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Sperm distribution and fertilization after unilateral and bilateral laparoscopic artificial insemination with frozen-thawed goat semen

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ABSTRACT

Generally, laparoscopic artificial insemination (LAI) provides a higher success rate than of cervical insemination in goats. However, the sperm distribution after LAI in goats remains unknown, particularly when frozen-thawed semen is used. This study evaluated the distribution of frozen-thawed goat spermatozoa after LAI and compared the effects of sperm numbers and deposition sites (unilateral and bilateral sites) on pregnancy rate. In experiment 1, the frozen-thawed spermatozoa were stained either with CellTracker Green CMFDA (CT-Green) or CellTracker Red CMPTX (CT-Red), and *in vitro* evaluations of viability and motility were performed. In experiment 2, the labeled spermatozoa were deposited *via* LAI into the left (CT-Green) and right (CT-Red) uterine horns (n = 4). After ovariohysterectomy (6 hours after insemination), the distributions of green- and red-colored spermatozoa were assessed *via* tissue section, flushing, and the oviductal contents were also collected. Experiment 3 was designed to test the pregnancy rates in a group of 120 does after LAI using different numbers of spermatozoa (60 and 120 $\times$ 10^6 sperm per LAI) and different deposition sites. The results demonstrated that the fluorochromes used in this study did not impair sperm motility or viability. Frozen-thawed goat spermatozoa can migrate transuterinally after LAI, as evidenced by the observations of both CT-Green– and CT-Red–labeled spermatozoa in both uterine horns. Lower numbers of spermatozoa (60×10^6) that are inseminated unilaterally (either ipsilateral or contralateral to the site of ovulation) can efficiently be used for LAI in goats (with a 56.67% pregnancy rate).

1. Introduction

Frozen-thawed semen is commonly used for genetic improvement of livestock animals. However, this semen must be deposited directly into the uterine lumen to increase pregnancy rates after artificial insemination (AI). In goats, laparoscopic artificial insemination (LAI), which involves the deposition of sperm directly into the uterine horns, provides a higher pregnancy rate compared with cervical AI [1–3]. Currently, LAI with frozen-thawed semen is an essential part of artificial breeding in goats because LAI provides valuable opportunities to improve reproductive efficiency and enhance genetic improvement with consistently high pregnancy rates [1]; however, there were a few reports about these techniques in goats [3,4].

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Laparoscopic artificial insemination technique in goat has been efficiently modified from sheep. However, the results of LAI in sheep have been variable and contradictory among the laboratories [5–9]. The majority of intrauterine inseminations using frozen-thawed goat semen are bilateral inseminations [3,10]. However, this technique is time-consuming, and extensive manipulation of the reproductive tracts may elevate uterine luminal levels of prostaglandin F2α, which affects embryo quality and pregnancy rates [11]. Moreover, pregnancy rates have been reported to be reduced when insufficient sperm numbers are used for AI [8,12]. Therefore, determination of the optimum number of spermatozoa is required to achieve the highest pregnancy rates in goats. A wide range of insemination doses (5×10^6 to 200×10^6 sperm) has been reported for frozen-thawed goat spermatozoa, and these doses depend largely on the AI technique that is employed. Additionally, the pregnancy rates that result from different insemination doses may also be influenced by sperm quality factors such as viability, motility, and longevity [1–3,13]. Research on the numbers of motile sperm and the sites of insemination (i.e., ipsilateral or contralateral to the site of ovulation) has been reported for frozen-thawed sheep spermatozoa, but little information is available for goats [3,4,14].

The single-uterine horn insemination without ovarian examination technique would be more practical for the goat industry. However, this procedure requires that spermatozoa are capable of transporting to both sides of the reproductive tract. Therefore, it is essential to investigate the distributions of sperm within the reproductive tracts of female goats after LAI. Several studies in dogs [15], pigs [16], and sheep [7,8] have indicated that spermatozoa can migrate throughout the uterus and oviducts even when semen deposition is limited to one uterine horn. However, the frozen-thawed sperm distributions after LAI in goats remain unknown. Several techniques have been used to identify sperm distributions in female genital tracts; for example, uterine flushing [16], mucosal imprint [15], histology [16], and the supravital staining of spermatozoa with nontoxic fluorochromes [17,18].

The objectives of this study were to evaluate the sperm distributions after intrauterine insemination with frozen-thawed spermatozoa that had previously been labeled with fluorochromes and to study the effects of sperm numbers and insemination procedures on pregnancy rates after laparoscopic insemination in goats.

2. Materials and methods

2.1. Experimental animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Chulalongkorn University (approval no.: 11310030). Post-pubertal crossbred Saanen (75%) goats between the ages of 2 and 4 year old were used. The animal facility was located at the CU network for Academic Opportunities and Services, Chulalongkorn University, Nan Province, Thailand (latitude 18 °N and longitude 100 °E). The goats were maintained in a semi-intensive system under preventive and clinical veterinary care. The goats were fed daily with concentrates (14% protein, wt/wt), *ad libitum* grass, and also had free access to mineral blocks and water.

2.1.1. Semen collection, cryopreservation, and thawing

Goat semen was collected from eight bucks using an artificial vagina; the semen was then frozen as a pooled sample that was used in all experiments. Tris–citric acid–fructose (TCF) solution (pH 7.0–7.2) consisting of 250 mM Tris (hydroxymethyl animomethane), 90 mM citric acid, 70 mM fructose (BDH, Poole, UK), 100 IU/mL penicillin G, and 100 μg/mL streptomycin was used as a basic extender. Each ejaculation was diluted with TCF (1:9 at 37 °C) and then centrifuged at 940× *g* for 10 minutes at room temperature. The supernatant was subsequently removed. After seminal plasma removal, the ejaculates from the eight bucks were pooled and diluted with TCF extender supplemented with 0.5% (vol/vol) Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA), 10% (vol/vol) glycerol (Sigma–Aldrich, St. Louis, MO, USA), and 10% (vol/vol) egg yolk. The total sperm was counted using a hemocytometer (Neubauer, Boeco, Germany) and then adjusted to 240×10^6 sperm per mL. The spermatozoa were frozen using the Styrofoam box method as previously described [19]. In brief, the diluted semen was equilibrated at 4 °C for 4 hours and then loaded into 0.25 mL straws (Minitüb, Landshut, Germany). The straws were placed horizontally in liquid nitrogen vapor at 4 cm above the liquid nitrogen for 10 minutes and then plunged into the liquid nitrogen. When required, the spermatozoa were thawed by placing the straw in a 37 °C water bath for 30 seconds.

2.1.2. Supravital dye labeling and assessment of sperm quality

All fluorescent probes were purchased from Invitrogen (Eugene, OR, USA). To prepare the stock solutions of CellTracker Green CMFDA (CT-Green) and CellTracker Red CMPTX (CT-Red), the lyophilized products were dissolved in DMSO (Sigma–Aldrich) to a final concentration of 10 mM. The stock solutions were further diluted in 10% egg yolk–TCF to a working concentration of 25 μM.

After thawing, the spermatozoa were evaluated for motility. The spermatozoa were further diluted with 10% egg yolk–TCF at a ratio of 1:5 and then centrifuged at 940× *g* for 10 minutes at room temperature (25 °C) to remove the glycerol and Equex STM paste, which may be toxic to spermatozoa [19,20]. The supernatant was discarded, and the spermatozoa were divided into three equal aliquots. The first aliquot was resuspended with 500 μL of 10% egg yolk–TCF as a control, and CT-Green (25 μM) or CT-Red (25 μM) in 500 μL of 10% egg yolk–TCF was added to the other two aliquots. After 1 hour of incubation, the spermatozoa were centrifuged to remove excess dye. The spermatozoa concentrations were adjusted to 60×10^6 sperm per 0.25 mL with 10% egg yolk–TCF extender and then incubated at 37 °C for the longevity tests.

2.1.3. Estrus synchronization, LAI, and ovariohysterectomy

The does were synchronized using intravaginal progesterone sponges (Sincro-gest sponges containing 65 mg of medroxyprogesterone; Laboratorios Ovejero, Leon, Spain) for 13 days. Upon sponge removal, 400 IU pregnant mare serum gonadotropin (Sincro-gest PMSG; Laboratorios Ovejero) was administered. Estrus was detected with