

Effect of capacitating agents on sperm pretreatment during in vitro fertilization for blastocyst production in caprines

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Received: 23.09.2015 • Accepted/Published Online: 13.05.2016 • Final Version: 15.12.2016

Abstract: The present study aimed at comparing the relative efficacy of different capacitating agents used for sperm pretreatment during in vitro fertilization (IVF) and subsequent embryo development of caprine oocytes. In experiment 1, the effect of different concentrations of heparin (0, 20, and 50 µg/mL) on sperm pretreatment for different periods of time (30 and 60 min) was studied. In experiment 2, the dose-dependent effect of calcium ionophore (0, 0.1, 0.2, and 0.5 µM) on sperm pretreatment was assessed. Experiment 3 validated and compared the effects of both capacitating agents on sperm pretreatment for capacitation based on the results of the first two experiments. The treated sperms from each group were used for in vitro fertilization and subsequent embryo development. The results indicated that in experiment 1, capacitation with heparin at 20 µg/mL for 60 min had significantly higher ($P < 0.05$) cleavage and blastocyst production. In experiment 2, capacitation with calcium ionophore at 0.1 µM had significantly more morulae ($P < 0.05$) and numerically more blastocyst production. In experiment 3, capacitation with heparin at 20 µg/mL had a significantly higher ($P < 0.05$) blastocyst production rate as compared to calcium ionophore treatment. Based on this study, heparin can be used to enhance capacitation of freshly collected sperms during in vitro fertilization and subsequent embryo development.

Key words: Caprine, capacitation, cleavage, blastocyst, in vitro fertilization

1. Introduction

In vitro embryo production has emerged as a better alternative to the in vivo embryo production technique for large-scale production of embryos to be used for assisted reproductive technologies. The reduced developmental competence of oocytes collected from abattoir ovaries limits the suitability of these oocytes for reproductive biotechnology research and retards the application of in vitro embryo production for commercial embryo technology.

During epididymal transit, sperms acquire the ability to move progressively; however, they are still incompetent in terms of fertilization (1). Fertilization capacity is gained after the sperm cells reside in the female reproductive tract for a definite period of time. In the female genital tract, mammalian spermatozoa undergo a unique maturational change, i.e. capacitation (2). Capacitation encompasses several cellular changes in the sperm, particularly in the distribution and composition of certain glycoproteins, protein tyrosine phosphorylation, and intracellular

Ca²⁺ and cAMP concentrations, as well as motility patterns (3). This phenomenon is an absolute prerequisite that spermatozoa must undergo in order to interact efficiently with the zona pellucida and to accomplish one of the last steps leading to fertilization, namely the acrosome reaction. Thus, capacitation is a critical biological process in spermatozoa to acquire the ability to fertilize the in vitro matured oocytes. It has been reported that chemically defined media with different capacitating agents, i.e. heparin, calcium ionophore, and caffeine (4–9), are able to induce capacitation of spermatozoa used for in vitro fertilization.

In vitro fertilization rates may be variable, depending upon the capacitating agent and concentration used. Therefore, efforts have to be made to standardize the semen capacitating process to improve the quality and quantity of in vitro-produced embryos. Selection of the capacitating agent, its concentration, and exposure time may play an important role in in vitro fertilization and subsequent in vitro embryo development. To date, meager attempts

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have been made to understand the dose-dependent effect of capacitating agents in small ruminants. Realizing the importance of this technology, the present study was carried out comparing different capacitating agents, doses of capacitating agents, and capacitation times to assess the impact of sperm pretreatment on in vitro fertilization and embryo developmental competence of caprine oocytes.

2. Materials and methods

All experimental procedures under study were undertaken according to the guidelines approved by the institutional animal ethics committee. All chemicals used were procured from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Collection and processing of ovaries

Caprine ovaries were obtained from a local slaughter house and transported to the laboratory in a thermos flask containing 0.9% sterile warm physiological normal saline solution supplemented with antibiotics. All the ovaries were then subjected to 5 or 6 serial washings with warm saline fortified with antibiotics and brought into a laminar flow for processing.

2.2. Oocyte collection and in vitro maturation

The oocytes were collected from each ovary into a petri plate containing oocyte collection media (Dulbecco's phosphate-buffered saline with 3 mg/mL BSA) by follicle puncture method with the help of a B.P. blade. Each petri dish containing oocytes was evaluated under a stereo zoom microscope and grade A, B, and C oocytes were chosen for in vitro maturation as per the method described by Kharche et al. (10). Selected oocytes were washed four or five times in oocyte holding medium (OHM- TCM-199 with HEPES modification, FBS 10%, sodium pyruvate 0.25 mM, gentamycin 50 µg/mL, glutamine 100 µg/mL, BSA 3 mg/mL). The oocytes were then washed three times with oocyte maturation medium and matured as per the method described by Kharche et al. (10).

2.3. Semen collection and in vitro capacitation

Semen ejaculate was collected from a fertile buck using an artificial vagina. Immediately after semen collection, ejaculate was assessed in terms of volume, color, consistency, and progressive and gross motility at a total magnification of 200 ×. Calcium ionophore A23187 (Sigma Chemical Co.) stock solution was prepared as stock in dimethyl sulfoxide and was frozen at -20 °C. Before use this was thawed and diluted with modified Tyrode's medium (TALP) as described by Parrish et al. (6) to make final concentrations of 0.1, 0.2, and 0.5 µmol. Similarly, heparin (Sigma Chemical Co.) stock solution was prepared as stock in ultrapure water (Sigma Chemical Co.) and frozen at -20 °C. Before use this was thawed and supplemented with modified TALP as described by Parrish

et al. (6) to make final concentrations of 20 and 50 µL/mL. The medium stock was prepared every week and was sterilized by Millipore filtration (pore size of 0.22 µm). The method of sperm pretreatment and in vitro fertilization was followed based on methodology described by Singh et al. (11) with minor modifications. First, 100 µL of semen ejaculate was diluted with 5 mL of sperm TALP medium and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the sperm pellet was resuspended in the same medium. This step was repeated three times to remove all seminal plasma. After a final washing, 50 µL of sperm pellet from each group was added to 900 µL of each group of fertilization medium (TALP solution containing 10% FBS, 8 mg/mL fatty acid-free BSA) with different doses of capacitating agents. The first experiment was conducted to assess the dose-dependent effect of heparin in sperm pretreatment for two different incubation periods (30 and 60 min) on in vitro embryo development potential. In experiment 1, sperms were capacitated as follows: Group 1, heparin 20 µg/mL for 30 min; Group 2, heparin 50 µg/mL for 30 min; Group 3, heparin 20 µg/mL for 60 min; Group 4, heparin 50 µg/mL for 60 min; Group 5 (control), heparin 0.0 µg/mL for 60 min.

Based on the results of the first experiment on incubation period, the second experiment was carried out to study the dose-dependent effect of calcium ionophore for 60 min of incubation for sperm pretreatment during in vitro fertilization. Experiment 2 investigated the effect of 60 min of calcium ionophore exposure at different concentrations (0, 0.1, 0.2, and 0.5 µM) on sperm pretreatment/capacitation. Sperm pellets were divided into four groups and capacitated as follows: Group 1, calcium ionophore (0.1 µM); Group 2, calcium ionophore (0.2 µM); Group 3, calcium ionophore (0.5 µM); Group 4 (control), calcium ionophore (0.0 µM) for 60 min. The third experiment was carried out based on the first two experiments to validate and compare the effect of the two different capacitating agents with optimum dose on sperm pretreatment for capacitation during in vitro fertilization as estimated in experiments 1 and 2 on sperm pretreatment. Sperm pretreatment was done for 60 min in fertilization TALP medium containing capacitating agents as follows: Group 1, heparin (20 µg/mL); Group 2, calcium ionophore (0.1 µM); Group 3, control (without any capacitating agent) in humidified atmosphere of 5% CO₂ at 38.5 ± 1 °C in a CO₂ incubator.

2.4. In vitro fertilization

After 27 h of in vitro maturation, oocytes were denuded in 0.1% hyaluronidase and passed repeatedly through a fine pipette for 3–5 min. After 27 h of culture, oocytes were evaluated for cumulus expansion and the presence of a first polar body. Only the mature oocytes were picked up

and denuded in 0.1% hyaluronidase by repeated pipetting through a fine pipette for 3–5 min. The denuded oocytes were washed 5 or 6 times in a fert-TALP medium and then transferred into drops of fert-TALP medium (50 μ L) under sterile mineral oil equilibrated for 2 h at 38.5 ± 1 °C in a CO₂ incubator. At the time of sperm insemination, 25 μ L of fertilization medium was removed from preincubated fertilization drops containing matured oocytes, and 25 μ L of capacitated semen from different groups was inseminated into each fertilization drop containing mature oocytes with denuded cumulus cell mass for 18 h under humidified atmosphere of 5% CO₂ at 38.5 ± 1 °C in a CO₂ incubator.

2.5. In vitro embryo culture

Eighteen hours after the insemination, presumptive zygotes were washed in order to separate adhering sperm cells. Presumptive zygotes from different groups were cultured in embryo development medium (mCR₂aa medium containing 10% FBS and 3 mg/mL BSA) for 8–10 days in humidified atmosphere of 5% CO₂ at 38.5 ± 1 °C in a CO₂ incubator.

2.6. Evaluation of in vitro embryo development

Presumptive zygotes were observed for embryo development and half of the culture medium was replenished with fresh medium at intervals up to 12 days. Embryos were morphologically evaluated under an inverted phase contrast microscope (TE 2000, Nikon, Japan).

2.7. Statistical analysis

All data were subjected to statistical analysis using the chi-square test (χ^2). Cleavage rates and embryo development between treatment groups were compared using the chi-square test. A probability of $P < 0.05$ was considered to be statistically significant (12).

3. Results

In the present study, the dose-dependent effect of different capacitating agents on cleavage rate and in vitro embryo development with special reference to blastocyst production was evaluated, validated, and compared. A total of 2795 oocytes from 1104 ovaries were recovered by follicle puncture technique, resulting in an average recovery rate of 2.53 culturable oocytes per ovary. Three independent experiments were conducted to assess the effect of sperm pretreatment on in vitro embryo development potential.

3.1. Dose-dependent effect of heparin with respect to different sperm incubation periods during in vitro fertilization

In the control group, no cleavage was observed; therefore, embryo development was not studied in this group. In experiment 1, the dose-dependent effect of heparin (0, 20, and 50 μ g/mL) with respect to two different sperm incubation times (30 and 60 min) was evaluated and summarized (Table 1). Cleavage rate differed significantly among treated groups and was significantly higher (49.46%, $P < 0.05$) in the sperm pretreatment group with 20 μ g/mL concentration of heparin for 60 min compared to the rest of groups. Similarly, blastocyst production among treated groups was significantly higher (11.68%, $P < 0.05$) in the sperm pretreatment group with 20 μ g/mL concentration of heparin for 60 min compared to the rest of groups. Significantly ($P < 0.05$) higher morula production rate was also observed in the group treated with heparin (20 μ g/mL) for 60 min compared to the groups treated with heparin (50 μ g/mL) for 30 min and heparin (50 μ g/mL) for 60 min. Findings indicate that a lower concentration of heparin (20 μ g/mL) for a longer duration (60 min) favored blastocyst production ($P < 0.05$) as compared to a higher concentration of heparin (50 μ g/mL) for a shorter

Table 1. Dose-dependent effect of heparin with respect to different sperm incubation period.

| Gr. | Capacitating agent | No. of ovaries | No. of oocytes | Cleavage | 2-cell | 4-cell | 8–16-cell | Morulae | Blastocysts |
|-----|------------------------------------|----------------|----------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| 1 | Heparin (20 μ g/mL) for 30 min | 70 | 187 | 6 ^a (3.21%) | 1 ^a (16.67%) | 2 ^a (33.33%) | 2 ^a (33.33%) | 1 ^a (16.67%) | 0 ^a (0.00%) |
| 2 | Heparin (50 μ g/mL) for 30 min | 89 | 250 | 21 ^b (8.40%) | 6 ^b (28.57%) | 4 ^b (19.05%) | 8 ^a (38.10%) | 2 ^b (9.52%) | 1 ^b (4.76%) |
| 3 | Heparin (20 μ g/mL) for 60 min | 96 | 277 | 137 ^c (49.46%) | 16 ^c (11.68%) | 27 ^b (19.71%) | 37 ^b (27.01%) | 41 ^c (29.93%) | 16 ^c (11.68%) |
| 4 | Heparin (50 μ g/mL) for 60 min | 79 | 232 | 94 ^d (40.52%) | 17 ^d (18.09%) | 13 ^c (13.83%) | 37 ^a (39.36%) | 23 ^c (24.47%) | 4 ^b (4.26%) |
| 5 | TALP (control) | 73 | 177 | 0 ^e (0.00%) | 0 ^e (0.00%) | 0 ^d (0.00%) | 0 ^e (0.00%) | 0 ^d (0.00%) | 0 ^a (0.00%) |

Values with different superscripts (a–d) in the same column are significantly different ($P < 0.05$).

duration (30 min). Based on the experiment, treatment of oocytes with heparin at 20 µg/mL for 60 min favored a higher percentage ($P < 0.05$) of blastocyst production in comparison to the rest of the groups. Results obtained from this protocol were used for its validation and confirmation in experiment 3.

3.2. Effects of different concentrations of calcium ionophore during in vitro fertilization

In the control group, no cleavage was observed; therefore, embryo development was not studied in this group. In experiment 2, the dose-dependent effect of different concentrations of calcium ionophore (0, 0.1, 0.2, and 0.5 µM) on sperm pretreatment was evaluated and summarized (Table 2). The cleavage rate was significantly higher ($P < 0.05$) with calcium ionophore at 0.2 µM as compared to other treated groups. A nonsignificant difference in the blastocyst formation rate was observed between groups treated with 0.1 and 0.2 µM calcium ionophore; numerically, more blastocyst formation was observed in sperm treated with 0.1 µM calcium ionophore. The study revealed a significantly lower percentage ($P < 0.05$) of 8–16-cell embryo arrest and higher morula production rates in the group treated with calcium ionophore (0.1

µM) compared to the other groups. Results obtained from this protocol were used for its validation and confirmation in experiment 3.

3.3. Validation and comparison of optimum dose of capacitating agents used in experiments 1 and 2 on sperm pretreatment during in vitro fertilization on blastocyst production

In the control group, no cleavage was observed; therefore, embryo development was not studied in this group. In experiment 3, the comparative efficacy of different capacitating agents with optimum doses of heparin (20 µg/mL) and calcium ionophore (0.1 µM) on sperm pretreatment for capacitation was evaluated (Table 3). Cleavage rate did not differ significantly between the calcium ionophore and heparin treatment groups ($P > 0.05$). However, blastocyst production was significantly higher ($P < 0.05$) in the heparin-treated group compared to the calcium ionophore-treated group.

4. Discussion

Capacitation is a crucial biological process that mammalian sperm must undergo in order to achieve fertilizing ability. Glycosaminoglycans, normally present

Table 2. Effects of different concentrations of calcium ionophore with standardized incubation period.

| Gr. | Capacitating agent | No. of ovaries | No. of oocytes | Cleavage | 2-cell | 4-cell | 8–16-cell | Morulae | Blastocysts |
|-----|----------------------------|----------------|----------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------|
| 1 | Calcium ionophore (0.1 µM) | 67 | 118 | 43 ^a (36.44%) | 8 ^a (18.60%) | 12 ^a (27.91%) | 11 ^a (25.58%) | 10 ^a (23.26%) | 2 ^a (4.65%) |
| 2 | Calcium ionophore (0.2 µM) | 62 | 169 | 82 ^b (48.52%) | 17 ^a (20.73%) | 19 ^a (23.17%) | 36 ^b (43.90%) | 8 ^b (9.76%) | 2 ^a (2.44%) |
| 3 | Calcium ionophore (0.5 µM) | 67 | 174 | 0 ^c (0.00%) | 0 ^b (0.00%) | 0 ^b (0.00%) | 0 ^c (0.00%) | 0 ^c (0.00%) | 0 ^b (0.00%) |
| 4 | TALP (control) | 71 | 173 | 0 ^c (0.00%) | 0 ^b (0.00%) | 0 ^b (0.00%) | 0 ^c (0.00%) | 0 ^c (0.00%) | 0 ^b (0.00%) |

Values with different superscripts (a–c) in the same column are significantly different ($P < 0.05$).

Table 3. Comparison of different capacitating agents with optimum concentration and incubation period on blastocyst development.

| Gr. | Capacitating agent | No. of ovaries | No. of oocytes | Cleavage | 2-cell | 4-cell | 8–16-cell | Morulae | Blastocysts |
|-----|----------------------------|----------------|----------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|
| 1 | Calcium ionophore (0.1 µM) | 142 | 377 | 171 ^a (45.36%) | 25 ^a (14.62%) | 43 ^a (25.15%) | 62 ^a (36.26%) | 35 ^a (20.47%) | 6 ^a (3.51%) |
| 2 | Heparin (20 µg/mL) | 157 | 297 | 144 ^a (48.48%) | 24 ^a (16.67%) | 34 ^a (23.61%) | 37 ^b (25.69%) | 35 ^a (24.31%) | 14 ^b (9.72%) |
| 3 | TALP (control) | 131 | 364 | 0 ^b (0.00%) | 0 ^b (0.00%) | 0 ^b (0.00%) | 0 ^c (0.00%) | 0 ^b (0.00%) | 0 ^c (0.00%) |

Values within different superscripts (a, b) in the same column are significantly different ($P < 0.05$).

in the oviductal fluid, have been regarded as potential *in vivo* capacitating agents (13). Proposed mechanisms of the induction of acrosome reactions by glycosaminoglycans are those of stimulation of passive or active calcium ion influx, or direct promotion of membrane fusion by other mechanisms (14). Calcium ionophore, heparin, caffeine, and other glycosaminoglycans are known to enhance this process (5–7,9). Spermatozoa of fresh semen require a period of incubation to induce the capacitation by complex transmembrane and intracellular signaling pathways. The present studies compared the effects of different sperm pretreatments, which are key determinants of efficiency of *in vitro* embryo production.

Heparin is often added to fertilization medium to induce capacitation *in vitro* using different concentrations ranging from 2 to 100 µg/mL (8,9,15,16). Addition of heparin at optimized concentration during sperm incubation for 15 min (15), 30 min (7), 45 min (15,17), and 60 min (4) was adequate for *in vitro* capacitation. The present study assessed and correlated the dose-dependent effects of different capacitating agents for two different incubation periods on sperm pretreatment to improve cleavage rate and blastocyst production in goats. In experiment 1, the dose-dependent effect of heparin (0, 20, and 50 µg/mL) for two different periods (30 min and 60 min) of sperm incubation was evaluated. Pretreatment of sperms with heparin for different periods of incubation revealed significantly different cleavage and embryo development rates. Statistical analysis revealed a significantly higher ($P < 0.05$) cleavage rate in sperm pretreatment with 60 min of incubation time in comparison to 30 min of incubation time for capacitation. Similarly, morula production was significantly higher ($P < 0.05$) with 20 µg/mL heparin for 60 min as compared to 50 µg/mL heparin for 30 min. Significantly higher ($P < 0.05$) blastocyst formation rate was observed in the sperm group treated with 20 µg/mL heparin compared to the sperm group treated with 50 µg/mL heparin for 60 min of incubation. This is consistent with the results of IVF studies stating that proper adjustment of both heparin concentration and sperm incubation time is crucial in the fertilization medium (6,18,19). The present findings are in agreement with an earlier report showing that sperm pretreatment with heparin for 15 min of exposure lowered the cleavage rate and *in vitro* development of embryos (20). The present findings also revealed no cleavage in the sperm pretreatment group with 0.0 µg/mL heparin (control), while oocytes were found to have cleaved in the sperm group pretreated with heparin. Similar to the present findings, heparin treatment of sperms improved cleavage and blastocyst rates compared to sperm not treated with heparin with unsorted sperm (21). Collectively, the present study revealed significantly higher ($P < 0.05$) cleavage and

morulae in the 20 µg/mL heparin-treated group compared to 50 µg/mL heparin treatments for 60 min of incubation time. Results indicated that 60 min of incubation for sperm pretreatment is required for obtaining an efficient cleavage rate and *in vitro* embryo development. This indicates that pre incubation may facilitate the process of capacitation. An earlier report also concluded that capacitation and the acrosome reaction of a portion of the sperm population can be induced within 60 min in medium with caffeine and heparin (22). Similarly, Zhou et al. (23) treated goat sperm with 50 µg/mL heparin for 0, 10, 20, 30, 45, 60, or 120 min. Although heparin treatments for 45 to 120 min did not differ significantly ($P > 0.05$) in capacitated sperm percentages, sperm motility and membrane integrity decreased significantly when sperm was treated with heparin for 120 min. They suggested that the optimal exposure time of heparin at 50 µg/mL for goat sperm capacitation could be 45 to 60 min. They also compared the effect of different doses (5, 10, 25, 50, and 100 µg/mL) of heparin treatment on acrosome reaction and suggested that the optimal dose of heparin could be 50 µg/mL for goat sperm capacitation. Other evidence also supports an efficient cleavage rate and blastocyst production yield in sperm pretreatment groups with 50 µg/mL of heparin (4,16).

Evaluation of the dose-dependent effect of capacitating agents on *in vitro* embryo development demonstrated that sperm pretreatment with 20 µg/mL heparin significantly enhances cleavage and blastocyst production rate compared to 50 µg/mL heparin under *in vitro* conditions. This may be due to the fact that a higher concentration of heparin might increase the chances of polyspermy. Possible effects of polyspermy may lead to reduced cleavage rate and *in vitro* embryo development potential. Evidence also supports efficient cleavage rate and blastocyst production yield in sperm pretreatment with 50 µg/mL heparin (4,15,23). Similar to the present study, Shi (18) found a significant increase in embryo yield when the heparin concentration was greater than 18 µg/mL. The present study observed no cleavage in a sperm pretreatment group with 0 µg/mL heparin, favorably supported by Shi (18).

Calcium ionophore A23187 has been shown to induce the acrosome reaction in mammalian spermatozoa (4). During this process acrosomal enzymes (hyaluronidase and acrosin) and motility of sperms are responsible for digestion of the ZP matrix. Once the two meet, a calcium influx occurs, causing a signaling cascade. This facilitates the penetration of the ovum and fertilization. Optimizing the dose of calcium ionophore during sperm preparation could be beneficial for sperm treatment. Experiment 2 was conducted to determine the optimal concentration of calcium ionophore for sperm pretreatment for capacitation during *in vitro* fertilization. Beneficial

effects of calcium ionophore on sperm pretreatment are seen in the present study. The present study revealed a significantly higher cleavage rate in oocytes fertilized with sperm pretreated with calcium ionophore compared to the control. Possibly, however, the better IVF results from calcium ionophore treatment may be related to the involvement of calcium ionophore A23187 and caffeine in the IVF medium. Spermatozoa undergo an acrosomal reaction when exposed to ionophore A23187, which increases intracellular calcium (24). The present findings are in agreement with those of Tanphaichitr and Hansen (25). They observed higher percentages of acrosome-free spermatozoa in A23187-treated samples in comparison to control samples incubated in culture medium only (25).

The cleavage rate following pretreatment of sperm with 0.2 μM calcium ionophore was significantly higher ($P < 0.05$) compared to 0.1 μM . However, in the present study, significantly higher ($P < 0.05$) morula production was observed in oocytes fertilized with sperms pretreated with 0.1 μM calcium ionophore. Similarly, numerically more cleaved oocytes reached the blastocyst stage in the sperm group treated with 0.1 μM calcium ionophore. However, no significant difference was observed in blastocyst production between 0.1 and 0.2 μM calcium ionophore concentrations. From the present findings it could be suggested that with increasing doses (0.1 μM to 0.2 μM) of calcium ionophore, embryo development potential decreases (Table 2). Earlier reports also support the present findings (4,26).

Treatment with calcium ionophore A23187 induces an increase in intracellular calcium and increased intracellular calcium has been functionally linked to sperm capacitation (5). As a result of their hydrophobic characteristics, calcium ionophores are able to transport ions across membranes. The increase in free calcium ionophore within the cell directly induces the acrosome reaction and leads to capacitation (19). Several researchers also reported beneficial effects of calcium ionophore treatment on in vitro fertilization, cleavage, and subsequent embryo development competence (27,28). In the present study fertilized oocytes did not cleave in the sperm pretreatment group containing 0.5 μM calcium ionophore, which suggested that a higher concentration (0.5 μM) of calcium ionophore has detrimental effects on cleavage rate and subsequent embryo development. The results suggest that calcium ionophore (0.1 μM) is effective for sperm pretreatment to induce capacitation during in vitro fertilization and subsequent in vitro development. Wang et al. (26) found higher cleavage, morula, and blastocyst production rates in sperm treatment with 0.1 μM of calcium ionophore+caffeine as compared to another sperm treatment (with 5% ewe estrous serum).

Finally, based on the results of experiments 1 and 2, a third experiment was conducted to validate and compare the optimum dose of heparin and calcium ionophore for sperm pretreatment during in vitro fertilization. Different capacitating agents were used in assisted reproductive technology to induce capacitation. In this study no significant difference ($P > 0.05$) was observed in the cleavage, 2-cell, 4-cell, and morula production rates between the heparin- and calcium ionophore-treated groups. Heparin apparently binds to sperm and plays a role in the sperm uptake of calcium (13). Other than heparin, calcium ionophore by passes calcium-dependent regulatory mechanisms by prompting a massive influx of calcium ions (29). However, significantly more embryos ($P < 0.05$) were arrested at the 8–16-cell stage in the calcium ionophore-treated group as compared to the heparin-treated group. Thus, the heparin group with an optimized concentration of 20 $\mu\text{g}/\text{mL}$ was found superior to the calcium ionophore-treated group for an incubation period of 60 min. The present study revealed significantly higher ($P < 0.05$) blastocyst production in the heparin-treated group than the calcium ionophore-treated group. This may be due to an inadequate massive influx of calcium ions during sperm treatment with calcium ionophore. Pereira et al. (27) evaluated the effect of heparin (5 IU), caffeine (5 mM), and calcium ionophore A23187 (0.1 mM) on motility and in vitro induction of the acrosome reaction in frozen-thawed bull and goat semen. Contrary to the present findings, Pereira et al. (27) demonstrated that the treatment of semen with calcium ionophore resulted in a significantly improved percentage of live spermatozoa with true acrosome reaction at all stages of incubation, both in bovines and caprines. The possible explanation behind this could be the different concentrations of capacitating agent, incubation periods, and frozen semen used in their study. Our results are in agreement with earlier studies that indicated that heparin may not only influence the fertilization process but may also affect subsequent embryo development (21,30).

The results of the present study led to the conclusions that supplementation of heparin (with 20 $\mu\text{g}/\text{mL}$ concentration for 60 min of incubation) in fertilization medium significantly increased ($P < 0.05$) the cleavage rate and blastocyst production as compared to other treated groups following in vitro fertilization by enhancing the sperm capacitation; sperm pretreatment with calcium ionophore was associated with a lower viability of embryos and blastocyst production; a higher concentration of calcium ionophore (0.1 μM concentration vs. 0.2 μM calcium ionophore) has detrimental effects on cleavage rate and subsequent embryo development as, with increasing doses of calcium ionophore, morula and blastocyst production decreases; and sperm pretreatment with 20

µg/mL heparin was found superior in terms of blastocyst production compared to calcium ionophore treatment for an incubation period of 60 min. Hence, heparin can be used to enhance capacitation of freshly collected sperms during *in vitro* fertilization and subsequent embryo development.

References

- Nandi P, Ghosh S, Jana K, Sen PC. Elucidation of the involvement of p14, a sperm protein during maturation, capacitation and acrosome reaction of caprine spermatozoa. PLoS ONE 2012;7: e30552.
- Austin CR. Observation on the penetration of the sperm into the mammalian egg. Aust J Sci Res 1951; 4: 581-596.
- Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Mol Cell Endocrinol 2002; 187: 139-144.
- Lv L, Yue W, Liu W, Ren Y, Li F, Lee KB, Smith GW. Effects of sperm pretreatments and *in vitro* culture systems on development of *in vitro* fertilized embryos derived from prepubertal Boer goat oocytes in China. Asian-Aust J Anim Sci 2009; 22: 969-976.
- Hanada A. *In vitro* fertilization in cattle with particular reference to sperm preparation with ionophore A 23187. Japanese J Anim Reprod 1985; 31: 56-61.
- Parrish JL, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38: 1171-1180.
- Kharche SD, Goel AK, Jindal SK, Yadav P, Sinha R, Sinha NK. Effect of serum albumin supplementation on *in-vitro* capacitation and fertilization of caprine oocytes. Small Rumin Res 2009; 81: 85-89.
- Kharche SD, Goel AK, Jindal SK, Goel P, Jha BK. Birth of twin kids following transfer of *in-vitro* produced goat embryos. Indian J Anim Sci 2011; 81: 1132-1134.
- Yadav P, Kharche SD, Goel AK, Jindal SK, Goel P. Assessment of nuclear maturation and subsequent *in vitro* embryo development of caprine oocytes with different supplementations in maturation medium. Indian J Anim Sci 2013; 83: 1048-1052.
- Kharche SD, Goel AK, Jindal SK, Sinha NK, Yadav P. Effect of somatic cells co-culture on cleavage and development of *in-vitro* fertilized caprine embryos. Indian J Anim Sci 2008; 78: 686-692.
- Singh KP, Saxena A, Kharche SD, Singh P. *In vitro* fertilization and cleavage rate of *in vitro* matured prepubertal and pubertal goat oocytes matured in maturation media with different supplementation. Indian J Anim Sci 2008; 78: 1078-1081.
- Snedecor GW, Cochran WG. Statistical Methods. 6th ed. Ames, IA, USA: Iowa State University Press; 1989.
- Parrish JL, Susko-Parrish JL, Handrow RR, Sims MM, First NL. Capacitation of bovine spermatozoa by oviduct fluid. Biol Reprod 1989; 40: 1020-1025.
- Lenz RW, Ball GD, Lohse JK, First NL, Ax RL. Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and *in vitro* fertilization. Biol Reprod 1983; 28: 683-690.
- Ksiazkiewicz-Katska L, Rynska B, Gajda B, Smorag Z. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production in goats. Theriogenology 2004; 62: 576-586.
- Lu KH, Seidel GE. Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically-sorted sperm. Theriogenology 2004; 62: 819-830.
- Izquierdo D, Villamediana P, Palomo MJ, Mogas T, Paramio MT. Effect of sperm capacitation and fertilization media on IVF and early embryo development of prepubertal goat oocytes. Theriogenology 1998; 49: 1501-1513.
- Shi DS. Studies related to factors affecting the yield of bovine embryos produced by *in vitro* techniques. PhD, National University of Ireland, Dublin, Ireland, 1987.
- First NL, Parrish JJ. Preparation of sperm for *in vitro* fertilization. In: Proceedings of the Symposium on Application of Egg and Embryo Technologies to Domestic Animals; 1991. pp. 33-35.
- Wang B, Baldassarre H, Tao T, Gauthier M, Neveu N, Zhou JF. Transgenic goats produced by DNA pronuclear microinjection of *in vitro* derived zygotes. Mol Reprod Dev 2002; 63: 437-443.
- Mendes JOB, Burns PD, De La Torre-Sanchez JF, Seidel GE Jr. Effect of heparin on cleavage rates and embryo production in four bovine sperm preparation protocols. Theriogenology 2003; 60: 331-340.
- Park CK, Ohgoda O, Niwa K. Penetration of bovine follicular oocytes by frozen thawed spermatozoa in the presence of caffeine and heparin. J Reprod Fertil 1989; 86: 577-582.
- Zhou JB, Wu YG, Liu LQ, Luo MJ, Chang ZL, Tan XW, Liu N, Tan JH. Studies on *in vitro* capacitation of goat spermatozoa by heparin treatment. Sheng Wu Gong Cheng Xue Bao 2004; 20: 252-256 (in Chinese with English abstract).
- Luconi M, Krausz C, Forti G, Baldi E. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. Biol Reprod 1996; 55: 207-216.

Acknowledgments

The authors wish to thank the ADG, NFBSFARA, New Delhi, India, for providing the funding and the Director, ICAR-CIRG, Makhdoom, Farah, Mathura, UP, India, for providing the facilities needed during the course of the study.

25. Tanphaichitr N, Hansen C. Production of motile acrosome reacted mouse sperm with nanomolar concentration of calcium ionophore A23187. *Mol Reprod Dev* 1994; 37: 326-334.
26. Wang S, Liu Y, Holyoak GR, Evans RC, Bunch TD. A protocol for in vitro maturation and fertilization of sheep oocytes. *Small Ruminant Res* 1998; 29: 83-88.
27. Pereira, RJ, Tuli RK, Wallenhorst S, Holtz W. The effect of heparin, caffeine and calcium ionophore A23187 on *in vitro* induction of the acrosome reaction in frozen-thawed bovine and caprine spermatozoa. *Theriogenology* 2000; 54: 185-192.
28. Ho HC, Suarez SS. An inositol 1,4,5-trisphosphatereceptor-gated intracellular Ca^{2+} store is involved in regulating sperm hyper activated motility. *Biol Reprod* 2001; 65: 1606-1615.
29. Watson PF, Plummer JM, Jones PS. The ionophore induced acrosome reaction differs structurally from the spontaneous acrosome reaction. *J Exp Zool* 1992; 264: 231-235.
30. Poulin N, Guler A, Pignon P, Cognie Y. In vitro production of goat embryos: heparin in IVF medium affect developmental ability. In: *Proceedings of the 6th International Conference on Goats*; 1996. pp. 838-840.