TLR7/8 Signalling Affects X-Sperm Motility via the GSK3 α/β -Hexokinase Pathway for the Efficient Production of Sexed Dairy Goat Embryos

Fa Ren

Northwest A&F University: Northwest Agriculture and Forestry University

Huaming Xi

Northwest A&F University: Northwest Agriculture and Forestry University

Yijie Ren

Northwest A&F University: Northwest Agriculture and Forestry University

Yu Li

Northwest A&F University: Northwest Agriculture and Forestry University

Fei Wen

Northwest A&F University: Northwest Agriculture and Forestry University

Ming Xian

Northwest A&F University: Northwest Agriculture and Forestry University

Mengjie Zhao

Northwest A&F University: Northwest Agriculture and Forestry University

Dawei Zhu

Northwest A&F University: Northwest Agriculture and Forestry University

Liqiang Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Anmin Lei

Northwest A&F University: Northwest Agriculture and Forestry University

Jianhong Hu (✉ 2008114994@nwafu.edu.cn)

Northwest Agriculture and Forestry University <https://orcid.org/0000-0002-5833-3444>

Research

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Abstract

Background

Goat milk is most similar to human milk because of its abundant nutrients and ease of digestion. To derive more economic benefits, farmers need to obtain offspring with more does; however, the buck to doe sex ratio of offspring is approximately 50%. At present, artificial insemination after separation of X/Y sperm using flow cytometry is the primary means to control the sex of livestock offspring. However, flow cytometry has not been successful in separating X/Y sperm for sexing control in dairy goats.

Results

In this study, a novel, simple goat sperm-sexing technology that activates the toll-like receptor 7/8 (TLR7/8), inhibiting X-sperm motility, was investigated. Results showed that TLR7/8 coding goat X-chromosome was expressed in approximately 50% of round spermatids in testis' cross-sections and sperm in the epididymis' cross-sections and ejaculate. Importantly, TLR7/8 was located at the tail of X-sperm. Upon TLR7/8 activation, phosphorylated forms of glycogen synthase kinase α/β (GSK3 α/β) and nuclear factor kappa-B (NF κ B) were detected in the X-sperm, reducing mitochondrial activity, ATP levels, and sperm motility. High-motility Y-sperm segregated to the upper layer and low-motility X-sperm to the lower layer. Following *in vitro* fertilisation using the lower layer TLR7/8-activated sperm, $80.52 \pm 6.75\%$ of the embryos were XX females. TLR7/8-activated sperm was used for *in vivo* embryo production via the superovulatory response, and 88.89% of the embryos were XX females.

Conclusions

Our study reveals a novel TLR7/8 signalling mechanism that affects X-sperm motility via the GSK3 α/β -hexokinase pathway, facilitating the efficient production of sexed dairy goat embryos.

Background

Sexing control is an important technology in livestock breeding, enabling female animals to produce offspring of specific sex according to the farmers' preference through particular interventions [1]. In livestock production, the demand for specific animal products results in an often biased need for sexing animal offspring [2]. For example, the production of goat milk requires more female dairy goats. Artificial insemination (AI) after separation of X/Y sperm is one of the primary means to achieve sexing control of livestock offspring [3]. In fact, scientists have always been interested in separating X- and Y-sperm [4, 5]. However, flow cytometry, taking advantage of sperm DNA differences to separate X-sperm and Y-sperm, is the only recognised and widely used method for sexing livestock [6]. At present, the production of sex-sorted sperm by flow cytometry has only been successfully applied to cattle [7]. Essentially, the chromosomal DNA content of X-sperm is $4.4 \pm 0.03\%$ higher than that of Y sperm in goats, making it, in principle, more amenable to flow cytometry than cattle sperm [8, 9]. Nonetheless, using flow cytometry to separate X/Y sperm in goats resulted in low sperm motility and pregnancy rate [10]. This outcome might be due to a species difference in mammalian sperm. Considering this, we aimed to find a new method to separate dairy goat X/Y sperm without flow cytometry.

Mammalian spermatogenesis is a highly complex and orderly process, which ultimately produces X-chromosome bearing sperm (X-sperm) and Y-chromosome bearing sperm (Y-sperm) that carry information determining offspring sex [6]. X-and Y-sperm are equally functional for *in vivo* or *in vitro* fertilisation (IVF) procedures. However, studies have shown several differences between X- and Y-sperm, such as size and density, DNA content, and sperm motility under environmental exposure [11, 12]. At present, there are a few convincing reports on X/Y sperm separation based on the basic and physiological differences between X- and Y-sperm. Recently, Umehara et al. [13] reported that TLR7 and TLR8, membrane-associated receptor proteins located at the sperm tail, could be used to separate X- and Y-sperm in mice. The work of Umehara and colleagues opens up exciting avenues for the possibility of further selection of offspring in mammals through sperm-surface molecular-specific protein [14]. In particular, it provides a new reference for the sexing control of livestock offspring. However, the effects of TLR7/8 signalling in X/Y sperm have not been elucidated in dairy goats and other livestock.

Goat milk has nutritional similarity to human milk because of its abundant nutrients and ease of digestion [15]. Human demand for goat milk is increasing, but the population of high production dairy does is insufficient. In addition, the value of buck kids is significantly lower than that of doe kids in dairy goat breeding. The proportion of buck and doe offspring from natural mating and reproduction is approximately 50%, and the excessive breeding of male dairy goats increases production costs [16]. If dairy goat sperm-sexing technology is available, more doe kids could be produced, reducing breeding costs. However, there are no reports on the practical and successful separation of X/Y sperm in dairy goats. In this study, the localisation of TLR7/8 in the testis, epididymis, and sperm of dairy goat was evaluated. After TLR7/8 ligand (R848) incubation, the effects of sperm motility parameters, mitochondrial activity, and ATP levels were evaluated. We sought to prove TLR7/8 was explicitly expressed in goat X-sperm. The mechanism by which TLR7/8 affects X-sperm motility was also investigated. Based on this finding, we developed a novel yet simple goat sperm-sexing technology that could be used to selectively separate X- or Y-sperm and efficiently produce sexed embryos in dairy goats.

Materials And Methods

Ethics statement

All experiments in the current study were approved by the Institutional Animal Care and Use Committee of the Northwest A&F University, Shaanxi, China.

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents used in the present study were purchased from Sigma-Aldrich China. The R848 (TLR7/8 ligand) was obtained from Novus Biologicals (Littleton, CO, USA).

Semen collection and Swim-up test

Semen was collected twice weekly from six Guanzhong dairy goat bucks (2–3 years old) using an artificial vagina at the Shaanxi Aonike Guanzhong Dairy Goat Breeding Farm, Fuping, Shaanxi, China. The ejaculate was transported to the lab, and sperm quality was assessed within 30 min. All semen samples with sperm motility >75% were mixed to avoid the influence of the individual bucks used in the study. The swim-up test is shown in Fig. 1, as described previously [13]. The sperm were incubated in a goat semen extender, as described previously

[17], containing different concentrations of R848 for 0, 15, 30, 45, and 60 min. Next, the percentage of sperm in the upper and lower layers was evaluated (number of upper or lower layer sperm/total sperm). The separated sperm were used for further analysis.

Sperm motility parameters assessment using the computer-aided sperm analysis (CASA) system

Sperm were incubated in goat semen extender containing 1 μ M R848 at 37°C for 30 min. Then, 10 μ L of semen was placed on a pre-warmed glass slide (CELL-VU[®] DRM-600, Millennium Sciences, New York, NY, USA) with a coverslip. Sperm motility parameters were evaluated using a computer-aided sperm analysis (CASA) system (HVIEW-SSAV8.0, FuZhouHongShiYe Software Technology Co., Ltd., China). The sperm motility parameters measured included sperm motility *per se*, average path velocity (VAP), average straight-line velocity (VSL), and average curvilinear velocity (VCL). All experiments were replicated five times.

Immunofluorescence (IF) of testis and epididymis

Testes and epididymis of adult dairy goat bucks were collected and fixed in 4% (w/v) paraformaldehyde overnight and embedded in paraffin. Sections were probed with an anti-TLR7 antibody (bs-6601; Bioss Antibodies, Woburn, MA, USA) and anti-TLR8 antibody (ab180610; Abcam, Cambridge, MA, USA) at a ratio of 1:200. After washing with PBS, the antigens were visualised using Cy3-conjugated goat anti-rabbit IgG (ab6939; Abcam), 30 μ L fluorescein isothiocyanate-peanut agglutinin (FITC-PNA, 100 μ g/mL in PBS), and 1 μ g/mL DAPI. Section staining was monitored and photographed using an epifluorescence microscope (Nikon 80i; Nikon, Tokyo, Japan). Three replications were performed.

Immunofluorescence of sperm

The sperm of the dairy goat bucks was spread over slides, air-dried, and fixed using 100% methanol for 10 min. After air-drying, the sample slides were rinsed with PBS three times for 5 min each time. The sperm samples were then permeabilised with 0.5% (v/v) Triton X-100/PBS at room temperature for 30 min. The sample slide was rinsed with PBS three times for 5 min each time. The sperm were incubated with blocking solution (5% BSA) at 37°C for 30 min. Sperm were probed with anti-TLR7 or anti-TLR8 antibodies at a ratio of 1:100, and incubated overnight at 4°C. After washing with PBS, the antigens were visualised with Cy3-conjugated goat anti-rabbit IgG (1:2000; ab6939, Abcam) and 1 μ g/mL DAPI. Sperm staining was monitored and photographed using an epifluorescence microscope (Nikon 80i; Nikon). Three replications were performed.

Analysis of sperm membrane integrity

Sperm membrane integrity was evaluated using SYBR-14/propidium iodide (PI) following the procedure described by [18]. Briefly, the sperm (100 μ L) was stained using 0.1 μ L of SYBR-14 working solution (100 μ M in DMSO) for 10 min at 37°C, and then 0.5 μ L propidium iodide (PI) working solution (2.4 M in water) was added for 10 min at 37°C. Sperm staining was monitored and photographed using an epifluorescence microscope (Nikon 80i; Nikon). More than 200 spermatozoa per slide were assessed. Three replicates were evaluated for each semen sample by the same observer.

Analysis of sperm acrosome integrity

Based on the method described by Ren et al. (2019) [17], acrosome integrity was measured using fluorescein isothiocyanate-peanut agglutinin (PNA-FITC) staining. Briefly, sperm (30 μL) was spread over slides, air-dried, and fixed using 100% methanol for 10 min. The fixed sperm were then stained using 30 μL PNA-FITC (100 $\mu\text{g}/\text{mL}$ in PBS), incubated at 37°C for 30 min, and rinsed thrice with PBS, and nuclear staining was performed using 1 $\mu\text{mol}/\text{L}$ DAPI. The surface dye was washed three times using PBS and air-dried in the dark. Spermatozoa staining was monitored and photographed using an epifluorescence microscope (Nikon 80i; Nikon). More than 100 spermatozoa per slide were assessed and requested five more fields per sample. Three replicates were evaluated for each semen sample by the same observer.

Analysis of sperm mitochondrial activity and ATP levels

The sperm mitochondrial activity changes were evaluated using a JC-1 (5,5',6, 6'-tetrachloro-1,1',3,3'-tetraethylimidazolcarbocyanine iodide) Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, Haimen, China), following the manufacturer's instructions. Briefly, sperm samples ($2 \times 10^6/\text{mL}$) were stained with 28 μL of JC-1 (stock solution) in PBS (final volume, 100 μL). After incubation at 37°C for 30 min in the dark, the samples were centrifuged at $600 \times g$ for 5 min, resuspended in JC-1 buffer, and placed on ice. Sperm samples were immediately analysed in a flow cytometer (FAC-SCalibur; BD Biosciences, San Jose, CA, USA) with excitation at 490 nm and emission at 530 nm (JC-1 monomer) and excitation at 525 nm and emission at 590 nm (JC-1 polymer). Sperm mitochondrial activity was calculated using the following equation:

Sperm mitochondrial activity (%) = the value of JC-1 polymer/(JC-1 monomer + JC-1 polymer)*100%.

At least three technical replicates were evaluated for each sperm sample.

ATP levels of dairy goat sperm were assessed using an ATP Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, 500 μL semen (8×10^7 sperm/mL) was centrifuged and resuspended in ATP assay lysate (200 μL) to release intracellular ATP at 4°C. The sperm was then centrifuged at $12,000 \times g$ at 4°C for 5 min, and the supernatant was aspirated. The ATP standard solution (0.5 mmol/L) was diluted to concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 $\mu\text{mol}/\text{L}$ in succession by ATP assay lysate. The supernatants (50 μL) and standards (50 μL) were added to a 100- μL ATP detection working solution in opaque 96-well plates. The samples' fluorescence intensity was detected using a multi-detection microplate reader (Bio Tek Synergy H1; Bio Tek, Winooski, VT, USA). At least three technical replicates were evaluated for each sperm sample.

***In vitro* fertilisation (IVF) using the lower layer TLR7/8-activated sperm**

Goat ovaries were obtained from a local abattoir and transported to the laboratory within 4 h in 0.9% sodium chloride solution at 38°C. Thirty to forty COCs were placed in a 200- μL drop of BO-IVM medium (IVF Bioscience, Falmouth, United Kingdom), and were incubated for 24 h (38.5°C, 5% CO_2). TLR7/8-activated sperm (50 μL , 2×10^7 spermatozoa/mL) was added to the 30–40 cumulus-oocyte complexes in a 500- μL six-well dish containing BO-IVF (IVF Bioscience) medium and mineral oil and incubated for 18 h of at 38.5°C and 5% CO_2 . Then, the unfertilised sperm and cumulus cells were removed from the zygotes using 0.1% hyaluronidase PBS. Next, the zygotes were transferred to BO-IVC medium (IVF Bioscience) for culture. Embryos were collected for sex determination.

Artificial insemination (AI) using the lower layer TLR7/8-activated sperm

A CIDR (CIDR[®], EaziBreed; Zoetis, Parsippany-Troy Hills, NJ, USA) was intravaginally placed in the donor Guanzhong dairy goat does ($n = 6$) on day 0. On day 14, the goats were injected with FSH (Folltropin-V[®]; Bioniche Animal Health, Belleville, Canada) from the morning. In total, 180 mg of FSH was injected for three days (50, 50, 30, 30, 10, and 10 mg) at 12 h intervals. The CIDR was removed from the does on day 17. After 24 h, the donor goats were intra-cervically inseminated with TLR7/8-activated sperm as described in our previous report [17]. Briefly, two inseminations after oestrus (12 h apart) were performed, and embryos were collected for sex determination after five days.

Sex determination of goat embryos by PCR

Each of the blastocysts *in vitro* was transferred to PBS in 0.01% BSA for washing, and then individually placed in a 0.2-mL enzyme-free centrifuge tube and dispensed with 2.5 μ L of genome extract (1 mg/mL Proteinase K, 0.5% Triton X-100, 50 mM Tris-HCl, pH = 8.0). The single blastocyst was then put in a Polymerase Chain Reaction (PCR) machine and incubated at 65°C for 3 h and at 95°C for 10 min. PCR analyses of single blastocysts were performed with KOD FX Neo (KFX-201, Toyobo Co., Ltd., Life Science Department, Osaka, Japan) according to the manufacturer's instructions. Primer sets recognising the Y- (*SRY*) and X-chromosome (*B-ACTIN*) were generated and used in PCR reactions (Table 1). The following PCR amplification conditions were used: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 40 s, with a final extension at 68°C for 5 min. Subsequently, the PCR products were visualised by 2% agarose gel electrophoresis.

Determination of X/Y sperm ratio by Flow cytometry

After incubating the sperm with 1 μ M R848 for 30 min at 37°C, the upper or lower sperm layer was centrifuged at 400 x *g* at 37°C for 5 min. The sperm samples were then diluted to a concentration of 2×10^8 sperm/mL with 500 μ L goat extender containing 8 μ L Hoechst 33342 (2 mg/mL) and were tailed off by ultrasound for 1 s. The sperm was incubated at 37°C for 50 min in the dark, mixing every 10 min. A high-speed sorting flow cytometer (BD FACSAria[™] III Cell Sorter; BD, Franklin Lakes, NJ, USA) was used to analyse the proportion of X/Y sperm.

Western blot analyses

As described in our previous study [19], to obtain total protein lysates, the sperm were lysed on ice for 30 min using a cell lysis buffer containing 250 μ L RIPA and 2.5 μ L PMSF. A BCA assay kit was used to determine the total protein concentrations, and then the protein samples with loading buffer were boiled at 100°C for 10 min. Protein samples (20 μ g protein each lane) were electrophoresed on 10% SDS-polyacrylamide gels. The proteins were separated by PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated with the indicated primary antibodies. Blots were incubated with primary antibodies overnight at 4°C. The primary antibodies used were: phospho-AKT (10176-2-AP; Proteintech Group, Inc., China), phospho-NF κ B (3031s; Cell Signaling Technology, Boston, MA, USA), phospho-GSK3 α/β (9331s; Cell Signaling Technology), total AKT (ab81283; Abcam), total NF κ B (8242s; Cell Signaling Technology), and total GSK3 α/β (5676s; Cell Signaling Technology). Finally, after washing with TBST, the blots were incubated with an alkaline phosphatase-conjugated secondary antibody (1:10000 dilutions in TBST) for 1 h at 37°C. The reactive proteins

were visualised with chemiluminescence (ECL) western blot reagents and quantified using ImageJ software. Protein levels were normalised to anti- α tubulin (ab7291; Abcam).

Statistical analyses

All experiments were replicated at least three times independently. All results are expressed as the mean $\pm$ standard error of the mean (SEM). Sperm motility, VAP, VCL, and VSL were compared using the independent-samples t-test. Other data were analysed by one-way ANOVA, and multiple comparisons with Duncan's multiple range tests were performed using SPSS version 20 for Windows (SPSS Inc., Chicago, IL, USA) (*, $P \leq 0.05$; **, $P \leq 0.01$).

Results

Localisation of TLR7 and TLR8 in dairy goat testis, epididymis, and sperm

The percentage and localisation of TLR7 and TLR8 in dairy goat testis, epididymis, and sperm are shown in Figs. 2 and 3. Using immunofluorescence techniques, TLR7⁺ cells were observed in approximately half of the round spermatids ($47.57 \pm 1.79\%$) and epididymis sperm ($43.95 \pm 1.02\%$) stained with PNA, a special dye that specifically binds to the acrosome (Fig. 2a, 2b, 2d, and 2e). In goat buck sperm collected by ejaculation, TLR7⁺ signals (red) were observed at the tail of the sperm and accounted for $46.99 \pm 1.83\%$ of the total sperm (Fig. 2c and 2f). On the other hand, TLR8⁺ signals (red) comprised $47.53 \pm 6.54\%$ and $47.54 \pm 1.87\%$ of PNA⁺ round spermatids and epididymis sperm, respectively (Fig. 3a, 3b, 3d, and 3e). Consistently, the percentage of TLR8⁺ sperm collected by ejaculation was approximately 50% (Fig. 3c and 3f).

TLR7/8 agonists inhibited goat sperm motility parameters

The effects of TLR7/8 agonists (R848) on sperm motility parameters were evaluated, and the results are shown in Fig. 4. As shown in Fig. 4a, the percentage of highly active goat sperm that swim to the upper layer was significantly reduced by the addition of R848 in a dose-dependent manner (from 0.3 to 4 μ M). The percentage of sperm in the upper layer of semen was decreased after incubation with 1 μ M R848 for more than 15 min, in a time-dependent manner (Fig. 4b). Sperm motility parameters were analysed using the CASA system, and the trajectories of goat sperm incubated with 1 μ M R848 were shorter than those of the control group (Fig. 4c). Sperm motility was also significantly reduced after incubation with 1 μ M R848 for 30 min (Fig. 4d). Additionally, after incubation for 30 min, 1 μ M R848 significantly decreased the VAP, VSL, and VCL of goat sperm compared with the control ($P < 0.05$) (Fig. 4e–g).

TLR7/8 agonists suppressed motility of the sperm bearing the X-chromosome

The sperm motility in the upper and lower layers was evaluated in 1 μ M R848 for 30 min. After the swim-up test, motility in the lower layer sperm was dramatically decreased (Fig. 5a). Interestingly, the membrane and acrosome integrity of the upper and lower-layer sperm were not reduced (Fig. 5a). Through observations using IF, the lower layer sperm had more TLR8⁺ signals than the upper layer (Fig. 5d). TLR7 (Gene ID 102171227) and TLR8 (Gene ID 100860909), from the National Coalition Building Institute (NCBI), encoded on the X-chromosome of dairy goats and was expressed in sperm. The percentage of Y- and X-sperm in the upper and

lower layer sperm were detected using flow cytometry, based on DNA content. The percentage of Y-sperm in the upper layer was $90.50 \pm 2.86\%$, and X-sperm in the lower layer was $80.30 \pm 2.91\%$ (Fig. 5e).

TLR7/8 agonists inhibited ATP levels and mitochondrial activity via NF κ B and GSK3 α/β phosphorylation

Mitochondria are important organelles as energy sources for sperm motility. Effects of the TLR7/8 ligand on ATP production and mitochondrial activity in sperm were detected. As shown in Fig. 6a, the ATP levels of sperm decreased after incubation with 1 μ M R848 for more than 30 min, in a time-dependent manner. Mitochondrial activity was decreased after incubation with 1 μ M R848 for over 15 min (Fig. 6c). After the swim-up test, ATP levels in the lower layer sperm were significantly reduced (Fig. 6b). Consistently, the lower layer sperm's mitochondrial activity was significantly lower than that of the upper layer sperm (Fig. 6d). Western blot analysis showed that increasing NF κ B and GSK3 α/β phosphorylation was detected in the sperm treated with R848 for 15 and 30 min. However, there was no effect on AKT phosphorylation (Fig. 6e and 6 g). On the other hand, these changes in proteins were observed in the lower layer sperm (X-sperm) after the swim-up test with 1 μ M R848 for 30 min (Fig. 6f and 6h). These data confirm that TLR7/8 agonists affect X-sperm motility via NF κ B and GSK3 α/β phosphorylation regulation of ATP levels and mitochondrial activity.

TLR7/8 ligand activation enabled sex preselection for IVF and AI

The operation method for separating dairy goat X- and Y-sperm is shown in Fig. 1. After the swim-up test, the X-sperm (lower layer) separated by R848 was collected, and used for IVF and AI. Forty-three embryos were obtained by IVF using the lower layer sperm. Thirty-five of these were XX embryos ($80.52 \pm 6.75\%$) and eight were XY embryos ($19.48 \pm 6.75\%$) (Fig. 7a). Using the lower layer sperm, six dairy goat does were subjected to superovulation and oestrus simultaneously, and five were artificially inseminated. Nine embryos were collected from the uterus of two does that conceived (40% pregnancy rate). Eight of these were XX embryos (88.89%), one was an XY embryo (11.11%) (Fig. 7b).

Discussion

Mammalian sperm are male germ cells that carry genetic information, fertilise oocytes, and determine offspring's sex. According to Mendelian separation, the ratio of X- to Y-sperm produced by meiosis in mammalian spermatogenesis is 1:1 [6]. Therefore, male and female individuals produced after natural mating are also expected to be equally divided. However, the economic benefits of animal husbandry are often associated with livestock gender. If farmers could determine the gender of livestock in advance, they could increase profitability [1]. Hence, the sex selection of offspring in livestock is crucial for scientific research and production practice. This study aimed to demonstrate a simple and novel dairy goat sperm-sexing technology that activates TLR7/8 inhibited X-sperm motility. Our results show for the first time that TLR7/8 is expressed in sperm carrying the X-chromosome in dairy goats, and treating X-sperm with TLR7/8 ligands (R848) could reduce the motility of X-sperm without affecting the Y-sperm. These results indicated that targeting X-sperm with TLR7/8 could be used to separate X/Y-sperm in dairy goat bucks.

TLRs, which are members of an evolutionarily ancient pattern-recognition receptor family, play a key role in mammalian innate immunity and its link to inflammation [20]. A previous report indicated that TLR signalling affects sperm motility and mitochondrial function in mice [21]. In the present study, TLR7 and TLR8 were

investigated to clarify their potential function in the sperm of dairy goat bucks. We found that TLR7/8 is located in the sperm's tail, and the proportion of TLR7⁺ or TLR8⁺ sperm in the testis, epididymis, and ejaculate of dairy goats was close to 50%. Umehara et al. (2019) have also demonstrated that the percentage of TLR7⁺ and TLR8⁺ sperm were approximately 50% in the cauda epididymis of mice, consistent with the results of the present study [13]. Furthermore, both *TLR7* (Gene ID 102171227) and *TLR8* (Gene ID 100860909) are specifically expressed on the X-chromosome in dairy goats. Therefore, we hypothesised that TLR7/8 might be expressed explicitly in X-sperm.

R848 (resiquimod), a small-molecule compound of imidazoquinoline, is an agonist of TLR7 and TLR8, currently used clinically to treat viral infectious diseases and tumours [22]. In the present study, we demonstrated that R848 reduces sperm motility, VAP, VSL, and VCL. These are crucial kinematic characteristics of sperm; significant correlations have been identified between VAP and the number of acrosome-reacted spermatozoa [23], VAP and VSL bear a positive correlation with fertility [24], and VCL has relevance to sperm concentrations and affects fertility [25]. Therefore, R848 might have a damaging effect on sperm movement through interference with the TLR7/8 pathway *in vitro*. However, after removing R848, sperm motility was restored, indicating that the inhibition of R848 on sperm motility characteristics is reversible, similar to the findings of Umehara et al. (2019) [13]. On the other hand, after incubating goat sperm with R848, sperm was separated into upper and lower layers for further analysis. Interestingly, goat sperm motility in the lower layer was significantly reduced, while the upper layer's motility remained unchanged. After labelling sperm with Hoechst 33342, flow cytometry distinguishes X/Y sperm with high precision [26]. Goat sperm in the upper or lower layers were identified by flow cytometry, and the proportion of Y- or X-sperm reached more than 80%. These results further support our hypothesis that TLR7/8 is expressed explicitly in X-sperm.

These findings indicate that R848 inhibits X-sperm motility by specifically activating the TLR7/8 pathway, which mediates motility functional differences between X/Y sperm in dairy goat bucks. As reported in our previous study, the membrane and acrosome integrity were significant parameters for evaluating sperm function and quality [17]. In the present study, the membrane and acrosome integrity of sperm in the upper or lower layers after incubation for 30 min was not significantly reduced by adding 1 μ M R848. Therefore, we speculated that the reduction of X-sperm motility by R848 might be related to mitochondrial function and ATP levels. Tourmente et al. (2019) reported that ATP is essential for sperm motility and increasing ATP content coevolved with faster sperm that were more efficient swimmers in mice [27]. The mitochondrial volume in goats has recently been positively correlated with sperm swimming velocities and ATP levels [28]. As the incubation time increased, the mitochondrial activity and ATP levels in the goat sperm were reduced by adding 1 μ M R848. Notably, only the mitochondrial activity and ATP levels of X-sperm (lower layer sperm) were significantly decreased by 1 μ M R848. Our results corroborate those of Umehara et al. [13]. Therefore, we provide the first evidence that TLR7/8 signalling affects X-sperm motility by direct regulation via ATP levels in dairy goat bucks. However, further studies are required to elucidate the mechanisms by which TLR7/8 regulates ATP levels in goat sperm.

The maintenance of sperm motility depends on complex interactions between different cell signalling systems, one of which is a network of signalling pathways involved in regulating sperm movement [29]. Phosphoinositide 3-kinases (PI3K), a family of lipid kinases, participate in multiple signalling pathways in cells, including cell proliferation, migration, survival, metabolism, and intracellular trafficking [30]. AKT is a key downstream stimulant of PI3K and is closely related to sperm motility [31, 32]. Li et al. (2020) focused on the relationship

between sperm motility and PI3K/AKT activity and found that sperm motility significantly decreased after blocking AKT activity in mice [33]. In the current study, goat sperm were incubated with R848, and motility decreased; however, AKT phosphorylation was unaffected. This observation could imply the possible involvement of other signalling pathways in R848 metabolism. The sperm motility in mice has been shown to decrease after TLR signal activation, but AKT was not phosphorylated, similar to our findings [26]. On the other hand, a previous report indicated that TLR7/8 signalling affects sperm motility and is associated with reduced ATP levels in mice [13] due to the phosphorylation of glycogen synthase kinase 3 α/β (GSK3 α/β) and nuclear factor-kappa B (NF κ B) activation, well-known downstream pathways of TLR7/8 [13]. We report this for the first time in goats. According to the results of western blotting, we hypothesised that TLR7/8 signal activation could improve the expression of phosphorylated GSK3 α/β and NF κ B protein through the TRAF6/PI3K/GSK3 α/β and TRAF6/NF κ B pathways, respectively. Moreover, they decreased ATP levels via the suppression of hexokinase activity in goat sperm (Fig. 8). However, further studies are needed to verify this hypothesis.

Based on these observations, R848 was used to reduce the ATP levels of X-sperm and inhibit its motility, Y-sperm motility was unaffected. This phenomenon could be used to separate X/Y sperm in dairy goats. We referred to the sperm separation method reported in mice by Umehara et al. (2019) and achieved similar results in dairy goats [13]. The ratio of X/Y sperm after sorting by this method could reach more than 80% in dairy goats, and the movement ability of X-sperm could be restored after the inhibition of X-sperm was released. In fact, scientists have been trying to find the difference between X- and Y-sperm to enable separation of X/Y sperms [6]. At present, flow cytometry is the most widely used method for separating sperm in livestock, with a separation accuracy of over 90% [34]. However, due to differences in sperm of different species, this method has only been commercially applied to separate X/Y sperm in cattle [7]. In other domestic species, including goats and pigs, this method has only been tested on a small scale [10, 35]. In the present study, the percentage of female embryos obtained by *in vitro* fertilisation with separated X-sperm was $80.52 \pm 6.75\%$, suggesting that the sex-sorted sperm could fertilise oocytes, and the resulting XX embryos had a similar ratio to that of sorted X-sperm. More encouragingly, the sorted X-sperm could produce more XX embryos after AI in the cervix. Since the AI experiment was conducted during the non-breeding season (winter) of goats and only nine embryos were obtained, it is difficult to draw strong conclusions on the X-sperm's true *in vivo* efficacy after sorting. However, this was within an acceptable range, considering the 88.89% XX embryos. Bathgate et al. (2013) indicated for the first time that sperm could be sex-sorted by flow cytometry in goats, successfully producing five female kids (83%) [36], consistent with our findings, and suggesting that sorted-sperm in our proposed method could pass through the cervix to fertilise oocytes *in vivo*. These data indicate that TLR7/8 signalling sufficiently affects X-sperm motility enough to separate X/Y sperm and produce sexed dairy goat embryos efficiently.

Conclusions

In conclusion, a novel, simple sperm-sexing method for dairy goats that activates TLR7/8-inhibited X-sperm motility to separate X/Y sperm was investigated. The ratio of X/Y goat sperm after sorting by this method was over 80%, and the movement ability of X-sperm could be restored after the inhibition of X-sperm was released. In addition, incubating sperm with TLR7/8 ligands permits easy separation of X/Y sperm obtaining more than 80% female embryos using X-sperm in IVF and AI. Our study reveals that TLR7/8 signalling affects X-sperm motility via the GSK3 α/β -hexokinase pathway, which is important for the efficient production of sexed dairy goat embryos.

Abbreviations

TLR7/8: Toll-like receptor 7/8; GSK3 α/β : glycogen synthase kinase α/β ; NF κ B: nuclear factor kappa-B; AI: Artificial insemination; X-sperm: X chromosome bearing sperm; Y-sperm: Y chromosome bearing sperm; PI: propidium iodide; IVF: in vitro fertilization; CASA: computer-aided sperm analysis; IF: immunofluorescence; PNA-FITC: fluorescein isothiocyanate-peanut agglutinin; PCR: Polymerase Chain Reaction; TLRs: Toll-like receptors; NCBI: National Coalition Building Institute.

Declarations

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Authors' contributions

HJH and RF conceived the idea, designed and oversaw the experimental study. RF undertook the analysis and wrote the draft. RF and XHM assessed the localization of TLR7/8 in testis, epididymis and sperm. RF and ZDW conducted assessments of the TLR7/8 agonists inhibited sperm motility parameters. RF and LAM were in charge of the IVF analysis. HJH, RF, LY, WF, XM and WLQ assessed the artificial insemination. RYJ and ZMJ assessed the western blot. HJH provided a critical revision of the article. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author by request.

Ethics approval and consent to participate

All experimental procedures were performed according to the Guide for Northwest A&F University Animal Care Committee. The experimental protocol was approved by the Departmental Animal Ethics Committee of Northwest A&F University. As suggested by the animal welfare protocol, all efforts were made to reduce animal suffering and to use only the number of animals required to produce dependable scientific data.

Consent for publication

Not applicable.

Competing interests

The authors have declared no conflict of interest.

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Tables

Table1 Amplification PCR primer sequences of the goat *SRY* and *β-ACTIN* gene

Gene	Accession Number	Forward Sequences (5'-3')	Reverse Sequences (5'-3')
<i>SRY-1</i>	XM_018044472.1	TGAACGAAGACGAAAGGTGGCT	CCTGGGTATTTGTCTCGGTGT
<i>SRY-2</i>	XM_018044472.1	GCTGGGATACGAGTGG	AAGCGATTTCTGTGGC
<i>β-ACTIN-1</i>	XM_018039831.1	CCTCTGAACCCCAAAGCCA	TTTCCCGCTCAGTCGTGGT
<i>β-ACTIN-2</i>	XM_018039831.1	GCCGAGCGGGAAATCGT	CCGTGTTGGCGTAAAGGT

Figures

Figure 1

The technical diagram of goat X/Y sperm separation method using R848. The goat semen were centrifuged at 800 rpm for 5 min, resuspended in separation extender and R848, and incubated on 37°C. Next, sperm in the upper (Y-sperm) and lower layers (X-sperm) was evaluated.

Figure 2

The percentage and localisation of TLR7 in goat testis, epididymis and sperm. (a) localisation of TLR7 in goat testis at 8 months. Cross-sections of goat testes were stained with TLR7 (red) or PNA (green), a marker of acrosome in round spermatid and sperm. Yellow arrows represent PAN+ and TLR7+ positive cells, white arrows represent PAN+ and TLR7- positive cells. Scale bar indicates 20 μ m. (b) localisation of TLR7 in goat epididymis at 8 months. Cross-sections of epididymis were stained with TLR7 (red) or PNA (green). Scale bar indicates 50 μ m. (c) localisation of TLR7 in goat sperm collected by ejaculation. Scale bar indicates 20 μ m. Yellow arrows represent TLR7 positive sperm (P), white arrows represent TLR7 negative sperm (N). (d) the percentage of TLR7

positive/negative cell in goat testis. (e) the percentage of TLR7 positive/negative sperm in goat spididymis. (f) the percentage of TLR7 positive/negative sperm in goat sperm. Bars represent the mean $\pm$ SEM (n = 3).

Figure 3

The percentage and localisation of TLR8 in goat testis, epididymis and sperm. (a) localisation of TLR8 in goat testis at 8 months. Cross-sections of goat testes were stained with TLR8 (red) or PNA (green), a marker of acrosome in round spermatid and sperm. Yellow arrows represent PAN+ and TLR8+ positive cells, white arrows represent PAN+ and TLR8- positive cells. Scale bar indicates 20 μ m. (b) localisation of TLR8 in goat epididymis at 8 months. Cross-sections of epididymis were stained with TLR8 (red) or PNA (green). Scale bar indicates 50

µm. (c) localisation of TLR8 in goat sperm collected by ejaculation. Scale bar indicates 20 µm. Yellow arrows represent TLR8 positive sperm (P), white arrows represent TLR8 negative sperm (N). (d) the percentage of TLR8 positive/negative cell in goat testis. (e) the percentage of TLR8 positive/negative sperm in goat epididymis. (f) the percentage of TLR8 positive/negative sperm in goat sperm. Bars represent the mean ± SEM (n = 3).

Figure 4

TLR7/8 agonists inhibit goat sperm motility parameters. (a) Percentages of swim-up sperm when sperm were cultured in extender with R848 for 1 hour at 37 oC. The numbers of sperm in the upper layer and the number of total sperm were counted at 1 hour. (b) Percentages of swim-up sperm when sperm were cultured in extender with 1 µM R848. Sperm were cultured in extender with 1 µM R848 for a maximum of 120 min. The numbers of sperm in the upper layer and total sperm were counted at each time point. The percent of swim-up sperm was then calculated. (c) Tracks of sperm incubated with 1 µM R848 for 1 h, determined using the CASA system. The white arrow indicated the sperm moving forward (progressive sperm). The yellow arrow indicated the sperm swinging in a circle (slow sperm). (d, e, f, and g) Sperm were incubated with 1 µM R848 for 1 h. Sperm motility,

average path velocity (VAP), average straight line velocity (VSL), and average curve line velocity (VCL) were measured using CASA system, respectively. Bars represent the mean $\pm$ SEM (n = 5). *P < 0.05 compared with the control.

Figure 5

The percentage of Y-sperm and X-sperm in the upper layer and lower layer sperm after TLR7/8 agonists treatment. (a) Comparison of sperm motility, membrane integrity, and acrosome integrity in the upper layer and lower layer sperm with 1 μ M R848 for 30 min (n=5). (b) Sperm were stained using SYBR-14/PI. White triangle indicates membrane integrity; yellow arrows indicates membrane damaged (Bars=20 μ m, n=5). (c) Sperm were

stained using FITC-PNA. White triangle indicates acrosome integrity; yellow arrows indicates acrosome damaged (Bars=20 μ m, n=5). (d) Localization of TLR8 in the upper layer and lower layer goat sperm after incubate with 1 μ M R848 for 30 min. Yellow arrows represent TLR8 positive sperm, white arrows represent TLR8 negative sperm (Bars=50 μ m). (e) The percentage of X-sperm and Y-sperm in the upper layer and lower layer sperm were measured using flow cytometer system, values are the mean $\pm$ SEM of three replicates. *P < 0.05 compared with the control.

Figure 6

TLR7/8 agonists inhibited ATP production via GSK3 α/β -hexokinase pathway. (a) The ATP levels of sperm after culture in extender with 1 μ M R848 for a maximum of 60 min. (b) The ATP levels of sperm in the upper layer and lower layer sperm with 1 μ M R848 for 30 min. (c) The mitochondrial activity of sperm after culture in extender with 1 μ M R848 for a maximum of 60 min. (d) The mitochondrial activity of sperm in the upper layer and lower layer sperm with 1 μ M R848 for 30 min. (e) Goat sperm were incubated with 1 μ M R848 for 0, 15, or 30 min, and 20 μ g of protein lysates was analysed for phospho-AKT, phospho-NF κ B, phospho-GSK3 α/β , total AKT, total NF κ B, and total GSK3 α/β expression by Western blotting. Tubulin was used as the loading control. Results are representative of three independent experiments. (f) Intensity of the phosphorylation of AKT, NF κ B, and GSK3 α/β in the upper layer and lower layer sperm induced by R848 for 30 min. (g, h) Columns represent the ratio of phosphorylated to total AKT, phosphorylated to total NF κ B or phosphorylated to total GSK3 α/β . Values are the mean $\pm$ SEM of three replicates. *P < 0.05 compared with the control.

Figure 7

TLR7/8 agonists inhibited X-sperm motility and enabled sex preselection for in vitro fertilization (IVF) and artificial insemination (AI). (a) Gel of duplex PCR products of DNA extracted from single embryo after IVF, using SRY-1 primers and B-ACTIN-2 primers. Lane M is 500 bp DNA ladders; lane Ma is the male goat DNA; lane Fe is the female goat DNA; lanes 1, 2, 3, 5, 6, 7, 8 and 11 are female embryos; lanes 4, 9, and 10 are male embryos. (b) Gel of duplex PCR products of DNA extracted from single embryo after AI, using SRY-1 primers and B-ACTIN-2 primers. Lane M is 600 bp DNA ladders; lanes 1, 2, 3, 5, 6, 7 and 9 are female embryos; lane 8 is male embryo.

Figure 8

TLR7/8 signalling mechanisms affect ATP production in X-sperm and Y-sperm of goat, respectively. In Y-sperm, mitochondrial and glycolytic ATP production occur regardless of the presence of R848. Thus, Y-sperm showed normal high-speed motility. In X-sperm, the activation of TLR7/8 localized in the sperm tail via phosphorylated NFκB and GSK3α/β, and via a PI3K/AKT-independent mechanism. Then suppressed the activity of hexokinase. As a result, ATP production in X-sperm was decreased under R848 condition. Thus, only X-sperm showed low-speed motility under R848 condition.