



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
Indexing: Embase
IJABR 2026; 10(9): 730-735
www.biochemjournal.com
Received: 26-06-2026
Accepted: 01-08-2026
Published: 26-09-2026

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Concentration-dependent effects of resiquimod on X-chromosome-bearing sperm enrichment in Vrindavani (*Bos taurus indicus*) bulls: Evaluation of a TLR7/8-associated motility-based sorting approach

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9i.10165>

ABSTRACT

Sex selection in bovine reproduction is an important tool to improve the efficiency of dairy and beef production by controlling the sex ratio of offspring. This study aimed to optimize the concentration of Resiquimod, a Toll-Like Receptor (TLR) 7/8 agonist, for the selective enrichment of X-chromosome bearing sperm in semen of Vrindavani bulls, an important crossbred dairy breed of India. The effect of Resiquimod on sperm motility, viability and concentration, the key parameters for effective sperm sorting, was assessed with semen samples treated with Resiquimod at concentrations of 0.4, 0.6 and 0.8 micromolar (μM). Of the concentrations tested, 0.6 μM was identified as optimal, resulting in maximal enrichment of X-sperm with high sperm quality. These results provide a basis for developing cost-effective and practical sex-sorting protocols that can be beneficial to Indian bovine breeding programs.

Keywords: Bull, Concentration, Motility, Resiquimod, Sperm

1. Introduction

Sex selection by sperm-sorting has been an important tool in bovine reproduction for increasing genetic gain and improve production efficiency. Various authors have attempted nanotechnological approach for sperm selection (Srivastava *et al.*, 2026) [1] or semen purification (Bisla *et al.*, 2020a; Rautela *et al.*, 2022) [2, 3]. Given unacceptable discard rate (Bisla *et al.*, 2022) [4] sperm selection procedure, once validated, may also help in utilizing border-line semen samples which might have been discarded otherwise. Studies showing distinct effects of TLR7/8 agonists on bovine X- and Y-bearing spermatozoa have made TLR7/8-mediated sperm sex selection a viable substitute for traditional flow-cytometric sperm sorting (Umehara *et al.*, 2020; Nistane *et al.*, 2026) [6, 5]. Sex control can be used by farmers to improve milk production with female calves or meat production with males. Crossbred dairy cattle play an important role in India and Vrindavani breed is one of such breed which needs optimized reproductive technologies to increase the productivity (Singh *et al.*, 2011) [7].

Existing sperm sorting techniques, including flow cytometry, based on difference in DNA content between X- and Y-bearing sperm, are expensive, technically challenging and potentially harmful to sperm viability (Wen *et al.*, 2023) [8]. Recent research has emphasized the potential of immunological approaches that exploit differential expression of receptors on the surface of sperm. In particular, Toll-Like Receptors (TLRs) 7 and 8 of the innate immune system are differentially expressed and can be activated by agonists such as Resiquimod to allow selective sperm sorting (Nistane *et al.*, 2026) [5].

Resiquimod is a synthetic TLR 7/8 agonist, and it has been shown to selectively induce functional changes in X-chromosome bearing sperm, due to the higher activity of these receptors on their surface (Umehara *et al.*, 2020) [6]. However, the optimum concentration of Resiquimod to maximize the enrichment of X-sperm without affecting the sperm quality has not been determined especially in Vrindavani bulls.

This study aimed to investigate the effect of three concentrations of Resiquimod (0.4, 0.6 and 0.8 μM) on sperm motility, viability and concentration to determine an optimum dose that enriches X-chromosome-bearing sperm while preserving sperm function. The aim of findings is to advance affordable and efficient sperm sorting technology suitable for Indian dairy farming conditions. This will help to establish a breed-specific concentration-response relationship for Resiquimod-mediated sperm sorting in Vrindavani bulls and to identify an optimum treatment concentration based simultaneously on X-sperm enrichment, sperm recovery and post-treatment sperm quality.

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2. Materials and Method

Preparation of working solution of resiquimod

In the present study, 10 mg Resiquimod (R848) (Cat No. SML0196) had been purchased from Sigma-Aldrich and stored at -20°C till further use. The working solutions were prepared by dissolving in DMSO and stored at -20°C to avoid repeated freeze-thaw cycles. To find out the most effective concentration at which best possible sorting occur, three different concentrations of Resiquimod (0.4, 0.6 and 0.8 µM) were prepared from the working solution prior to every experiment. For treatment experiments, each of these three different concentrations of resiquimod were freshly dissolved in 3 ml modified human tubal fluid extender (Cat # 90,126) purchased from Irvine Scientific. To account for any potential impact of the solvent, the Control group was given an equivalent volume of DMSO to that provided in the Resiquimod-treated samples.

Semen collection and initial evaluation

A total of 24 semen ejaculates were collected from three healthy Vrindavani bulls aged 5-10 years that were maintained at the Germplasm Centre, Division of Animal Reproduction, ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India, under standard feeding and management conditions. The frequency of semen sample collections was two times per week over a period of 10 weeks using an artificial vagina prewarmed at 42°C. Each ejaculate was immediately placed in a sterile collection tube and kept at the proper temperature to reduce cold shock. After that, ejaculates were subjected to routine macroscopic and microscopic evaluation to record volume, colour, consistency, mass activity following which samples were selected based on concentration, progressive motility and viability (Srivastava *et al.*, 2013, Bisla *et al.*, 2020b) [9].

Motility based separation of spermatozoa

After Resiquimod treatment, spermatozoa were subjected to motility based separation to enrich the sperm population according to their different motility response to treatment. Modified Swim-up method was used to carry out the separation process.

This procedure was carried out in four round bottom glass tubes each containing 3 ml of modified human tubal fluid with different concentrations of Resiquimod dissolved in treatment groups and DMSO in the Control group. Volume of freshly collected semen ejaculates to be added was calculated based on concentration and motility of ejaculates and were mixed in each of the tubes. The tubes are vertically aligned and kept undisturbed in an incubator maintained at 37°C for 40 minutes. After this 1 ml each of the uppermost and lowermost fraction was collected in 1.5 ml Eppendorf tubes placed in dry bath maintained at 37°C and subjected for assessment of concentration, motility, viability and abnormalities (Gupta *et al.*, 2022) [10].

Assessment of sperm concentration

Following the motility-based separation, sperm concentration was measured in the recovered upper and lower fractions. Samples were diluted in normal saline solution and sperm concentration was determined using automated sperm analyser.

The concentration of spermatozoa recovered from each semen sample was expressed as $\times 10^6$ spermatozoa/ml. If required, the total sperm concentration of the recovered fraction was determined by multiplying sperm concentration by the volume of recovered fraction (Singh *et al.*, 2015; Perumal *et al.*, 2017) [12, 11].

Assessment of sperm motility

The motility of both upper and lower separated fractions was assessed by placing 10 µl of fractionated sample on a clean glass slide and covered with coverslip. The total motile spermatozoa were counted and progressive sperm motility was assessed individually for each semen sample using a phase contrast microscope (Leica DM 500, Schweiz) equipped with a heated stage maintained at 37°C. For evaluation of progressive motility 4-5 randomly selected microscopic fields were evaluated and the percentage of progressively motile spermato-

zoa were recorded (Pande *et al.*, 2015; Ponraj *et al.*, 2016; Srivastava and Pande, 2017) [13, 14, 15].

Assessment of sperm viability and abnormalities

Sperm viability was assessed in the post separation semen samples using eosin-nigrosin staining described by Kumar *et al.*, (2017) [16] and Pande *et al.* (2019) [17]. An aliquot of the sperm suspension was combined with the dye to create a thin smear on clean grease free glass slide and stained with a solution of eosin (1.67%) and nigrosine (10%) staining. After drying, spermatozoa were examined under a light microscope (Leica DM 500, Schweiz) equipped with a heated stage maintained at 37°C at 40X magnification. A minimum of 200 spermatozoa were evaluated per sample. Spermatozoa with intact plasma membranes that were not stained and appeared colourless were considered as viable and those that were stained and appeared pink were considered as non-viable. Simultaneously, morphological abnormalities of spermatozoa in head and tail region were identified and recorded (Srivastava *et al.*, 2017, Kumar *et al.*, 2021) [18].

3. Results

Effect of resiquimod concentration on sperm concentration

Resiquimod treatment resulted in a concentration-dependent distribution of spermatozoa between the upper and lower fractions following swim-up separation. The average concentration of sperm cells in the fresh stage after sorting in the 0.4 µM, 0.6 µM and 0.8 µM treatment group samples were 115.9 ± 12.23 , 112.8 ± 9.56 and 103.6 ± 10.81 million/ml in the lower fraction *i.e.* expected X sperm population and 87.96 ± 9.25 , 98.38 ± 10.73 and 64.19 ± 7.07 million/ml in the upper fraction (expected Y sperm population) respectively. The overall difference among the concentrations was not statistically significant in the upper fraction ($P = 0.426$) and resulted in statistically significant in lower fraction ($P = 0.02$) (Table 1).

Effect of resiquimod concentration on sperm motility

Sperm motility changed in a concentration dependent manner after treatment with resiquimod. The 0.4, 0.6 and 0.8 µM concentration significantly decreased the total and progressive motility in the lower sperm fraction when compared to the untreated control ($P < 0.05$) (Table 1). Treatment with the TLR7/8 agonist R848 induced a significant, dose-dependent reduction in total motility across all processing stages (fresh, pre-freeze, and post-thaw). In the control group (0 µM R848), fresh semen exhibited the highest total motility ($87.50 \pm 0.60\%$), which declined progressively through pre-freeze ($76.96 \pm 0.59\%$) and post-thaw ($61.83 \pm 1.30\%$) stages (Table 1). Superscript annotations indicate that all R848-treated groups differed significantly from control ($P < 0.05$), with the 0.4 µM group showing intermediate values ($80.67 \pm 0.19\%$ fresh; $72.96 \pm 0.62\%$ pre-freeze; $50.92 \pm 1.46\%$ post-thaw), while the 0.6 µM and 0.8 µM groups exhibited further reductions, with the 0.8 µM group displaying the lowest post-thaw total motility ($40.83 \pm 1.02\%$).

Progressive motility followed an identical dose-response pattern. Control samples maintained $82.79 \pm 0.50\%$ progressive motility in fresh semen, declining to $72.00 \pm 0.43\%$ pre-freeze and $48.33 \pm 1.55\%$ post-thaw. R848 treatment at 0.4 µM reduced fresh progressive motility to $80.00 \pm 0.50\%$, with further declines at 0.6 µM ($78.00 \pm 0.53\%$) and 0.8 µM ($75.00 \pm 0.58\%$). Post-thaw progressive motility was most severely affected in the 0.8 µM group ($35.00 \pm 1.04\%$), representing a 27.6% absolute reduction compared to control.

While the higher percentage showed somewhat greater motility preservation, the amount of the loss increased with increasing Resiquimod concentration.

Effect of resiquimod concentration on sperm viability

Viability assessments revealed a similar dose-dependent trend, though with comparatively smaller absolute declines than motility parameters. Fresh viability in the Control group was $88.75 \pm 0.40\%$, decreasing to $80.54 \pm 0.62\%$ pre-freeze and $64.08 \pm 1.00\%$ post-thaw. R848 treatment at 0.4 µM reduced fresh viability to $87.04 \pm 0.37\%$, with

progressive declines at 0.6 μM ($83.13 \pm 0.49\%$) and 0.8 μM ($73.92 \pm 0.31\%$) (Table 1). Post-thaw viability in the 0.8 μM group ($45.75 \pm 0.72\%$) was significantly lower than control ($64.08 \pm 1.00\%$), indicating that higher R848 concentrations exacerbate cryopreservation-induced membrane damage.

4. Discussion

Our results show that R848 suppresses sperm motility in a dose-dependent manner, with cryopreservation amplifying this effect. At 0.4 μM , fresh total motility was $\sim 92\%$ of control, whereas 0.8 μM maintained similar fresh motility but dropped to $\sim 66\%$ of control after thawing, indicating that freeze-thaw stress sensitizes sperm to TLR7/8-mediated inhibition (Wen *et al.*, 2023) [8].

The steeper decline in progressive versus total motility at higher doses (e.g., post-thaw $\sim 35\%$ vs $\sim 41\%$ at 0.8 μM) fits an energy-limitation model: progressive movement requires sustained ATP for flagellar beating, while non-progressive motility can persist when metabolism is compromised (Wen *et al.*, 2023; Huang *et al.*, 2022) [8, 19]. Mechanistically, R848 activates TLR7/8 on X-sperm, engaging PI3K-dependent pathways (PI3K/GSK3 α/β and PI3K/NF- κB) that reduce hexokinase activity and impair glycolysis and mitochondrial ATP production (Wen *et al.*, 2023 [8]; recent work also implicates LKB1/AMPK in suppressing glycolysis and fatty acid β -oxidation) (Wen *et al.*, 2023).

In our dataset, 0.6 μM was a critical inflection point: beyond this concentration, post-thaw motility and viability fell sharply. This aligns with optimal ranges reported for immunological sex-sorting in live-stock ($\sim 0.5 \mu\text{mol/L}$ in cattle; 0.4–0.6 μM in dogs; 0.3–0.5 μM in goats), where Y-sperm enrichment is achieved while preserving functional semen quality (Wen *et al.*, 2023; Shattock *et al.*, 2026; Huang *et al.*, 2022) [8, 20, 19]. At 0.6 μM , post-thaw total motility ($\sim 48\%$) and viability ($\sim 53\%$) remained compatible with artificial insemination, whereas 0.8 μM approached subfertility thresholds (Wen *et al.*, 2023) [8]. However, incorporation of antioxidants such as alpha-lipoic acid loaded nanoliposomes (Jackson *et al.*, 2026) [21] to improve semen quality parameters and reduce embryonic mortality (Jerome *et al.*, 2012) [22] can further improve the utility of sorted spermatozoa.

5. Conclusion

The present findings support the potential use of Resiquimod as a TLR7/8-associated motility-based approach for sperm enrichment in Vrindavani bulls. Among the concentrations evaluated, 0.6 μM represents a promising candidate for further optimization because it may provide a favorable compromise between differential motility and preservation of sperm viability. Direct molecular validation of X/Y enrichment and subsequent fertility assessment are required before practical application.

Table 1. Effect of Resiquimod (RQ) concentration on total motility, progressive motility and viability of spermatozoa at fresh, pre-freeze and post-thaw stages in Vrindavani Cattle bull semen (Mean±SE, N = 24)

R848 µM	Total motility (%)		Total motility (%)		Total motility (%)		Progressive motility (%)		Progressive motility (%)		Progressive motility (%)		Viability (%)		Viability (%)	
	Fresh	Pre-Freeze	Post-thaw	Fresh	Post-thaw	Fresh	Pre-Freeze	Post-thaw	Fresh	Post-thaw	Fresh	Pre-Freeze	Fresh	Post-thaw	Fresh	Post-thaw
Control	87.50 ± 0.60 ^a	76.96 ± 0.59 ^a	61.83 ± 1.30 ^a	82.79 ± 0.50 ^a	72.00 ± 0.43 ^a	48.33 ± 1.55 ^a	80.54 ± 0.62 ^a	88.75 ± 0.40 ^a	80.54 ± 0.62 ^a	64.08 ± 1.00 ^a						
0.4RQ	80.67 ± 0.19 ^b	72.96 ± 0.62 ^b	50.92 ± 1.46 ^b	80.00 ± 0.50 ^b	70.00 ± 0.50 ^b	43.75 ± 1.00 ^b	75.88 ± 0.55 ^b	87.04 ± 0.37 ^b	75.88 ± 0.55 ^b	56.92 ± 1.00						
0.6RQ	80.00 ± 0.12 ^c	71.71 ± 0.68 ^c	47.92 ± 1.20 ^c	78.00 ± 0.53 ^c	68.00 ± 0.53 ^c	41.25 ± 1.00 ^c	74.00 ± 0.50 ^c	83.13 ± 0.49 ^c	74.00 ± 0.50 ^c	52.75 ± 0.76 ^c						
0.8RQ	80.63 ± 0.46 ^c	68.46 ± 0.63 ^c	40.83 ± 1.02 ^d	75.00 ± 0.58 ^d	65.04 ± 0.59 ^d	35.00 ± 1.04 ^d	70.00 ± 0.57 ^d	73.92 ± 0.31 ^d	70.00 ± 0.57 ^d	45.75 ± 0.72 ^d						

Note: Control (no RQ), 0.4RQ, 0.6RQ and 0.8RQ are different groups supplemented with 0, 0.4 µM, 0.6 µM, 0.8 µM concentration of resiquimod respectively in the extender. (a-d) Different letters indicated significant differences between columns in each sperm parameter (p < 0.05)

6. Acknowledgement

The authors are thankful to the Director, ICAR-Indian Veterinary Research Institute, Izatnagar, India, for providing necessary facilities and financial assistance to carry out this experiment.

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How to cite this article: Yadav U, Khan M, Srivastava N, Kumar B, Srivastava P, Yadav L, et al. Concentration-dependent effects of resiquimod on X-chromosome-bearing sperm enrichment in Vrindavani (*Bos taurus indicus*) bulls: Evaluation of a TLR7/8-associated motility-based sorting approach. *International Journal of Advanced Biochemistry Research*. 2026;10(9):730-735. doi: <https://www.doi.org/10.33545/2617469.3.2026.v10.i9i.10165>

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