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Original Article

Sorting effects of X/Y sperm on in vitro fertilization of Belgian blue bulls

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Abstract

There is a large market in China for the production of Belgian blue bulls, which would benefit from semen flow sorting for animal sex control. The sperms of the Belgian blue bulls were separated by flow cytometry, and then the quality of sorted sperm was tested by in vitro fertilization (IVF) and artificial insemination (AI). The fresh semen of 8 Belgian blue bulls was individually collected and processed for sorting. Sperm sorting was carried out using MOFLO cell sorter and cryopreservation. There were no significant differences in sperm motility, acrosome integrity, and DNA integrity between sex-sorted and non-sorted sperm ($p > 0.05$). The purified sperm with higher viability was fertilized with mature oocytes in vitro, co-cultured to cleavage and blastocyst stage, embryo sex was identified by nested PCR amplification of *AMEL* gene, and there was no significant difference between sorted sperm and non-sorted sperm ($p > 0.05$). For artificial insemination, the pregnancy rate using non-sorted sperm was higher than for sorted sperm ($p < 0.05$). After delivery, the sex ratio of offspring using X- and Y-sperm was 100% and 90.9%, respectively, with a significant deviation from conventional semen ($p < 0.05$). The birth weight of male and female calves in the control group was similar to that in the sex-sorted sperm ($p > 0.05$). In summary, after artificial insemination using sex-sorted sperm, normal Belgian blue calves with the predicted gender can be produced. It is of great significance to improve the commercial promotion and production efficiency of semen after sorting Belgian blue bulls.

Keywords: Belgian blue bull, Flow cytometry, In vitro fertilization, Artificial insemination, Sex ratio

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Introduction

Artificial insemination (AI) is a crucial technology for enhancing genetic improvement in livestock and enabling the efficient use of semen over long distances and also enhances productively (Yousuf,

2021). Productivity not only in the beef cattle industry but also in the dairy cattle industry is important for economic growth and improvement. Productivity is mainly defined as the number and weight of weaned calves, considering the reproductive performance and milk yield of the cows, as well as the mortality and growth rate of calves (Fiems and Ampe, 2015; Khan et al., 2021; Ezzat et al., 2023). The economic traits of interest in livestock production are directly related to the sex, if the producer predetermine the sex of the offspring, then sure have increased economic benefits. Producers of animals such as cows, goats, etc. prefer female calves to produce milk and beef cattle producers prefer male calves for higher meat production (He et al., 2022). Belgian blue bulls, a popular breed on the Belgian meat market, are characterized by strong adaptability, early sexual maturity, and good muscular development (Kouamo and Nyonga, 2022). Semen-sorted flow cytometry and sex ratio adjustment for offspring is the leading technology to improve the reproductive efficiency and production performance of Belgian blue bulls (Naniwa et al., 2019; Guo et al., 2023).

In 2017, China began to develop the Belgian blue bull industry using imported frozen semen for artificial insemination and embryonic transfer, increasing stock and breeding scale (Huang et al., 2023). With the development of scientific research and the market economy, sperm sorting has become the most effective method to control the sex of offspring. In mammals, the DNA contents of X sperm chromosomes are about 3.6-4.0% more than that of Y sperm (Penfold et al., 1998). Moreover, Hoechst33342 fluorescent dye can specifically bind to living cells, the prepared cell suspension passes through the flow cytometer sorting chamber at high speed to achieve the purpose of sorting (De Geyter et al., 2019; González-Marín et al., 2021). Johnson (2000) first reported the isolation of live X and Y sperm from male rabbits and performed intrauterine surgical insemination on female rabbits. The results obtained by him showed that the accuracy of females and males reached 94 and 81%, respectively. It has been found that the sperm of multiple species can be sex-sorted, and can be combined with technologies such as AI, IVF, and ICSI (O'Brien et al., 2009; Squires, 2020; Zaki and Alkhail, 2023; Wen et al., 2023; Bebas et al., 2023).

Fourth-generation flow cytometer has improved classifying bovine sperm carrying X or Y chromosomes (Rahman and Pang, 2020; Pflug et al., 2020). Research shows that the average separation speed can reach 5000-6000/s (Xiong et al., 2021), which greatly improves the separation efficiency and population size. Therefore, this technical improvement is essential for the application of sorted semen, and the use of sex identification technology has been proven to be an effective method for the use of low-dose spermatozoa in cow artificial insemination (Garner and Seidel, 2008). According to our knowledge, after artificial insemination with a small number of frozen-thawed sperms sorted by the presence of X- or Y-chromosomes, Belgian blue calves of pre-determined sex can be produced. Few similar reports have yet been published (Healy et al., 2013; Vishwanath and Moreno, 2018). The purpose of this study was to successfully sort sperm with X and Y chromosomes and evaluate the normality of offspring of Belgian blue cattle using *in vitro* fertilization and artificial insemination techniques.

Material and Methods

Ethics approval

All animal experiments were conducted according to the Regulations and Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China, revised in 2004). The present study was approved by the Institutional Animal Care and Use Committee of Tarim University.

Semen collection

Eight healthy Belgian blue bulls with an average age of 4 years were chosen, and their semen was collected with the help of an artificial vagina and sorted into X and Y semen. The semen of an individual bull was evaluated for quality; if it met the sorting conditions, i.e., vitality ≥ 70%, and density of 0.8-1.5×10^9/mL, then processed further. Each experiment was repeated at least 3 times. The semen quality test results of any three bulls are shown in Table 1. Each bull's semen was individually diluted in equal proportions with a special diluent (Dairy Center, Beijing, China). Semen was stored in an electric refrigerated box (constant temperature, 17°C), and was quickly sent to the sorting laboratory.

Table-1. Bull semen quality test results.

Bull ear tag	Motility %	Density (10^8/mL)
750	86.5±1.3	9.8±0.5
720	84.4±1.1	8.7±0.2
705	81.7±0.7	9.1±0.3

Sperm staining

Each semen sample was diluted to 1×10^8 sperm/mL using the modified TALP medium (Revah et al., 2000). They were then incubated with Hoechst33342 (Sigma-Aldrich, St. Louis, MO, USA) at 34°C for 45min. An equal volume of X/Y TALP (containing 4% egg yolk) and 0.002% food dye was added (Hollinshead et al., 2002). The sperm staining solution was filtered with a 50μm nylon mesh filter, and any condensed sperm or debris was removed.

Flow cytometry sorting

An improved high-speed cell sorter (SX-MOFLO, Beckman Coulter, Brea, CA, USA), sorting and operating with preheated Tris-based sheath at 50 psi, was adopted to analyze and separate the bull sperms (Schenk et al., 1999). The packing dose of X- and Y-sperm was 2.0×10^6 sperm/0.25mL. The sperm temperature was gradually reduced from -4°C to -140°C using a programmed cryostat (IMV Inc., Paris, France), and then transferred to liquid nitrogen for storage.

Re-analysis of sorted sperms

The classified sperms were irradiated with ultrasound for 4-10s, and the sperm tail was removed to improve the efficiency of sperm orientation. The sperms were stained with Hoechst33342, and the classification purity of sperm samples was detected by flow cytometry reanalysis (Welch and La, 1999).

Evaluation of motion parameters

All frozen sperm samples were thawed in a 37°C water bath for 30s and diluted proportionally with saline. Evaluation of sperm viability and motility properties was performed under a microscope, the density was measured by a sperm densitometer and the number of effective sperm was measured by a blood cell counting plate (Sari et al., 2021). Each experiment was repeated at least 3 times and each replicate was processed individually.

Acrosome integrity rate evaluation

Then sperm smears were prepared, treated with formalin phosphate fixative for 15min. The sperm samples were washed with ultrapure water and air-dried. The smears were then stained with Giemsa stain solution (prepared with a 1:4 ratio of dye and buffer) for 90min, air-dried, and examined microscopically (Tsouloufi et al., 2020).

DNA integrity assessment

The sperm DNA fragments were detected by the sperm chromatin dispersion (SCD) method (Zhai et al., 2017). Sperm samples were stained with Wright's solution (1:2 mixture of dye and buffer) and washed and dried after microscopic examination.

In vitro fertilization (IVF)

Cattle ovaries were collected from the local slaughterhouse, stored in sterile saline solution at 37°C, and brought to the laboratory immediately. The cumulus-oocyte complex was obtained by extraction and transferred to a 5% CO_2 incubator (MC0-15A, Osaka, Japan) at 38.5°C and saturated humidity for incubation, until the first polar body discharges (Opiela et al., 2019).

We used the upstream method to purify sperms (Yang et al., 2019). Briefly, 1mL semen sample were centrifuged at 800×g 5min, discarded the supernatant, and repeated centrifugation again. Placed mature oocytes into 50μL droplets coated with paraffin oil (each group has C, X, Y, 156, 152, and 149 oocytes for fertilization). The purified semen was added to fertilized microdroplets (sperm density, 1×10^6 sperm/mL), and continued to culture in the incubator for 18 hours. Washed the fertilized eggs in embryo development solution, the granulosa cells, and fertilized eggs were cultured at 38.5°C with 5% CO_2, and 100% humidity. The number of embryonic divisions at 36 hours of development was recorded. The culture medium was replaced every second day. Finally, the number of blastocysts was recorded on the 6th day of development.

Table-2. Nested PCR primers used in the present study.

Primer	Sequence form 5′to 3′	Annealing temperature °C	Fragment length
AMEL-F-out	CATGGTGCCAG CTCAGCAG	62	X：349bp Y：289bp
AMEL-R-out	CCGCTTGGTCTT GTCTGTTGC		
AMEL-F-in	CAGCAACCAAT GATGCCAGTTC	62	X：311bp Y：251bp
AMEL-R-in	GTCTTGTCTGTT GCTGGCCA		

Embryo sexing

Based on the cattle *AMEL* gene sequence (*AMEL*-X, GeneBank accession no: NM_001014984; *AMEL*-Y, GeneBank accession no: NM_174240), nested primers were designed by BLAST and Clustal W program. Primer sequence information is shown in Table 2.

Added 5μL of embryo lysate to each PCR tube, then X, Y, and conventional embryos were added for lysis, nested PCR amplification of embryonic genomic DNA (Mizia et al., 2023). Mixed and centrifuged the PCR tubes with reagents (Table 3) at PCR reaction conditions: pre-denaturation (95°C, 3min), denaturation (95°C, 30s), annealing (62°C, 30s), extension (72°C, 30s, 35cycles), final extension (72°C, 5min) and preservation at 4°C. The second round of PCR products was electrophoresed on a 2% agarose gel, 100voltage for 25min and then observed results in the gel imaging system (Colley et al., 2008; Xiao et al., 2021).

Table-3. The first and second round of PCR reaction system.

First Round		Second Round	
Reagent	System (μL)	Reagent	System (μL)
2× PreMix	12.5	2× PreMix	12.5
AMEL-F-out	0.5	*AMEL-F-in*	0.5
AMEL-R-out	0.5	*AMEL-R-in*	0.5
DNA	5	ddH2O	11
ddH_20	6.5	The first round of PCR dilution product	0.5
Total	25	Total	25

Artificial insemination

A total of 66 healthy Belgian blue cattle were provided by Fabrika Farming in Xinjiang, China. Animal management in this study was approved by China Agriculture Association. The estrus synchronization was performed by injecting PG and placing CIDR. Post 48-54h of CIDR and intramuscular injection of GnRH, two semen insertions 10h apart into the deep horn of the uterus were performed. The control group used a 1.8×10^7 dosage form, and the X- and Y sperm groups used a 2.0×10^6 dosage form for insemination (Morotti et al., 2022). At 60 days, the rectal examination was carried out to know the pregnancy rate (%), and offspring sex ratio (%) while birth weight (kg) of each calf was recorded after parturition.

Statistical analysis

Sperm quality was presented as mean±SE, then the variance analysis was used to test the difference between treatments (SPSS software; SPSS, Inc., Chicago, IL, USA). The blastocyst rate, pregnancy rate, and sex ratio data were analyzed using the Chi-square test. The significance level of 5% was considered as significant.

Results

Detection of frozen-thawed sperm quality

Data on sperm viability, effective sperm count, acrosome integrity, and DNA integrity after thawing are presented in Table 4. There was non-significant difference in post-thaw sperm motility between the control (non-sorted), X, and Y-sorted semen samples ($p > 0.05$). The effective sperm count in the control group was 10 times that of the sorted sperm. The acrosome integrity rate and DNA integrity rate after thawing of sorted sperm showed higher values, which aligned with the experimental results. The results of sperm acrosome staining, and sperm DNA fragmentation detection are presented in Figure 1.

Table-4. Post-freezing viability, acrosome integrity, DNA integrity, and effective sperm count of different types of semen.

Type of semen	Post-thaw motility (%)	Effective sperm count (10^6 sperm)	Acrosome integrity rate (%)	DNA integrity (%)
Control group(n=8)	46.0 ± 1.1^a	11.1 ± 0.6^a	70.7 ± 0.6^a	72.9 ± 1.1^a
X-sperm (n=6)	43.7 ± 0.5^a	1.5 ± 0.3^b	72.3 ± 1.0^a	73.3 ± 0.5^a
Y-sperm (n=6)	43.9 ± 1.6^a	1.4 ± 0.4^b	70.9 ± 1.3^a	73.7 ± 0.6^a

Values (mean±SE) bearing different letters in a column differ significantly ($p < 0.05$).

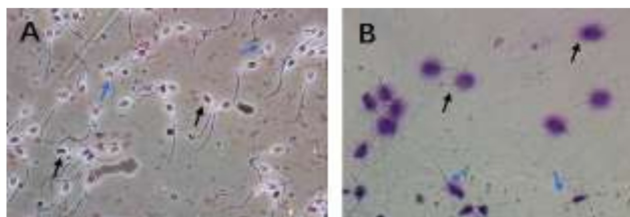

Figure-1. A) Sperm acrosome staining results (200×). Black represents sperm acrosome integrity, and blue represents sperm acrosome loss. B) Sperm DNA fragment detection results (200×). Black arrow represents sperm DNA integrity, blue arrows represent sperm DNA damage.

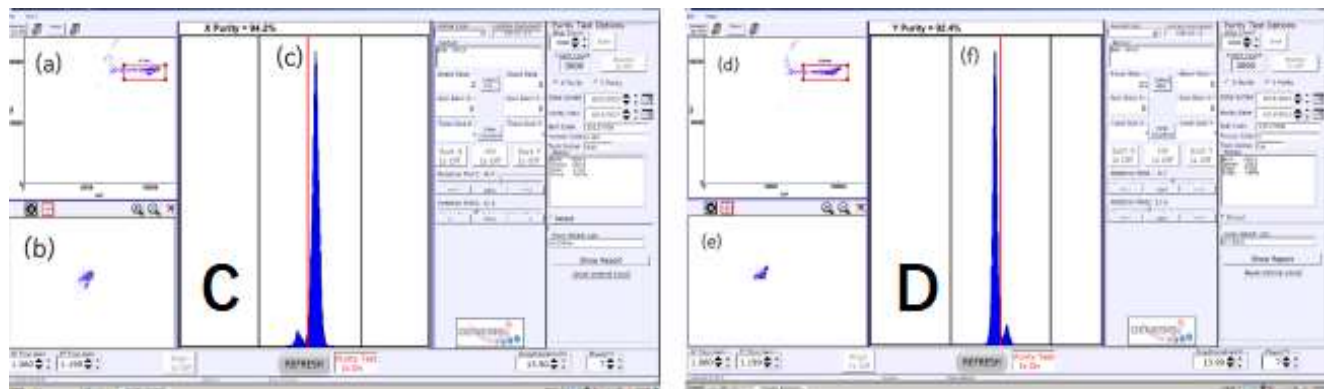

Figure-2. C) X sperm separation accuracy. In the figure, (a) shows the correct orientation of sperm; (b) shows the dead sperm rate; (c) shows that the accuracy of X sperm separation is 94.2%. D) Y sperm separation accuracy. In the figure, (d) shows the correct orientation of sperm; (e) shows the dead sperm rate; (f) shows that the accuracy of Y sperm separation is 92.4%.

Sperm sorting results and purity detection

The semen of bulls that met the sorting requirements was isolated, and a total of 426 X-semen and 464 Y-semen were produced. The average sorting rate of X- and Y-sperms reached 4800/s. The accuracy of the separation of X- and Y-sperm was tested (Figure 2). We found the accuracy of X and Y sperm separation to be 94.2 and 92.4%, respectively.

Analysis of in vitro fertilization results

Using the control, the data of embryonic development to cleavage and blastocyst stage after IVF with X- and Y-sperm are shown in Table 5. The embryo division rate and blastocyst rate of in vitro fertilization with sorted sperms were non-significantly ($p > 0.05$) different from those with conventional sperms (control group). Mature oocytes were cultured *in vitro* (Figure 3E), and embryonic development status was assessed on day 6 after fertilization (Figure 3F).

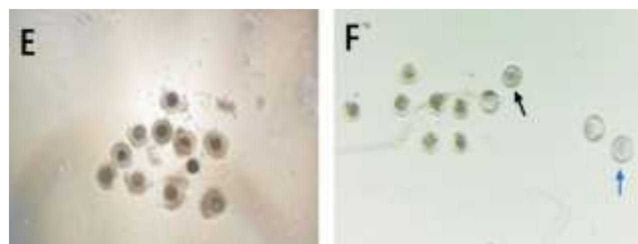

Figure-3. E) Matured oocyte (100×). The oocytes were cultured in IVM for 24 h, discharge the first polar body, to reach the mature stage. F) The fertilized egg developed to the blastocyst stage (100×). The fertilized eggs developed to day 6 in IVC, black arrows represent blastocysts, blue arrows represent expanded blastocysts.

Table-5. Sperm sorting and conventional sperm in vitro fertilization and embryo development.

Type of semen	Number of oocytes	Cleavage rate (%)	Blastocyst rate (%)
Control group	156	66(75.3)[a]	21(31.8)[a]
X-sperm	152	61(74.3)[a]	17(30.6)[a]
Y-sperm	149	56(73.7)[a]	15(30.5)[a]

Values (%) bearing same letters in a column differ non-significantly ($p > 0.05$).

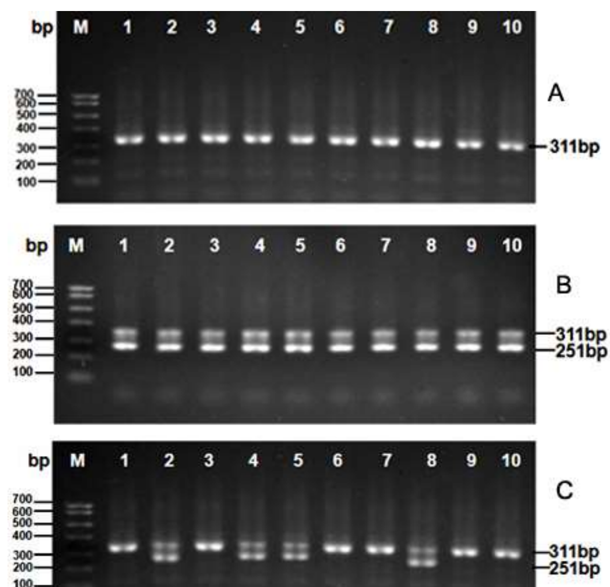

Figure-4. Nested amplification results of *AMEL* gene fragment of cattle genomic DNA. M: DNA Marker (700 bp); A) 1-10: Injected X sperm; XX one belt; B) 1-10: Injected Y sperm; XY two belts and C) 1-10: Injected of conventional sperm; XX one belt, XY two belts.

Analysis of embryo sex determination

After cleavage of a single blastocyst, and nested PCR amplification using the *AMEL* gene, the results of the embryo sex verification were shown in Figure 4.

Table-6. Comparison of results of intrauterine insemination using sex-sorted and routinely thawed sperm.

Type of semen	Number of inseminated cows	60-d pregnancy rate (%)	Gender proportion of descendants (%)	Sex ratio of sperm (%)
Control group	25	19 (76.0)[a]	10:9 (52.6M: 47.4F)[a]	50:50(expected not tested)
X-sperm	18	8(44.4)[b]	0:8 (0M:100F)[b]	94.2 (5.8:94.2)
Y-sperm	23	11(47.8)[b]	10:1 (90.9M:9.1F)[b]	92.4 (92.4:7.6)

Values (%) bearing different letters in a column differ significantly ($p < 0.05$).

Table-7. Mean birth weight of calves and the number of calves after insemination with control or sex-sorted thawed sperm.

Type of semen	Male birth weight (kg)	Female birth weight (kg)	Gestation period (d)
Control group	46.7±0.5 (n=10)[a]	45.4±0.7 (n=9)[a]	280±1.5(n=19)[a]
X-sperm	-	44.9±0.6(n=8)[a]	280±1.2(n=8)[a]
Y-sperm	45.6±1.1 (n=10)[a]	-	279±0.6(n=11)[a]

Values (mean±SE) bearing different letters in a column differ significantly ($p < 0.05$).

Analysis of artificial insemination results

Data on the number of inseminated cows, pregnancy rate, and sex of the resulting calves are presented in Table 6. The pregnancy rate (76.0%) of the control group on the 60th day of artificial insemination was significantly ($p < 0.05$) higher than that of X-sperms (44.4%) and Y-sperms (47.8%), while there was non-significant ($p > 0.05$) difference in the pregnancy rates of X- and Y-sperms, we used split-time artificial insemination (STAI) to improve the pregnancy rate of cows. There was significant ($p < 0.05$) difference in the sex ratio among the off-springs of sorted X-sperm (100% female; purity 94.2%), Y-sperm (90.9% male; purity 92.4%), and non-sorted sperm (50:50 male and female).

The mean birth weight and gestation period of calves born in the control and X/Y-sorted sperms groups are shown in Table 7. The gestation period of all cows was between 270 and 290 days. There was a non-significant difference between the birth weights of control calves and calves born to cows inseminated with sorted X- or Y-sperm. Similarly, the sex ratio of calves born did not varied significantly ($p > 0.05$).

Discussion

Sorting mammalian X and Y sperm can maximize the potential of exceptional breeding animals and contribute to the economic growth of animal husbandry. The commercialization and widespread use of flow cytometry for sorting sperm in cattle has already been reported (Quelhas et al., 2021). Sorted semen is used for *in vitro* fertilization and early embryo sex identification. Additionally, artificial insemination is employed to detect indicators of offspring production after delivery (Zwiefelhofer et al., 2021; Fuentes et al., 2022). In the present study, the average sorting rate of X-and Y-sperms reached 4800/s, with a sorting purity >92%, both sorted sperm quality assessment and artificial insemination offspring indicators met the experimental requirements.

With the development of scientific research, detection techniques, such as sperm sorting have been successfully tested, which met several standards, and can accelerate the upgrading of experimental equipment (Umehara et al., 2019). Sperm loss and damage during sperm sorting, centrifugation, and packaging have been identified as persistent issues (Gao et al., 2010). Other than these, sheath fluid pressure, laser intensity, dye concentration, and environmental conditions need further investigations for the elimination of the shortcomings (Santiani et al., 2023; Mukhopadhyay et al., 2023).

According to Chowdhury et al. (2019), Y-spermatozoa can be agglutinated and interfere with oocyte motility, thus fertilization can be inhibited, thereby affecting the developmental potential of the embryo. However, X-sperms have a typical motility pattern and are more likely to be involved in the fertilization of oocytes (Chowdhury et al., 2019; Quelhas et al., 2021; Pozdyshev et al., 2023). From semen sorting to the cryopreservation process, sperm viability may decrease by 30-40% (Palma et al., 2007). After IVF using non-sorted and sex-sorted frozen-thawed sperms, comparing the 3rd day and 17th day of embryonic development, there was a non-significant difference in cleavage rate (76.8 vs.

75.4%) and blastocyst development rate (32.2 vs. 26.9%) (Underwood et al., 2010). In our study, the percentage of embryos obtained from X-sperms at the cleavage and blastocyst stages was slightly higher than that of Y-sperms, and there was a non-significant difference in embryonic development to the cleavage and blastocyst stages using sex-sorted and non-sorted frozen-thawed sperm. According to various reports, compared with non-sorted sperms, sex-sorted sperms used for IVF (Schenk et al., 2009) and embryonic transfer (Hayakawa et al., 2008) were associated with lower fertility. Low fertility could be due to the damage to the sperms during the sorting process, including dilution, centrifugation, culture, staining, high pressure, laser, and charging (Frijters et al., 2009; Metz et al., 2019; Da Costa et al., 2021; Llavanera et al., 2022). In our study, the use of sex-sorted sperms did not affect normal fertility compared with non-sorted sperms.

The best use of the sperm sorting method requires effective technologies and management (Magopa et al., 2023). When using sex-segregated sperms for insemination, estrus, and pregnancy rates can be improved by STAI and delaying the late time point (Grèze et al., 2019; Ketchum et al., 2021). We used delayed insemination time point that could improve the pregnancy rate of cows. Ketchum et al. (2021) found that the pregnancy rate of fertilized cows may be related to the sperm type (59% in the conventional group and 48% in the sorted sperm group), but not to the insemination method. Anel-López et al. (2018) found that the average pregnancy rate of X- (51.3%) and Y- (49.8%) sperms after sorting was lower than that of conventional sperms (77.6%) (Seidel and Dejarnette, 2021). We also found that the viability, acrosome integrity rate, and DNA integrity of frozen-thawed sperm were similar to the control group, while the conception rate with sorted sperms was lower, could be due to the low dose of sex-sorted sperms per insemination (Maicas et al., 2020). Therefore, achieving higher pregnancy rates using sex-sorted frozen-thawed sperms may require higher insemination doses of sex-sorted sperms than those used in the present study (Osada et al., 2019).

Studies have shown that using Hoechst 33342 dye to label sperm cells and exposing them to ultraviolet laser light has no toxic effect (Parrilla et al., 2004; Winters et al., 2018). Eddaoudi et al. (2018) measured DNA and RNA contents in living cells by flow cytometry, combined with immunophenotyping analysis. They indicated that Hoechst 33342 has no obvious effect on the genetic material of cells. However, the use of Hoechst 33342 staining and laser irradiation in the flow sorting process tend to increase the incidence of chromosomal aberrations (Garner, 2006; Long et al., 2023). Although the sperm sorting process can cause damage and declined fertility, however, offspring are usually normal (Drake et al., 2020; Priyanto et al., 2023). No genetic abnormalities of offspring derived from sorted sperm were recorded in this study. In the present study, the results of early embryo sex identification were consistent with the purity of sperm sorting. There were non-significant differences in birth weight (46.1 vs 45.6 vs 44.9%) and gestation period (280 vs 280 vs 279 days) between calves born with sorted sperms and control calves. Therefore, our preparation of sex-controlled frozen semen for Belgian blue bull can be used for production and is economical, convenient, and environmental friendly.

Conclusion

This study successfully sorted high-purity X- (94.2%) and Y- (92.4%) sperms. The results of sperm quality detection, early embryonic development, and sex identification all met the test requirements. Application of sorted sperms for artificial insemination trials produces normal Belgian blue cattle progeny of predicted sex.

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Contribution of Authors

Niu P: Conceived idea, designed research methodology, collected & analyzed data and wrote draft of manuscript
Huang F, Wang J, Gao Q, Liu B & Fang D: Acquired funds for project, performed laboratory experiments, wrote & edited the manuscript
Liu X: Interpreted the data, critically edited & revised the manuscript and approved final draft
Khan A: Performed laboratory experiments, wrote & edited the manuscript

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