Characteristics of Pasundan Cattle Sperm Activated by Toll-Like Receptor 7/8 (TLR 7/8) for Sexing Purposes

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(received 25-04-2025; revised 22-07-2025; accepted 18-09-2025)

ABSTRAK

Hastuti, Karja NWK, Arifiantini RI, Purwantara B, Prasetyaningtyas WE. 2025. Karakteristik sperma sapi pasundan dengan aktivasi Toll-Like Receptor 7/8 (TLR 7/8) untuk tujuan sexing. JITV 30(4):185-192. DOI:http://dx.doi.org/10.14334/jitv.v30 i4.3554.

Menentukan jenis kelamin sperma sangat penting untuk meningkatkan efisiensi produksi ternak. Metode konvensional masih sering mengalami kendala, seperti penurunan motilitas pada sperma setelah proses sexing. Penelitian ini bertujuan untuk mengkaji karakteristik sperma yang telah di-sexing pada semen sapi dengan mengaktifkan Toll-Like Receptor 7/8 (TLR 7/8) menggunakan resiquimod 848 (R848). Penelitian telah dilakukan selama tujuh bulan di Unit Pelaksana Teknis Daerah Pusat Pembibitan dan Pengembangan Inseminasi Buatan (UPTD-BPPIB) Sapi Potong di Ciamis. Penelitian ini menggunakan tiga ekor sapi jantan pasundan umur produktif. Sperma diinkubasi dalam berbagai konsentrasi R848 (0, 0,03, 0,3, dan 0,6 µM) selama 30 dan 45 menit. Sperma pada lapisan atas diencerkan dan dibekukan. Parameter yang dievaluasi meliputi motilitas progresif, viabilitas, dan integritas membran plasma sperma, baik sebelum maupun setelah thawing. Hasil penelitian menunjukkan bahwa kualitas semen segar bervariasi antar individu, dan kualitas semen setelah inkubasi juga berbeda secara individu. Data menunjukkan bahwa konsentrasi R848 sebesar 0,03 µM selama 30 menit menghasilkan kualitas semen setelah pembekuan yang hampir sama dengan kontrol. Konsentrasi tertinggi (0,6 µM) secara signifikan menurunkan semua parameter kualitas semen. Aktivasi TLR 7/8 dengan konsentrasi 0,03 µM R848 selama 30 menit terbukti sesuai untuk proses sexing sperma pada sapi pasundan.

Kata Kunci: Resiquimod 848, Motilitas Sperma, S*exing* Sperma, Viabilitas Sperma, Toll-Like Receptor 7/8

ABSTRACT

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Determining the sex of sperm is vital for boosting the efficiency of livestock production. Conventional methods, however, often struggle with issues like reduced motility in sexed sperm. This study set out to examine the characteristics of sexed sperm in cattle by activating Toll-Like Receptor 7/8 (TLR 7/8) with resiquimod 848 (R848). Conducted over a period of seven months at the Regional Technical Implementation Unit of the Artificial Insemination Breeding and Development Center (UPTD-BPPIB) for Beef Cattle in Ciamis. Three productive-age Pasundan bulls were used in this study. The study involved incubating sperm with different concentrations of R848 (0, 0.03, 0.3, and 0.6 µM) for 30 and 45 minutes. The study evaluated parameters such as progressive motility, viability, and the integrity of the sperm plasma membrane, both before and after thawing. The results indicated that the quality of fresh semen varied among individuals, and the quality of semen after incubation also differed individually. Overall, the data demonstrated that a concentration of 0.03 µM R848 for 30 minutes resulted in semen quality after freezing that was nearly identical to the control. In contrast, the highest concentration (0.6 µM) significantly decreased all semen quality parameters. The activation of TLR 7/8 with 0.03 µM R848 for 30 minutes is suitable for sperm sexing in Pasundan cattle.

Key Words: Resiquimod 848, Sperm Motility, Sperm Sexing, Sperm Viability, Toll-Like Receptor 7/8

INTRODUCTION

Pasundan cattle are a local cattle breed and become the germplasm of the pride of West Java province, which has been maintained for generations and is an important part of the lives of farmers. Pasundan cattle are designated as a cattle breed that needs to be preserved based on the Decree of the Minister of Agriculture Number 1051/Kpts/SR.120/10/2014. The development of Pasundan cattle is very important in order to fulfill meat needs, especially in West Java. The Pasundan cattle breed is notable for its use in the production of beef, which leads to a comparatively higher demand for bulls than cows. This phenomenon can be attributed to the growth efficiency of bulls in beef cattle production systems, which results in a higher market preference for bulls compared to cows (Almakmum et al. 2021). Concurrently, the demand for bull cattle experiences a marked increase, driven by the need for sacrificial animals in preparation for Eid al-Adha. Sex selection,

otherwise known as sperm sexing, is a necessary procedure that can be conducted at artificial insemination centers (AICs). These facilities produce frozen semen for artificial insemination (AI) in the field.

Sperm sexing technology began in the 1980s (Moore & Hasler 2017). This technology has become one of the most important innovations in the field of animal reproduction. A good sexing method can improve the fertilization ability of sexed sperm (Moore & Hasler 2017). Sperm sexing methods can be categorized into two distinct classifications: conventional and advanced. Conventional sexing methodologies encompass Percoll gradient, bovine serum albumin (BSA), density gradient sedimentation, and free-flow electrophoresis (Yata 2022). The utilization of flow cytometry has been demonstrated to achieve a separation of X sperm with a success rate exceeding 95% (Balao da Silva et al. 2016 ; Diskin 2018). The cost of this technology is exorbitant due to the requirement for the AIC to pay royalties for each dose of sexed frozen semen produced, resulting in a significantly elevated selling price for farmers. In Indonesia, AICs currently utilize two methods: the Percoll gradient (Susilawati 2014) and BSA, which has been developed since 2012. However, the consistency of the desired gender of the offspring remains unsatisfactory. The accuracy of sexing dairy cows in Bogor and Bandung using the BSA method was 58.91%. A report by dairy farmers in Pujon, East Java, who utilized the Percoll gradient method, yielded a result of 58.14% (Achjadi et al. 2022). The inconsistent accuracy of current sperm sexing methods, specifically Percoll gradient and BSA, in Indonesia, where the success rates for producing offspring of the desired gender remain below 60%, despite decades of technological advancement and the availability of more precise methods like flow cytometry.

The most recent method of sex determination is the immunological approach. This method utilizes scFv antibodies present in Y chromosome sperm (Sringarm et al. 2022). The swim-up method is one of the alternative methods that is easy to perform in evaluating X and Y sperm. This method involves the activation of Toll-like receptors (TLRs), which utilize a group of pattern recognition receptors (PRRs). Toll-like receptor 7/8 (TLR7/8) is one of the TLRs expressed on the tail of X sperm (Wen et al. 2023). TLR7/8 is a receptor that is closely associated with TLR and is activated by resiquimod 848 (R848) (Hemmi et al. 2002). As demonstrated in prior research, the activation of TLR signaling has been observed to induce mitochondrial dysfunction, resulting in a decline in sperm motility in both murine and human subjects (Zhu et al. 2016). Recent studies in mice have demonstrated that TLR7/8 is localized on X sperm and responds to a specific agonist (R848) that only affects X sperm motility (Umehara et al. 2019). Research on TLR has led to the development of methods for sexing X and Y sperm in cattle. The development of a cost-effective sperm sexing method that minimizes cell damage and requires minimal equipment is imperative. The objective of this study is to characterize sexed sperm in Pasundan cattle semen through TLR 7/8 activation with R848 and to determine the optimal R848 concentration and incubation time in the sexing process.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, Bogor Agricultural University, with the approval number 194/KEH/SKE/III/2024.

Research replication and location

The research was conducted with three replicates, using three bulls, each of which underwent semen collection three times as replicates, resulting in a total of nine ejaculations. The research was conducted at two locations: the Regional Technical Implementation Unit, Artificial Insemination Breeding and Development Centre (UPTD-BPPIB) for Beef Cattle in Ciamis, and the Primate Study Centre, IPB University.

Preparation of diluent

The diluent utilized in this study was a commercial (Andromed, Minitube Germany). The Andromed diluent was diluted with distilled water at a ratio of 1:4. The diluent was then divided into four separate tubes, with each tube containing a specific concentration of resiquimod 848 (R848): 0 µM, 0.03 µM, 0.3 µM, and 0.6 µM, respectively. Subsequently, the diluent was stored at 34°C.

Semen collection

Semen samples were collected from three Pasundan cattle with 3-5 years of age. Semen was collected using an artificial vagina twice a week. The semen underwent a thorough assessment to determine its quality. This evaluation was carried out in two stages: initially through a macroscopic examination, followed by a microscopic analysis (Arifiantini 2012).

Fresh semen analysis

A macroscopic analysis of the semen was conducted, encompassing parameters such as volume, color, pH level, and consistency. Microscopic analysis included sperm motility, concentration, sperm viability, and sperm plasma membrane integrity. Sperm motility was assessed using computer-assisted sperm analysis

(CASA) (Del Prete et al. 2022). The concentration of sperm is calculated using a photometer SDM 6 (Minitube, Germany). The evaluation of sperm viability is conducted through the utilization of eosin nigrosin staining, as previously outlined by Pardede et al. (2020). The sperm plasma membrane integrity is evaluated using the hypoosmotic swelling test (HOST) (Santoso et al. 2021).

Semen dilution

Semen is composed of four distinct tubes. Each semen tube was diluted in Andromed according to the resiquimod (R848) treatment with different concentrations (0 µM, 0.03 µM, 0.3 µM, and 0.6 µM). Each specimen was meticulously divided into two separate tubes and subjected to an incubation period of 30 minutes and 45 minutes at a temperature of 37°C. Following the designated incubation period, the sperm layer was meticulously separated into three distinct layers: the upper layer, which contained Y chromosomes; the middle layer, which was discarded; and the lower layer, which contained X chromosomes (Wen et al. 2023). The sperm motility from the upper layer was evaluated.

Semen freezing after incubation

Subsequently, semen from the upper layer is packaged into 0.25-ml straws and equilibrated for a period of four hours. Semen is cryopreserved in liquid nitrogen vapor for a period of 10 minutes, following which it is stored in a liquid nitrogen container maintained at a temperature of -196°C.

Frozen-thawed sperm sexing testing

The evaluation encompassed assessments of sperm motility, sperm viability, and sperm plasma membrane integrity. The testing method employed was analogous to that utilized for fresh semen, with certain modifications.

Data analysis

The analysis of research data was conducted using analysis of variance (ANOVA) with SPSS 20 software. In instances where substantial disparities were identified ($P<0.05$), the Duncan test was implemented, with a 95% confidence interval.

RESULTS AND DISCUSSION

Characteristic of Pasundan cattle fresh semen

The characteristics of fresh Pasundan cattle semen are presented in Table 1. As indicated by the data presented in Table 1, the quality of Pasundan cattle semen is suitable for further processing. The standard for normal semen quality is established as sperm motility exceeding 70% (Santoso et al. 2021).

Progressive motility of Pasundan cattle sperm after incubation with R848

The progressive motility of sperm from the upper layer was evaluated. The mean progressive motility of sperm following incubation exhibited no significant variation among the different groups, including the interaction between male subject and R848 concentrations or incubation times. The mean progressive motility of sperm exhibited variability among individual bulls. The study's findings indicated that R848 concentrations ranging from 0 to 0.3 µM, with incubation times of 30–45 minutes, did not demonstrate a significant difference in bull 1, exhibiting progressive sperm motility ranging from 82.43±1.84% to 84.67±2.70%. These values were higher than those obtained with an R848 concentration of 0.6 µM and an incubation time of 30–45 minutes. Bull number 2 exhibited variability in the values of progressive sperm motility. The R0–30 and R0.03–30 treatments exhibited higher values compared to the other treatments. No significant variations in progressive sperm motility values were observed among the other treatments. The progressive sperm motility exhibited by bull number 3 demonstrated nearly identical values between the control, 0.03 µM, and 0.6 µM concentrations, with incubation times of 30 and 45 minutes, ranging from 73.60±7.15 to 80.13±3.62% (Table 2).

Frozen-thawed quality of Pasundan cattle Semen after sexing with R848

Pasundan cattle are beef cattle; therefore, it is expected that the semen used for insemination will be Y sperm. A Y sperm is in the upper layer because it is lighter than X sperm, therefore, semen quality testing is only performed on the upper layer. The progressive motility of Pasundan cattle semen frozen-thawed in the upper layer showed that R848 treatment at 0 to 0.3 µM did not differ significantly with incubation times of 30 minutes and 45 minutes ($P>0.05$). These values were higher ($P<0.05$) compared to other treatments. The concentration of R848 0.3 µM with an incubation time of 45 minutes and the concentration of R848 0.6 µM with an incubation time of 30 minutes were not significantly different. The R848 concentration of 0.6 µM with an incubation time of 45 minutes showed progressive sperm motility post-thawing of only 30.42±10.28%, the lowest compared to all treatments (Table 3).

The viability of frozen-thawed Pasundan cattle sperm of the upper layer showed that treatment with R848 0 to 0.3 µM did not differ with incubation times of

Table 1. Characteristics of fresh semen from Pasundan cattle

Variable	Bull 1	Bull 2	Bull 3
Color	Creamy white	Creamy white	Creamy white
Consistency	moderate	moderate	moderate
pH	6.4	6.4	6.4
Semen Volume (ml)		7.1±2.52a	

Different superscripts in the same row indicate significant differences (P<0.05)

Table 2. Progressive motility of Pasundan cattle sperm in the upper layer after the administration of R848 at different concentrations and incubation times.

Concentration and incubation time	Bull number			Mean±SD
	1	2	3	
R0-30	82.83±2.38$^{abcd\ A}$	78.33±0.90$^{ab\ A}$	74.80±6.55$^{ab\ A}$	78.65±3.28^{a}
R0-45	84.67±2.70$^{ab\ A}$	76.73±3.14$^{abc\ B}$	78.93±2.25$^{a\ AB}$	80.11±2.70^{a}
R0,03-30	84.47±3.15$^{ab\ A}$	78.50±3.73$^{ab\ A}$	79.20±7.62$^{a\ A}$	80.72±4.83^{a}
R0,03-45	83.03±4.24$^{abcd\ A}$	74.60±3.05$^{bcd\ B}$	80.13±3.62$^{a\ AB}$	79.25±3.64^{a}
R0,3-30	82.43±1.84$^{abcde\ A}$	75.70±1.39$^{abc\ B}$	76.80±0.80$^{a\ B}$	78.31±1.62^{a}
R0,3-45	83.63±1.63$^{abc\ A}$	71.70±4.20$^{cd\ B}$	75.93±5.75$^{ab\ AB}$	77.09±3.86^{a}
R0,6-30	77.67±7.67$^{de\ A}$	74.43±2.45$^{bcd\ A}$	75.03±5.10$^{ab\ A}$	75.71±5.07^{a}
R0,6-45	78.70±4.95$^{cde\ A}$	73.03±7.10$^{bcd\ A}$	73.60±7.15$^{ab\ A}$	75.11±6.40^{a}

R0-30= R848 concentration 0 µM (control) incubated for 30 minutes; R0-45= R848 concentration 0 µM (control) incubated for 45 minutes; R0.03-30= R848 concentration 0.03 µM incubated for 30 minutes; R0.03-45= R848 concentration 0.03 µM incubated for 45 minutes; R0.3-30= R848 concentration 0.3 µM incubated for 30 minutes; R0.3-45= R848 concentration 0.3 µM incubated for 45 minutes; R0.6-30= R848 concentration of 0.6 µM incubated for 30 minutes; R0.6-45= R848 concentration of 0.6 µM incubated for 45 minutes. Different lowercase superscript letters following the same column number indicate significant differences (P<0.05). Different uppercase superscript letters following the same row number indicate significant differences (P<0.05)

Table 3. Progressive sperm motility of post-thawed Pasundan cattle sperm in the upper layer after the administration of R848 at different concentrations and incubation times.

Concentration and incubation time	Bull number			Mean±SD
	1	2	3	
R0-30	58.39±5.83$^{a\ A}$	57.32±5. 54$^{abc\ A}$	44.27±1.39$^{ab\ B}$	53.33±4.25^{a}
R0-45	54.18±9.40$^{abc\ A}$	56.33±5.00$^{abc\ A}$	39.54±10.26$^{abc\ A}$	50.02±8.22^{a}
R0,03-30	59.24±4.82$^{a\ A}$	60.98±0.51$^{a\ A}$	48.36±4.06$^{a\ B}$	56.19±3.13^{a}
R0,03-45	53.41±3.81$^{abc\ A}$	49.97±1.59$^{cde\ A}$	39.08±13.90 $^{abc\ A}$	47.49±6.43^{ab}
R0,3-30	57.03±9.62$^{ab\ A}$	47.63±5.96$^{de\ AB}$	29.33±15.50$^{bcde\ B}$	44.66±10.3^{ab}
R0,3-45	53.43±8.61$^{abc\ A}$	38.34±3.13$^{fg\ AB}$	26.63±9.57$^{cde\ B}$	39.47±7.10^{b}
R0,6-30	48.36±13.11$^{bcd\ A}$	42.98±0.55$^{ef\ A}$	26.39±12.04$^{cde\ A}$	39.24±8.57^{b}
R0,6-45	37.79±11.06$^{e\ A}$	33.93±4.02$^{g\ AB}$	19.54±15.75$^{e\ B}$	30.42±10.28^{c}

R0-30= R848 concentration 0 µM (control) incubated for 30 minutes; R0-45= R848 concentration 0 µM (control) incubated for 45 minutes; R0.03-30= R848 concentration 0.03 µM incubated for 30 minutes; R0.03-45= R848 concentration 0.03 µM incubated for 45 minutes; R0.3-30= R848 concentration 0.3 µM incubated for 30 minutes; R0.3-45= R848 concentration 0.3 µM incubated for 45 minutes; R0.6-30= R848 concentration of 0.6 µM incubated for 30 minutes; R0.6-45= R848 concentration of 0.6 µM incubated for 45 minutes. Different lowercase superscript letters following the same column number indicate significant differences (P<0.05). Different uppercase superscript letters following the same row number indicate significant differences (P<0.05).

30 minutes and 45 minutes (P>0.05) with values ranging from 53.96 7.77% to 64.04±4.26%. These values differed significantly (P<0.05) from other treatments. The concentration of R848 at 0.3 µM with an incubation time of 45 minutes, and the concentration of R878 at 0.6 µM with an incubation time of 30 minutes did not differ significantly. The concentration of R878 at 0.6 µM with an incubation time of 45 minutes was significantly different from the other treatments (P<0.05) with a value of 39.53±11.47%. This value was the lowest among all post-incubation sperm viability values (Table 4).

The integrity of the upper layer of after frozen-thawed Pasundan bull sperm membranes showed that treatment with R848 at concentrations ranging from 0 to 0.3 µM did not differ significantly between incubation times of 30 minutes and 45 minutes (P>0.05), with values ranging from 47.38±7.14% to 58.02±4.67%. These values differed significantly (p<0.05) from other treatments. The concentrations of R848 at 0.3 µM and R878 at 0.6 µM, with incubation times of 45 minutes and 30 minutes, respectively, showed no significant difference. The concentration of R878 at 0.6 µM with an incubation time of 45 minutes was the lowest compared to the other treatments (P<0.05), with a value of 34.18±11.52% (Table 5).

DISCUSSION

The results demonstrated that the progressive motility of Pasundan bull sperm following incubation in the upper layer did not exhibit any significant differences between the various treatments. However, as illustrated in Table 2, significant variations in response to concentration and incubation time were observed among individual bulls. The progressive motility of sexed Pasundan bull sperm exhibited a maximal response at a concentration of R848 0.03 µM, with an incubation time of 30 minutes, and a minimal response at a concentration of R848 0.6 µM, with an incubation time of 45 minutes. These findings corroborate earlier reports in the field of dairy goat research, indicating that concentrations greater than 0.4 µM, when incubated for 40 minutes, result in a decline in sperm motility (Huang et al. 2022). Sperm exhibit optimal motility at a temperature of 37°C. Prolonged incubation has been demonstrated to result in a decline in sperm motility (Gong et al. 2017).

It is important to understand that progressive sperm motility after thawing is key to successful fertilization because sperm must be able to move toward the egg (Byeun et al. 2024). The results of the study demonstrated that progressive sperm motility in the upper layer exhibited nearly identical results across all treatments. The administration of Resiquimod 848 at a concentration of 0.03 µM has been observed to induce a stimulatory effect on progressive sperm motility, which is associated with sperm cell activity. Conversely, a concentration of Resiquimod at 848 µM has been observed to exert an opposite effect. Furthermore, Wen et al. (2023) observed a substantial decline in sperm motility when dairy bull sperm was incubated with R848 at a concentration of 0.6 µM for a duration of 30 minutes. The optimal R848 concentration varies among different livestock species. The optimal concentration for goat semen has been determined to be 0.2 µM (Huang et al. 2022), while for sheep semen, it has been established as 0.3 µM (Setiawan et al. 2024).

The viability and plasma membrane integrity of prozen-thawed sexed Pasundan cattle sperm in the upper

Table 4. Sperm viability of post-thawed Pasundan cattle sperm in the upper layer after the administration of R848 at different concentrations and incubation times

Concentration and incubation time	Bull number			Mean ±SD
	1	2	3	
R0-30	68.44±3.79$^{ab\ A}$	63.31±7.04$^{abc\ B}$	52.06±3.06$^{ab\ C}$	61.27±4.63^{a}
R0-45	63.47±5.29$^{abcd\ A}$	63.55±8.24$^{abcd\ A}$	46.91±9.97$^{abc\ B}$	57.98±7.83^{a}
R0,03-30	69.44±3.27$^{a\ A}$	67.38±4.92$^{a\ A}$	55.29±4.58$^{a\ B}$	64.04±4.26^{a}
R0,03-45	62.44±02.49$^{abcd\ A}$	57.89±3.37$^{cde\ A}$	48.17±14.8$^{abc\ A}$	56.17±6.89^{a}
R0,3-30	65.63±8.26$^{abc\ A}$	58.57±1.03$^{bcd\ A}$	37.67±14.02$^{bcde\ B}$	53.96±7.77^{a}
R0,3-45	63.49±8.68$^{abcd\ A}$	45.79±2.58$^{fg\ B}$	35.64±10.61$^{cde\ B}$	48.31±7.29^{b}
R0,6-30	55.46±11.84$^{def\ A}$	50.81±6.11$^{ef\ A}$	34.66±13.04$^{cde\ B}$	46.98±10.33^{b}
R0,6-45	48.80±11.89$^{f\ A}$	42.65±6.66$^{g\ AB}$	27.13±15.86$^{e\ B}$	39.53±11.47^{c}

R0-30= R848 concentration 0 µM (control) incubated for 30 minutes; R0-45= R848 concentration 0 µM (control) incubated for 45 minutes; R0.03-30= R848 concentration 0.03 µM incubated for 30 minutes; R0.03-45= R848 concentration 0.03 µM incubated for 45 minutes; R0.3-30= R848 concentration 0.3 µM incubated for 30 minutes; R0.3-45 = R848 concentration 0.3 µM incubated for 45 minutes; R0.6-30 = R848 concentration of 0.6 µM incubated for 30 minutes; R0.6-45= R848 concentration of 0.6 µM incubated for 45 minutes. Different lowercase superscript letters following the same column number indicate significant differences (P<0.05). Different uppercase superscript letters following the same row number indicate significant differences (P<0.05)

Table 5. Sperm plasma membrane integrity of frozen-thawed Pasundan bull sperm in the upper layer after administration with R848 at different concentrations and incubation times

Concentration and incubation time	Bull number			Mean ±SD
	1	2	3	
R0-30	62.74±4.82$^{a\ A}$	57.58±9.40$^{ab\ A}$	45.82±3.09$^{ab\ B}$	55.38±5.77^{a}
R0-45	58.60±6.41$^{\mathbf{acde\ A}}$	58.40±6.86$^{\mathbf{ab\ A}}$	42.25±10.79$^{\mathbf{abc\ B}}$	53.08±8.02^{a}
R0.03-30	62.32±4.08$^{\mathbf{ab\ A}}$	62.32±5.06$^{\mathbf{a\ A}}$	49.43±4.87$^{\mathbf{a\ B}}$	58.02±4.67^{a}
R0.03-45	56.19±2.04$^{\mathbf{abcde\ A}}$	52.77±2.92$^{\mathbf{bc\ A}}$	42.44±13.77$^{\mathbf{abc\ A}}$	50.47±6.24^{ab}
R0.3-30	59.68±6.72$^{\mathbf{abcd\ A}}$	52.01±2.52$^{\mathbf{bcd\ A}}$	30.45±12.18$^{\mathbf{bcd\ B}}$	47.38±7.14^{ab}
R0.3-45	56.52±8.22$^{\mathbf{abcde\ A}}$	42.02±3.27$^{\mathbf{ef\ AB}}$	31.13±10.31$^{\mathbf{bcd\ B}}$	43.22±7.27^{b}
R0.6-30	50.71±11.14$^{\mathbf{ef\ A}}$	45.07±6.31$^{\mathbf{de\ A}}$	28.69±11.03$^{\mathbf{cd\ B}}$	41.49±9.49^{b}
R0.6-45	41.99±12.082$^{\mathbf{g\ A}}$	36.52±6.73$^{\mathbf{f\ AB}}$	24.03±15.75$^{\mathbf{d\ B}}$	34.18±11.52^{c}

R0-30= R848 concentration 0 µM (control) incubated for 30 minutes; R0-45= R848 concentration 0 µM (control) incubated for 45 minutes; R0.03-30= R848 concentration 0.03 µM incubated for 30 minutes; R0.03-45= R848 concentration 0.03 µM incubated for 45 minutes; R0.3-30= R848 concentration 0.3 µM incubated for 30 minutes; R0.3-45= R848 concentration 0.3 µM incubated for 45 minutes; R0.6-30= R848 concentration of 0.6 µM incubated for 30 minutes; R0.6-45= R848 concentration of 0.6 µM incubated for 45 minutes. Different lowercase superscript letters following the same column number indicate significant differences (P<0.05). Different uppercase superscript letters following the same row number indicate significant differences (P<0.05)

layer exhibited variation across treatments. Sperm viability in the upper layer at R848 concentrations ranging from 0 µM to 0.3 µM with a 45-minute incubation time was nearly identical. Conversely, an R848 concentration of 0.6 µM, with a 45-minute incubation time, exhibited an opposing effect. The integrity of the plasma membrane of sexed sperm in the upper and lower layers of goat semen at R848 concentrations of 0.3 µM, 0.6 µM, and 0.9 µM did not differ, ranging from 79.84% to 84.76% (Maulana et al. 2024).

The integrity of the sperm plasma membrane is imperative for maintaining sperm motility and viability. The plasma membrane fulfills the role of a barrier, thereby protecting the internal components of sperm and regulating the exchange of substances between the cell and the external environment (Glazar & McCue. 2021; Prihantoko et al. 2020). Damage to the plasma membrane, particularly in the midpiece, which is abundant in mitochondria, can result in a variety of adverse consequences. Mitochondria, in particular, are the primary energy source for sperm, playing a crucial role in the production of ATP (adenosine triphosphate) through the process of cellular respiration. Prolonged exposure to external stressors can result in the disruption of the plasma membrane, thereby facilitating the release of enzymes that are typically confined within the cell.

These enzymes, which are vital for metabolic processes, are released into the extracellular environment when the plasma membrane becomes compromised. This enzyme release has been demonstrated to disrupt biochemical processes within the sperm, which can lead to apoptosis or cell death. Consequently, this can further reduce the number of healthy sperm. A decline in ATP production, resulting in plasma membrane damage, leads to diminished sperm motility. This phenomenon has been demonstrated to exert a substantial influence on the sperm's capacity to navigate towards the egg during the process of fertilization. Therefore, the integrity of the sperm's plasma membrane is indicative of sperm health and efficiency, ensuring optimal function (Prihantoko et al. 2020). Maintaining the integrity of the plasma membrane of sexed sperm is imperative for both physiological and reproductive purposes, ensuring the seamless progression of the reproductive process.

The TLR7/8 mechanism in X sperm is activated by specific ligands, such as Resiquimod 848, which binds to TLR7/8, which is only present in X sperm. In X sperm, TLR7 is positioned in the tail, while TLR8 resides in the midpiece. This activation is specific to X sperm and does not impact Y sperm, as these receptors are absent in Y sperm (Umehara et al. 2019). These binding triggers intracellular reactions that inhibit the Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6) protein through the Phosphatidylinositol-3-Kinase (PI3K) and Nuclear Factor-Kappa B (NFκB) pathways. This inhibition has been shown to reduce the activity of hexokinase, an enzyme that plays a critical role in glucose metabolism. Consequently, this results in a decrease in the formation of adenosine triphosphate (ATP), which is the primary energy source for sperm motility (Umehara et al. 2019; Wen et al. 2023).

Other studies have shown that TRAF6 activation triggers two main pathways. The TRAF6 → PI3K → GSK3α/β → inhibition of hexokinase activity pathway, and the TRAF6 → NF-κB → regulation of gene expression and mitochondrial activity pathway. Inhibition of hexokinase reduces the conversion of glucose to ATP, thereby decreasing energy production in X sperm (Ren et al. 2021). This reduction in hexokinase

activity leads to decreased ATP production in X sperm. Since ATP is the primary energy source required for sperm motility, the decrease in ATP directly results in reduced motility or sperm movement speed (Wen et al. 2023). X sperm move more slowly than Y sperm, whose motility is not affected by TLR7/8 activation. Slow X sperm typically remain at the bottom during sperm sexing using the swim-up method, while Y sperm remain at the top.

The present study demonstrated the individual effects on all variables of fresh semen quality, post-incubation with R848, or post-thawing. This observation was corroborated by Baharun et al. (2025), who reported similar findings in the same cattle. A concentration of R848 at 0.03 µM, with an incubation time of 30 minutes, yielded values that closely resembled the control values. Therefore, this concentration and incubation time can be employed in future studies. This study constitutes an inaugural investigation into the development of a sperm sexing method employing the swim-up principle with R848 in Pasundan cattle, with a primary focus on the quality of sexed semen. The necessity of validation is twofold: first, to confirm the suitability of X-chromosome-carrying and Y-chromosome-carrying sexed sperm through rt-PCR analysis; and second, to ascertain the success of artificial insemination using the sexed semen.

The limitations of this study lie in the evaluation of the effects of Resiquimod 848 (R848) concentration and incubation time on the quality of sexed Pasundan bull semen, specifically regarding progressive motility and plasma membrane integrity of spermatozoa in the upper layer during the swim-up method. This research focuses on optimizing incubation conditions—namely, an R848 concentration of 0.03 µM and 30 minutes of incubation—which showed the best sperm response, consistent with control values, without diminishing motility or damaging membrane integrity. However, higher R848 concentration (0.6 µM) and longer incubation time (45 minutes) resulted in decreased sperm performance, aligning with previous findings in other livestock species such as goats and sheep. Additionally, the study acknowledges general limitations that optimal responses to R848 vary across species and individuals, making the results specific to the Pasundan population and not broadly validated. Major limitations also include the absence of molecular validation (rt-PCR) to confirm accurate sex separation and the lack of functional testing via artificial insemination to demonstrate reproductive success, which are critical aspects for advancing sex separation methods based on swim-up and R848.

CONCLUSION

The present study concluded that the characteristics of Pasundan cattle sperm are influenced by individual factors. A resiquimod 848 concentration of 0.03 µM and an incubation time of 30 minutes have been determined to be optimal for activating TLR 7/8 in the sexing process of Pasundan cattle sperm. Further research is needed to validate the accuracy of sexing results in vitro using PCR and in vivo through artificial insemination.

ACKNOWLEDGEMENT

We express our gratitude to the Ministry of Education, Culture, Research, and Technology, and the Ministry of Finance of the Republic of Indonesia for funding this research under contract number: 006/C3/DT.05.00/PL/2025. We also thank the Regional Technical Implementation Unit, Artificial Insemination Breeding and Development Centre (UPTD-BPPIB) for Beef Cattle in Ciamis, West Java Province.

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