

RESEARCH ARTICLE

Heparin induces more pronounced capacitation-associated changes than progesterone in goat spermatozoa assessed using sperm quality indexes



Alberto J. Cardenas-Padilla¹ , Xana S. Martinez-Morales² , Luis F. Hernandez-Mentado³ and Alfredo Medrano¹

1. Unidad de Investigación Multidisciplinaria (L2-Reproducción Animal), Departamento de Ciencias Pecuarias, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México (UNAM), Cuautitlán Izcalli 54714, México.
2. Especialización en Producción de Ovinos y Caprinos, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México (UNAM), Cuautitlán Izcalli 54714, México.
3. Maestría en Ciencias de la Producción y de la Salud Animal, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México (UNAM), Cuautitlán Izcalli 54714, México.

ABSTRACT

Background and Aim: Optimization of sperm capacitation protocols is important for improving assisted reproductive technologies (ARTs) in goats. Heparin and progesterone are used as capacitating agents in mammalian spermatozoa; however, direct comparisons of their effects on goat sperm functional dynamics during *in vitro* incubation remain limited. This study aimed to compare heparin and progesterone during *in vitro* capacitation of goat spermatozoa using conventional functional parameters and sperm quality indexes (SQIs).

Materials and Methods: Semen was collected from two Saanen bucks during three independent collection sessions. After individual quality assessment, ejaculates were pooled within each session, generating three independent experimental replicates. Each pool was divided into control (CG), heparin-supplemented (HG; 20 µg/mL), and progesterone-supplemented (PG; 2 µg/mL) groups. Spermatozoa (100×10^6 sperm/mL) were incubated at 37°C under 5% CO₂ for 4 h and evaluated at 0, 1, 2, and 4 h. Progressive motility, plasma membrane integrity, morphology, acrosome integrity, capacitation status, and plasma membrane fluidity were assessed. SQI-1 and SQI-2 were calculated from the evaluated functional variables.

Results: Significant treatment $\times$ incubation period interactions were detected for the non-capacitated CTC F pattern, membrane hyper-fluidity, and SQI-2. HG produced the most pronounced capacitation-associated changes, including a decrease in the F pattern and increases in capacitated and acrosome-reacted spermatozoa and membrane hyper-fluidity. Direct between-treatment comparisons further showed significantly higher SQI-2 values in HG than in PG at T2 and T4 (adjusted $p < 0.05$). SQI-2 in HG increased from $5.8 \pm 1.79\%$ at 0 h to $29.3 \pm 1.02\%$ at 4 h and was overall higher than in CG and PG. SQI-1 did not differ significantly among groups. PG induced more gradual capacitation-associated changes, whereas sperm quality progressively declined in CG during incubation.

Conclusion: Heparin induced more pronounced functional changes compatible with sperm capacitation than progesterone under the evaluated *in vitro* conditions. SQIs provided an integrative assessment of temporal sperm functional dynamics and may complement conventional parameters when optimizing goat sperm capacitation protocols for ARTs.

Keywords: acrosome reaction, assisted reproductive technologies, goat spermatozoa, heparin, *in vitro* capacitation, membrane fluidity, progesterone, sperm quality indexes.

INTRODUCTION

Assisted reproductive technologies (ARTs) are widely used in animal breeding to improve reproductive efficiency, increase offspring production, enhance genetic selection, and contribute to species preservation. In goats, recent advances in ARTs have focused on improving gamete competence and *in vitro* embryo production systems [1]. However, the efficiency of these technologies remains strongly dependent on sperm quality and functional competence under *in vitro* conditions [2]. Therefore, optimization of sperm preparation and capacitation protocols remains an important objective in reproductive biotechnology. Recent studies on goat *in vitro* fertilization have highlighted the importance of appropriate capacitation conditions and supplementation strategies for improving sperm functionality and embryo production outcomes [3].

Corresponding Author: Alberto J. Cardenas-Padilla

E-mail: acardenasp@cuautitlan.unam.mx

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Co-authors: XSMM: seyinx@gmail.com; LFHM: mvz_lfernandohm@unam.edu; AM: amedrano@unam.mx

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Freshly ejaculated spermatozoa are not immediately capable of fertilizing oocytes and must undergo a series of physiological and biochemical changes within the female reproductive tract collectively known as sperm capacitation [4]. Capacitation involves membrane remodeling, cholesterol efflux, increased membrane fluidity, and activation of intracellular signaling pathways that prepare spermatozoa for the acrosome reaction and subsequent fertilization [5, 6]. The acrosome reaction involves fusion of the plasma membrane with the outer acrosomal membrane, resulting in the release of proteolytic enzymes required for zona pellucida penetration [7, 8]. Thus, capacitation and the acrosome reaction are essential processes for successful mammalian fertilization.

Under *in vitro* conditions, capacitation can be induced using various supplements added to fertilization media, including albumin, heparin, and steroid hormones [9]. Among these agents, heparin is extensively used to promote sperm capacitation in mammals. Heparin supplementation induces functional changes associated with capacitation, including membrane remodeling, increased membrane fluidity, and the acrosome reaction [10, 11]. In goats, heparin has been successfully used for *in vitro* sperm capacitation, with progressive increases in capacitated and acrosome-reacted spermatozoa reported during incubation periods of 1–4 h [8, 12].

Progesterone also plays an important role in sperm physiology within the female reproductive tract. It has been associated with sperm activation, calcium influx, hyperactivation, and induction of the acrosome reaction through receptor-mediated mechanisms [13–15]. Previous studies have indicated that progesterone can induce capacitation-associated responses in goat spermatozoa, although the magnitude and temporal dynamics of these responses may differ from those induced by heparin [16]. These observations suggest that heparin and progesterone may produce distinct patterns of functional changes during *in vitro* sperm capacitation.

The efficiency of sperm capacitation is also influenced by incubation conditions and sperm-handling procedures. Different sperm preparation techniques can affect sperm recovery, plasma membrane integrity, motility, and functional competence, thereby influencing the response to capacitating agents [8]. Consequently, assessment of temporal changes in sperm function during incubation is important for improving the reproducibility and efficiency of *in vitro* fertilization protocols in goats. Evaluation of individual sperm parameters can characterize specific aspects of sperm function; however, an integrative assessment using sperm quality indexes (SQIs) may provide complementary information on the overall balance between sperm quality preservation and acquisition of capacitation-associated characteristics during incubation.

Although heparin and progesterone have individually been evaluated as capacitating agents in goat spermatozoa, direct comparisons of their effects under identical experimental and incubation conditions remain limited. Furthermore, previous investigations have generally emphasized individual sperm functional parameters or specific incubation periods, providing limited information on the temporal progression of capacitation-associated changes. Consequently, the comparative dynamics of membrane remodeling, capacitation status, acrosomal changes, and overall sperm functional quality in response to heparin and progesterone remain insufficiently characterized. In addition, the use of SQIs to integrate multiple functional parameters throughout *in vitro* capacitation has received limited attention. To our knowledge, no previous study in goats has directly compared heparin and progesterone across multiple incubation periods while simultaneously evaluating conventional sperm parameters, capacitation-associated markers, and SQI-1 and SQI-2. A direct time-dependent comparison using conventional sperm functional measurements together with SQIs could therefore provide a more comprehensive assessment of the responses induced by these capacitating agents.

This study aimed to compare the effects of heparin and progesterone on the temporal functional dynamics of goat spermatozoa during *in vitro* capacitation. Specifically, progressive motility, plasma membrane integrity, sperm morphology, acrosome integrity, capacitation status, and plasma membrane fluidity were evaluated at defined incubation periods. In addition, SQI-1 and SQI-2 were used to integrate the evaluated sperm variables and characterize changes associated with the preservation of sperm quality and acquisition of capacitation-associated characteristics. It was hypothesized that heparin and progesterone would induce distinct temporal patterns of functional changes during *in vitro* capacitation.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving the animals, including semen collection, were conducted in accordance with the regulations of the Institutional Subcommittee for Care of Animals in Experimentation (Subcomité Institucional para el Cuidado de Animales en Experimentación), Universidad Nacional Autónoma de México (UNAM), Mexico. The experimental protocol was reviewed and approved by the Institutional

Subcommittee for Care of Animals in Experimentation, UNAM (Approval No. SICUAE.DC-2020/3-3; approved October 5, 2020).

Study period and location

The study was conducted during November and December 2025 during the natural breeding season at the Animal Reproduction Laboratory, Multidisciplinary Research Unit, Faculty of Higher Studies Cuautitlán, UNAM, Mexico. The bucks were housed in the postgraduate pens at the Education Centre for Agriculture and Animal Breeding (Centro de Enseñanza Agropecuaria [CEA]), Faculty of Higher Studies Cuautitlán. During the study period, the average photoperiod was 10.9 h light:13.1 h dark.

Study design

The study used an experimental *in vitro* design to compare the effects of heparin and progesterone on the temporal functional dynamics of goat spermatozoa during capacitation. Semen was collected from the same two Saanen bucks during three independent collection sessions performed on different days, with one ejaculate obtained from each buck during each session. Following individual semen quality assessment, eligible ejaculates were pooled within each collection session, resulting in three independent pooled semen samples. Each pooled sample represented an experimental replicate and was divided into control group (CG), heparin-supplemented group (HG), and progesterone-supplemented group (PG). Sperm functional characteristics were evaluated during 4 h of incubation at T0, T1, T2, and T4.

Animals and housing

Two sexually mature Saanen bucks, 4–5 years of age, with a body condition score of 2.5/5 and an approximate body weight (BW) of 95 kg, were used for semen collection. Both bucks had previous reproductive experience, including routine semen collection. The animals received a uniform diet consisting of concentrate feed containing 17% crude protein, 4% crude fat, 10% crude fiber, 9% ash, and 48% nitrogen-free extract, together with alfalfa hay, mineral blocks, and water *ad libitum*. Daily feed intake was equivalent to approximately 3% of live BW on a dry-matter basis.

Materials and reagents

Incubation was performed using an Incusafe MCO-18AC CO₂ incubator (SANYO Electric Co., Ltd., Osaka, Japan). Microscopic analyses were conducted using a Leica DM1000 LED differential interference contrast microscope and a Leica DMLS epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). Unless otherwise specified, reagents used for semen processing and sperm functional assessments were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Semen collection and preliminary sperm assessment

Semen was collected using an artificial vagina. Three independent semen collection sessions were conducted on different days using the same two bucks, with one ejaculate collected from each buck during each session. Immediately after collection, each ejaculate was macroscopically evaluated for volume, color, appearance, and the presence of abnormal contaminants, including blood, pus, or debris. Samples were protected from light and transported to the laboratory at 37°C in a dry container. On arrival, each ejaculate was maintained in a water bath at 37°C and evaluated microscopically before pooling. Only ejaculates with $\geq 70\%$ progressive motility, $\geq 2000 \times 10^6$ sperm/mL, and $< 20\%$ morphologically abnormal spermatozoa were included.

For microscopic assessment, each sample was diluted 1:100 (v/v) in 0.9% NaCl, except for sperm concentration determination. Spermatozoa were assessed for progressive motility, plasma membrane integrity, morphological abnormalities, acrosome integrity, capacitation status, and plasma membrane fluidity [17].

Progressive motility was evaluated using a Leica DM1000 optical microscope at 10× and 20× magnification and expressed as the percentage of spermatozoa exhibiting forward movement. Plasma membrane integrity and sperm morphology were evaluated using Eosin–Nigrosin staining. A total of 200 spermatozoa were examined to determine plasma membrane integrity, with unstained spermatozoa classified as viable and stained spermatozoa as nonviable, and to classify sperm morphology as normal or exhibiting primary or secondary abnormalities [18].

For sperm concentration determination, 10 μ L of each fresh ejaculate was diluted 1:200 (v/v) in 0.4% formalin saline solution. Spermatozoa were counted using a Neubauer chamber under a Leica DM1000 optical microscope at 40× magnification. Acrosome integrity was assessed using phase-contrast microscopy after diluting samples 1:1 (v/v) in 0.4% glutaraldehyde, with 200 spermatozoa evaluated per sample.

Capacitation status was assessed using the chlortetracycline (CTC) assay. Briefly, 100 μ L of each sample was

diluted 1:1 (v/v) in CTC solution containing 130 mM NaCl, 20 mM Tris, 805 mM CTC-HCl, and 5 mM cysteine (pH 7.8). The mixture was fixed with 22 μ L of 0.2% glutaraldehyde, placed on a microscope slide, and supplemented with a drop of 1,4-diazabicyclo[2.2.2]octane (DABCO). After examination of 200 spermatozoa in different fields using a Leica DMLS fluorescence microscope at 100 $\times$ magnification, CTC fluorescence was classified into three patterns: F, uniform fluorescence over the entire sperm head, indicating non-capacitated, acrosome-intact spermatozoa; B, a fluorescence-free band in the post-acrosomal region, indicating capacitated, acrosome-intact spermatozoa; and AR, almost no fluorescence over the sperm head except for a fluorescent band in the equatorial segment, indicating acrosome-reacted spermatozoa.

Plasma membrane fluidity was assessed using the merocyanine 540 (MC540) assay. A 10 μ L aliquot of MC540 working solution, prepared from 495 μ L Tyrode's albumin lactate pyruvate (TALP) and 5 μ L MC540 stock solution (14 mg MC540 in 5 mL dimethyl sulfoxide), was added to 140 μ L of diluted semen. The mixture was fixed with 22 μ L of 0.4% glutaraldehyde, placed on a microscope slide, and supplemented with a drop of DABCO. A total of 200 spermatozoa were evaluated using a Leica DMLS fluorescence microscope at 100 $\times$ magnification and classified as opaque, indicating low membrane fluidity, or brilliant, indicating high membrane fluidity. The CTC and MC540 assays were previously validated for goat spermatozoa by Cardenas-Padilla *et al.* [17], based on the methodologies described by Green and Watson [19] and Harrison *et al.* [20], respectively.

Goat sperm *in vitro* capacitation

Following the initial microscopic assessment, each sample was diluted 1:5 (v/v) in washing medium containing 250 mM Tris, 28 mM glucose, 104 mM citric acid, 0.05% streptomycin, and 500 U/mL penicillin (pH 7.4) at 37°C and centrifuged at 750 $\times g$ for 10 min. The supernatant containing seminal plasma was removed, and the sperm pellet was resuspended in (TALP; pH 7.2–7.4) medium to a concentration of 200×10^6 sperm/mL.

TALP was prepared using Tyrode's salts (9.6 g/L; Sigma-Aldrich, T2145), providing final concentrations of 136.9 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4 , 1.8 mM CaCl_2 , 1.1 mM MgCl_2 , and 5.6 mM D-glucose and supplemented with 11.9 mM NaHCO_3 , 1.0 mM sodium pyruvate, 5 mg/mL bovine serum albumin, and 20 mg/L phenol red.

After processing, equal volumes of the standardized sperm suspensions from the two bucks were then combined to obtain each pooled sample, ensuring equal contribution from both bucks within each collection session to generate three independent pooled semen samples. Each pool was considered an experimental replicate and divided into CG, HG, and PG.

Heparin and progesterone preparation

Low-molecular-weight heparin (enoxaparin sodium, Sigma-Aldrich, USA, E0180000) was diluted 1:100 (w/v) in deionized water and stored at 5°C until use. On each experimental day, a working solution was freshly prepared by further dilution in TALP. A subsequent dilution in TALP was prepared to obtain a heparin concentration of 40 μ g/mL before its final dilution in the experimental treatment.

Water-soluble progesterone (Sigma-Aldrich, P7556), supplied as a cyclodextrin-complexed preparation, was diluted 1:10 (w/v) in deionized water, aliquoted, and stored at –20°C until use. No additional organic solvent was used. On each experimental day, an aliquot was thawed and further diluted in TALP to prepare the working solution. A subsequent dilution in TALP was performed to obtain a progesterone concentration of 4 μ g/mL before its final dilution in the experimental treatment.

Treatment groups

CG consisted of spermatozoa diluted 1:1 (v/v) in TALP without additional supplementation to obtain a final concentration of 100×10^6 sperm/mL.

HG consisted of spermatozoa diluted 1:1 (v/v) in TALP containing 40 μ g/mL heparin, resulting in a final sperm concentration of 100×10^6 sperm/mL and a final heparin concentration of 20 μ g/mL, as previously described [21].

PG consisted of spermatozoa diluted 1:1 (v/v) in TALP containing 4 μ g/mL progesterone, resulting in a final sperm concentration of 100×10^6 sperm/mL and a final progesterone concentration of 2 μ g/mL, as previously described [22].

Each treatment was subsequently subdivided into separate aliquots independently assigned to T0, T1, T2, and T4. T0 corresponded to the initial assessment performed after preparation of the treatment groups and addition of the respective supplements, before the scheduled incubation period. The final incubation volume was 1 mL for each treatment at each incubation period.

Assessment of sperm parameters during incubation

Spermatozoa from CG, HG, and PG were incubated for 4 h at 37°C under 5% CO₂ to induce *in vitro* capacitation and the acrosome reaction. Sperm functional characteristics were evaluated at T0, T1, T2, and T4. For each assay, 200 spermatozoa were evaluated independently for each replicate–treatment–time combination. The assessed parameters included progressive motility, plasma membrane integrity, morphological abnormalities, acrosome integrity, capacitation status determined using CTC, and plasma membrane fluidity determined using MC540. The experimental workflow is presented in Figure 1. Microscopic assessments were performed with the observer blinded to the treatment group, although the observer was aware of the corresponding incubation time.

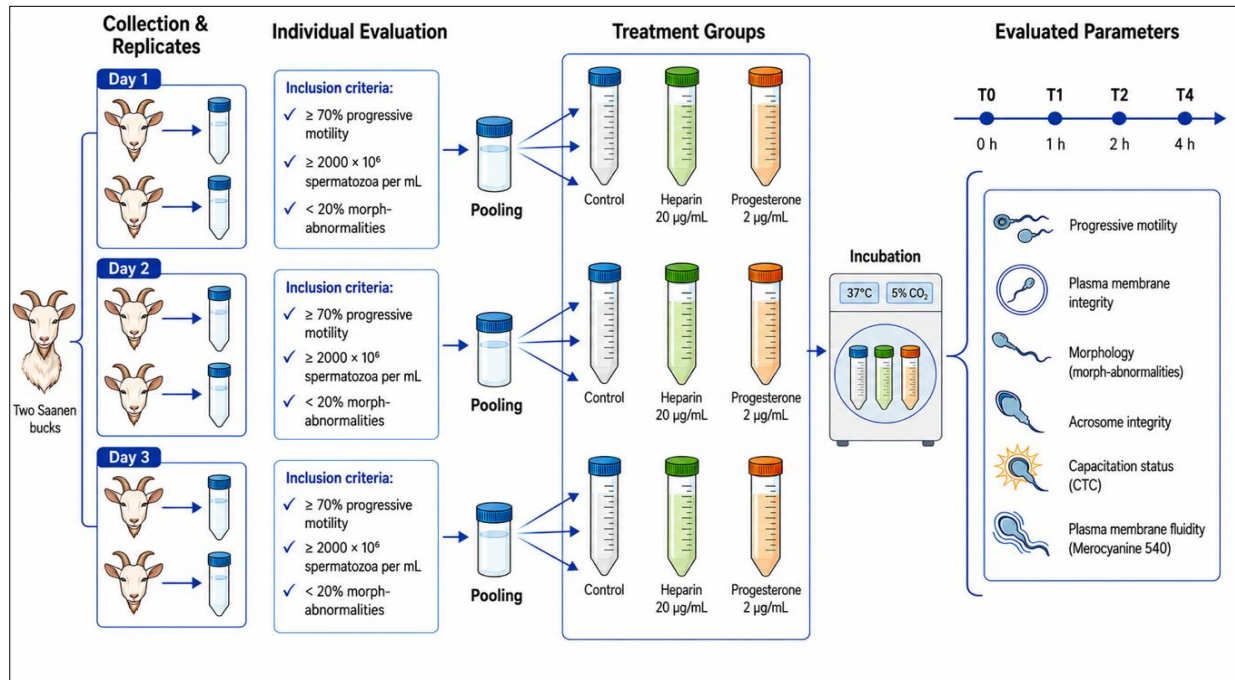


Figure 1: Schematic representation of the experimental design for *in vitro* capacitation of goat spermatozoa. Semen was collected from two Saanen bucks during three independent collection sessions conducted on different days. Following individual semen quality assessment, eligible ejaculates were pooled within each collection session, resulting in three independent experimental replicates. Each pooled sample was divided into CG, HG (20 µg/mL heparin), and PG (2 µg/mL progesterone) and evaluated at T0, T1, T2, and T4. CG = Control group, HG = Heparin-supplemented group, PG = Progesterone-supplemented group, T0 = 0 h, T1 = 1 h, T2 = 2 h, T4 = 4 h.

SQI

Data obtained from the sperm functional assessments were additionally integrated using the previously reported SQIs [23]. SQI-1 incorporated variables expected to decrease during incubation, namely progressive motility, plasma membrane integrity, the CTC F pattern, and acrosome integrity. SQI-2 incorporated variables expected to increase during capacitation, namely the CTC B and AR patterns and membrane hyper-fluidity.

$$SQI1 = \frac{(\text{Progressive motility \%}) + (\text{Plasma membrane integrity \%}) + (\text{F pattern \%}) + (\text{Acrosome integrity \%})}{4}$$

$$SQI2 = \frac{(\text{B pattern \%}) + (\text{AR pattern \%}) + (\text{Hyper – fluid membranes \%})}{3}$$

All component variables were expressed as percentages, and no differential weighting factors were applied.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Normality was assessed using the Shapiro–Wilk test applied to model residuals, and homogeneity of variance was evaluated using Levene’s test. The effects of treatment, incubation period, and the treatment × incubation period interaction were analyzed using two-way analysis of variance (ANOVA). When significant effects were detected, multiple comparisons were performed using Tukey’s post hoc test. The three independent pooled semen samples represented the experimental replicates. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of treatment and incubation period on sperm functional characteristics

Two-way ANOVA revealed significant treatment $\times$ incubation period interactions for the CTC F pattern ($p = 0.011$), membrane hyper-fluidity assessed using MC540 ($p < 0.001$), and SQI-2 ($p < 0.001$). No significant treatment $\times$ incubation period interactions were detected for the remaining sperm functional parameters or SQI-1 ($p > 0.05$).

Table 1 presents the functional characteristics of goat spermatozoa during the 4-h incubation period in CG, HG, and PG. Progressive motility, sperm morphology, and acrosome integrity remained relatively stable throughout incubation ($p > 0.05$). A significant main effect of incubation period on plasma membrane integrity was detected, with significantly lower values at T2 ($p = 0.005$) and T4 ($p = 0.001$) compared with T0, when considering all treatment groups collectively. However, comparisons among incubation periods within each treatment group (CG, HG, and PG) did not reveal statistically significant differences ($p > 0.05$). The combined temporal variation in these parameters and the CTC F pattern was further reflected in SQI-1.

Table 1: Functional characteristics of goat spermatozoa during *in vitro* incubation in CG, HG, and PG.

Group	Incubation period (h)	Progressive motility (%)	Viability (%)	Normal morphology (%)	F (%)	B (%)	AR (%)	Hyper-fluid membranes (%)	Acrosome integrity (%)
CG	T0	80.0 $\pm$ 0.00	78.7 $\pm$ 2.73	95.7 $\pm$ 0.88	81.3 $\pm$ 5.61	13.0 $\pm$ 4.00	5.7 $\pm$ 2.19 ^a	2.3 $\pm$ 1.20 ^a	90.7 $\pm$ 1.20
	T1	80.0 $\pm$ 2.89	76.0 $\pm$ 8.50	96.3 $\pm$ 1.45	83.0 $\pm$ 1.53	13.0 $\pm$ 3.21	4.3 $\pm$ 1.86 ^a	4.3 $\pm$ 2.19 ^{ab}	91.7 $\pm$ 1.45
	T2	76.7 $\pm$ 6.01	60.3 $\pm$ 3.71	95.3 $\pm$ 1.67	75.7 $\pm$ 1.20	16.0 $\pm$ 2.64	8.3 $\pm$ 1.45 ^{ab}	7.7 $\pm$ 0.67 ^{ab}	86.7 $\pm$ 3.53
	T4	70.0 $\pm$ 5.77	59.0 $\pm$ 7.00	92.0 $\pm$ 3.06	74.7 $\pm$ 1.45	12.0 $\pm$ 0.58	13.3 $\pm$ 2.03 ^b	10.3 $\pm$ 1.67 ^b	84.3 $\pm$ 0.88
HG	T0	73.3 $\pm$ 3.33	80.3 $\pm$ 1.76	96.0 $\pm$ 0.58	86.7 $\pm$ 3.48 ^a	11.3 $\pm$ 3.48 ^a	2.7 $\pm$ 0.33 ^a	3.3 $\pm$ 1.67 ^a	91.0 $\pm$ 2.08
	T1	75.0 $\pm$ 2.89	61.7 $\pm$ 1.20	94.0 $\pm$ 1.53	66.0 $\pm$ 3.21 ^b	23.0 $\pm$ 5.03 ^{ab}	11.0 $\pm$ 2.65 ^{ab}	34.7 $\pm$ 2.67 ^b	91.7 $\pm$ 2.96
	T2	73.3 $\pm$ 3.33	62.0 $\pm$ 6.51	94.0 $\pm$ 3.06	58.7 $\pm$ 4.06 ^b	28.0 $\pm$ 3.21 ^b	13.3 $\pm$ 0.88 ^b	38.7 $\pm$ 1.54 ^b	88.0 $\pm$ 2.89
	T4	75.0 $\pm$ 2.89	60.0 $\pm$ 6.67	94.0 $\pm$ 1.00	53.0 $\pm$ 0.58 ^b	29.7 $\pm$ 2.03 ^b	17.3 $\pm$ 2.40 ^b	41.0 $\pm$ 2.65 ^b	84.7 $\pm$ 2.19
PG	T0	71.7 $\pm$ 4.41	74.7 $\pm$ 7.69	94.3 $\pm$ 2.03	81.0 $\pm$ 5.29	15.7 $\pm$ 4.63	3.3 $\pm$ 0.88 ^a	4.7 $\pm$ 0.88 ^a	89.7 $\pm$ 2.33
	T1	75.0 $\pm$ 5.00	69.0 $\pm$ 5.13	95.0 $\pm$ 2.08	80.3 $\pm$ 4.63	15.0 $\pm$ 4.36	4.7 $\pm$ 0.88 ^{ab}	6.7 $\pm$ 2.85 ^{ab}	90.3 $\pm$ 1.33
	T2	78.3 $\pm$ 4.41	64.7 $\pm$ 6.17	93.7 $\pm$ 2.33	72.7 $\pm$ 4.06	19.0 $\pm$ 4.16	8.33 $\pm$ 0.67 ^{bc}	16.3 $\pm$ 2.91 ^{bc}	86.3 $\pm$ 1.20
	T4	75.0 $\pm$ 5.00	58.7 $\pm$ 4.41	94.3 $\pm$ 1.45	69.0 $\pm$ 0.00	19.7 $\pm$ 1.45	11.3 $\pm$ 1.46 ^c	24.0 $\pm$ 1.00 ^c	87.7 $\pm$ 1.20

Values are presented as mean $\pm$ SEM. Within each treatment group and column, values with different superscript letters (^a, ^b, ^c) differ significantly ($p < 0.05$). Progressive motility was assessed visually; viability and morphology were assessed using eosin–nigrosin staining; capacitation status was assessed using the CTC assay; membrane hyper-fluidity was assessed using MC540; and acrosome integrity was assessed using phase-contrast microscopy.

CG = Control group, HG = Heparin-supplemented group, PG = Progesterone-supplemented group, T0 = 0 h, T1 = 1 h, T2 = 2 h, T4 = 4 h, CTC = Chlortetracycline, F = Non-capacitated acrosome-intact pattern, B = Capacitated acrosome-intact pattern, AR = Acrosome-reacted pattern, MC540 = Merocyanine 540, SEM = Standard error of the mean.

Capacitation-associated changes in CG

In CG, the proportion of spermatozoa exhibiting the CTC F pattern gradually decreased, whereas the proportion exhibiting the B pattern increased; however, these changes were not statistically significant ($p > 0.05$). In contrast, the AR pattern increased significantly from 5.7 $\pm$ 2.19% at T0 to 13.3 $\pm$ 2.03% at T4, whereas membrane hyper-fluidity increased significantly from 2.3 $\pm$ 1.20% at T0 to 10.3 $\pm$ 1.67% at T4 ($p < 0.05$).

Capacitation-associated changes in HG

HG showed pronounced temporal changes in capacitation-associated parameters. The proportion of spermatozoa exhibiting the F pattern decreased significantly from 86.7 $\pm$ 3.48% at T0 to 66.0 $\pm$ 3.21% at T2, whereas membrane hyper-fluidity increased markedly from 3.3 $\pm$ 1.67% at T0 to 34.7 $\pm$ 2.67% at T1 ($p < 0.05$). The B pattern increased from 11.3 $\pm$ 3.48% at T0 to 28.0 $\pm$ 3.21% at T2, whereas the AR pattern increased from 2.7 $\pm$ 0.33% to 13.3 $\pm$ 0.88% over the same period ($p < 0.05$).

Capacitation-associated changes in PG

In PG, the proportion of spermatozoa exhibiting the F pattern decreased and the proportion exhibiting the B pattern increased during incubation; however, these changes were not statistically significant ($p > 0.05$). The AR pattern increased significantly from 3.3 $\pm$ 0.88% at T0 to 8.33 $\pm$ 0.67% at T2. Membrane hyper-fluidity also increased progressively from 4.7 $\pm$ 0.88% at T0 to 16.3 $\pm$ 2.91% at T2 and reached 24.0 $\pm$ 1.00% at T4 ($p < 0.05$).

Changes in SQI-1 during incubation

Table 2 presents SQI-1 and SQI-2 for goat spermatozoa during the 4-h incubation period in CG, HG, and PG. A significant overall effect of incubation period was detected for both indexes. For SQI-1, significant differences were observed between T0 and T2 ($p = 0.004$), T0 and T4 ($p < 0.001$), and T1 and T4 ($p = 0.007$).

No significant overall differences in SQI-1 were detected among CG, HG, and PG ($p > 0.05$). Within CG, SQI-1 decreased significantly from $82.6 \pm 2.30\%$ at T0 to $72.0 \pm 1.80\%$ at T4 ($p < 0.05$). Within HG, SQI-1 decreased significantly from $82.8 \pm 0.94\%$ at T0 to $73.6 \pm 1.74\%$ at T1 ($p < 0.05$). In contrast, SQI-1 remained relatively stable throughout incubation in PG ($p > 0.05$).

Table 2: Sperm quality indexes of goat spermatozoa during *in vitro* incubation in CG, HG, and PG.

Index	Treatment	T0	T1	T2	T4
SQI-1	CG	82.6 ± 2.30^x	82.7 ± 2.96^x	74.8 ± 2.09^{xy}	72.0 ± 1.80^y
	HG	82.8 ± 0.94^x	73.6 ± 1.74^y	70.5 ± 2.27^y	68.2 ± 1.18^y
	PG	79.2 ± 3.03	78.7 ± 3.74	75.5 ± 3.13	72.6 ± 2.06
SQI-2	CG	7.0 ± 2.27	7.2 ± 1.18^a	10.7 ± 0.19^a	11.9 ± 0.22^a
	HG	5.8 ± 1.79^x	22.9 ± 1.28^{by}	26.7 ± 1.15^{byz}	29.3 ± 1.02^{bz}
	PG	7.9 ± 1.93^x	8.8 ± 2.42^{ax}	14.6 ± 2.32^{axy}	18.3 ± 0.33^{cy}

Values are presented as mean $\pm$ SEM. Different superscript letters within columns (^a, ^b, ^c) indicate significant differences among treatments within the same incubation period, whereas different superscript letters within rows (^x, ^y, ^z) indicate significant differences among incubation periods within the same treatment ($p < 0.05$).

CG = Control group, HG = Heparin-supplemented group, PG = Progesterone-supplemented group, SQI-1 = Sperm quality index 1, SQI-2 = Sperm quality index 2, T0 = 0 h, T1 = 1 h, T2 = 2 h, T4 = 4 h, SEM = Standard error of the mean.

Changes in SQI-2 during incubation

For SQI-2, significant overall differences were detected between T0 and T2 ($p = 0.015$) and between T0 and T4 ($p = 0.002$). A significant treatment effect was also observed, with overall SQI-2 values being significantly higher in HG than in CG ($p < 0.001$) and PG ($p = 0.008$). No significant overall difference was detected between CG and PG ($p = 0.475$).

Significant between-treatment differences in SQI-2 were observed at T1, T2, and T4. Specifically, HG exhibited higher SQI-2 values than PG at T1 ($22.9 \pm 1.28\%$ vs. $8.8 \pm 2.42\%$), T2 ($26.7 \pm 1.15\%$ vs. $14.6 \pm 2.32\%$), and T4 ($29.3 \pm 1.02\%$ vs. $18.3 \pm 0.33\%$) ($p < 0.05$, according to the post hoc comparisons presented in Table 2). These findings show that SQI-2 responds differently over time to heparin supplementation than CG and PG.

DISCUSSION

Comparative effects of heparin and progesterone

The present study demonstrated that incubation time and supplementation with capacitating agents significantly influenced the functional status of goat spermatozoa under *in vitro* conditions. Among the evaluated treatments, HG induced the most pronounced capacitation-associated changes, characterized by reductions in non-capacitated spermatozoa together with increases in membrane hyper-fluidity, capacitated spermatozoa, acrosome-reacted spermatozoa, and SQI-2 values. In contrast, PG produced a more gradual response while maintaining relatively stable SQI-1 values throughout incubation. These findings indicate that both agents induced functional changes compatible with sperm capacitation, although with different magnitudes and temporal patterns.

Heparin induced capacitation-associated responses

The responses observed in HG are consistent with the recognized role of heparin as an effective capacitating agent in mammalian spermatozoa [5, 10]. Heparin supplementation promoted a progressive reduction in spermatozoa exhibiting the F pattern together with increases in the B and AR patterns, consistent with membrane remodeling events associated with sperm capacitation and the acrosome reaction. These findings agree with previous studies demonstrating the capacitating effect of heparin in goat spermatozoa.

Using the same concentration evaluated in the present study ($20 \mu\text{g/mL}$), Goel *et al.* [21] reported that 60 min of sperm pretreatment resulted in significantly greater cleavage and blastocyst production than other heparin concentration–time combinations. Although their study assessed subsequent *in vitro* fertilization outcomes rather than membrane-associated changes, the findings support the functional relevance of this heparin concentration in goats. In the present study, $20 \mu\text{g/mL}$ heparin induced an early increase in membrane hyper-fluidity from 3.3% at T0 to 34.7% at T1, followed by progressive changes in CTC patterns. Sahoo *et al.* [12] similarly reported progressive capacitation and acrosomal responses during the first hours of heparin exposure, although differences in concentration and experimental conditions limit direct quantitative comparisons. The marked increase in membrane hyper-fluidity observed in HG is consistent with membrane remodeling processes associated with sperm capacitation [6, 11].

Progesterone-induced capacitation-associated responses

Progesterone supplementation also induced capacitation-associated changes, although these responses were less pronounced than those observed in HG. The gradual increases in acrosome-reacted spermatozoa and membrane hyper-fluidity indicate that progesterone modulated sperm functional activation during incubation. Previous reports have shown that progesterone participates in sperm activation and the acrosome reaction through calcium-dependent physiological responses [13–15].

Previous reports have described progesterone-induced capacitation and acrosomal responses in goat spermatozoa [16]. However, differences in experimental design should be considered when comparing results across studies. Somanath *et al.* [22] reported that 2 µg/mL progesterone produced the greatest acrosomal response among the concentrations evaluated. In the present study, the same concentration induced a more gradual response, with acrosome-reacted spermatozoa increasing from 3.3% at T0 to 8.3% at T2 and membrane hyper-fluidity increasing from 4.7% at T0 to 24.0% at T4. These findings suggest that progesterone can induce capacitation-associated changes in goat spermatozoa, but at a slower temporal pace than heparin under the conditions evaluated.

Temporal dynamics of membrane remodeling

The distinct temporal responses observed between HG and PG may reflect differences in the physiological processes influenced by these agents. Heparin, a glycosaminoglycan, has been associated with membrane remodeling and downstream signaling events involved in capacitation, whereas progesterone has been linked to sperm activation through calcium-dependent signaling pathways [10–15].

A notable finding was the marked increase in membrane hyper-fluidity during the first hour of incubation, particularly in HG, whereas changes in CTC fluorescence patterns developed more gradually across the evaluated time points. This temporal difference may reflect the distinct membrane-associated events detected by the two assays. MC540 is sensitive to early alterations in membrane lipid organization and phospholipid packing associated with increased membrane fluidity, whereas CTC fluorescence patterns reflect subsequent membrane-associated changes that develop progressively during capacitation [6, 20]. Therefore, early detection of increased MC540-positive spermatozoa at T1 may reflect an earlier stage of membrane remodeling that precedes the progressive transition from the F pattern to the B and AR patterns.

Sperm quality preservation during incubation

Plasma membrane integrity declined overall during incubation, whereas progressive motility remained relatively stable. The reduction in plasma membrane integrity may reflect progressive membrane remodeling under *in vitro* incubation conditions, although it could also reflect deterioration. Similar declines in sperm quality during prolonged incubation have been reported in small ruminants and may accompany physiological changes related to sperm activation and capacitation [25, 26].

In contrast, the relative stability of progressive motility suggests that this parameter was less responsive to the short-term incubation conditions than membrane-associated functional variables. Recent advances in goat semen biotechnology have similarly emphasized the importance of plasma membrane integrity and oxidative stability for maintaining sperm functionality during *in vitro* handling and semen preservation [27, 28].

Utility of sperm quality indexes

A key aspect of the present study was applying SQI-1 and SQI-2 to characterize sperm functional dynamics during capacitation. Individual sperm parameters provide information on specific aspects of sperm function, whereas SQIs integrate multiple variables and may help interpret the balance between preserving sperm quality and acquiring capacitation-associated characteristics.

SQI-1 did not differ significantly among treatment groups, although temporal reductions were observed in CG and HG. In contrast, SQI-1 remained relatively stable in PG. SQI-2 showed a clearer treatment-dependent response and was significantly higher overall in HG than in CG and PG. The increase in SQI-2 in HG reflected the concurrent increase in capacitated spermatozoa, acrosome-reacted spermatozoa, and membrane hyper-fluidity during incubation.

Although SQIs were originally proposed to evaluate frozen–thawed goat semen [23], these findings suggest they may also provide useful complementary information during *in vitro* capacitation. In particular, SQI-2 appeared useful for integrating capacitation-associated functional changes that might otherwise be evaluated separately.

Practical implications

From a practical perspective, the different responses observed between HG and PG may matter when selecting capacitation conditions based on the desired balance between sperm activation and preservation of sperm functional quality during incubation. Under the present experimental conditions, heparin induced a faster and more pronounced capacitation-associated response, whereas progesterone produced a more gradual pattern.

Previous studies in mammalian spermatozoa have suggested that heparin and progesterone may exert complementary effects during *in vitro* fertilization by influencing sperm release, membrane fluidity, and fertilizing competence under capacitating conditions [24]. Based on the distinct temporal responses observed in the present study, sequential or combined exposure to these agents may warrant further investigation. However, such an approach was not evaluated in the present study, and no conclusions regarding potential additive or synergistic effects can be drawn.

Study limitations

This study has several limitations. Although the observer was blinded to treatment allocation during microscopic assessments, the incubation time was known; therefore, a potential influence of observer bias related to incubation time cannot be completely excluded. The experimental design assessed sperm functional responses under *in vitro* conditions without evaluating fertilization, cleavage, blastocyst development, or other embryo production outcomes. Therefore, the observed capacitation-associated changes cannot be assumed to translate directly into improved fertilizing capacity.

Moreover, the application of SQIs to *in vitro* capacitation represents an extension beyond the context in which these indexes were originally proposed for frozen–thawed goat semen. Therefore, the changes observed in SQI values should be interpreted as integrative descriptors of the functional variables included in each index rather than as validated indicators of fertilizing competence.

In addition, biological replication was limited to three independent pooled semen samples obtained during separate collection sessions from the same two bucks. Consequently, buck-specific responses could not be determined, and the limited number of semen donors restricts the generalizability of the findings. Furthermore, the study focused primarily on functional and membrane-associated endpoints and did not evaluate molecular markers or intracellular signaling pathways involved in capacitation.

Future perspectives

Future studies should include more bucks to assess individual variability and improve the generalizability of the findings. Incorporation of *in vitro* fertilization and embryo-development outcomes would be important for determining whether the observed functional changes and SQI responses are associated with fertilization competence and subsequent embryo development. Evaluation of molecular and biochemical markers of capacitation may also help clarify the signaling pathways associated with the distinct temporal responses induced by heparin and progesterone.

The potential sequential or combined use of heparin and progesterone should also be investigated experimentally to determine whether their different temporal effects can be used to optimize sperm preparation protocols without compromising sperm functional quality.

Overall interpretation

Overall, the present findings provide comparative evidence of distinct temporal functional responses of goat spermatozoa to heparin and progesterone during *in vitro* capacitation. Under the evaluated conditions, HG produced more pronounced capacitation-associated responses than PG, particularly in membrane hyper-fluidity, CTC patterns, and SQI-2. In contrast, PG induced more gradual functional changes while maintaining relatively stable SQI-1 values. The use of SQIs provided an integrative approach for evaluating sperm functional dynamics during incubation and may complement conventional sperm assessments when optimizing *in vitro* capacitation protocols in goats.

CONCLUSION

Heparin and progesterone produced distinct temporal patterns of functional change in goat spermatozoa during *in vitro* incubation. Under the concentrations and experimental conditions evaluated, heparin induced more pronounced capacitation-associated responses than progesterone, particularly in membrane hyper-fluidity,

CTC patterns, and SQI-2, whereas progesterone produced more gradual functional changes and maintained relatively stable SQI-1 values. The combined assessment of conventional sperm parameters, capacitation-associated markers, and SQIs provided an integrative description of sperm functional dynamics across incubation. These findings are restricted to the concentrations, incubation conditions, and functional endpoints evaluated in the present study and should not be interpreted as evidence of improved fertilizing competence. Although SQIs provided complementary information for describing sperm functional responses, their relationship with fertilization, cleavage, and embryo-development outcomes remains to be established. Further studies incorporating these reproductive outcomes are required to determine the biological and predictive relevance of SQI responses during *in vitro* capacitation.

DATA AVAILABILITY

The data supporting the findings of this study are included within the article. The dataset used for the statistical analyses are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

The authors declare that no generative artificial intelligence tools were used in the preparation of this manuscript.

AUTHORS' CONTRIBUTIONS

AJCP: Conceptualization, investigation, methodology, data analysis, writing – original draft, and writing – review and editing. XSMM and LFHM: Investigation, methodology, and writing – review and editing. AM: Study design, supervision, data analysis, and writing – review and editing. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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