

APPLICATION OF R848 LIGAND TO IMPROVE THE EFFICIENCY OF SEX SEPARATION IN RAM SPERMATOOA

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Abstract

The present study aims to evaluate the application of the TLR7/8 agonist – Resiquimod (R848) to improve the efficiency of sex separation of ram spermatozoa. Semen samples collected from five clinically healthy rams (*Ovis aries*) of the Lacon breed were treated with the R848 ligand to separate spermatozoa carrying X and Y chromosomes into separate fractions (RX and RY). Kinematic parameters were analyzed using a computer-assisted sperm analyzer (CASA), and sperm vitality was assessed by staining with eosin-nigrosin. Real-time PCR tracked the expression of genes specific to X and Y chromosomes – TLR7 and SRY. The results showed successful separation of ram spermatozoa by sex, with expression of TLR7 and SRY in the RX or RY fraction, respectively ($p < 0.05$). An enrichment trend was recorded for X-carrying (84.9%) and Y-carrying (86.5%) fractions. In conclusion, the use of ligand R848 in this study showed possible application in reproductive medicine of small ruminants as well as introducing a novel approach to molecular sex determination based on gene expression analysis. This highlights the need for future studies and the application of sorted semen fractions in in vivo experiments.

Key words: sperm separation, ram, TLR7/8 agonist, real-time PCR, ligand R848

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Introduction. Agriculture is an important economic sector of human activity that ensures the production of food and raw materials. In the modern world, with the growing demand for high-quality and safe food, the main goals of animal husbandry are to increase productivity and optimize the use of resources while minimizing negative impacts on the environment [1]. This reflects the primary goal of reproductive biotechnologies, as they provide the opportunity for selective breeding according to the needs of a specific farm or enterprise. One of the current biotechnologies in the field is sperm sexing, through which semen is fractionated based on chromosomes to produce offspring of the desired sex during subsequent artificial insemination of animals, depending on the specific needs of the breeding programme [2]. The benefits associated with the use of this technology include effective utilization of male breeders, genetic selection, reduced breeding costs, and a decrease in the risk of transmitting diseases.

Flow cytometry has become the gold standard in sperm sexing in cattle, but it requires expensive equipment and causes some serious damage to the gametes during laser exposure [3]. For this reason, in recent years, new approaches have been sought for separating semen without negatively impacting the viability and motility of the sperm, to implement the technology into artificial insemination agriculture programmes.

It was discovered that the TLR7/8 receptor, localized on the cell surface, is expressed exclusively in sperm carrying the X chromosome, but not in those with the Y chromosome [4]. An agonist of these receptors is the ligand Resiquimod (R848), which reduces the motility of X chromosome-carrying sperm by modulating mitochondrial metabolic pathways without affecting Y sperm, enabling effective sex separation [4]. This method has proven effective in mice, cattle, and goats [5], but there is currently insufficient literature on its application in rams.

Therefore, the aim of this study is to investigate the application of ligand R848 on the efficiency of sex separation of ram spermatozoa by implementing real-time PCR to track gene expression of SRY for the presence of the Y chromosome and TLR7 for the presence of the X chromosome.

Materials and methods. Sample collection and primary assessment. The studies were conducted at the Institute of Biology and Immunology of Reproduction “Acad. Kiril Bratanov” – Bulgarian Academy of Sciences (BAS).

Semen samples from the species *Ovis aries* were collected from five clinically healthy rams of the Lacon breed, at breeding age, from an agricultural facility near Sofia-city. The samples were regularly collected three times from each animal, in accordance with the species sexual cycle ($n = 15$).

After an initial evaluation with a computer-assisted sperm analysis (CASA), the ejaculates were divided into three groups: a control group (C), which was not treated, and two experimental groups – X-containing (RX fraction) and Y-containing (RY fraction), obtained after treatment with the ligand R848. Each sample was analyzed twice to minimize errors.

Separation of sperm with ligand R848. A basic medium was prepared with R848 (Sigma, USA) and creatine, which was used to separate spermatozoa. For this purpose, R848 and creatine were added to a base medium containing glucose, various salts, as well as the antibiotics streptomycin and penicillin added to prevent bacterial contamination [6].

The seminal plasma was separated from the cell component by centrifugation at $700\times g$ for 5 min, and the resulting supernatant was removed.

Each semen sample was divided into two parts and incubated for 60 min at 37°C in a CO_2 incubator. After subsequent incubation R848 ligand was added, as a result of which gradient separation into three clear fractions was observed. One milliliter of upper and lower fractions were pipetted and transferred to new tubes. In the intermediate fraction, the presence of both types as well as defective cells is assumed [4], but this was not an object of the present study.

Kinematic analysis with computer sperm analyzer (CASA). The kinematic parameters of the spermatozoa were analyzed to confirm their quality using a computer-assisted sperm analyzer (CASA) with the SCA system (Microptic, Spain). This method was applied both before and after the sorting of the gametes in order to check their fertilization capacity. Disposable chamber slides of the Leja type (Microptic, Spain) were used to count spermatozoa and analyze their motility, heated on a table, part of the system to maintain a constant temperature of 37°C . For observation, we used a light phase-contrast microscope with a magnification of $10\times$ and a green filter, as well as a camera attached to the system. The settings of the software for operation are set according to the manufacturer's requirements as follows: animal species – ram; lens – Nikon BM $10\times$; Ph – “–”. The motility and concentration of the $10\ \mu\text{L}$ sperm sample were analyzed, with more than 200 gametes evaluated [7].

Study of the vitality of spermatozoa. The vitality of the gametes was assessed by preparing smears, which were stained with BrightVit kit by the company Microptic S.L. (Spain). Two smears of each sample containing $10\ \mu\text{L}$ of semen and $50\ \mu\text{L}$ of colouring solution were prepared according to the manufacturer's instructions. The slides were analyzed under an Olympus BX microscope (magnification $40\times$). On one slide, 100 or more sperm cells were evaluated and recalculated as a ratio of live:dead spermatozoa, presented in percentage (%).

Genomic DNA isolation. Genomic DNA (gDNA) was extracted from the samples using the standard protocol with chloroform, which involves working with the interphase [8]. The DNA was washed twice with a 0.1 M sodium citrate solution in 10% ethanol. The resulting pellets were then dried and dissolved in $20\ \mu\text{L}$ of nuclease-free H_2O . After isolation, a spectrophotometric analysis was performed using a Jenway Genova Nano (UK) at a wavelength of 260 nm to quantitatively determine the extracted gDNA. For each sample, the measurement was performed in duplicate, and the average value was calculated, expressed in ng/ μL .

Real-time PCR for the validation of the results from the gamete sorting. Each gDNA sample was analyzed separately for the two target genes – SRY (on the Y chromosome) and TLR7 (on the X chromosome), as well as the housekeeping gene GAPDH (Table 1). In addition to the chromosome-separated samples, unsorted samples were also analyzed, which served as controls.

T a b l e 1
Primers used for real-time PCR

Gene	Sequence of the forward primer	Sequence of the reverse primer	Melting temperature (T _m), (°C)
TLR7	CTGGACCATCTGGTGGAGAT	GCTGGTTTCCATCCAGGTAA	62
SRY	TGAATAGAACGGTGCAATCGT	TTGAACATAAGCGCAAGAAAG	62
GAPDH	GTCCGTTGTGGATCTGACCT	TGCTGTAGCCGAATTCATTG	62

Each tube contained, in addition to 2 µL of gDNA, 1 µL of MgCl₂, 1 µL of forward primer, and 1 µL of reverse primer for the corresponding gene (Sigma, USA), as well as 5 µL of mastermix (Genaxxon, Germany), which provides deoxynucleotide triphosphates (dNTPs), Taq DNA polymerase, and SYBR and ROX fluorescent dyes. The total volume of each sample was 20 µL. The samples were analyzed in duplicate.

Statistical analysis. The data were presented as a mean ± standard deviation (SD). The statistical analysis was performed using SPSS v.27, and statistical significance was reported at $p < 0.05$.

Results. Analysis of sperm parameters. The semen samples from the three experimental groups (control, RX, and RY fractions) were analyzed with CASA after sorting with the ligand R848. The data for total motility (%) of the gametes showed no significant differences ($p > 0.05$) among the three groups (Fig. 1).

When tracking progressive motility, a tendency ($p > 0.05$) was observed for a decrease in the percentage of progressively motile sperm in the RX group (50.9 ± 10.4) compared to the RY group (54.4 ± 10.55) after sorting the treated samples.

Sperm vitality test. To assess whether the ligand R848 affects the integrity of the cell membranes, a vitality test was performed. For the RX and RY fractions, a non-significant decrease ($p > 0.05$) in vitality was observed compared to the control group, with the following values: 80.01 ± 2.8 for the control group; 76.1 ± 3.1 for the RX fraction; and 78.9 ± 2.7 for the RY fraction.

Real-time PCR for validation of sorting results. Since the main difference between sperm carrying the X and Y chromosomes lies in their DNA content, most sex-sorting techniques in farm animals are based on this characteristic. This

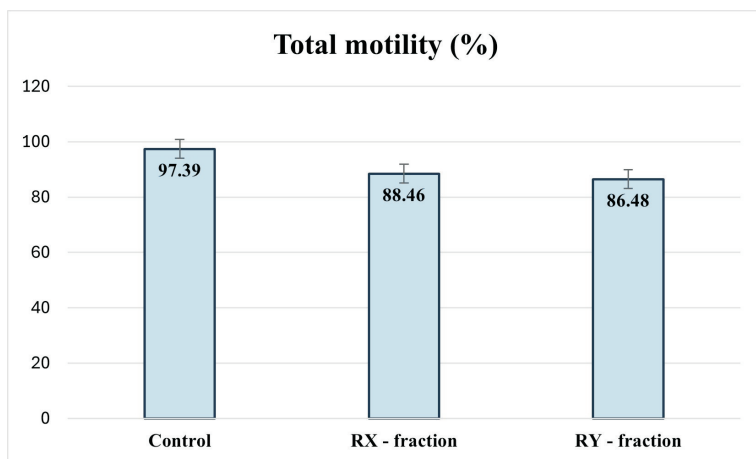


Fig. 1. Total motility of the three examined groups

is why real-time PCR was used to demonstrate the successful separation of sperm by sex [9].

To confirm the chromosomal identity of sorted semen samples using the ligand R848 in this study, we used real-time PCR based on SYBR® Green. The TLR7 gene was selected as the target, as it encodes receptors located on the X chromosome that act as sensors and could confer functional differences to X-bearing sperm compared to Y-bearing ones. To validate the sperm sex-sorting protocol, an analysis of the SRY gene was performed on ram sperm as a verification step, while the GAPDH gene was amplified as a control for expression and for normalization of the results as presented in Fig. 2.

A new approach of this study is the clear significant expression of TLR7 observed in RX samples, while SRY was not amplified. Conversely, the activity of gene SRY was statistically relevant in the RY samples in comparison with RX ($p < 0.05$). The control group, which was not selected, showed mixed genetic profile. As seen in Table 2, the summed values of RX and RY groups clearly deviate from those of the control group and are statistically significant among themselves ($p < 0.05$), highlighting the method's effectiveness.

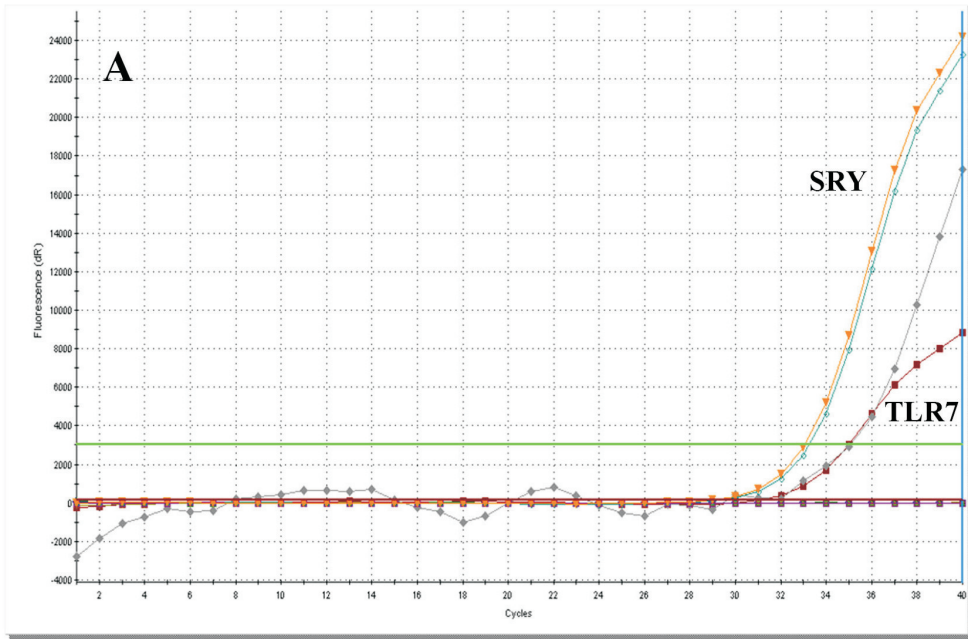
T a b l e 2

Percentage of X- and Y-bearing spermatozoa in the analyzed samples

Group	X chromosomes (%)	Y chromosomes (%)
Control	54.40 ± 15.2	50.20 ± 15.2
RX	$84.87 \pm 10.5^*$	15.33 ± 10.5
RY	13.25 ± 6.1	$86.46 \pm 6.1^*$

*significant $p < 0.05$

Amplification Plots



Dissociation Curve

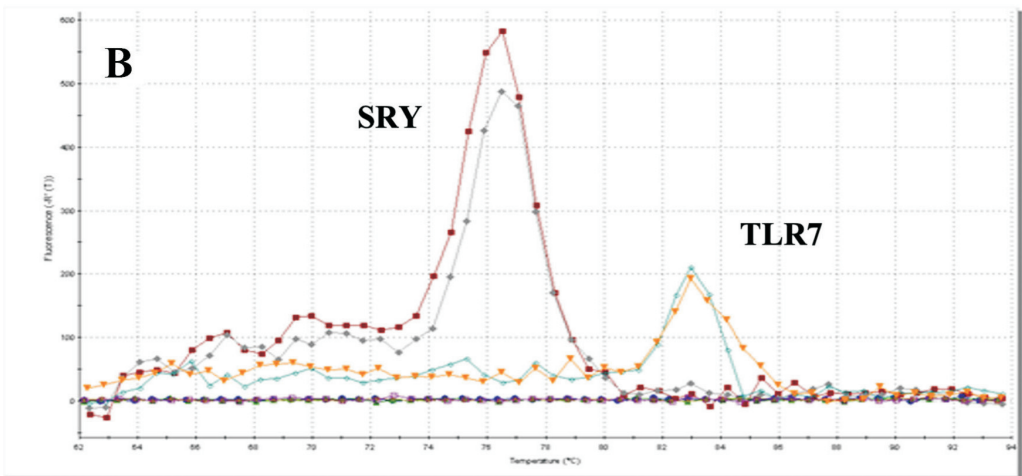


Fig. 2. Real-time PCR results: A – Amplification curve of RX fraction as a positive expression of TLR7 gene and of RY fraction as a result of expression of SRY gene; B – Dissociation curve for both target products, demonstrating the success of the analysis; two distinct products are clearly visible as peaks

These results, obtained from the real-time PCR analysis, indicate for the first time successful sex separation of ram samples using the ligand R848.

Discussion. The slight change in motility in the treated fractions compared to the control is likely because of the ligand used to decrease ATP levels, as reported by other authors [10]. Upon activation of TLR7, which is localized in the tail of sperm, cytoplasmic glycolysis is suppressed, and the motility of the flagellum is blocked [4, 10]. This effect is due to the modulation of signalling pathways that regulate mitochondrial function and the consequent decrease in ATP production.

In a previous study with the same species it was found that activation of TLR7/8 by Resiquimod (R848) leads to a decrease in the motility of X sperm compared to Y sperm [11], which aligns with the current data. The trend toward a reduction in the percentage of progressive motile sperm in the RX fraction compared to the RY fraction after sorting the treated samples corresponds to the results of YOTOV et al. [12]. About vitality, the results confirm that ligand-mediated sperm sorting with R848 represents a less invasive alternative to the widely used flow cytometry [13].

In this study, SRY was chosen as the target gene to confirm the presence of the Y chromosome, as it is crucial for both male development and spermatogenesis, as well as for the maintenance and survival of sperm carrying the Y chromosome [14, 15]. TLR7, which selectively binds to the TLR7/8 molecular markers, was analyzed in X-carrying sperm [5, 16]. Therefore, analyzing the two fractions based on independent genes allowed for their distinct separation for the first time in ram, which was missing in previous research [17]. Examination of both sex chromosomes confirmed the accuracy and reliability of the sex determination procedure.

The X and Y chromosome ratio obtained in the control sample was approximately 1:1, as expected due to meiotic division, which confirms the correct functioning of the protocol [18]. The present results confirm that SRY is expressed in ram sperm, allowing reliable differentiation between the two analyzed fractions, RX and RY. The same methodology has also detected transcription of this gene in mice [19].

Conclusion. In conclusion, the use of ligand R848 in this study demonstrated a possible application in reproductive medicine of small ruminants as well as introducing a novel approach to molecular sex determination based on gene expression analysis. The results showed successful separation of ram spermatozoa by sex, with expression of TLR7 and SRY in the RX or RY fraction, respectively ($p < 0.05$). An enrichment trend was recorded for X-carrying (84.9%) and Y-carrying (86.5%) fractions. This highlights the need for future studies and the application of sorted semen fractions in in vivo experiments.

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