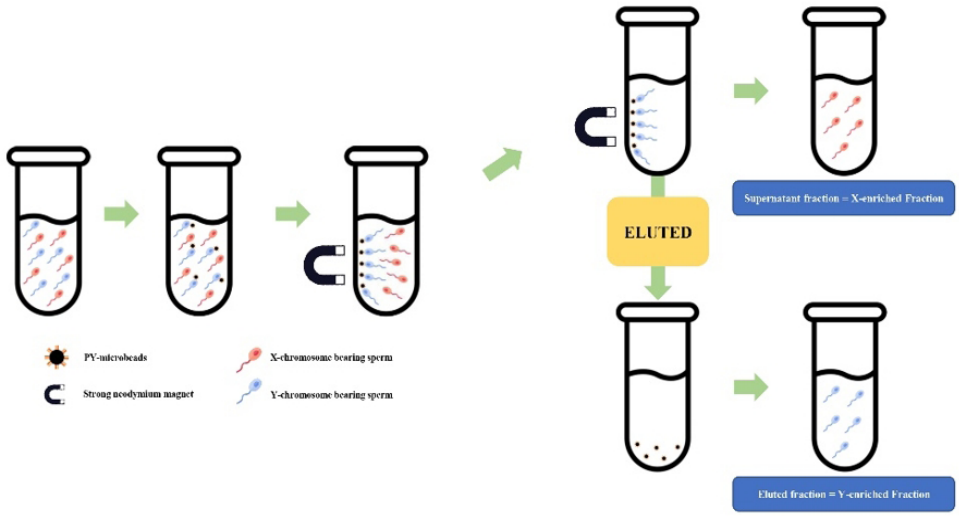

Figure 1 A schematic of magnetic-activated cell sorting conjugated with Y-scFv antibodies (M-Zlex).

MATERIALS AND METHODS

Ethics approval

Animal experiments were conducted according to the guidelines and rules for the care of animals established by the Animal Care and Use for Science and Technology Research, Institutional Animal Care and Use Committee (Agricultural Animals), Chiang Mai University, Chiang Mai, Thailand (approval no. RAGIACUC005/2564).

Animals and Semen Collection

Semen samples were collected from eight sexually mature Brahman bulls (*Bos indicus*) at the Standard Semen Production Center (Chokwassana farm, Nakhon Phanom, Thailand). Fresh semen samples from a total of 26 ejaculates were collected one time per week via an artificial vagina (2-4 ejaculates/bull; n=26). Only fresh semen samples exhibiting a sperm concentration exceeding 6.5×10^8 cells/mL, a total motility exceeding 80%, and an abnormal sperm count below 20% were utilized in the experiments.

Production of Frozen Sexed Bull Sperm by PY-microbeads

The M-Zlex sexing method was performed using the produced PY-microbeads following Sringarm et al. (2022). After preparation of magnetic microbeads coupled with Y-scFv antibodies (PY-microbeads) and then using PY-microbeads in M-Zlex process.

Fresh semen samples from each ejaculated were divided into two equal parts to prepare for production: conventional semen (CONV), which was diluted in a Tris-egg yolk-based extender (tris 3.025% w/v, citric acid 1.7% w/v, D-fructose 0.2% w/v, egg yolk 20% w/v, glycerol 8% v/v, gentamycin 0.3 mg/mL, and penicillin 1000 IU) to a final concentration of 8.0×10^7 cells/mL; and sexed semen using the M-Zlex process. In the M-Zlex sexing process, fresh semen was diluted to 1.0×10^9 cells in 5 mL of Tris-citric acid-based extender before 100 mg of PY-microbeads were added and incubated for 15 min at 37 °C under gentle shaking. The PY-microbeads were then trapped in the bottom of the tube by resting on a strong neodymium magnet for 5 min at room temperature. The supernatant fraction, or unbound PY-microbeads, was removed and placed in new tubes. This fraction contained a high concentration of X-sperm and was referred to as M-Zlex sexed semen. The freezing procedure, a Tris-egg yolk-based extender was used to dilute CONV and M-Zlex semen to a final concentration of 8.0×10^7 cells/mL. The extended semen was packed in 0.25 mL straws, allowed to adjust at 4 °C for 4 h, and then cooled at -140 °C for 15 min before being frozen in liquid nitrogen (-196 °C). A diagram of the steps involved in sexing bovine semen production using magnetic-activated cell sorting conjugated with Y-scFv antibodies and the evaluation of post-thaw bull semen in the present study was shown in Figure 2.

Semen collection using artificial vagina
(2-4 ejaculated/bull; 8 bull; n=26)

Sexed by using MACS with Y-scFv antibodies (M-zlex)

Incubated sperm together with PY-microbeads at 37 °C for 15 min

Trap PY-microbeads with strong neodymium magnet at RT for 5 min

Collected supernatant fraction
(high number of X-sperm)

Conventional semen
(CONV)

Diluted with Tris-egg extender
to 8.0×10^7 cells/mL

Frozen semen

Sperm motility

Kinematic variables

X-/Y-sperm ratio

Figure 2 A diagram of the steps involved in sexing bovine semen production using magnetic-activated cell sorting conjugated with Y-scFv antibodies and the evaluation of post-thaw bull semen in the present study.

Evaluation of post-thaw sperm motility and kinematic variables by CASA

The frozen semen samples from CONV and M-Zlex were thawed in a water bath at 37 °C for 30 s. The assessment of semen quality was performed using the HT CASA II Software Manual (Hamilton Thorne Inc., Beverly, MA, USA) connected to a Nikon Eclipse Ci series optical microscope (Nikon Instruments Inc., Melville, NY, USA) using a "Bull Nikon Ci 10X BM-Leja" set-up specifically for evaluating the quality of bull sperm. Semen aliquots (5.5 µL) were placed on a stage warmer (MiniTherm®, Hamilton Thorne Inc., Beverly, MA, USA) and covered with a 22 mm x 22 mm coverslip before immediate analysis via CASA. A variety of variables were calculated and statistically analyzed: total sperm motility (TM; %), progressive sperm motility (PM; %), and kinematics; average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), straightness (STR; %), linearity (LIN; %), amplitude of lateral head displacement (ALH; µm), and wobble (WOB; %) were calculated and statistically analyzed (Sringarm et al., 2022).

Discrimination of the X-/Y-sperm ratio in post-thaw sexed semen by Imaging Flow Cytometry

Sperm samples were thawed in a water bath at 37 °C for 30 s, centrifuged at 269 × g for 8 min, diluted to 1.0×10^6 cells/mL in PBS with 1.2 µL of 50 mg/mL Hoechst 33342, and then incubated for 10 min at 37 °C in the dark. The intensity of Hoechst 33342 staining is greater in X-sperm than in Y-sperm. After that, the number of X- and Y-sperm was detected and distinguished by imaging flow cytometry (FlowSight, Seattle, WA, USA) with a 405-nm laser at 10 mW. Histograms were generated to assess the fluorescence of Hoechst 33342, and IDEAS version 6.2 (Amnis, Seattle, WA, USA) software was used to analyze the data (Thongkham et al., 2021).

Statistical analysis

The sperm motility, kinematic variables, and percentages of X- and Y-sperm between CONV and M-Zlex semen were analyzed by a paired sample t-test using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). p-Values less than 0.05 were considered statistically significant. (Thongkham et al., 2021).

RESULTS

Sperm motility of post-thaw CONV and M-Zlex semen evaluated by CASA

The sperm motility in the post-thaw CONV and M-Zlex semen is shown in Figure 3. After sexing, the total sperm motility of M-Zlex semen (55.07%) did not differ from that of CONV (55.78%) ($p > 0.05$). In addition, the progressive sperm motility did not differ between CONV (28.21%) and M-Zlex semen (26.58%) ($p > 0.05$).

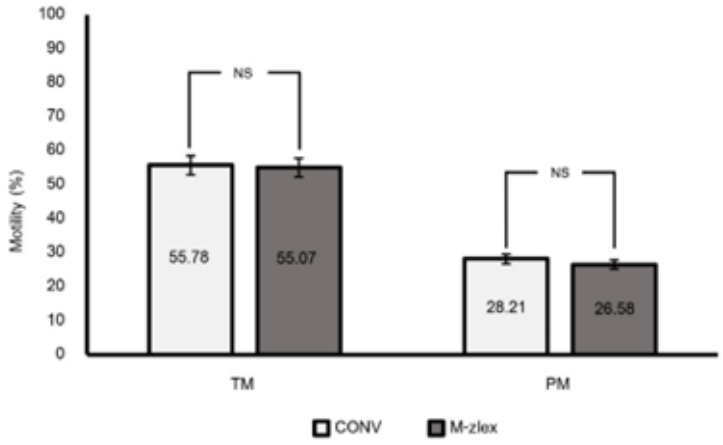

Figure 3 The sperm motility of post-thaw CONV and M-Zlex semen by PY-microbeads CONV: conventional semen; M-Zlex: semen sexed by PY-microbeads; TM: total sperm motility; PM: progressive sperm motility; NS: non-significant; p-value < 0.05 is statistically significant.

Kinematics of post-thaw CONV and M-Zlex semen evaluated by CASA

The results for the kinematics variables in the post-thaw CONV and M-Zlex semen was shown in Figure 4. The kinematics variables of M-Zlex semen (VAP = 70.42 µm/s; VSL = 56.77 µm/s; VCL = 127.20 µm/s; STR = 79.00 %; LIN = 45.25 %; ALH = 6.47 µm; WOB = 55.95 %) did not differ from those of CONV (VAP = 75.99 µm/s; VSL = 60.12 µm/s; VCL = 137.46 µm/s; STR = 77.83 %; LIN = 44.42 %; ALH 6.65 µm; WOB = 55.35 %) (p > 0.05).

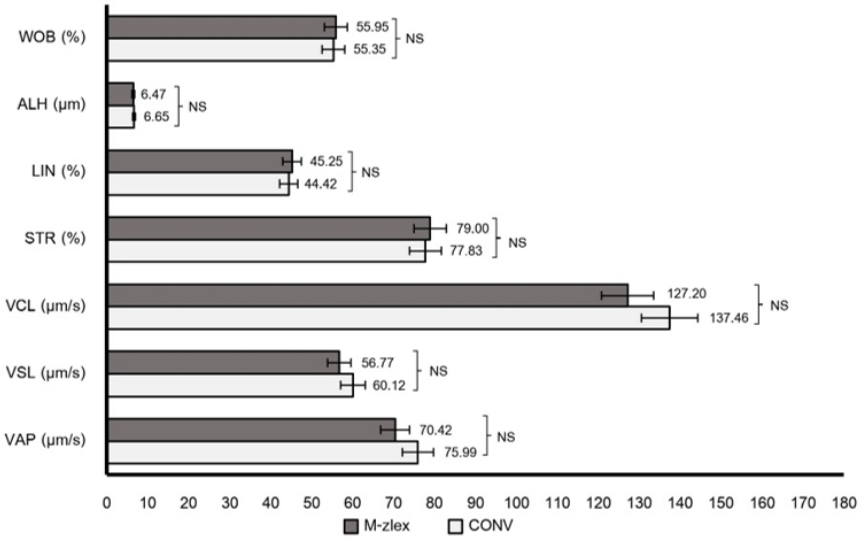

Figure 4 The kinematics variables of sperm of post-thaw CONV and M-Zlex semen by PY-microbeads. CONV: conventional semen; M-Zlex: semen sexed by PY-microbeads; VAP: average path velocity; VSL: straight line velocity; VCL: curvilinear velocity; STR: Straightness; LIN: Linearity; ALH: Amplitude of lateral head; WOB: Wobble; NS: non-significant. p-value < 0.05 is statistically significant.

Discrimination of the X-/Y-sperm ratio of post-thaw CONV and M-Zlex semen evaluated by imaging flow cytometry

The percentage of the X-/Y-sperm ratio of post-thaw CONV and M-Zlex semen is shown in Figure 6. A significant difference was observed between M-Zlex and CONV in the proportion of X-sperm ($p < 0.05$) in eight bulls. 76.10%–78.78% X-sperm was present in M-Zlex, while the range of CONV was between 50.60% and 51.35%. However, the percentage of Y-sperm was significantly lower in the M-Zlex than in CONV semen ($p < 0.05$).

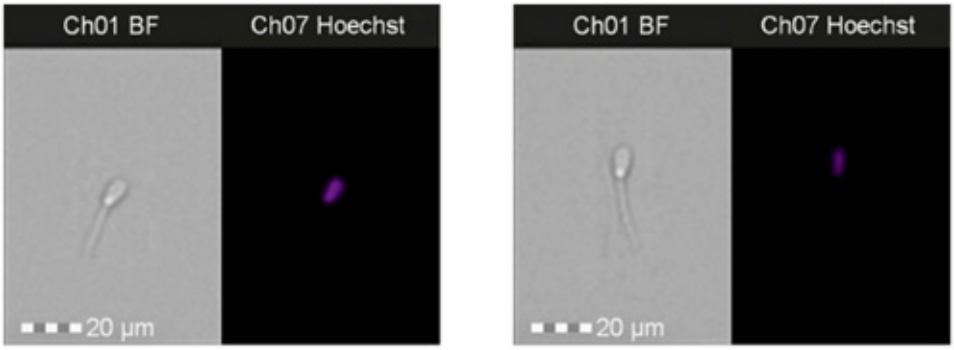

Figure 5 The difference between X- and Y-sperm that is stained Hoechst 33342 and evaluated by imaging flow cytometry (FlowSight, Seattle, WA, USA) with a 405-nm laser at 10 mW. (a) Patterns of X-sperm were observed with Hoechst 33342; (b) Patterns of Y-sperm were observed with Hoechst 33342.

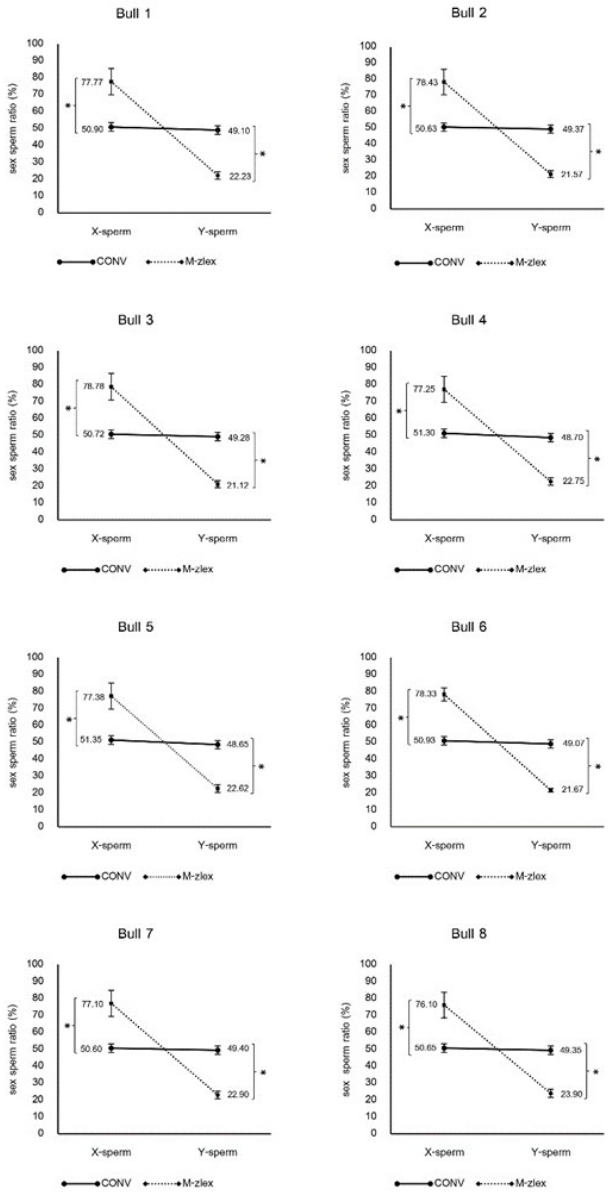

Figure 6 The sex sperm ratio of CONV and M-Zlex semen by PY-microbeads. CONV: conventional semen; M-Zlex: semen sexed by PY-microbeads; X-sperm: X-chromosome-bearing-sperm; Y-chromosome-bearing sperm. p-Value < 0.05 is statistically significant.

DISCUSSION

The CASA has the ability to provide a more accurate fertility projection than the common microscopic approach to sperm assessment since it can precisely and objectively examine huge quantities of spermatozoa in a short time. (Jo et al., 2021; Bahmid et al., 2023; Qi et al., 2024). CASA has a variety of sperm motility parameters that provide vital information about the physiological state of sperm. Sperm motility is an important component of sperm fertility since it is required for sperm transportation for fertilization in the female reproductive tract (Sringarm et al., 2022). The total sperm motility is determined by velocity classification, which includes all sperm traveling faster than 5 μm/s. Moreover, progressive sperm motility comprises any sperm traveling at more than 20 μm/s, which is necessary to enter an oocyte (Bahmid et al., 2023). In the present study, the M-Zlex semen showed TM and PM that did not differ from those of CONV semen, indicating that the M-Zlex sexing procedure had no effect on the motility of sperm. This result accorded with those of Sringarm et al. (2022), who reported that the same sexing approach had no effect on the motility of sexed semen in Holstein Friesian bulls.

Furthermore, CASA analyzed kinematic variables that impact overall sperm motility and are associated with sperm fertility, namely three velocities (μm/s): VAL, VSL, and VCL; three dimensionless motility indices (%): STR, LIN, and WOB; and ALH (μm) (Jo et al., 2021). The most frequently reported sperm movements are VAP, VSL, and VCL, whose velocity is connected to fertilization ability. The VCL is the velocity of sperm on their trajectory and shows the strength of the movement, not the course and direction. Sperm with a VCL value greater than or equal to 70 μm/s were the optimal values and counted as hyper-activated, including ALH, which indicates sperm hyper-activation. Hyper-activation, which is characterized by asymmetrical flagellar beating, is an essential aspect of sperm function because it influences the ability of sperm to move and achieve the region of fertilization. Moreover, the VSL demonstrates important characteristics of sperm function, while the VAP values can be utilized to estimate the fertilization potential of post-thaw bull semen (Akyol et al., 2018; Pfeifer et al., 2019; Jo et al., 2021; Bahmid et al., 2023). The current study, compared to CONV semen, proved that the M-Zlex sexing approach had no influence on velocity (VAP, VSL, and VCL) or ALH values. The VCL corresponds to sperm penetration via the mucus in the cervical cavity, while the VSL is linked to bull fertility (Bahmid et al., 2023). In addition, the LIN denotes progressive motility, the STR denotes movement pattern, and the WOB is the maximal quantity of sperm per second. Sperm motility in a straight path with an average LIN of >35% and STR of >50%. This sexing technique had no effect on LIN, STR, and WOB, according to the present results, due to not differing with CONV semen. A high LIN and STR value suggested a progressive motility pattern of the sperm, whereas a low LIN indicated hyper-activation. Velocity and linearity constitute essential sperm function parameters (Bahmid et al., 2023).

The result of this study suggests that producing sexed semen using the M-Zlex sexing technique does not damage sperm that is unbound by PY microbeads. As a result, such sexed semen has the efficient ability to move to achieve fertilization with oocytes, which is not different from conventional

semen or non-sexed semen. The M-Zlex sexing approach decreases the detrimental effects on sperm quality that might occur following the sexing process and so fulfills the semen quality criteria for producing sexed semen to be applied in the Brahman breeder farm.

Flow cytometry is a practical approach for examining cell physiology and function that has applications across many cell types and has become widely utilized for sperm analysis, gradually replacing time-consuming and error-prone methods. Additionally, flow cytometry has the ability to analyze numerous sperm parameters at once, which makes it intriguing for novel methods of sperm quality analysis that seek to integrate several tests to gain a better knowledge of sperm function. One well-known use of flow cytometry technology is "sexing" mammalian sperm based on differences in sperm DNA content. Flow cytometry can not only separate the X- and Y- sperm populations, but it can also verify the data to guarantee that the two cell populations were separated (Martínez-Pastor et al., 2010; Purdy et al., 2022). Thongkham et al. (2021) employed image flow cytometry in conjunction with Hoechst 33342 staining to assess the proportions of X- and Y-sperm. Due to the fact that the DNA content of bovine X-sperm is 3.8% higher than that of Y-sperm, Hoechst 33342 dye is more concentrated in X-sperm than in Y-sperm, allowing fluorescent dyes to be used to assess the presumed X- or Y-sperm. A double Gaussian distribution can be shown on a histogram, representing the variations between X- and Y-sperm. After the sexing process, the M-Zlex semen from eight Brahman bulls had a greater proportion of X-sperm, over 76.10% up to 78.78%, which is higher than the normal ratio of X-sperm in conventional semen, which is 50%, or a ratio of 1:1 when compared to Y-sperm. The results found that the M-Zlex sexing approach can be used to efficiently separate Y-sperm from X-sperm in Brahman bulls, with high purity of X-sperm due to the specificity of scFv antibodies to Y-sperm. Moreover, it has been proven that the scFv antibodies are specific to the Y-sperm of both Bos taurus and Bos indicus. As a result of the previous study, Sringam et al. (2022) created an M-Zlex technique for sexing sperm in Holstein Friesian bulls and discovered that the proportion of X-sperm after sexing was 80.24%, as measured by image flow cytometry. It is regarded as another success for the M-Zlex sexing technique to produce sexed semen in Brahman bulls. Nevertheless, the sex-sorted semen produced through flow cytometry cell sorting is able to distinguish between X- and Y-sperm with up to 90% accuracy, but the high cost, number of sperm per unit, quality, and fertility of post-thaw semen remain a constraint (DeJarnette et al., 2009; Oosthuizen et al., 2021; Quelhas et al., 2021). Therefore, the M-Zlex sexing technique is another effective option for sexed Brahman bull semen production because of its low cost, high concentration per straw, lack of sperm destruction, and comparable quality to conventional semen. Furthermore, sexed semen produced by the M-Zlex increases the number of female calves to pass on good genetics from the superior sire and serves to swiftly and efficiently expand cow herds.

CONCLUSIONS

The M-Zlex sexing approach used MACS coupled to the Y-scFv antibodies to produce sexed semen from Brahman bulls. CASA was utilized to evaluate the sperm motility and kinematic variables, and M-Zlex semen did not differ from those of CONV semen. This method of sexing has no negative effects on the quality or fertility of the sexed sperm. Moreover, the proportion of X-sperm was assessed using imaging flow cytometry. A significant difference was observed between M-Zlex and CONV in the proportion of X-sperm ($p < 0.05$) in eight bulls. 76.10%–78.78% X-sperm was present in M-Zlex, while the range of CONV was between 50.60% and 51.35%. Thus, the M-Zlex technique could increase the number of female calves in Brahman cattle.

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CONFLICT OF INTEREST

Phanuwit Paitoon: Methodology, Investigation, Data curation, Writing – original draft.
Apinya Sartsook: Methodology, Investigation, Writing—review and editing.
Marninphan Thongkham: Methodology, Data curation, Writing – original draft.
Onpreeya Chot: Methodology, Data curation, Writing – original draft.
Anucha Sathanawongs: Formal analysis, Resources, Writing—review and editing.
Wiwat Pattanawong: Validation, Formal analysis, Resources, Writing—review and editing.
Korawan Sringarm: Conceptualization, Funding acquisition, Project administration, Data curation, Writing – original draft.

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