Original Article

Effect of antibodies binding to Y chromosome-bearing sperm conjugated with magnetic nanoparticles on bull sperm characteristics

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ABSTRACT The immunological sperm separation method is economical compared to the existing sorting method, and it is promising for the development of new technologies by reducing sperm damage. Wholemom (WM) is a sex-regulating protein that comprises on immunoglobulin G coupled with magnetic nanoparticles (MNPs) that responds to surface proteins derived from the Y chromosome in cattle. Y sperms are restricted in motility as the WM aggregates them, and the magnet could separate the non-aggregated cells. This study was conducted to investigate the effect of WM treatment on the characteristics of bull sperm. After treating sperm with WM and incubation for 6 h, the motility parameters including total motility, progressive motility, velocity average path, velocity straight line, amplitude of lateral head displacement, and linearity were significantly higher in the WM treatment group than in the control group ($p < 0.05$). Sperm viability and acrosome reaction rates were similar in both groups during each incubation period ($p > 0.05$). In conclusion, the immunological sperm sexing procedure using a monoclonal antibody conjugated with MNPs did not affect the characteristics of bull sperm. This study suggests that compared to other techniques, the immunological method for sperm sexing could classify sperm quickly and efficiently without the use of expensive equipment.

Keywords: immunological method, sperm characteristics, sperm sexing, Y specific antibody

INTRODUCTION

Production of offspring of the desired sex by artificial regulation is considered critical to improve livestock productivity and increase economic efficiency in the livestock industry. Dairy experts consider dairy products very important from a food security and nutritional perspective (Al-Dulaimi and Al-Timimi, 2018; Safari et al.,

2018). In addition, rapid economic growth and population growth have led to the expansion of livestock product consumption and the securing of various distribution channels.

In mammals, the sex of the offspring is determined by the sex chromosomes in the sperm. Altering the normal 1:1 ratio of X or Y sperm to favor conception of one sex or the other is the most logical approach to control the sex ratio.

The artificial sex-separation of spermatozoa is representative of reproductive biotechnology for livestock industry species. These techniques are based on the physiological differences in X- or Y-bearing sperm. In cattle, X chromosome-bearing sperm contains approximately 4% more DNA than Y chromosome-bearing sperm (Garner et al., 1983; Johnson and Clarke, 1988), and Y sperms swim faster than X sperms (Penfold et al., 1998). In addition, other differences include the size (Cui, 1997), and surface charge on sperm (Kaneko et al., 1983). Several studies have shown that flow cytometric measurement of DNA content in individual sperm allows the resolution of X and Y populations (Seidel Jr, 2012; Laxmivandana et al., 2021). Other commercially used methods of sorting X- or Y-sperms have been reported, including swim-up (Han et al., 1993), H-Y antigen (Bennett and Boyse, 1973), and percoll gradient centrifugation (Koundouros and Verma, 2012), that based on physiological differences. However, the sperm separation methods developed to date have resulted in significantly reduced sperm motility and viability owing to the isolation process (laser, high pressure, fluorescence, and electrical stimulation) despite the high cost (Seidel Jr, 2012). In addition, the sperm concentration of the separated semen is reduced compared to that of conventional semen and this reduction could lower the pregnancy rate of livestock, causing economic loss to farmers. These limitations have prompted the establishment of efficient and non-invasive approaches for sperm sorting.

Across species, the observed differences in DNA between X and Y-bearing sperm led to the possibility that these differences might result in protein differences. Based on this concept, immunological sex separation methods for sperm using different proteins present on the surface of X- and Y-bearing sperm have been developed. The H-Y antigen is a male-specific antigen on the sperm surface and is separated from the sex-determining region Y (SRY). It has been reported that the H-Y antigen preferentially binds the anti-H-Y antibody to Y sperms (Zavos, 1983), enabling the identification and isolation of sperm using the H-Y antigen. However, there was no change in the sex ratio of the offspring even after using this antibody, and Prasad et al. (2010) reported that the H-Y antigen was present in the cell membranes of both X- and Y-bearing sperm. Therefore, further research is needed to isolate semen using the H-Y antigen. A proteomics approach has been developed based on the protein profile differentially expressed in X- and Y-bearing sperm (De

Canio et al., 2014). It has been shown that sex-dependent protein expression in cattle can affect sperm function, phenotype, and interactions between sperm and oocytes (Li et al., 2016). These proteins could be used as potential molecular markers to differentiate and classify X- and Y-bearing sperm. Sperm sorting based on immunological methods is a reproducible, economic and convenient approach. Immunological methods are promising for the development of new technologies to overcome existing limitations, and are expected to benefit the agricultural industry.

Wholemom (WM) is sex-regulating protein comprising an immunoglobulin G coupled with magnetic nanoparticles (MNPs) that responds to surface proteins derived from the Y chromosome in cattle. The WM binds to the plasma membrane of Y-bearing sperm, and the agglutinated sperm settles on the bottom or is fixed with a magnet. This technique aims to produce female individuals by allowing only X-bearing sperm to migrate to the fertilization site without loss in motility. This study hypothesized that a method based on the physiological differences of sperm with X and Y would lead to efficient sperm isolation. This study was conducted to investigate the effect of WM treatment on the characteristics of bull sperm.

MATERIALS AND METHODS

Chemicals

All reagents used in this study were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

Preparation of sexed sperm

Frozen semen from Korean native cattle (Hanwoo) was obtained from the Hanwoo Improvement Center (Nong-Hyup Agribusiness Group Inc., Chungcheongnam-do, Korea). Eight frozen semen straw of same bull were used for each replication and 3 to 5 animals were used in each experiment. One frozen semen straw was thawed at 37°C for 1 min. Then, the sperm were suspended in 5.0 mL of KO solution (sperm washing medium; 0.105 g/L magnesium chloride hexahydrate, 0.249 g/L calcium chloride, 4.68 g/L sodium chloride, 0.3 g/L potassium chloride, 0.129 g/L sodium phosphate monobasic dihydrate, 5.96 g/L HEPES, 2.518 g/L D-glucose, 0.06 g/L L-cystine, 1.06 g/L caffeine, 8.0 mg/L phenol red, 3.108 g/L sodium bicarbon-

ate) containing 0.5% BSA. Both the control group and the WM treatment group were prepared using the same washing media.

Sperm motility was visually confirmed under a stereoscopic microscope and the solution was centrifuged at $430 \times g$ for 5 min. After removing the supernatant including the cryopreservation solution, 1.0 mL of KO solution and 1 vial of WM (Nuri Science, Gyeonggi-do, Korea) was added to the sperm pellet and allowed to stand at 37°C for 20 min. Because the MNPs can sink, the tube containing the sperm treated with WM was periodically rotated to react with the target cells of the WM antibody. After 20 min, the WM-treated sperm were transferred to a 5.0 mL round tube surrounded by a magnet (STEMCELL, CA-BC, Canada), and KO solution was added so that the total volume was 3.0 mL. After reacting for 5 min under a magnetic environment, 1.0 mL of the supernatant was carefully recovered using a pipette so as to not touch the sperm aggregated on the wall of the round tube. The transferred sperm were centrifuged at $430 \times g$ for 5 min with KO solution and the sperm was used to analyze sperm characteristics. Control sperm were centrifuged twice times during the preparation of the WM treatment group and used in each experiment.

Computer-assisted sperm analysis (CASA)

Sperm motility was assessed using the CASA system (Hamilton Thorne, Beverly MA, USA). This program consists of a phase-contrast microscope, a digital camera, a thermal stage warmer, and a computer that stores and analyzes the data. For approximately 200 sperm sample, sperm motility was measured at 0, 2, 4, and 6 h under $100 \times$ magnification. Ten microliters of sperm diluted to a concentration of 1.0×10^6 sperm/mL was mounted in a Standard Count 4 Chamber Slide (IMV Technologies, L'Aigle, France), and microscopic fields of five randomly selected sections were scanned and used for statistical analysis. Nine sperm parameters including total motility (TM; %), progressive motility (PM; %), velocity average path (VAP; $\mu\text{m/s}$), velocity straight line (VSL; $\mu\text{m/s}$), curvilinear velocity (VCL; $\mu\text{m/s}$), the amplitude of lateral head displacement (ALH; μm), beat-cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %) were analyzed using CASA program.

Fluorescent staining and flow cytometry analysis

Flow cytometry analysis was performed using a FACS-Calibur flow cytometer (Becton Dickinson, NJ, USA). Sex-separated sperm presumed to be X chromosomes were centrifuged for 5 min at $430 \times g$, and then, the supernatant was removed. Sperm samples were resuspended to be 1.0×10^6 sperm/mL with 1.0 mL of PBS (-), and sperm viability and acrosome integrity were analyzed using a fluorescent dye.

A LIVE/DEAD Sperm Viability Kit (L7011, Molecular Probes, CA, USA) was used to detect sperm viability. The final concentration of SYBR-14 was adjusted to 0.78 nM in diluted sperm samples and incubated for 5 min in a dark room at 38°C . Then, propidium iodide (PI) was added to be 37.5 nM in the samples and cultured under the same conditions. After staining, the sperm were centrifuged for 5 min at $430 \times g$. The supernatant was removed and resuspended in 300 μL PBS (-). A total of 10,000 cells were analyzed by flow cytometry. Flow cytometry data were analyzed using Flowing Software (Version 2.5.1, Turku, Finland).

The acrosome integrity of sperm was evaluated after staining the sperm with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; L7381) and PI double staining. The FITC-PNA (2.5 $\mu\text{g/mL}$) was added to the sperm samples and incubated for 5 min in a dark room at 38°C . Then, PI was added to sperm samples until 37.5 nM and cultured under the same conditions. After removing the supernatant, 300 μL of PBS (-) was added. A total 10,000 sperms were analyzed with flow cytometry and data were analyzed with Flowing Software.

Bull sperm were stained and examined using a fluorescence microscope for visual confirmation. To determine the viability of sperm, sperm samples were diluted to 5.0×10^6 sperm/mL in 1.0 mL of PBS (-). 1.0 μL of SYBR-14 (1.0 mM, v/v) and 2.5 μL of PI (2.4 mM, v/v) were mixed with sperm samples and incubated at 38°C in a dark room for 5 min. After staining, the samples were centrifuged for 5 min at $430 \times g$, and the sperm pellet was resuspended in 100 μL of PBS (-). Acrosome integrity of sperm was confirmed by staining 1.0 μL of FITC-PNA (5 mg/mL, v/v) and 1.0 μL of Hoechst 33342 (1 mg/mL, v/v) in 1.0 mL of PBS (-) containing 5.0×10^6 sperm/mL of cells. After incubation under the same conditions and centrifugation, the pellet was resuspended in 100 μL of PBS (-). A labeled 10 μL sperm sample was placed on a microscope

slide and covered with a cover glass. Slides were observed and recorded under a fluorescence microscope (Olympus BX 50; Olympus, Tokyo, Japan) at 400 × magnification. Live sperm were stained green by SYBR-14 whereas the membrane-damaged sperm were stained red with PI. Entire sperm were stained with Hoechst 33342 as blue fluorescence, and acrosome-reacted sperm were stained with FITC-PNA as green fluorescence.

Statistical analysis

The data were analyzed using Statistical Analysis System Software (SAS; version 9.4, NC, USA) and expressed as mean ± standard error of the mean (SEM). When the data were percentages, we arcsine transformed the data to improve the approximation to normality. A *p*-value of < 0.05, was considered indicative of a statistically significant difference. Differences between groups were assessed using the Student's *t*-test followed by Duncan's multiple range test using one-way analysis of variance (ANOVA).

RESULTS

Effect of WM treatment on the agglutination of bull sperm

Sperm in the upper layer presumed to have an X chromosome did not exhibit aggregation, however, it was vi-

usually confirmed that the sperm aggregates and attached to the magnet by WM were head-to-head aggregation. Aggregated sperm are presumed to be cells with a Y chromosome, and it was observed that they had less motility than sperms in the supernatant owing to WM treatment (Fig. 1).

Effect of WM treatment on the characteristics of bull sperm

The motility parameters recorded during incubation after the treatment of sperm with WM are shown in Table 1. The TM, PM, and VCL were reduced in both sperm groups after 2 h. After 6 h of incubation, all parameters, except VCL, BCF, and STR were significantly higher in the WM treatment group than in the control group (*p* < 0.05). No difference was observed in BCF and STR, regardless of WM treatment during the incubation period. Sperm viability and acrosome reaction rates did not differ between the both sperm groups at each incubation time (Fig. 2).

DISCUSSION

The purpose of this study was to analyze changes in the motility, viability, and acrosome damage of sex-separated bull sperm by treatment with WM, a protein that regulates sex. The WM treatment group showed higher motility

Fig. 1. Morphology of agglutinated spermatozoa of Korean native cattle (Hanwoo) at 25 min after Wholemom (WM) treatment, pre-treatment of bull sperm (A, D), non-agglutinated sperm (B, E; assumed X-sorted sperm), and agglutinated sperm with WM (C, F; assumed Y-sorted sperm), black scale bar: 400 μ m, white scale bar: 200 μ m.

Table 1. Computer-assisted sperm analysis (CASA) of bull sperm during different incubation time with or without Wholemom (WM) treatment

Parameters	Treatment group	Period of sperm incubation time (hours)			
		0	2	4	6
TM (%)	Control	37.6 ± 6.4 ^A	8.4 ± 1.9 ^B	7.0 ± 3.0 ^B	0.6 ± 0.2 ^{a, B}
	WM	27.6 ± 3.1 ^A	8.0 ± 2.3 ^B	8.8 ± 2.6 ^B	5.8 ± 1.2 ^{b, B}
PM (%)	Control	20.8 ± 3.9 ^A	3.4 ± 0.9 ^B	3.0 ± 1.4 ^B	0.0 ± 0.0 ^{a, B}
	WM	13.4 ± 1.4 ^A	3.4 ± 1.7 ^B	3.4 ± 0.8 ^B	2.2 ± 0.2 ^{b, B}
VAP (μm/s)	Control	83.3 ± 5.4 ^A	67.4 ± 7.6 ^{AB}	53.9 ± 5.0 ^B	29.0 ± 7.9 ^{a, C}
	WM	76.2 ± 1.6	54.7 ± 5.7	61.8 ± 12.5	53.5 ± 3.2 ^b
VSL (μm/s)	Control	61.6 ± 4.2 ^A	50.5 ± 9.2 ^{AB}	36.0 ± 4.5 ^{AB}	17.5 ± 5.9 ^{a, C}
	WM	59.0 ± 3.0	40.3 ± 7.0	48.0 ± 10.5	42.0 ± 3.4 ^b
VCL (μm/s)	Control	144.6 ± 11.2 ^A	109.4 ± 11.0 ^{AB}	101.4 ± 7.4 ^B	70.1 ± 19.1 ^B
	WM	117.8 ± 3.9 ^A	88.3 ± 4.5 ^B	95.7 ± 13.7 ^{AB}	88.8 ± 5.6 ^B
ALH (μm)	Control	7.7 ± 0.5 ^A	5.2 ± 1.2 ^{AB}	6.5 ± 0.5 ^A	2.0 ± 1.6 ^{a, B}
	WM	6.1 ± 0.6	4.8 ± 0.5	6.4 ± 0.6	6.4 ± 0.7 ^b
BCF (Hz)	Control	29.5 ± 1.6	29.8 ± 2.0	32.2 ± 2.5	33.3 ± 1.9
	WM	28.1 ± 1.0	30.1 ± 1.4	29.6 ± 2.0	26.0 ± 1.4
STR (%)	Control	68.6 ± 1.9	73.4 ± 2.5	67.0 ± 3.5	49.2 ± 16.2
	WM	71.8 ± 2.8	70.4 ± 5.2	71.6 ± 2.0	78.4 ± 2.5
LIN (%)	Control	44.2 ± 2.1 ^A	51.6 ± 5.2 ^A	42.6 ± 3.2 ^A	25.8 ± 9.1 ^{a, B}
	WM	51.4 ± 3.4	50.6 ± 4.9	50.6 ± 3.7	55.8 ± 2.3 ^b

^{a, b}Small letters indicate a significant difference between treatment groups in the same parameter ($p < 0.05$).

^{A-C}Large letters indicate a significant difference with incubation time in the same treatment group ($p < 0.05$).

TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

Fig. 2. Viability (A) and acrosome reaction rates (B) of bull sperm treated with different periods of Wholemom (WM) treatment.

than the control group after 6 h. The viability and acrosome reaction rates of sperm and the development rate of embryos were not affected by WM treatment.

The CASA procedure is a very useful technique for evaluating sperm motility and fertility. Although evaluating sperm motility using conventional microscopic methods is subjective, CASA can precisely analyze large numbers of spermatozoa in a short period. Assessment of sperm motility is efficient in predicting the potential fertility of sperm, as it plays an important role in the progression of sperm to the cervical mucus and penetration into the

zona pellucida of oocytes (Verstegen et al., 2002). These motility and velocity parameters depend on factors such as collection time, age, temperature, pH, and time between ejaculations (Wallach and Blasco, 1984).

Sperm motility has been used to predict fertility in livestock (Kang et al., 2020). In the present study, after 6 h of WM treatment PM and TM were significantly higher than in the control group, which showed a positive correlation with the velocity parameters VAP and VSL. Perumal et al. (2014) reported a similar result that during the same period, ALH and LIN of the WM treatment group showed

higher values than those of the control group, and the effect of WM was not observed on VCL, BCF, and STR. Parameters such as PM, VAP, VSL, ALH, and BCF contribute to the total motility of spermatozoa and consequently correlate with sperm fertility (Kumar Yata et al., 2020). The VCL and ALH indicate hyperactivation of spermatozoa, and LIN indicates a measure of linearity, which is essential for fusion with oocytes (Perumal et al., 2014). Therefore, sperm motility was not inhibited by WM and could maintain motility longer than that of conventional sperm after a long period (≥ 6 h). It could be expected that the process of separating sperm that is not aggregated with MNPs is similar to the swim-up method so that low-quality sperm could be filtered out.

Cryopreservation of semen increases the number of sperm exhibiting acrosome reaction and damage to the sperm membrane, which reduces viability and fertility (Felipe-Pérez et al., 2008; Almubarak et al., 2021). There is a need for an efficient approach to minimize damage to sperm during sex separation of frozen-thawed semen. Sperm capacitation and acrosome reactions are essential biochemical processes that allow sperm to penetrate the zona pellucida of oocytes (Breitbart, 2002). This study indicates that sex separation of sperm by WM treatment does not affect the proportion of viable and acrosome-reacted sperm. Thongkham et al. (2021) reported that immunological sexing did not negatively affect sperm acrosome integrity but lowered the percentage of live sperm.

In general, frozen semen straw of Hanwoo contain 1.8 to 2.5×10^7 sperm. In artificial insemination (AI) in cattle, sperm numbers ranging from 0.5 to 1.5×10^7 sperm per insemination dose have been found to be optimal for fertility (Sullivan and Elliott, 1968). Sperm counts 0.5×10^6 sperm/straw and 5.0×10^6 sperm/straw were considered suboptimal for liquid and frozen semen, respectively (Mohanty et al., 2018). Also, a study on effects of two different sperm doses showed that fertilization rates were improved for both fresh and frozen sperm does, however, the percentages of motile to viable sperm was not affected by the increased sperm concentration (Nadir et al., 1993).

Barsuren et al. (2019) reported that sex-controlled calves were produced without a decrease in pregnancy rate and no increase or decrease in duration of pregnancy when using WM not combined with MNPs. According to a previous report, it was possible to successful sperm sexing

and control of embryos using WM which MNPs were not combined (Heo et al., 2018). With the use of WM, the concentration of sperm will be halved due to sex segregation, and it is assumed that the reduced concentration would not affect fertility of sperm or pregnancy during AI. In addition, it is thought that the economic efficiency of farms could be improved through sex pre-select by applying WM to embryo transfer in cattle.

CONCLUSION

The monoclonal antibody conjugated with MNPs used for immunological sperm discrimination caused aggregation of Y sperm in response to WM treatment and did not negatively affect the characteristics of bull sperm. These results suggest that compared to other techniques, the immunological method for sex separation of sperm can classify sperm quickly and efficiently without use of expensive equipment.

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