ABSTRACT

A large number of female goats are needed for the dairy goat industry; therefore, the development of a method to ensure the birth of more females than males in a single pregnancy will lead to economic benefits. Increasing the number of X-sperm would be an effective way to increase the proportion of female offspring. In this study, goat semen was incubated at pH 7.4 in alkaline diluent combined with resiquimod (R848) and the number of X-sperm was enriched by the swim-up method. The percentage of X-sperm was determined using the double TaqMan qPCR method. Sperm total motility, progressive motility, average path velocity, straight-line velocity, and curvilinear velocity were measured using a computer-aided sperm analysis system, and the functional parameters of the sperm plasma membrane, the acrosome, mitochondrial activity, ATP content, and reactive oxygen species levels were also measured. Lastly, the ratio of female embryos was determined by in vitro fertilization, and the number of female kids and the pregnancy rate of does was assessed by artificial insemination. The results showed that dilution of semen in an alkaline buffer containing R848 could enrich the number of X-sperm to 85.57% ± 3.27%. The progressive motility, average path velocity, straight-line velocity, curvilinear velocity, mitochondrial activity, and ATP level of the collected X-sperm-enriched semen were significantly reduced, but its total motility, plasma membrane, and acrosome were not affected. The in vitro fertilization experiments showed that the rate of female embryo production using X-sperm-rich seminal fluid could reach 83.25% (174/209), which was significantly higher than the proportion of female embryos in the control group, 47.71% ± 1.80% (104/218). As determined by artificial insemination, the number of female kids in the test group increased by 62.79% (243/387), which was significantly higher than that in the control group (47.65%, 193/405). There was no significant difference in pregnancy rate between the test group and the control group (71.71% vs. 78.48%). Therefore, this study demonstrated that use of a pH 7.4 diluent containing R848 is a simple and effective method of X-sperm enrichment for dairy goat production. Its application would allow does to produce more female offspring for herd expansion and milk production.

Key words: dairy goat, alkaline, R848, X/Y sperm separation

INTRODUCTION

Goat milk is increasingly preferred over cow milk, and demand for goat milk products is increasing in both traditional and emerging markets because of the easier digestion and absorption of goat milk protein and its higher mineral content and utilization (Pulina et al., 2018; Prosser, 2021). The fecal microbiota of infants fed goat milk formula was closer to that of breast-fed infants than that of infants given formula made from cow milk (Tannock et al., 2013). During the decade from 2007 to 2017, the total global goat milk production increased by 16%, and the breeding population increased by almost 22% (Miller and Lu, 2019). The economic growth potential of the goat milk industry is very high. The main goal of breeders is to obtain more female offspring to derive greater economic benefit, so a new technology for producing a higher number of females is required.

Sex-control technology can accelerate the livestock breeding process and produce great socioeconomic benefits for livestock development (Xie et al., 2020). Given the risk of embryonic nonviability and damage associated with invasive procedures for sexing embryos, the best method for selecting the sex of offspring is by separation of X- from Y-bearing sperm (Douglas and...
Turner, 2020). Over the past decades, many studies have been conducted based on differences between X- and Y-sperm, including density (Hossain et al., 2001), DNA content (Johnson et al., 1989), viability (Ericsson et al., 1973; Sarkar et al., 1984), surface charge (Blottner et al., 1994), and sex-specific antigens (Greenfield and Koopman, 1996); however, the efficiency of sex preselection using these methods remains debatable. With the development of molecular biology and cell biology, gene editing (Fernández et al., 2017) and RNA interference techniques (Xi et al., 2019; Wei et al., 2020) are frequently used during spermatogenesis to separate X and Y spermatozoa in the testis. The use of flow cytometry is the most reliable method, with a separation accuracy of up to 90% (Garnier and Seidel, 2008; Xie et al., 2020). Although these methods can produce the desired offspring with high probability, gene-editing techniques require highly skilled personnel, RNA interference can increase the risk of infertility in offspring, and sorting the sperm by flow cytometry can reduce fertilization ability (Dejarnette et al., 2011; Mikkola and Taponen, 2017). Furthermore, flow cytometry requires expensive equipment and long sorting times (Seidel, 2007; Vishwanath and Moreno, 2018). Therefore, the development of a simple and inexpensive method for the selection of female dairy goat offspring is needed to enhance profits in the dairy goat industry.

An intriguing recent study by Umehara et al. (2019) highlighted the discovery of a cell-surface marker, toll-like receptor 7/8 (TLR7/8), which was expressed on X-bearing but not Y-bearing sperm. The imidazoquinoline, resiquimod (R848), is an effective synthetic agonist of TLR7/TLR8. Treatment of murine sperm and frozen-thawed bovine sperm with TLR7/8-specific ligands (R848) could affect oxidative phosphorylation and glycolysis of mitochondria, thereby dramatically reducing X-sperm energy and motility without affecting that of the Y-sperm (Umehara et al., 2020). Subsequently, Ren et al. (2021) also revealed that TLR7/8 was only located in the tail of X-sperm in Guanzhong dairy goat semen and affected the motility of X-sperm through the GSK3 α/β-hexokinase pathway. Because of the difference in sperm motility, the incubation of sperm with R848 made it easier to separate the X-sperm (lower layer) from the Y-sperm (upper layer), resulting in an 80.3% enrichment of X-sperm.

Studies have shown that the pH of the vagina during the process of fertilization may influence the migration of X- and Y-bearing sperm, leading to skewness in the sex of the offspring. Wakim (1972) reported that the pH of the cervix of rabbits was positively correlated with the proportion of male offspring. Oyeyipo et al. (2017) incubated human sperm in an alkaline diluent and found that the percentage of Y-sperm in the upper-layer sperm increased by 8.9% compared with the control group. He et al. (2021) incubated dairy goat semen with pH 7.4 alkaline diluent, isolated about 70% of Y-sperm in the upper layer, and obtained about 70% of male embryos by in vitro fertilization (IVF). Those studies showed that Y-sperm were more enriched in the upper layer by incubating semen in alkaline pH diluent.

These studies showed that a pH 7.4 diluent could promote the motility of Y-sperm and inhibit the motility of X-sperm, whereas R848 inhibited the motility of X-sperm but did not affect the motility of Y-sperm. Thus, in this study, we aimed to achieve sex control in dairy goats through treatment with R848 in an alkaline diluent to maximize the difference between X- and Y-sperm motility. We combined the 2 elements and determined the minimum effective dose of R848 at pH 7.4 for optimal X- and Y-sperm sorting from semen of Saanen dairy goats without reducing the intrinsic fertilizing ability of the sperm. To prove this, we tested the fertilization ability of the treated sperm and determined the numbers of female embryos and female offspring by using the isolated sperm for IVF and AI experiments.

**MATERIALS AND METHODS**

**Experimental Dairy Goats**

All experimental procedures in this study complied with the experimental animal management regulations and ethical requirements and were approved by the Experimental Animal Ethics Committee of the College of Animal Medicine, Northwest A&F University. The experimental bucks were selected from the Saanen dairy-goat-breeding farm of Yangling Keyuan Clone Co. Ltd. in Shaanxi Province, China, and the semen was collected from 8 healthy 2-yr-old bucks from the breeding farm. The ovaries of dairy goats used for IVF were collected from a slaughterhouse in Baoji, Shaanxi Province, China, and the age-appropriate breeding does for AI were from a large-scale dairy goat farm in Shaanxi Province. All test goats were fed and managed according to the management standards of dairy goats, and roughage and concentrated feeds were supplied according to the established nutritional needs of dairy goats.

**Reagents and Diluent**

The R848, was obtained from Novus Biologicals (Alt Catalog #IMG-2208). The SYBR-14/PI (cat. no. GMS14057) and the PNA-FITC (cat. no. GMS14015.1.1) were purchased from Genmed Scientifics. The enhanced...
assay kit with JC-1, the reactive oxygen species (ROS) assay kit, and the enhanced ATP assay kit were purchased from Beyotime Biotechnology. Other chemicals and reagents were obtained from Sigma-Aldrich. The main components of our diluent are glucose, fructose, and lactose. The buffer pair used for the acidity and alkalinity of the diluent was citric acid plus sodium citrate. Hydrochloric acid was used to adjust the pH to 6.8 and NaOH was used to adjust it to 7.4.

**Optimal R848 Concentration and Timing of Incubation of Goat Sperm in the Weakly Alkaline Diluent**

Semen was collected twice a week using an artificial vagina from 8 Saanen bucks (approximately 2 yr old) that were healthy with medium body condition score and high libido. The semen was collected and placed into a small temperature-controlled container at 35°C and quickly (within 30 min) brought back to the laboratory for routine evaluation. Semen with progressive motility over 70%, no abnormal odor, and milky-white color was selected for the experiment. Ejaculates were processed and diluted separately to 5 × 10⁷/mL with semen diluent at pH 6.8, and then the diluted semen was centrifuged at 400 × g for 5 min at 37°C and the supernatant was discarded. A range of concentrations of R848 solution (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 μg/mL) were added to semen diluent at pH 7.4, and sperm were resuspended in test tubes (17.5 mm × 118.5 mm) at 3 mL/group (one concentration per group) at 37°C in weakly alkaline diluent using the Genmed dual fluorescence (SYBR-14/PI) kit. First, 10 μL of SYBR14 (reagent B) was gently mixed and incubated at 37°C for 10 min, and then 5 μL of PI (reagent C) was added and incubated at 37°C for 10 min. Double-stained sperm (20 μL) were pipetted onto a slide, covered with a coverslip, and immediately observed and counted under a fluorescence microscope (BX63, Olympus) with 200× magnification (200 sperm were counted in each visual field). The stained sperm samples were additionally analyzed by flow cytometry (CytoFLEX, Beckman) for SYBR-14 [excitation/emission (Ex/Em) = 485/535] and PI (Ex/Em = 525/590).

Plasma membrane integrity was examined by staining the upper and lower sperm layers (100 μL) treated with R848 in alkaline diluent using the Genmed dual fluorescence (SYBR-14/PI) kit. First, 10 μL of SYBR14 (reagent B) was gently mixed and incubated at 37°C for 10 min, and then 5 μL of PI (reagent C) was added and incubated at 37°C for 10 min. Double-stained sperm (20 μL) were pipetted onto a slide, covered with a coverslip, and immediately observed and counted under a fluorescence microscope (BX63, Olympus) with 200× magnification (200 sperm were counted in each visual field). The stained sperm samples were additionally analyzed by flow cytometry (CytoFLEX, Beckman) for SYBR-14 [excitation/emission (Ex/Em) = 485/535] and PI (Ex/Em = 525/590).

The Genmed peanut agglutinin fluorescence labeling (PNA-FITC) staining kit was used to detect the acrosome integrity of the upper- and lower-layer sperm treated with R848 in alkaline diluent. The reagent E (PNA-FITC, 200 μL) was added and samples were incubated for 20 min at room temperature protected from light. Afterward, sperm were washed twice (5 min each time) and collected by centrifugation at 400 × g for 5 min at room temperature, then mixed with 200 μL of PI working solution (0.4 μg/mL) and incubated for 5 min at room temperature in the dark. After washing twice, the sperm (3 replicates) were resuspended in 1 mL of reagent C and immediately assayed by flow cytometry; more than 10,000 sperm cells were observed at 500 cells per second. The above sperm were also stained with 1 μM 4',6-diamidino-2-phenylindole and washed twice, and 20-μL sperm smears on slides were air-dried. The reagent D fixative from the genmed kit (200 μL) was pipetted onto the surface and allowed to
stand for 1 min at room temperature. Then 200 μL of PNA-FITC was added to cover the surface, and slides were incubated for 20 min at room temperature, washed twice, covered with a coverslip, imaged, and counted under a fluorescence microscope (BX63, Olympus) with 200× magnification. Throughout the process, the slides were protected from light. A total of 200 spermatozoa were observed in each visual field, and 5 visual fields in each sample were randomly selected for evaluation. Five replicate observations were performed for each semen sample.

Changes in mitochondrial activity in the upper and lower spermatozoa treated with the alkaline R848 solution were detected using the fluorescent JC-1 enhanced mitochondrial membrane potential assay kit (Beyotime Biotechnology). The spermatozoa were stained with 100 μL of JC-1 working solution for 20 min at 37°C in the dark, and the spermatozoa were resuspended with JC-1 buffer after washing twice with centrifugation at 400 × g for 5 min at 37°C. The sperm samples were immediately analyzed by flow cytometry. The excitation and emission wavelengths were 490 nm and 525 nm for the JC-1 monomer and 530 nm and 590 nm for the JC-1 polymer, respectively. Sperm mitochondrial activity was calculated as follows: sperm mitochondrial activity (%) = JC-1 polymer/(JC-1 monomer + JC-1 polymer) × 100%. There were 3 replicates for each sperm sample.

**Determination of Sperm ATP Content**

The measurement of sperm ATP content was performed with the alkaline ATP assay kit (Beyotime Biotechnology). Sperm were incubated at 37°C for 0, 10, 20, 30, 40, 50, and 60 min in alkaline diluent with or without 0.2 μg/mL R848. Aliquots of the upper and lower semen (200 μL, diluted to 2 × 10⁷ sperm/mL) were resuspended in 200 μL of ATP lysis solution, lysed on ice for 15 min, then centrifuged at 12,000 × g for 5 min at 4°C. Aliquots (20 μL) of the sample supernatants and standards at different ATP concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM) were added to 100 μL of luciferase reagent in an opaque 96-well plate. The luminescence intensity of the samples was detected using a microplate luminometer (GloMax Navigator). Each sperm sample was repeated 3 times, and the concentration of ATP in the sample was calculated according to the standard curve.

**Determination of Sperm ROS Content**

Sperm ROS levels were conducted using an ROS assay kit. Spermatozoa were incubated at 37°C in pH 7.4 diluent containing 0.2 μg/mL R848 for 0, 20, 40, 60, and 80 min. Samples of 200 μL of the upper and lower semen layers (diluted to 1 × 10⁷ sperm/mL) were centrifuged at 400 × g for 5 min at 37°C each time point, and the supernatant was discarded. The sperm were resuspended in 200 μL of 2',7'-dichlorodihydrofluorescein diacetate probe working solution (final concentration, 10 μM), incubated for 20 min at 37°C in the dark, washed twice by centrifugation at 400 × g at 37°C after incubation (5 min each time), and analyzed by flow cytometry. The ROS level was proportional to the intensity of FITC fluorescence (Ex/Em = 488/525). A total of 10,000 sperm cells were assessed for each sample.

**Sperm Sorted by Incubation with R848 Under Weakly Alkaline Conditions for IVF and Embryo Sex Determination**

Dairy goat ovaries collected from abattoirs were placed in saline containing penicillin and streptomycin at 25°C and transported to the laboratory within 2 to 4 h. Adherent surface tissues were removed from the ovaries with autoclaved surgical scissors, cleaned with saline, quickly disinfected with one rinse of 75% alcohol, and washed 4 to 6 times with sterile saline. The ovaries were placed in a 60-mm dish with 4 mL of warmed PBS egg collection buffer, and the 3-to-6-mm follicles on the ovarian surface were punctured with a needle and gently pressed to squeeze out the cumulus-oocyte complexes (COC) with the follicular fluid. The COC with at least 3 layers of granulocytes were picked up with a self-made egg transfer needle under a stereomicroscope, and approximately 50 COC were transferred to a 4-well plate containing 500 μL of oocyte maturation medium per well and cultured at 38.5°C for 24 h in 5% CO₂. For the IVF procedure, we slightly modified it from our previous report (Deng et al., 2019). The cultured mature oocytes were gently pipetted and washed to separate them before being placed into drops containing 100 μL BO-IVF medium (IVF Bioscience). The X-enriched spermatozoa (50 μL, 1 × 10⁷ sperm/mL) isolated from the treated sperm were added to the drop with 30 to 40 COC and incubated for 12 to 16 h at 38.5°C in a 5% CO₂ incubator. The fertilized COC were transferred into TL-HEPES buffer with an egg transfer needle, washed to remove adhering sperm and cumulus cells, and then digested in TCM199 containing 0.5% hyaluronidase for 3 min to completely remove the cumulus cells. The fertilized COC were then transferred into G1 cleavage embryo culture medium (Vitrolife) covered with mineral oil and cultured in an incubator at 38.5°C with 5% CO₂.
After 7 d of culture, individual blastocysts were transferred to 200-μL enzyme-free centrifuge tubes containing 5 μL of lysis solution and placed on ice for 10 min to lyse. The lysis products were used for PCR amplification under the following conditions: 3 min pre-denaturation at 95°C; denaturation at 95°C for 30 s, annealing at 58°C for 20 s, extension at 72°C for 20 s, 35 cycles; and final extension at 72°C for 5 min. Polymerase chain reaction products were electrophoresed on 2% agarose gels at 110 V for 20 min, digitally imaged, and analyzed. Female embryos were identified by the production of one band (GAPDH, 395 bp), and male embryos by the production of 2 bands (GAPDH, 395 bp; and SRY, 199 bp).

**Weakly Alkaline Semen Diluent Combined with R848 Allowed Isolation of X-enriched Sperm for AI**

From September 2020 to November 2021, age-appropriate (1-to-5-yr-old) female breeding goats were selected from a herd of healthy Saanen dairy goats for AI. The AI procedure was carried out according to Leethongdee et al. (2021) with minor modifications. Female dairy goats that were in heat. Females were cervically inseminated with lower-layer (X-enriched) sperm treated with 0.2 μg/mL R848 at pH 7.4 and sperm without R848 treatment at pH 6.8 after does came into estrus. Artificial insemination was performed twice, the first AI taking place 8 to 12 h after identification of estrus by bucks. The interval between the first insemination and the second insemination was 8 to 12 h. The AI volume was 0.5 mL per doe (5 × 10⁷ sperm each time). The does were tested for pregnancy by ultrasound (Changchun) at approximately 35 to 45 d, and the female offspring ratio of female and male kidding was calculated after the does had given birth at full term based on gestation records.

**Statistical Analysis**

All data are presented as the mean ± standard error of the mean from at least 3 independent experiments. All data were first checked for normality and homogeneity of variances. Following this, the data of double TaqMan qPCR were analyzed with a statistical package (IBM SPSS for Windows, version 26.0) using ANOVA, and Duncan’s test for multiple pairwise comparisons. Other statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc.). Data were analyzed by one-way ANOVA and 2-way ANOVA. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**Efficiency of Enrichment of X-sperm by R848 in Weakly Alkaline Diluent**

To verify the efficacy of R848 combined with alkaline pH diluent to enrich X-sperm, sperm were co-incubated in different concentrations of R848 and diluent at pH 7.4 (3 mL/group, 5 × 10⁷ sperm/mL). The results are shown in Figure 1A. After 40 min of incubation of sperm in the diluent, the addition of R848 (from 0.1 to 1.6 μg/mL) was found to significantly reduce the percentage of highly active sperm swimming to the upper 1-mL layer, in a dose-dependent manner, relative to the 2 controls (pH 7.4 and pH 6.8). The percentage of X-sperm in the upper, middle, and lower layers was determined by dual real-time qPCR, and results (Figure 1B) showed that the control group (without R848) at pH 7.4 had 33.65% ± 3.20% and 69.87% ± 4.80% of X-sperm in the upper and middle layers, which was significantly higher than the percentage of X-sperm in the upper and middle layers with R848 treatment, 11.37% ± 2.19% (P < 0.01) and 55.70% ± 3.45% (P < 0.05); however, the percentage of X-sperm in the lower layer of the control group (49.08% ± 4.81%) was significantly lower than that in the treated group (85.57% ± 3.27%, P < 0.01). The number of sperm swimming to the upper 1-mL layer decreased in a time-dependent manner with incubation at the optimal R848 concentration compared with the control (Figure 1C). The sperm were incubated with 0.2 μg/mL R848 in alkaline diluent for 40 min, and the percentage of X-sperm in the lower layer reached 85.62% ± 2.37%, which was significantly higher than for incubation at 0 min (47.80% ± 3.85%, P < 0.01, Figure 1D). We determined the optimal concentration of R848 in combination with alkaline diluent and incubation time for enriching X-sperm in the lower sperm layer.

**R848 Inhibits X-sperm Motility**

Analysis of sperm motility parameters using the CASA system revealed that treatment of sperm with 0.2 μg/mL R848 at pH 7.4 for 40 min did not significantly decrease the total motility of either upper- or lower-layer sperm compared with the control group at pH 7.4 or pH 6.8 (P > 0.05, Figure 2B), indicating that this concentration and incubation time with R848 did not affect the sperm survival rate. Interestingly, 40-min incubation with 0.2 μg/mL R848 not only shortened the motility trajectory of the lower-layer sperm (Figure 2A) but also significantly reduced its progressive motility, straight-line velocity, average path velocity, and curvilinear velocity compared with the pH-7.4 and
pH-6.8 controls and the upper-layer sperm treated with R848 ($P < 0.05$, Figure 2C–F); this suggests that the majority of the lower-layer sperm were in rotational motion.

**Effect of Weakly Alkaline Diluent Combined with R848 on Sperm ATP Level and Mitochondrial Activity**

Sperm require ATP for motility; therefore, the effect of TLR7/8 ligands on sperm ATP content was evaluated in this study. The ATP standard curve coefficient of determination ($R^2$) was 0.997, indicating high reliability (Figure 4A). The ATP content of the spermatozoa was derived from the standard curve formula. After incubation with 0.2 μg/mL R848 for 30 min at pH 7.4, the ATP content of the lower-layer sperm decreased gradually with time, which was significantly different from the upper-layer sperm and the control group ($P < 0.05$).
In contrast, the ATP content of the upper-layer sperm and the control did not differ significantly ($P > 0.05$) with time, which was consistent with the results of the sperm motility assay. Mitochondrial activity was closely related to ATP production and was quantified by flow cytometry. After incubation with 0.2 μg/mL of R848 for 40 min, the mitochondrial activity of the lower-layer sperm was 82.63% ± 2.84%, which was significantly lower than the 92.48% ± 1.54% of the upper-layer sperm and the 91.63% ± 2.13% of the control sperm ($P < 0.05$, Figure 4C).

**Effect of Treatment with R848 at Weakly Alkaline pH on Mitochondrial ROS Levels in Sperm**

Reactive oxygen species are a by-product of oxidative phosphorylation by sperm. Sperm were incubated in pH 7.4 diluent containing 0.2 μg/mL R848 at 37°C for different times. Flow cytometry (Figure 5) revealed that the mitochondrial ROS content of lower-layer sperm was not significantly different ($P > 0.05$) at 0, 20, and 40 min but significantly increased at 60 and 80 min, and the same was true for the upper-layer sperm. Interestingly, after incubation for 60 min, the ROS content of the upper-layer sperm was significantly higher than that of the lower-layer sperm.

**Sex Ratio After IVF and AI**

Sperm were treated with 0.2 μg/mL R848 in weakly alkaline solution for 40 min, and the lower-layer sperm were washed and used for IVF after the dead sperm were removed by density gradient centrifugation. There was no significant difference in fertilization, cleavage, and blastocyst rates of the lower-layer sperm compared with controls ($P > 0.05$, Table 1). The sex of blastocysts was determined using dual PCR (Figure 6). The percentage of female embryos in the group treated with lower-layer sperm was 83.25% ± 2.77% (174/209), which was significantly higher than that in the control group at 47.71% ± 1.80% (104/218) ($P < 0.05$, Table 1). Artificial insemination was performed on 488...
does in natural estrus in the experiment, including 237 does in the control group and 251 does in the treated group. Table 2 shows that the proportion of pregnant animals (71.71%) and birth rate (215.00%) for AI with R848-treated lower-layer sperm (after wash) were not significantly different from those of the control group (78.48%, 217.74%, \( P > 0.05 \), Table 2). The treated group produced 387 kids, including 243 females (62.79%). In the control group, 405 kids were produced, of which 193 were females (47.65%). The proportion of females in the treatment group was significantly increased compared with the control (\( P < 0.05 \), Table 2).

**DISCUSSION**

The male-to-female sex ratio in higher animals is stabilized at 1:1 under long-term natural selection to maintain the sex balance of the population. Since Painter (1923) proposed the existence of X and Y spermatozoa in mammals, researchers have been interested in the differences between these 2 types of sperm. Although the intercellular bridges formed during spermatogenesis allow the sharing of expression products between sperm cells with different genotypes while ensuring the simultaneous developmental maturation of all connected
sperm cells (Erickson, 1973; Greenbaum et al., 2011; Rahman and Pang, 2020), Ventela et al. (2003) found that only 28% of small particles were actually transported to other cells via intercellular bridges in rats. After continuing research, many differentially expressed proteins were identified between X- and Y-bearing sperm populations, and these proteins were associated with energy metabolism, the structural cytoskeleton of flagella, calmodulin, serine activity, glycolytic enzymes, and mitochondrial activity (Chen et al., 2012; Quelhas et al., 2021). Based on the differences in protein expression in X- and Y-sperm, immunological methods for sexing sperm have been developed (Rahman and Pang, 2020).

Toll-like receptors are important pattern-recognition receptors first identified in the innate immune response (Yadav et al., 2021). In 2016, it was reported that TLR signaling regulated mitochondrial membrane potential and ATP levels in spermatozoa through activation of the MyD88/PI3K/GSK3α pathway, thereby reducing sperm motility (Zhu et al., 2016). Recent studies have demonstrated that the TLR7/8 protein encoded by the X sex chromosome gene in mammals (mouse, cow,}

Figure 4. Sperm ATP content and mitochondrial activity. (A) ATP standard curve. (B) ATP content at different time points of upper- and lower-layer sperm incubated at pH 7.4 with and without R848. (C) Statistical plot of mitochondrial activity. (D) Mitochondrial activity of sperm detected by flow cytometry. JC-1 monomer: green fluorescence indicates low sperm mitochondrial activity; JC-1 aggregates: red fluorescence indicates high sperm mitochondrial activity. Dot plots indicate mean ± standard deviation (n = 3); * indicates P < 0.05 compared with control. Bar plots indicate mean ± standard deviation (n = 3); different letters indicate significant differences (P < 0.05).

Figure 5. Sperm mitochondrial reactive oxygen species (ROS) content. The ROS content of mitochondria in upper- and lower-layer sperm treated with resiquimod (R848) at pH 7.4 for different lengths of time by flow cytometry. The bars indicate mean ± standard deviation (n = 3), and different letters indicate significant differences (P < 0.05). MFI = mean fluorescence intensity; AU = absorbance unit.
goat) exists only in sperm bearing the X chromosome (Umehara et al., 2019; Ren et al., 2021) and that R848 can specifically activate MyD88-PI3K-GSK3α/β and MyD88-TRAF6/NF-κB signaling pathways induced by TLR7/8 on X-sperm, resulting in slow motility of X-sperm due to decreased ATP production. A simple and convenient method for sperm sex control based on TLR7/8 activation on X-sperm was developed to obtain female offspring with skewed high sex ratios after using treated sperm for IVF (Ren et al., 2021).

Because adding R848 can inhibit the motility of X-sperm, we tried to maximize the difference in motility between X- and Y-sperm by increasing the motility of Y-sperm using an alkaline diluent. Previous studies have shown that sex ratios are affected by environmental stress (Xie et al., 2020), and sperm are very sensitive to slight changes in the pH of their environment (Mishra et al., 2018). Motility, viability, mitochondrial activity, and metabolic activity of bovine spermatozoa have been reported to be optimal in the physiological pH range of 7.0 to 7.5, with pH values below 6.5 and above 8.0 causing a significant decrease in those parameters (Conti et al., 2013). Oyeyipo et al. (2017) detected 62% of human X-sperm in the upper layer of an acidic diluent at pH 5.5. Park et al. (2021) stored boar sperm under acidic conditions (pH 6.2) for 2 d and produced an optimal X-to-Y sperm ratio (1.2:1) without affecting sperm function or fertility-related protein expression. After incubating goat sperm with diluents at different pH for 40 min, He et al. (2021) screened the acidic (pH 6.2) diluent and alkaline (pH 7.4) diluent for enrichment of X- and Y-sperm in the upper layer. We found that the percentage of X-sperm in the upper, middle, and lower layers in the weakly alkaline diluent (without R848) at pH 7.4 was 33.65% ± 3.20%, 69.87% ± 4.80%, and 49.08% ± 4.81%, respectively, indicating that the weakly alkaline diluent significantly enhanced the upstream ability of Y-sperm and inhibited the motility of X-sperm, but this effect on X-sperm enrichment was still unsatisfactory. The percentage of X-sperm in the lower layer was increased to 85.57% with the addition of 0.2 μg/mL (equivalent to 0.6 μM) R848 to the diluent. The optimal concentration (0.6 μM) that we selected was higher than that (0.3 μM)

### Table 1. Proportion of female embryos after in vitro fertilization

| Group     | COC  | Fertilization rate (%) | Cleavage rate (%) | Blastocyst rate (%) | Female embryos (%) |
|-----------|------|------------------------|-------------------|---------------------|--------------------|
| Control   | 659  | 88.01 ± 5.88a          | 66.03 ± 3.81a     | 37.59 ± 2.82a       | 47.71 ± 1.80a      |
| Low²      | 659  | 86.19 ± 6.19a          | 65.49 ± 4.29a     | 36.80 ± 3.01b       | 83.25 ± 2.77b      |

²Within a column, values with different superscript letters indicate significant differences (P < 0.05).

³Data are expressed as mean ± SEM (n = 6). COC = cumulus-oocyte complex.

²Low = lower-layer (X-enriched) sperm treated with 0.2 μg/mL R848 at pH 7.4 (after washing).

Figure 6. Double PCR electropherogram for sex determination of partial embryos. The double-stranded PCR products of single embryonic DNA after in vitro fertilization using SRY (199 bp) and GAPDH (395 bp) primers were subjected to gel electrophoresis. Lane M, 2,000-bp marker; lane Ma, male goat DNA; lane Fe, female goat DNA; lane 7, negative control; lanes 1, 4, 5, 6, and 8, female embryos; and lanes 2 and 3, male embryos.
In this study, the results of both flow cytometry and fluorescence microscopy showed no significant difference (\( P > 0.05 \)) between plasma membrane (>90%) and acrosome (>80%) integrity of upper- and lower-layer sperm treated with a weakly alkaline solution of 0.2 \( \mu \text{g/mL} \) R848 and the control group, indicating that the fertilization ability of R848-treated sperm was not significantly affected. Aerobic metabolism of sperm necessarily produces ROS as a by-product (Wagner et al., 2017); in this study, the ROS in the upper and lower layers of sperm treated with R848 was found to accumulate with increasing incubation time and was significantly higher after 60 min (\( P < 0.01 \)). The ROS have physiological and pathological functions, and sperm require small amounts of ROS activation during motility, hyperactivation, capacitation, and acrosome reaction (Asadi et al., 2021). The results of this study showed that the lower-layer sperm had lower ROS content than the upper-layer sperm at 60 min and 80 min due to reduced motility and energy consumption, but ROS content also accumulated slowly with time, so sperm should not be incubated any longer than necessary.

R848 may impair sperm movement by interfering with the TLR7/8 pathway in vitro. However, it has been shown that receptor degradation can be induced by activation with specific ligands, and receptor turnover occurs with many types of receptors (Burden, 1977). Umehara et al. (2020) found that the expression of TLR7/8 in the sperm was decreased after treatment with R848 and demonstrated that the negative effects of R848 were transient; X-sperm recovered motility and fertilization ability after the removal of R848. According to the description of Umehara et al. (2020), we used diluent (without R848) to wash the treated sperm, then performed IVF and AI experiments. The IVF results showed that the fertilization rate, cleavage rate, and blastocyst rate of the treated group were not significantly different from those of the control group (\( P > 0.05 \)). The results of AI showed that the proportion of pregnant does and birth rate of the treated group were not significantly different from those of the control group (\( P > 0.05 \)). The results of AI showed that the motility and fertilization ability of X-sperm can be restored after the removal of R848.

### Table 2. Proportion of female offspring after AI

| Group   | Female goats | Proportion pregnant (%) | Birth rate (%) | Female kids (%) |
|---------|--------------|-------------------------|----------------|----------------|
| Control | 237          | 78.48\(^a\) (186/237)   | 217.74\(^a\) (405/186) | 47.65\(^a\) (193/405) |
| Low\(^1\) | 251          | 71.71\(^b\) (180/251)   | 215.00\(^b\) (387/180) | 62.79\(^b\) (243/387) |

\(^a\)Within a column, values with different superscript letters indicate significant differences (\( P < 0.05 \)).
\(^b\)Low = lower-layer (X-enriched) sperm treated with 0.2 \( \mu \text{g/mL} \) R848 at pH 7.4 (after washing).

reported by Umehara et al. (2019), probably due to species difference and the differences between fresh and frozen semen. However, our optimal concentration (0.6 \( \mu \text{M} \)) was lower than that (1 \( \mu \text{M} \)) determined by Ren et al. (2021). It may be that the combined effect of alkalinity resulted in double inhibition of X-sperm, thereby reducing the dose of R848 needed.

In mammals, motility is a characteristic of mature sperm and reflects sperm health. The solution pH has significant effects on sperm viability, motility, and energy acquisition (Zhou et al., 2015). In alkaline pH, sperm are hyperactivated by tyrosine phosphorylation, cholesterol efflux, inward calcium ion flow, and alkalization of cytoplasmic pH (Lindemann and Kanous, 1989; Vredenburgh-Wilberg and Parrish, 1995; Sun et al., 2017), but the results of our study showed that the various motility parameters were not significantly different (\( P > 0.05 \)) between the control groups (pH 6.8 and pH 7.4), which is consistent with the results of He et al. (2021). When R848 was added to the weakly alkaline sperm medium, however, we found that the other motility parameters of the lower-layer sperm were significantly lower than those of the upper-layer sperm and the 2 controls (\( P < 0.05 \)), except for total sperm motility, indicating that the slowed motility of the lower sperm was influenced by R848. This may be related to TLR7/8 signaling specific to the X chromosome. In the present study, we also measured the ATP content and mitochondrial membrane potential in the lower sperm sample after R848 incubation for 40 min. The results suggested that specific binding of R848 to TLR7/8 and activation of the signaling pathway could reduce the mitochondrial membrane potential and ATP levels in X-sperm, leading to reduced motility, which is consistent with the results of Umehara et al. (2019) and Ren et al. (2021).

Sperm plasma membrane and acrosome integrity are essential for successful oocyte fertilization, and damage to the sperm plasma membrane can lead to an imbalance in the sperm internal environment and reduce sperm survival, whereas the sperm acrosome plays an important role in sperm capacitation, acrosome reaction, and zona pellucida binding (Tanga et al., 2021). In this study, the results of both flow cytometry and
ingly, we obtained 243 female offspring (62.79%) by AI with the enriched X-sperm. However, Ren et al. (2021) obtained only 8 female embryos after AI, so their AI experiments made it difficult to draw strong conclusions about the true in vivo potency of sorted X-sperm compared with our data. However, there was a significant difference in female embryo rate by IVF and the female offspring rate produced by AI of the enriched X-sperm. The mechanism is still under study. We aimed to increase the female kid’s birth rate after AI, and although we did not achieve the desired goal, we did increase the percentage of female offspring by 15.14% (62.79% vs. 47.65%) compared with the control group.

CONCLUSIONS

This study demonstrated that a weakly alkaline diluent at pH 7.4 combined with R848 could enrich X-sperm, with the ratio of X-sperm reaching 85.57%. Using mature oocytes for IVF, the female embryo rate could reach 83.25%; for AI, the female kid rate of the does flock could reach 62.79%; and the does’ pregnancy rate was not affected.

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