RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 12: 2054-2060 (December 2025)

Effects of TLR7/8 Activator on Sperm Quality, Energy Metabolism and the Separation Efficiency of X- and Y-Chromosome-bearing Sperm in Frozen-thawed Bull Semen

Jingchun Li[1#], Yulun Song[1#], Yingying Dong[1], Qing Guo[1], Hechuan Wang[1], Yanbing Li[1] 10.18805/IJAR.BF-1965

ABSTRACT

Background: This study investigated the effects of Requimod (R848), a TLR7/8 activator on sperm quality, energy metabolism and the separation efficiency of X- and Y-chromosome-bearing sperm in frozen-thawed bull semen.

Methods: Bull semen samples were divided into six groups: A control group and five experimental groups treated with different concentrations of R848 (0.05, 0.5, 1, 2 and 4 µmol/L). Following incubation at 37°C for 60 min by upstream methods, sperm quality parameters were assessed for upper- and lower-layer sperm. Further evaluations included acrosomal integrity, plasma membrane integrity, mitochondrial activity and ATP content.

Result: The results demonstrated that treatment with 0.5 µmol/L R848 significantly reduced sperm quality parameters in lower-layer X-sperm. This concentration also inhibited mitochondrial activity and ATP content in X-sperm. However, no significant differences in acrosomal or plasma membrane integrity were observed between the control and experimental groups. Additionally, the R848 treatment had no significant effects on sperm quality parameters, acrosomal integrity, or plasma membrane integrity of the upper-layer Y-sperm compared to the control group. Flow cytometric analysis of separated sperm indicated that 76.13% of the sperm in the upper layer were Y-sperm, while 61.78% of the sperm in the lower layer were X-sperm. These findings suggest that 0.5 µmol/ LR 848 effectively enhances the separation of X- and Y-sperm in frozen-thawed bull semen.

Key words: Bull sperm, R848, Sperm quality, Tlr7/8 activator.

INTRODUCTION

At present, numerous methods have been developed for separating X- and Y-chromosome-bearing sperm, including flow cytometry sorting, Percoll and albumin gradient centrifugation, upstream methods and immunological techniques (Azizeddin *et al.,* 2014; Bhat *et al.,* 2020; Kusumawati *et al.,* 2019). Among these, flow cytometry remains the most reliable and effective method for sorting X and Y-sperm (Cottle *et al.,* 2018). This technology achieves separation based on differences in DNA content between X- and Y-sperm and has been commercialized in the dairy industry. However, significant limitations, such as high costs and the potential for sperm damage during processing, have restricted its broader adoption in animal husbandry (Lu *et al.,* 2013). Consequently, there is an urgent need for an alternative sperm sorting method that is cost-effective, efficient and minimally harmful to sperm, particularly to accelerate the breeding of high-yielding cows and advance the dairy industry. Immunological methods (Ren *et al.,* 2021; Umehara *et al.,* 2019), which leverage protein differences between X- and Y-sperm, have emerged as a promising alternative to flow cytometry.

Toll-like receptors (TLRs), specifically TLR7 and TLR8, play a critical role in reproductive physiology (Saeidi *et al.,* 2014). Umehara *et al.* (2020) demonstrated that TLR7/8 is expressed in mouse sperm and activation of these receptors significantly inhibited the motility of both mouse and human sperm. Furthermore, Umehara *et al.* (2019) identified TLR7/8 as receptor proteins encoded by the X chromosome, expressed in approximately 50% of sperm. Notably, TLR7 is localized in the sperm tail, while TLR8 resides in the sperm midpiece and is associated with mitochondria. These findings revealed that co-incubation with TLR7/8 agonists selectively reduced the motility of X-sperm without affecting the Y-sperm. Similarly, in bovine sperm, TLR7/8 expression was found to be X-sperm specific, with a positive rate of approximately 50% (Umehara *et al.,* 2019). Building on this foundation, Ren *et al.* (2021) demonstrated that co-incubation of goat sperm with TLR7/8 activators reduced the motility of lower-layer X-sperm. These findings suggest the potential of a sperm

[1]College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, 163319, P.R. China.
[#]These authors contributed equally to this work.

Corresponding Author: Jingchun Li, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, 163319, P.R. China. Email: ljchun112@163.com

How to cite this article: Li, J., Song, Y., Dong, Y., Guo, Q., Wang, H. and Li, Y. (2025). Effects of TLR7/8 Activator on Sperm Quality, Energy Metabolism and the Separation Efficiency of X- and Y-Chromosome-bearing Sperm in Frozen-thawed Bull Semen. *Indian Journal of Animal Research*. **59(12):** 2054-2060. doi: 10.18805/IJAR.BF-1965.

Submitted: 27-01-2025 **Accepted:** 10-03-2025 **Online:** 04-04-2025

sorting method based on TLR7/8 activation. However, additional research is necessary to optimize this method for practical applications and improve its separation efficiency in various species.

Resiquimod (R848), a member of the imidazoline-quinoline family, is a small organic molecule with potent antiviral and anticancer properties (Morgan *et al.,* 2021). Currently, R848 functions as an agonist of TLR7 and TLR8 in humans and cattle and recent studies have explored the effects of R848 on sperm physiology (Agier *et al.,* 2020). Umehara *et al.* (2019) reported that R848 selectively inhibits ATP production via the GSK3α/β-hexokinase pathway, reducing the motility of X-sperm. Similarly, Ren *et al.* (2021) demonstrated that R848-mediated activation of TLR7/8 signaling pathways, including TRAF6/PI3K/GSK3α/β and TRAF6/NFκB, increased the phosphorylation levels of GSK3α/β and NFêB proteins. This cascade inhibits hexokinase activity, leading to reduced ATP production and decreased motility in X-sperm (Zhou *et al.,* 2021). These studies highlight the inhibitory effect of R848 on X-sperm motility, offering a potential tool for sperm sorting. Despite these advances, limited studies have explored the application of R848 for isolating X- and Y-sperm in frozen-thawed bull semen. This study aims to investigate the effects of the TLR7/8 activator R848 on frozen-thawed bull sperm quality, evaluate its role in sex-controlled sperm separation and elucidate the underlying mechanisms of its action.

MATERIALS AND METHODS

The experiment was conducted *rabi* session of 2022-10 and 2024-12 at the Animal reproduction laboratory, college of animal science and veterinary medicine, Heilongjiang Bayi Agricultural University.

Preparation of bull semen and sex-controlled semen

Frozen semen from dairy bulls was obtained from the Animal Husbandry Bureau of Daqing City, Heilongjiang Province. Post-thaw, the sperm motility rate exceeded 0.6 and the malformation rate was ≤15%, meeting the criteria for this study. Sex-controlled semen, consisting of X-chromosome-bearing sperm from Holstein bulls, had a post-thaw motility rate of >0.3 and a malformation rate ≤15%.

Test reagent

R848 was sourced from TargetMOI. SYBR-14, PI, FITC-PNA and JC-1 were procured from Thermo Fisher Technologies. Hoechst 33342 was purchased from Sigma Inc. and the ATP assay kit was obtained from Biyuntian Biotechnology Co., Ltd.

Incubation and isolation of sperm

Frozen semen was removed from liquid nitrogen storage and thawed in a 38.5°C water bath for 13 s. The sperm suspension was centrifuged at 800× g for 7 min to remove the diluent. The resulting sperm pellet was resuspended in a culture medium containing R848 at varying concentrations (0, 0.05, 0.5, 1, 2 and 4 µmol/L). The samples were incubated at 37°C for 1 h in a 5% CO_2, incubator, using the upstream method as outlined in Umehara's protocol (As shown in Fig 1). Each sample had a sperm concentration of 1×10^8 sperm /mL and 3 mL of suspension was placed in test tubes for incubation. Following a 30-minute incubation in the sperm separation medium containing R848, the samples were centrifuged and the sperm numbers in the upper and lower layers were determined. Sperm from these layers were subsequently used for further analysis.

Evaluation of motility and velocity parameters

After the 30 min incubation, 500 µL of sperm suspension from both the upper and lower layers of each group was collected and placed in 1.5 mL centrifuge tubes. A 10 µL aliquot from each concentration group was deposited onto a coverslip. Sperm motility and velocity parameters, including straight-line velocity (VSL), curvilinear velocity

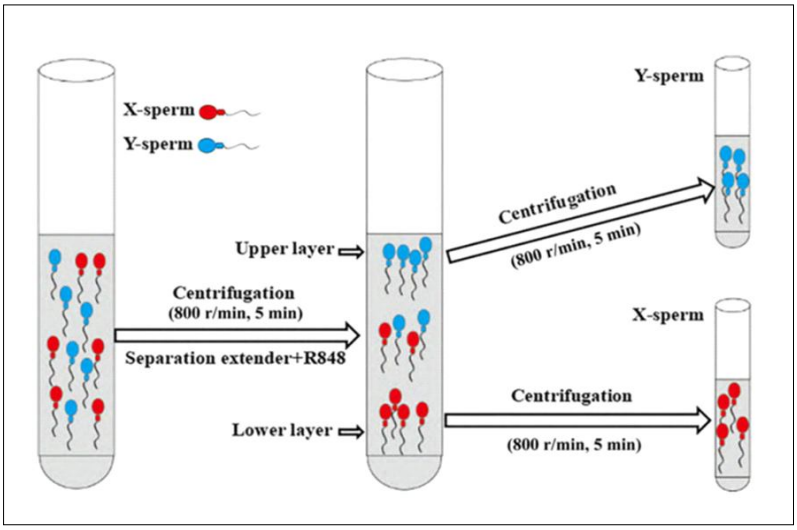


Fig 1: Schematic diagram of X- and Y-sperm isolation in dairy cows using R848 (Umehara *et al.,* 2020).

(VCL) and average path velocity (VAP), were assessed using a computer-assisted sperm analyzer (CASA) system. The system consisted of a phase-contrast microscope with a heated stage (37°C) equipped with Sperm Class Analyzer software (CASA, Nanning Song Jing Tianlun Biotechnology Co., Ltd.). Each sample was analyzed in five replicates (Li *et al.,* 2020; Li *et al.,* 2021).

Sperm plasma membrane integrity test

Following incubation, 250 µL of each sperm sample was centrifuged at 800 × g for 7 min and the supernatant was discarded. The pellet was resuspended in 500 µL of HEPES-buffered saline. Sperm in each group were stained with 5 µL of PI and incubated at 37°C for 5 min. Subsequently, 5 µL of SYBR-14 was added and samples were incubated for an additional 5-10 min. A 10-µL aliquot of the stained sperm was placed on a slide, covered with a coverslip and examined under a fluorescence microscope. Five replicates were conducted per sample, with a minimum of 200 sperm assessed per replicate and the results were recorded.

Analysis of sperm acrosome integrity

Acrosome integrity was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining in a procedure slightly modified from the method described by Aboagla and Maeda (2011).

Analysis of sperm mitochondrial activity

Following incubation, 250 µL of sperm suspension was centrifuged at 800 × g for 7 minutes. The pellet was resuspended in 500 µL of HEPES-buffered saline and 10 µL of JC-1 dye was added. Samples were incubated at 37°C for 30 min. A 10-µL aliquot was placed on a slide, covered with a coverslip and examined under a fluorescence microscope and video graphed. There were 5 replicates for each semen sample and the sperm count of each sample was observed to exceed 200 and the data were recorded.

Analysis of ATP content of sperm

ATP content in sperm samples was measured using the ATP assay kit, following the manufacturer's instructions.

Accuracy of sperm isolation by flow cytometry

Sperm samples were analyzed after incubation using the protocol described by Ribeiro (2013). Hoechest 33342 staining was performed and flow cytometry was used to evaluate the efficiency of sperm separation.

Statistical analysis

The experimental data were pre-processed using Excel 2019 and then analyzed with SPSS 26.0 software. Results are presented as mean±standard deviation. Statistical significance was determined by differences in means with distinct superscript letters in the same column (*P<0.05*).

RESULTS AND DISCUSSION

Effects of different concentrations of R848 on sperm quality in the lower layer

As shown in Table 1, the motility of lower-layer sperm in all experimental groups was significantly lower than in the control group under varying incubation conditions of R848 (*P<0.05*). Among the experimental groups, the sperm motility in the 0.05 µmol/L R848 group was significantly higher than that in the 0.5, 1, 2 and 4 µmol/L groups (*P<0.05*). No significant differences in sperm motility were observed among the 0.5, 1, 2 and 4 µmol/L groups (*P>0.05*). Additionally, the sperm motility in all R848-treated groups was significantly lower than in the control group (*P<0.05*). Among the treated groups, the 0.05 µmol/L group exhibited significantly higher motility compared to the higher concentration groups. No significant differences were noted in sperm motility among the 0.5, 1 and 2 µmol/L R848 groups (*P>0.05*). Under different concentrations of R848, there were no significant differences in path velocity (VAP), linear velocity (VSL) and curvilinear velocity (VCL) between the 0.05 ìmol/L group and the control group (*P>0.05*). However, in the 0.5 µmol/L group, these parameters were significantly different compared to the 1, 2 and 4 µmol/L groups (*P<0.05*). With increasing R848 concentrations, the viability and motility of lower-layer sperm showed a clear downward trend. However, no significant differences were observed among the 1, 2 and 4 µmol/L groups for these parameters (*P>0.05*). These results indicate that the addition of R848 to bull semen does not affect the viability and motility of upper-layer sperm. However, in the lower layer, sperm motility and viability began to decrease significantly when the R848 concentration reached 0.5 µmol/L. This suggests that R848 selectively inhibits the motility

Table 1: Effects of different concentrations of R848 on sperm quality in lower-layer.

Concentrations of R848 (µmol/L)	Sperm viability (%)	Sperm motility (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
0	62.19±1.95^{a}	46.25±2.45^{a}	44.03±0.68^{a}	45.57±0.96^{a}	45.74±0.97^{a}
0.05	46.56±5.97^{b}	32.62±3.26^{b}	43.64±1.48^{a}	44.95±1.98^{a}	45.07±2.04^{a}
0.5	23.93±2.81^{c}	17.92±2.42^{c}	42.27±0.66^{b}	43.20±0.94^{b}	43.23±0.94^{b}
1	22.37±6.46^{c}	16.90±5.63^{cd}	38.00±1.04^{c}	39.43±1.30^{c}	39.52±1.68^{c}
2	19.70±3.27^{c}	14.11±3.39^{cd}	34.90±0.97^{d}	38.28±0.71^{c}	38.25±0.47^{c}
4	19.48±2.57^{c}	12.74±1.4^{3d}	34.40±0.51^{d}	37.82±1.00^{c}	37.88±0.76^{c}

Data are given as mean±SD from five replicated experiments.

Values with different superscripts within columns are significantly different (*P<0.05*).

of X-chromosome-bearing sperm in the lower layer. The findings of this study align with those reported by Ren (2021) and Umehara (2020), further supporting the selective inhibitory effects of R848 on sperm motility in the lower layer.

Effects of R848 on sperm quality after elution

Table 2 shows that sperm viability, motility, VAP, VSL and VCL before elution were significantly lower in the experimental groups compared to the control group (*P<0.05*). After elution, significant improvements were observed in sperm viability and motility compared to the control group (*P<0.05*). However, there were no significant differences in VAP, VSL and VCL between the 0.5(-) group and the control group (*P>0.05*). The elution treatment significantly enhanced sperm quality parameters in the bull sperm samples compared to their pre-elution state. These findings suggest that the inhibitory effects of R848 on X-sperm motility and velocity are reversible and the inhibition can be effectively eliminated through elution. Building on the studies by Umehara *et al*. (2019) and Ren *et al.* (2021), this study further improved the efficiency of the upstream separation method by incorporating R848. Following co-incubation with frozen-thawed bull sperm, it was observed that the proportion of upper-layer sperm was significantly reduced. The reduction in upper-layer sperm was directly proportional to the concentration and incubation time of R848, indicating that R848 selectively inhibits the motility of certain sperm, leading to a lower proportion of motile sperm migrating upstream. The results of this study are consistent with previous findings. Umehara *et al.* (2019) reported a significant reduction in the proportion of highly motile sperm in the upper layer when R848 was co-incubated with mouse sperm. Similarly, Ren *et al*. (2021) observed comparable outcomes in dairy goats, further validating the selective inhibitory effect of R848 on sperm motility.

Effects of 0.5 µmol/L R848 on acrosomal integrity and plasma membrane integrity of sperm

The acrosomal and plasma membrane integrity of upper-layer sperm (0.5 µmol/L, referred to as 0.5 up) did not differ significantly from those of lower-layer sperm (0.5 µmol/L, referred to as 0.5 low) or the control group under incubation with 0.5 µmol/L R848 (*P>0.05*) (Fig 2). Given that R848 can inhibit X-sperm motility, it was necessary to determine whether it adversely affects sperm quality. Acrosomal integrity and plasma membrane integrity are key indicators of sperm quality, as these parameters are closely linked to motility in bovine sperm (Kanno *et al.* 2016). In this study, sperm were incubated with 0.5 µmol/L R848 for 1 h, after which FITC-PNA and SYBR-14/PI staining were employed to assess acrosomal and plasma membrane integrity in the control, upper-layer and lower-layer sperm groups. The results indicated that while sperm motility in the lower layer was significantly lower than that in the control and upper-layer groups, there were no significant differences in acrosomal or plasma membrane integrity between the control and upper-layer groups. These findings align with previous reports. Shi *et al.* (2016) observed that while TLR agonists (TLR1, TLR7/8 and TLR9) reduced sperm motility after 6 hours of incubation with mouse sperm, they did not significantly increase apoptotic sperm counts. Similarly, Umehara *et al*. (2020) demonstrated that R848 inhibited X-sperm motility in mouse sperm, but the inhibition was reversible following centrifugation. Based on these observations, it is speculated that R848 reduces X-sperm motility by binding to the TLR7/8 protein receptor on the sperm. However, R848 does not significantly impair the integrity of the plasma membrane or acrosome. Furthermore, the degradation or removal of the TLR7/8 receptor during centrifugation may restore the motility of sperm previously inhibited by R848 (Varshney *et al.,* 2021).

Effects of 0.5 µmol/L R848 on mitochondrial activity and ATP content of sperm

Following incubation with 0.5 µmol/L R848, the mitochondrial activity of sperm in the control group was significantly higher than that of upper-layer sperm (0.5 up) and lower-layer sperm (0.5 low) (Fig 3). Furthermore, the mitochondrial activity of upper-layer sperm was significantly greater than that of lower-layer sperm. Similarly, the ATP content of sperm in the control group was significantly higher than that of both upper-layer and lower-layer sperm, with the ATP content in the upper-layer sperm exceeding that of the lower-layer sperm.

The results suggest that TLR7/8 is a specific protein associated with X-sperm in cattle and R848 reduces the motility of X-sperm by binding to the TLR7/8 protein. This inhibition may occur due to the dual action of R848, suppressing hexokinase activity and impairing mitochondrial function, which ultimately disrupts sperm motility *via* the TLR7/8 signaling pathway. Consequently, ATP production in sperm is reduced (Zhu *et al.,* 2019),

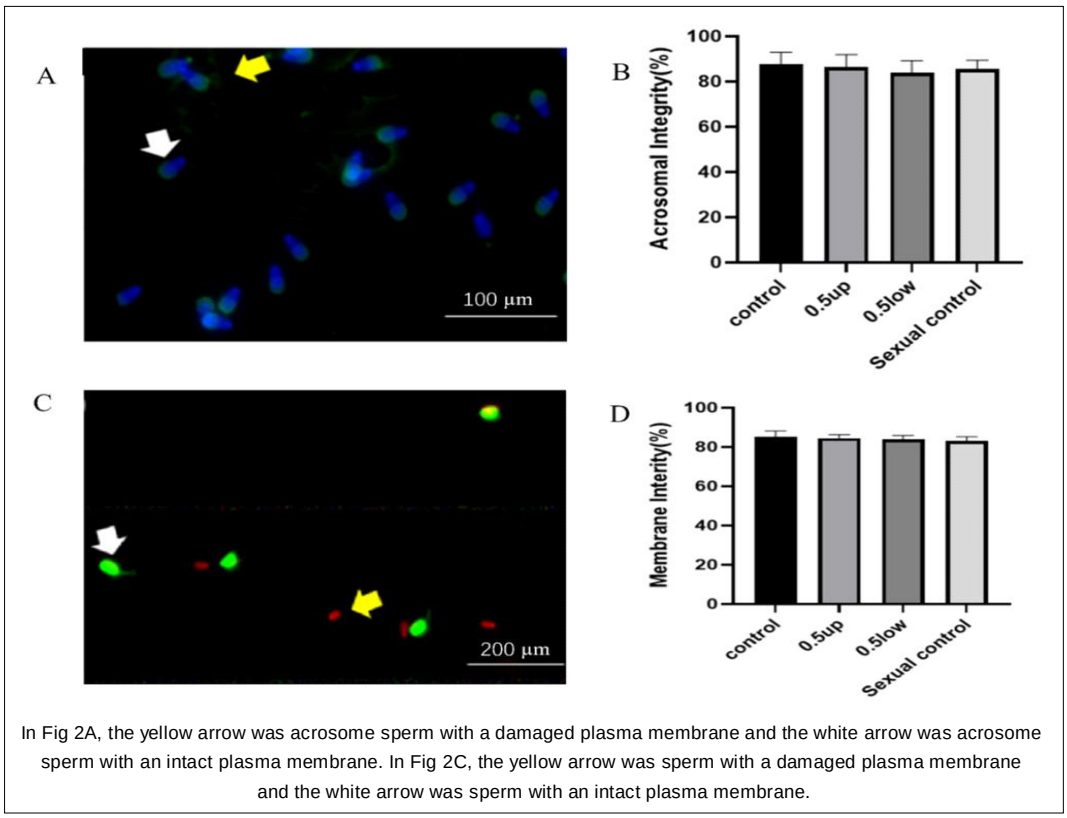
Table 2: Effects of R848 on sperm quality after elution.

Concentrations of R848 (µmol/L)	Sperm viability (%)	Sperm motility (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
0 (Control)	58.94±2.80^{a}	44.00±2.15^{a}	44.49±0.72^{a}	46.17±0.81^{a}	46.40±1.02^{a}
0.5(+)	19.52±2.59^{c}	13.35±2.10^{c}	41.38±0.24^{b}	41.91±0.36^{b}	41.95±0.34^{b}
0.5(-)	53.81±2.71^{b}	37.41±2.35^{b}	43.80±2.08^{a}	45.23±3.03^{a}	45.32±3.04^{a}

Data are given as mean±SD from five replicated experiments.

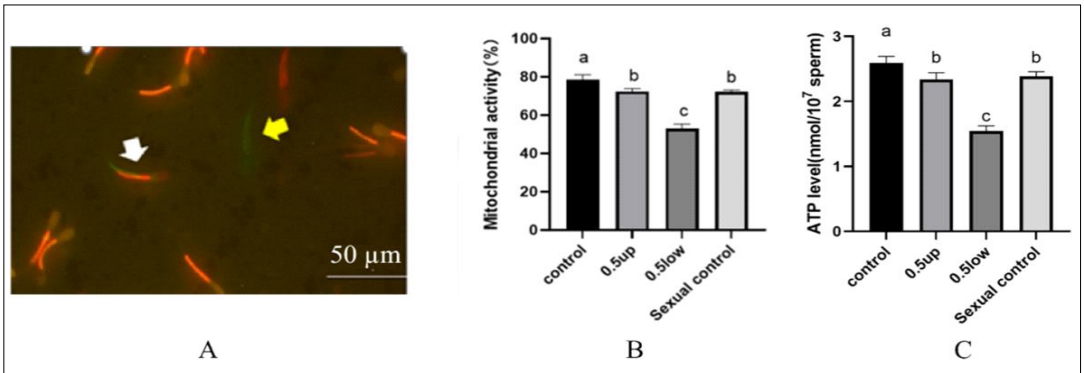
Values with different superscripts within columns are significantly different (*P<0.05*).

thereby impairing motility. Sperm motility is a fundamental characteristic of mature sperm and is influenced by multiple factors, including pH, temperature, intracellular calcium levels and cAMP concentrations. In most cells, mitochondria serve as the primary site for aerobic respiration, supplying the energy necessary for cellular activities (Bornhövd *et al.,* 2006). Thus, disruptions to mitochondrial function can significantly impact cell motility. In this study, the effects of R848 on sperm motility were examined by evaluating ATP levels and mitochondrial membrane potential. The findings revealed that both ATP content and mitochondrial membrane potential were significantly lower in the lower-layer sperm of the flotation system compared to the upper-layer Y-sperm and the control group. These observations are consistent with previous studies, such as Shi *et al.* (2016), who reported reductions in ATP content and mitochondrial membrane potential in sperm following incubation with multiple TLR agonists. Additionally, this



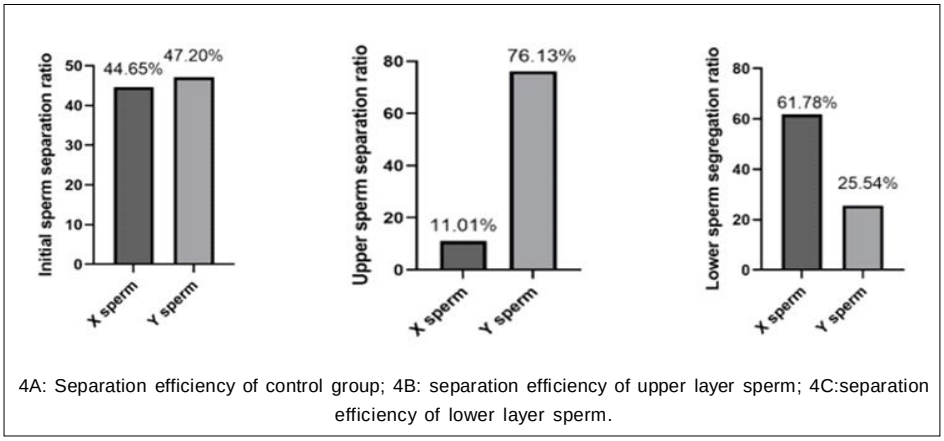
In Fig 2A, the yellow arrow was acrosome sperm with a damaged plasma membrane and the white arrow was acrosome sperm with an intact plasma membrane. In Fig 2C, the yellow arrow was sperm with a damaged plasma membrane and the white arrow was sperm with an intact plasma membrane.

Fig 2: The effects of 0.5 μmol/L R848 on acrosomal integrity (A, B) and plasma membrane integrity (C, D) of sperm.



Note: The yellow arrows showed high mitochondrial active sperm and the white arrows showed low mitochondrial active sperm in Fig 3A.

Fig 3: Effects of 0.5 μmol/L R848 on mitochondrial activity of sperm (A, B), ATP(C) content of sperm.



4A: Separation efficiency of control group; 4B: separation efficiency of upper layer sperm; 4C:separation efficiency of lower layer sperm.

Fig 4: Separation efficiency of bovine sperm by flow cytometry.

study demonstrated that decreased mitochondrial membrane potential in X-sperm corresponded with reduced motility. This reduction may occur due to the action of R848 on X-sperm mitochondria, leading to a decline in mitochondrial membrane potential. This decline may reverse the proton pump and drive ATP synthase to hydrolyze ATP, ultimately lowering ATP levels and inhibiting motility. Consequently, sperm viability and motility are compromised.

The effects of 0.5 μmol/L R848 on separation efficiency of X- and Y-sperm

The separation ratio of X- and Y-sperm, as determined by flow cytometry, was initially 44.65% and 47.20%, respectively (Fig 4). After incubation with 0.5 μmol/L R848, the proportions shifted significantly. In the upper layer, the X- and Y-sperm separation ratio was 11.01% and 76.13%, respectively, while in the lower layer, the X- and Y-sperm separation ratio was 61.78% and 25.54%, respectively. To confirm the purity of X- and Y-sperm following the separation process, a reanalysis was conducted using flow cytometry. The results revealed that Y-sperm constituted 76.13% of the upper layer, whereas X-sperm accounted for 61.78% of the lower layer (Fig 4). The results of our study were similar to those of Wen's, who reported that the separation ratio of Y sperm was 88.6% and that of X sperm was 72.5% (Wen *et al.,* 2023), The results of this study were slightly lower than Wen's results, mainly due to the frozen semen in this study.

CONCLUSION

The TLR7/8 activator, R848, selectively inhibited the viability and motility parameters of the X-sperm in the lower layer without significantly affecting the Y-sperm in the upper layer. Following treatment with 0.5 ìmol/L R848 for 60 min, the viability and motility of X-sperm in the lower layer declined but were restored after elution. This inhibition was associated with reduced ATP content and mitochondrial activity in X-sperm, while acrosomal integrity and plasma membrane integrity remained unaffected. Flow cytometry analysis demonstrated a separation efficiency of 76.13% for Y-sperm in the upper layer and 61.78% for X-sperm in the lower layer, highlighting the potential of R848 as an effective tool for improving sperm separation efficiency.

ACKNOWLEDGEMENT

The present study was supported by the Natural Science Foundation of Heilongjiang Province of China (No. LH2022C068).

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Aboagla M.E. and Maeda T. (2011). Arbutin's suppression of cryodamage in goat sperm and its mechanism of cryoprotection. *Theriogenology*. **76(3):** 538-546.

Agier, J., Brzezińska-Błaszczyk, E., Witczak, P., Kozłowska, E. and elechowska, P. (2021). The impact of TLR7 agonist R848 treatment on mast cell phenotype and activity. *Cellular Immunology*. **359:** 104241.

Azizeddin, A., Ashkar, F.A., King, W.A. and Revay, T. (2014). Enrichment of Y-chromosome-bearing bull spermatozoa by swim-up through a column. *Reproduction in Domestic Animals*. **49(1):** 1-4.

Bhat, Y., Sharma, M. (2020). X-sperm enrichment of bovine semen by percoll density gradient method and Its effect on semen quality, sex ratio and conception rate. *Indian Journal of Animal Research*. **54(10):** 1181-1187. doi: 10.18805/ijar.B-3823.

Bornhövd, C., Vogel, F., Neupert, W. and Reichert, A.S. (2006). Mitochondrial membrane potential is dependent on the oligomeric state of F1F0-ATP synthase supracomplexes. *The Journal of Biological Chemistry*. **281(20):** 13990-13998.

Cottle, D.J., Wallace, M., Lonergan, P. and Fahey, A.G. (2018). Bioeconomics of sexed semen utilization in a high-producing Holstein-Friesian dairy herd. *Journal of Dairy Science*. **101(5):** 4498-4512.

Kanno, C., Kang, S.S., Kitade, Y., Yanagawa, Y., Takahashi, Y. and Nagano, M. (2016). Simultaneous evaluation of plasma membrane integrity, acrosomal integrity and mitochondrial membrane potential in bovine spermatozoa by flow cytometry. *Zygote* (Cambridge, England). **24(4):** 529-536.

Kusumawati, D.E., Isnaini, N., Yekti, A.P.A., Luthfi, M., Susilawati, T. (2019). The motility and ratio of X and Y sperm filial ongole cattle using different sexed semen methods. *American Journal of Animal Veterinary Sciences*. **14(2):** 111-114.

Li, J.C., Li, Q., Li, Y.B., Wei, G.S., Sun, D.B. (2020). Effects of negative pressure applied before storage (liquid storage, 17°C) on boar semen quality and fertilization ability. *Indian Journal of Animal Research*. **54(10):** 1201-1205. doi: 10.18805/ijar.B-1042.

Li, Y., Li, J., Zhang, Q., Wang, Q., Guo, M., Li, Q. and Wei, G. (2021). The Effects of negative pressure on the gene expression of motility related proteins in boar spermatozoa during Liquid Storage at 17°C. *Indian Journal of Animal Research*. **55(5):** 511-516. doi: 10.18805/IJAR.B-1337.

Lu,K., Lu,Y., Zhang,M., Yang, X. (2013). Sperm sexing in buffalo using flow cytometry. *Buffalo Bulletin*. **32(3):** 22-30.

Morgan, F., Tejas,P., Danie, J.L. (2021). Resiquimod: A new topical immune-response modifier for the treatment of actinic keratosis. *Expert Opinion on Emerging Drugs*. **26(4):** 433-434.

Ren, F., Xi, H.M., Ren,Y.J., Li, Y., Hu, J.H. (2021). TLR7/8 Signalling Affects X-Sperm Motility via the GSK3*α/β*-Hexokinase pathway for the efficient production of sexed dairy goat embryos. *Journal of Animal Science Biotechnology*. **12(1):** 89-95.

Ribeiro, S.C., Sartorius, G., Pletscher, F., De Geyter, M., Zhang, H. and de Geyter, C. (2013). Isolation of spermatozoa with low levels of fragmented DNA with the use of flow cytometry and sorting. *Fertility and Sterility*. **100(3):** 686-694.

Sariadi, S., Dasrul, D. and Akmal, M. (2014). Rasio jenis kelamin kelahiran anak kambing peranakan ettawa (pe) hasil inseminasi buatan menggunakan spermatozoa swim up. *Journal Agripet*. **14(2):** 132-138.

Shi,D., Zhu, X., Zhou,H., Wu, F.J., Gong, W. (2016). TLR signalling affects sperm mitochondrial function and motility *via* phosphatidylinositol 3-kinase and glycogen synthase kinase-3 alpha. *Cellular Signalling*. **24(4):** 529-536.

Umehara,T., Tsujita, N., Shimada, M. (2019). Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. *PLoS Biology*. **17(8):** e3000398.

Umehara,T., Tsujita, N., Zhu, Z., Ikedo, M., Shimada, M. (2020). A simple sperm-sexing method that activates TLR7/8 on X sperm for the efficient production of sexed mouse or cattle embryos. *Nature Protocols*. **15(8):** 2645-2667.

Varshney, D., Qiu, S.Y., Graf, T.P., Mchugh, K.J. (2021). Employing drug delivery strategies to overcome challenges using TLR7/8 agonists for cancer immunotherapy. *The AAPS Journal*. **23(4):** 1-5.

Wen, F., Liu,W., Li, Y., Zou, Q., Xian, M., Han, S., Zhang, H., Liu,S., Feng, X., Hu,J. (2023). TLR7/8 agonist (R848) inhibit bovine X sperm motility *via* PI3K/GSK3α/β and PI3K/NFκB pathways. *Int. J. Biol. Macromol*. **232:** 123485.

Zhou,Y., Chen,X.J., Cao, Z.J., Li, J.L., Sun, Y. (2021). R848 Is Involved in the antibacterial immune response of golden pompano (*Trachinotus ovatus*) through TLR7/8-MyD88-NF-κB-Signaling Pathway. *Frontiers in Immunology*. **11(5):** 12-18.

Zhu, Z., Li, R., Wang, L., Zheng, Y., Hoque, S.A. M., Lv, Y. and Zeng, W. (2019). Glycogen synthase kinase-3 regulates sperm motility and acrosome reaction *via* affecting energy metabolism in goats. *Frontiers in Physiology*. **10(1):** 966-968.