BIOLOGY OF REPRODUCTION 51, 795–799 (1994)

Effect of Macromolecules from Oviductal Conditioned Medium on Bovine Sperm Motion and Capacitation[1]

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ABSTRACT

The effect of macromolecules from oviductal conditioned medium (CM) on sperm motility and capacitation was studied. Sperm pooled from three bulls was incubated in either luteal isthmic CM, luteal ampullary CM, estrual isthmic CM, estrual ampullary CM, or control medium (no CM) for 4 h. Sperm capacitation and motility were assessed at 10 min and 4 h. Estrual isthmic CM capacitated significantly more spermatozoa at 4 h than estrual ampullary CM or control medium. CM also affected lateral head movement (ALH) and beat cross-frequency (BCF) of sperm. In a second experiment, the glycosaminoglycan (GAG) content of the different types of oviductal CM was quantified. Estrual isthmic CM contained more GAG than estrual ampullary CM. Among luteal samples, no difference in GAG concentration between the isthmic and ampullary CM was found. Heat treatment (100°C) of oviductal CM before coincubation with sperm significantly reduced, but did not eliminate, the capacitating ability. Because heat treatment denatures proteins and decreases the capacitating ability of certain GAG, we concluded that the capacitating effect of estrual isthmic CM may be associated with proteins, GAG, and proteoglycans in the CM. Isthmic secretions may play a major role during in vivo sperm capacitation, given that bovine spermatozoa may reside in the oviduct isthmus for up to 18 h before fertilization.

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INTRODUCTION

Bovine oviductal fluid (ODF) capacitates spermatozoa and sustains motility in vitro [1, 2]. Spermatozoa also are capacitated more rapidly in nonluteal than luteal ODF. Glycosaminoglycans (GAG) have been suggested to be a capacitating factor present in oviductal fluid (ODF) at the time of estrus [2]. GAG are generally found covalently attached to a core protein as a proteoglycan rather than as free GAG. Proteins in ODF also appear to capacitate the sperm. Both in vivo- and in vitro-derived estrus-associated proteins have been shown to capacitate spermatozoa [3], influence sperm motility [4], and improve fertilizing ability [3]. In addition, oviduct-specific proteins, synthesized by the bovine oviductal epithelium [5] and in ODF at estrus [6], bind to spermatozoa [7]. However, the effects of regional and staged oviductal conditioned medium (CM) on sperm function have not been examined.

The present study was undertaken to determine whether sperm capacitation and motility are affected by CM of oviductal explants from different stages of the estrous cycle and different regions of the oviduct. If capacitating ability differs with stage of the estrous cycle and region of the oviduct, these differences may be attributable to the relative amount of GAG or proteins in the CM. To test this hypothesis, we investigated whether or not sperm capacitation and motility were affected by CM of ampullary and isthmic explants from cows at different stages of the estrous cycle. We also examined whether the GAG content associated with in vitro-synthesized oviductal protein was affected by region of the oviduct and stage of the estrous cycle. Finally, we determined whether or not heat treatment of CM affected the ability of CM to capacitate sperm.

MATERIALS AND METHODS

Culture of Oviductal Explants

Cows with normal estrous cycles that were killed either at behavioral estrus (n = 4) or 12–14 days later (n = 4). Blood samples were taken at slaughter and later assayed for serum progesterone by RIA to confirm the hormonal status of each cow. Oviducts were removed, trimmed of surrounding tissue, and divided into ampulla and isthmus. Each region was cut open longitudinally and dissected into 3–4-mm pieces of tissue containing epithelium, stroma, and muscle layers. To maintain intercellular associations and simulate in vivo conditions, the epithelium was not removed. Ten pieces of tissue were cultured in 5 ml of RPMI 1640 (Sigma Chemical Co., St. Louis, MO)/Dulbecco's Minimum Essential Medium (Gibco, Grand Island, NY) serum-free culture medium supplemented with glucose (17 $mmol \cdot l^{-1}$), glutamine (2 $mmol \cdot l^{-1}$), epidermal growth factor (25 $ng \cdot ml^{-1}$), and insulin-transferrin-sodium selenite (5 $\mu g \cdot ml^{-1}$, 5 $\mu g \cdot ml^{-1}$, 5 $ng \cdot ml^{-1}$, respectively; Sigma) in 100 × 35-mm petri dishes at 37°C in humidified 5% CO_2:95% air. After 16–18 h, 2 ml of fresh medium was added to each dish, and explants were cultured for an additional 24 h. Conditioned culture medium was centrifuged (2000 × g; 30 min) to pellet cells and recover the supernatant.

Accepted June 13, 1994.
Received September 30, 1993.
[1]This study was supported in part by USDA grant #91-37203-6554.
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Effect of Oviductal CM on Sperm Capacitation and Motility

After ampullary and isthmic explants were recovered and cultured from four cows in diestrus and four cows in estrus, the CM proteins were treated by ultrafiltration (Centriprep; Amicon Corp., Danvers, MA), which concentrated macromolecules greater than 10 kDa. Protein concentration was determined by the method of Lowry [8]. The treatments tested were luteal isthmic CM, luteal ampullary CM, estrous isthmic CM, estrous ampullary CM, and modified Tyrode's medium with 1 mg/ml polyvinyl alcohol (MTM) [2] containing no protein. CM treatments contained 0.5 mg protein (equal amounts of protein from each cow) per tube, and the macromolecules were concentrated using a Speed Vac (Savant Instruments, Farmingdale, NY).

Semen was collected from three Holstein bulls by use of an artificial vagina. Aliquots of semen containing equal numbers of sperm from each bull were pooled in 10 ml of protein-free MTM for a final concentration of 20×10^6 sperm/ml. Sperm were washed by centrifuging for 10 min at $500 \times g$ and then resuspending the pellet in 10 ml of MTM.

One-half milliliter of sperm suspension (10×10^6 sperm) was added to each of five incubation tubes, making the final CM protein concentration 1 mg/ml. All tubes were incubated in 5% CO_2:95% air for 4 h. Aliquots were removed at 10 min and 4 h for assessment of capacitation status (percentage live sperm able to undergo the acrosome reaction). Sperm motility was assessed at 4 h with a computer-assisted, Hamilton Thorn Motion Analyzer (Hamilton Thorn Research Inc., Danvers, MA). This experiment was replicated three times on 3 days.

Lysophosphatidylcholine (LPC; Sigma L-5004), a fusogenic lipid, was used to induce the acrosome reaction in capacitated sperm. Sperm (100 μl; 1×10^6 sperm) from each of the treatments were added to tubes containing 30 μg/ml LPC and 50 mg/ml BSA (fraction V; Sigma #A-2153), gently mixed, and then incubated for 10 min at 39°C to induce the acrosome reaction in capacitated sperm. Sperm smears were stained with Fast Green FCF-Eosin B [9] and assessed by differential interference contrast microscopy for percentage of acrosome-reacted live (RL), acrosome-reacted dead (RD), intact live (IL), and intact dead (ID) spermatozoa. The percentages of total live (TL), total dead (TD), total reacted (TR), and total intact (TI) spermatozoa also were calculated.

Within 30 sec after the sperm and treatments were combined, a 5-μl sample of MTM-treated sperm suspension (control) was assessed for motility. After a 4-h incubation, 5 μl of sperm suspension from each treatment was pipetted into a 20-μl-deep Microcell disposable counting chamber (Fertility Technologies, Inc., Natick, MA) maintained at 39°C. Sperm motion parameters were evaluated microscopically under negative phase optics (Olympus model CH-2; Hi-Tech Instruments, Philadelphia, PA). The microscope field was immediately videotaped for 10 sec. This procedure was repeated with a second 5-μl sample. The microscope was equipped with a Panasonic WV-D5100 video camera (Panasonic Communications & Systems Co., Secaucus, NJ). An MOS color monitor (MicroImage Video Systems Co., Boyertown, PA) was attached to the microscope via an MTV-3 adapter, and a video cassette recorder (model VC-A5640U, Sharp, Denver, CO) was used to record movement.

The video recorder was connected to a Hamilton Thorn Motion analyzer (HTM). Videotapes were played through a computer-assisted semen analysis system to assess motility parameters. A Panasonic Color Video Data Monitor (#CT-1400MG) was viewed to scan each field from each sample at each time point. Each field was scanned for 0.66 sec, and each scan consisted of 20 frames. Each motile cell track (path) was analyzed by the HTM. Sperm cells were tracked by comparing successive frames. The motility parameters determined were as follows: VSL = straight line velocity (μm/sec); VCL = track speed (μm/sec); VAP = average path velocity (μm/sec); ALH = amplitude of lateral head displacement (μm); STR = straightness (%); LIN = linearity (%); and BCF = beat cross frequency (Hz).

The HDATA program (Hamilton Thorn Research Inc.) was run on a personal computer and was used to capture and manage the data generated by the HTM-C analyzer. The equipment settings suggested by the manufacturer were optimized for accurate assessment of the concentration of sperm as determined by cytometer and assessment of motility as determined by visual observation.

GAG Concentrations Associated with Oviductal Proteins

To determine whether the GAG content of in vitro-synthesized oviductal CM, treated by ultrafiltration to contain primarily molecules > 10 kDa, differed between region of the oviduct and stage of the estrous cycle, CM from isthmic and ampullary explants of four cows in estrus and four luteal cows were prepared. GAG content of the luteal isthmic, luteal ampullary, estrous isthmic, and estrous ampullary CM adjusted to 2 mg/ml protein was determined using the colorimetric method of Whiteman [10], as modified by Kubajak and Ax [11], with chondroitin sulfate (Sigma C-8529) as standard. Chondroitin sulfate was 70% chondroitin sulfate A; the balance was chondroitin sulfate C. The interassay coefficient of variation was 7.31%, and the intraassay coefficient of variation was 3.54%.

Effect of Heat Treating Oviductal Macromolecules

Oviductal CM proteins (2 mg) and associated macromolecules > 10 kDa were prepared from isthmic and ampullary explants of four estrous cows and four luteal cows; an equal amount of CM protein from each cow was combined into luteal isthmic, luteal ampullary, estrous isthmic, and estrous ampullary pools. Four replicates of each pool

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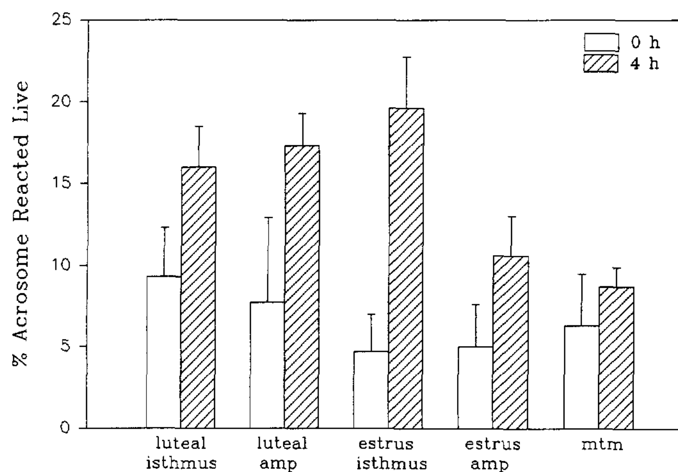

FIG. 1. Effect of time and treatment on percentage RL sperm. The percentage RL was higher at 4 h than at 0 h across the sperm treatments: luteal/isthmic, luteal/ampullary, estrous/isthmic, estrous/ampullary CM, and MTM. At 4 h the percentage RL among the sperm treated with estrous/isthmic CM was significantly greater than that of estrous/ampullary CM-treated spermatozoa. The percentages RL among sperm treated with CM from luteal/ampullary and estrous/isthmic explants were significantly greater than for sperm incubated in MTM.

were prepared by pipetting 50 μl of CM (350 μg protein) and 50 μl of MTM (control) into incubation tubes. Half of these samples were boiled for 30 min to inactivate the proteins.

Aliquots of semen from three bulls were pooled by adding an equal number of washed spermatozoa from first ejaculates to 10 ml of protein-free MTM for a final concentration of 1.3×10^8 sperm/ml. Another pool was prepared by combining equal numbers of spermatozoa from the second ejaculates.

The medium of CM-treated spermatozoa contained 2 mg/ml protein and had a final volume of 150 μl. All tubes were incubated at 39°C for 4 h in 5% CO_2 in air. A subsample was removed from each tube at 4 h for assessment of the capacitation status (percentage of live sperm able to acrosome-react).

Statistical Analysis

The factors examined in experiment 1 were five treatments and two times of incubation (0 and 4 h). This experiment was replicated three times (three days). The response variables measured included capacitation (percentage RL, RD, IL, ID, TL, TD, TR, and TI) and motility (VSL, VCL, VAP, ALH, STR, LIN, and BCF). Capacitation was assessed at 0 and 4 h of incubation, and motility was assessed at 4 h. Repeated measures analysis on the Statistical Analysis System (SAS) was used to examine the data [12]. Bonferonni multiple comparison tests were used to identify treatment group differences. Least square means were used to determine the p values of treatment group differences. A significance level of $p = 0.05$ was used for all statistical testing.

Experiment 2 was designed to determine whether the GAG content of oviductal protein varied according to region of the oviduct and stage of the estrous cycle. The factors considered were two stages of the estrous cycle (luteal and estrous) and two regions of the oviduct (ampulla and isthmus). Eight cows nested within stage were replicates. Within each stage, paired t-tests were conducted on the differences in GAG content between the ampullary and isthmic proteins across cows.

The aim of experiment 3 was to determine whether boiling oviductal proteins and the associated macromolecules (> 10 kDa) before the 4-h incubation decreased the number of capacitated spermatozoa. The experiment was replicated using pools of samples from three bulls for each of two ejaculates. Therefore, the two factors considered were two processing treatments (boiled or control oviductal proteins) across four pooled types of oviductal CM. Capacitation (percentage RL) was assessed at 4 h and normalized on the percentage RL in the corresponding MTM controls. A paired t-test was conducted to test for differences in the number of capacitated spermatozoa (percentage RL) between heat-treated CM and control CM spermatozoa across the types of oviductal protein.

RESULTS

Effect of Oviductal CM on Sperm Capacitation and Motility

Capacitation. Time (0 and 4 h) had a significant effect ($p < 0.05$) on the percentages IL, RL, RD, TI, TR, TL, and TD among sperm treated with oviductal CM. At 4-h incubation, the percentage IL (68.5 ± 2.8) was lower than ($p = 0.0001$) the percentage IL at 0 h (81.1 ± 2.2) across all treatments.

The percentage RL at 4 h (14.4 ± 1.4) was higher than ($p = 0.0001$) at 0 h (6.6 ± 1.4) across all treatments (Fig. 1). A significant ($p = 0.038$) treatment effect was observed at 4 h. At this time the percentage RL in sperm treated with estrous isthmic CM (19.7 ± 3.2) was greater than ($p = 0.02$) that of sperm treated with estrous ampullary CM (10.6 ± 2.4), and the percentages RL in sperm treated with luteal ampullary CM (17.3 ± 2.0; $p = 0.03$) and estrous isthmic CM (19.7 ± 3.2; $p = 0.008$) were greater than those for sperm incubated in MTM (8.7 ± 1.2).

The percentage RD spermatozoa at 4 h (15.9 ± 2.3) was higher than ($p = 0.02$) at 0 h (9.9 ± 1.35) across all treatments. The percentage TI spermatozoa at 4 h (69.7 ± 2.5) was lower than ($p = 0.0001$) at 0 h (83.4 ± 2.3), while percentage TR spermatozoa at 4 h (30.4 ± 2.6) was higher than ($p = 0.0001$) at 0 h (16.5 ± 2.3). The percentage TL spermatozoa at 4 h (73.3 ± 8.7) was lower ($p = 0.03$) than at 0 h (89.7 ± 7.0).

Motility. Analysis of variance showed that oviductal explant CM had a significant ($p < 0.05$) effect on sperm ALH ($p = 0.02$) and BCF ($p = 0.03$) after 4 h of incubation (Fig.

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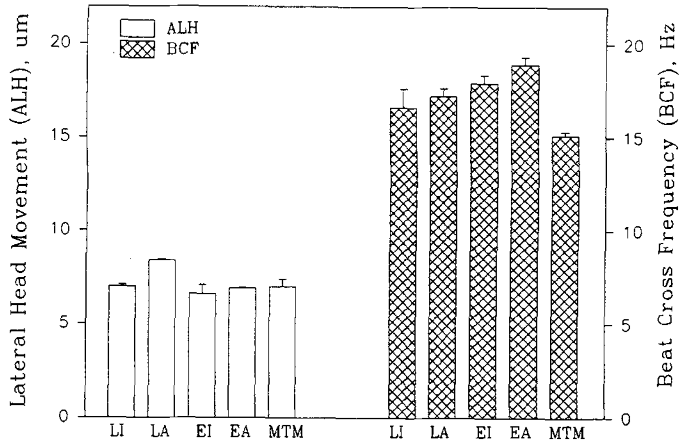

FIG. 2. Effect of treatment on sperm ALH and BCF at 4 h. After 4 h of incubation, oviductal explant CM had an effect on sperm ALH and BCF. The ALH of sperm treated with luteal/ampullary CM (LA) was higher than for sperm treated with luteal/isthmic (LI), estrous/isthmic (EI), and estrous/ampullary (EI) CM or with MTM. The BCF of spermatozoa incubated in MTM was less than for those treated with LA, EI, and EA proteins.

2). ALH, the mean width of the head oscillation as the cell swims, is the maximum value of the distance of any point on the track from the corresponding five-point average, multiplied by two to give the full width. BCF was the number of times the sperm head crossed the path per second. ALH of spermatozoa treated with CM from luteal ampullary explants (8.4 ± 0.05) was higher than that of spermatozoa treated with CM of luteal isthmic (7.0 ± 0.1; p = 0.01), estrous isthmic (6.6 ± 0.5; p = 0.006), and estrous ampullary explants (6.9 ± 0.05; p = 0.009) and with MTM (7.0 ± 0.4; p = 0.02). The BCF of spermatozoa incubated in MTM (15.1 ± 0.2) was less than that of spermatozoa treated with luteal ampullary (17.2 ± 1.0; p = 0.04), estrous isthmic (17.9 ± 0.5; p = 0.02), and estrous ampullary CM (18.9 ± 0.4; p = 0.005).

GAG Concentrations Associated with Oviductal Proteins

The GAG concentration (mean ± SEM, ng/μg protein) in CM of estrous isthmic explants was 254 ± 18 ng/μl; this was higher than the value in CM of estrous ampullary (209 ± 20 ng/μl), luteal isthmic (211 ± 25 ng/μl), and luteal ampullary explants (207 ± 18 ng/μl). A paired Student's t-test showed that among media from estrous explants, isthmic CM contained significantly (p = 0.016) more GAG per microgram protein than the ampullary CM. Among the luteal explants, there was no difference in GAG between CM from the two regions.

Effect of Heat Treating Oviductal Macromolecules

After 4 h of incubation, the number of spermatozoa capacitated (percentage RL = 9.75 ± 1.37) in vitro by oviductal CM was significantly (p = 0.02) reduced when CM macromolecules were heat-treated (6.12 ± 1.28), but capacitation was not fully prevented.

DISCUSSION

The effects of oviductal fluid (ODF) [1, 2] or oviductal proteins [3, 4] on sperm function have been examined in several studies, but the effects of regional and staged concentrated macromolecules have not been investigated. A principal finding of the present study was that at estrus, isthmic CM capacitated more spermatozoa at 4 h than ampullary CM or control medium (no protein). These results support the findings of Parrish [2] showing that bovine ODF capacitated significant numbers of spermatozoa in vitro by 4 h and that the capacitating activity of ODF peaked at estrus and declined during the luteal phase. Likewise, McNutt and Killian [1] found that spermatozoa were capacitated more rapidly in nonluteal than in luteal ODF. The results of the present study suggest that macromolecules produced by the isthmus at estrus may play a major role in capacitation. These observations may be of special significance because spermatozoa in vivo are believed to reside in the bovine isthmus for up to 18 h before fertilization [13].

At 4 h of incubation, oviduct explant CM had an effect on ALH and BCF. The ALH of sperm treated with luteal ampullary proteins was higher than for those treated with luteal isthmic, estrous isthmic, and estrous ampullary CM or with control medium. The BCF of spermatozoa incubated in control medium with no CM was less than for sperm treated with luteal ampullary, estrous isthmic, or estrous ampullary CM. The physiological importance of these findings is unknown. However, significant correlations between objectively measured sperm motion endpoints and fertility have been reported for bovine spermatozoa [14, 15]. Changes in sperm motion endpoints are believed to be indicative of either metabolic or structural changes in the sperm.

Parrish et al. [2] suggested that GAG are a capacitation factor present in ODF at the time of estrus. Proteoglycans may be another capacitating factor within the oviduct. Proteoglycans consist of GAG covalently bound to a core protein. For example, heparin sulfate is a GAG that is generally found covalently attached to a core protein as a proteoglycan rather than as free GAG [2]. The increased capacitating ability of estrual isthmic proteins may be due to an increased relative concentration of GAG covalently bound to these proteins. The results of our studies support this hypothesis, because among the estrous samples, isthmic explant CM was associated with significantly more GAG, when normalized for protein concentration, than was ampullary explant CM. Among the luteal samples, no difference in GAG content between the regions was found. Parrish et al. [2] found that proteolysis or a 30-min heat (60°C) treatment of ODF had no effect on its capacitating ability as judged by the ability of LPC to induce acrosome reactions in bovine sperm. However, we found that when proteins and macromolecules greater than 10 kDa were heat-treated (100°C) for 30 min, sperm capacitation by oviductal CM was significantly reduced, but not eliminated. The discrepancy be-

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tween these two studies may be attributable to the different temperatures. Treating CM for 30 min at 100°C may have inactivated both the GAG and the protein. To determine whether 30-min heat treatments could decrease the capacitating ability of GAG, we conducted an additional experiment. We found that after heparin or chondroitin sulfate was heat-treated for 30 min at either 60°C or 100°C, the sperm-capacitating ability of heparin was reduced in samples from two of three bulls. Chondroitin sulfate did not capacitate the sperm of any bull. Therefore, the heat treatments used in the current study could have reduced the capacitating ability of both proteins and certain types of GAG. Furthermore, it is likely that more than one capacitation mechanism exists—one mediated by GAG and another mediated by proteins. It also is possible that both the GAG and the core protein of the proteoglycan must be functional in order for optimal capacitation to occur.

In summary, estrual isthmic CM capacitated more spermatozoa at 4 h than estrual ampullary CM or control medium. Because estrual isthmic CM contained significantly more GAG normalized by protein than estrual ampullary CM, one of the capacitating factors associated with estrual isthmic CM may be a proteoglycan. Given that ODF contains both GAG [16] and proteins [3], which individually have been shown to capacitate bovine sperm, it is possible that both molecules are needed in order for optimal capacitation to occur in vivo. In addition, GAG are a component of the extracellular matrix of most cells and are likely to be present in the extracellular matrix of oviductal epithelial cells to which sperm are exposed during in vivo capacitation [2]. Considering that spermatozoa may reside in the bovine isthmus for up to 18 h before fertilization, macromolecules secreted by the isthmus at estrus probably play a major role in capacitation.

ACKNOWLEDGMENT

The authors thank Amy Way and the support staff at the Dairy Breeding Research Center.

REFERENCES

1. McNutt TL, Killian GJ. Influence of bovine follicular and oviduct fluids on sperm capacitation in vitro. J Androl 1991; 12:244–252.
2. Parrish JJ, Susko-Parrish JL, Handrow RR, Sims MM, First NL. Capacitation of bovine spermatozoa by oviduct fluid. Biol Reprod 1989; 40:1020–1025.
3. King RS, Anderson SH, Killian GJ. Bovine estrus-associated protein facilitates sperm capacitation and fertilizing ability. Proc 12th Int Congr Anim Reprod 1992; 1:487–489.
4. Anderson SH, King RS, Killian GJ. In vivo and in vitro derived oviductal estrus-associated protein affect sperm capacitation, motility, and agglutination. Proc 12th Int Congr Anim Reprod 1992; 1:396–398.
5. Wegner CC, Killian GJ. Origin of oestrus-associated glycoproteins in bovine oviductal fluid. J Reprod Fertil 1992; 95:841–854.
6. Gerena RL, Killian GJ. Electrophoretic characterization of proteins in oviduct fluid of cows during the estrous cycle. J Exp Zool 1990; 256:113–120.
7. McNutt TL, Rogowski LA, Vasilatos-Younken R, Killian GJ. Adsorption of oviductal fluid proteins by the bovine sperm membrane during capacitation. Mol Reprod Dev 1992; 33:313–323.
8. Lowry O, Rosengrough N, Farr A, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265–275.
9. Aalseth EP, Saacke RG. Vital staining and acrosomal evaluation of bovine sperm. Gamete Res 1986; 15:73–81.
10. Whiteman P. The quantitative measurement of alcian blue—glycosaminoglycans complexes. Biochem J 1973; 131:343–350.
11. Kubajak CL, Ax RL. Measurement of glycosaminoglycans in ovarian follicular fluid using alcian blue dye. In: Toft D, Ryan R (eds.), Proceedings of the Fifth Biennial Ovarian Workshop. Champaign: Ovarian Workshop; 1985: 497–501.
12. SAS. SAS User's Guide, The GLM Procedure. Cary, NC: Statistical Analysis System Institute, Inc.; 1985.
13. Hawk HW. Transport and fate of spermatozoa after insemination of cattle. J Dairy Sci 1987; 70:1487–1503.
14. Parrish JJ, Foote RH. Quantification of bovine sperm separation by a swim-up method: relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. J Androl 1987; 8:259–266.
15. Budworth PR, Amann RP, Chapman DL. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J Androl 1988; 9:41–54.
16. Parrish JJ, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.

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