Biol Res 39: 631-639, 2006

Effect of heparin on *in vitro* capacitation of boar sperm

DORA G DAPINO[1, 2]; PATRICIA E MARINI[2, 3] and MARCELO O CABADA[2, 4]

[1]Cátedra de Fisiología. Facultad de Ciencias Veterinarias. UNR [2] División Biología del Desarrollo (IBR-CONICET) y Área Biología. Facultad de Ciencias Bioquímicas y Farmacéuticas. UNR. [3] Member of the research career (CIC-UNR). [4] Member of the research career (CONICET).

ABSTRACT

Chlortetracycline (CTC) fluorescent pattern, the ability to undergo acrosome reaction (AR) upon exposure to 10 μM calcium ionophore A23187 and vitality estimation were used to investigate the effect of the sulfated glycosaminoglycan heparin on the *in vitro* capacitation of porcine spermatozoa. Sperm incubation in capacitating medium (CM) supplemented with 10 mM heparin for up to 120 min, showed an increase in the number of capacitated sperm (B pattern) and acrosome reacted sperm (AR pattern), without affecting their viability. In this condition, spermatozoa were incubated in CM depleted of albumin, calcium, bicarbonate or combinations, in the presence of heparin. In either calcium or bicarbonate-free media, capacitation was only basal and did not show variations in the presence of heparin. In absence of albumin the presence of calcium and bicarbonate stimulated capacitation, which was further increased by the addition of heparin. These results suggest that heparin enhances *in vitro* capacitation of porcine sperm only under capacitating conditions. Additionally, when sperm were incubated with 100 μg/ml biotinylated heparin in the presence or absence of unlabeled heparin, we observed that heparin binding sites were located mostly on the acrosomal region of boar sperm in an specific and saturable manner. The *in vitro* effect of heparin described in this work indicates that sulfated glycosaminoglycans, which are normally present in the female reproductive tract, might play an important role in the fertilization process in porcines.

Key terms: boar sperm; capacitation; heparin; sulfated glycosaminoglycans

INTRODUCTION

Glycosaminoglycans (GAGs) are essential components of the extracellular matrix, contributing to cell recognition, cellular adhesion and growth regulation. Individual GAGs are characterized by their sugar residues and other features, such as sulfation. Four main groups can be distinguished: a) the non-sulfated GAG, hyaluronan, b) the sulfated GAG keratan sulphate, c) heparin/heparan sulfate and d) the sulfated galactosaminoglycans chondroitin sulfate/ dermatan sulfate (Thientai et al. 2000).

Heparin has been extensively used *in vitro* to study the endogenous role of heparin-like GAGs secreted by the epithelium of the female reproductive tract. Its capacitating effect on bovine (Parrish et al., 1989) and human (Valencia et al., 1984) sperm has been established. However, its action on pig sperm physiology is still unclear.

In cattle, heparin is thought to promote capacitation by binding to and removing seminal plasma proteins that are adsorbed to the sperm plasma membrane, and would inhibit capacitation (Miller et al., 1990). In this mammal, heparin also produces a rise of sperm intracellular pH and Ca^{++} concentration (Parrish et al., 1989), protein phosphorylation and modification of motility parameters (Chamberland et al., 2001).

In pigs, the role of sulfated GAGs as sperm capacitating agents, remains uncertain.

The aim of the present work is to study the effect of the sulfated glycosaminoglycan heparin on the *in vitro* capacitation of pig spermatozoa.

Correspondence author: Marcelo O. Cabada, Area Biología, Suipacha 531, Rosario (S2002LRK), Argentina. Tel.: (0054) 341 4804601; Fax: (0054) 341 4804601; e-mail: cabada@ibr.gov.ar

Received: December 29, 2005. Accepted: July 25, 2006

MATERIALS AND METHODS

Media

Sperm capacitating medium (CM), (Tyrode's medium, TALP; Parrish et al., 1988) contains 96 mM NaCl , 3.1 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4 , 20 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$ and 3 mg/ml BSA). Medium prepared with neither $CaCl_2$, $NaHCO_3$ nor BSA, is called non-capacitating medium (NC). Variations of NC were prepared adding 2.0 mM $CaCl_2$ (NCC), 15 mM $NaHCO_3$ (NCB) or both (NCCB), according to experimental requirements.

To avoid acrosome alterations by osmotic shock, the osmolarity of all of these solutions was maintained at 285 - 315 mosm/l with NaCl.

Semen collection and treatments

Semen was collected from adult fertile boars by the glove-hand method. Sperm rich fraction was diluted in Sperm plus L.C.® (Laboratorio Productos Agropecuarios, I. Sobrino S.L., San Juan de Mozarripar, Zaragoza, España), and conserved at 16 ºC until used (no more than 24 hours from collection).

Quality of the samples was established by evaluating motility, viability, concentration, acrosomal and morphological parameters (Althouse, 1997).

Motile spermatozoa were separated by centrifugation at 50 x g during 10 minutes at 20ºC. The supernatant was centrifuged at 700 x g for 5 minutes for sperm recovery. The pellet was resuspended to 10^7 sperm/ml in different media according to the experiment, as stated in each case.

Evaluation of the effect of heparin concentration on the physiology of boar spermatozoa

Spermatozoa (10^7 sperm/ml) were suspended in CM and separated in three 1 ml aliquots. Heparin from porcine intestinal mucosa *(Sigma-Aldrich, USA)* was added to two aliquots, at final concentrations of 10 and 100 μg/ml, respectively. Samples were incubated at 39º C in a 5% CO_2 atmosphere. Aliquots were taken at 0, 30, 60, 90 and 120 minutes and evaluated for sperm maturational state by CTC and Wells & Awa staining assays and for viability by eosin-nigrosin staining method.

Evaluation of the effect of heparin on spermatozoa under capacitating and non capacitating conditions

Sperm were suspended in different media (CM, NC, NCC, NCB, NCCB) and incubated with 10 μg/ml heparin at 39° C in a 5% CO_2 atmosphere for 120 min. Capacitation was assessed by CTC staining assay and by the ability of capacitated sperm to undergo the AR in presence of calcium ionophore A23187. Sperm viability was also analysed by eosin-nigrosin staining.

Assessment of heparin binding on boar sperm plasma membrane

The topography of heparin – binding proteins on boar sperm was studied by indirect inmunofluorescence microscopy according to Debbage et al., (1988). Briefly, 10 μl of capacitated spermatozoa suspension (10^6/ml) was spread on slides, air-dried, fixed for 15 min in methanol, and incubated with PBS buffer containing 5% (w/v) BSA for 2 h at 39ºC, followed by incubation with biotinylated heparin (100 μg/ml) in PBS/BSA buffer in the presence or absence of unlabeled heparin. After washing with PBS/BSA buffer, both samples were incubated with 100μg/ml FITC-labelled streptavidin (*Sigma-Aldrich, USA*) dilution, and washed exhaustively with PBS/BSA buffer. Finally, 10 μl of PBS:glycerol (1:9, v/v) was added to the samples, which were then covered with glass slides, and observed under a fluorescence microscope (Nikon E800). Images were registered with CCD Spot RT, Digital Instruments.

Chlortetracycline staining assay

CTC was used to determine sperm maturational status (Wang et al., 1995). In

brief, CTC (750 μM) was prepared in 20 mM Tris buffer containing 130 mM NaCl and 5 mM DL-cysteine (final pH 7.8). Sperm suspension (5μl) was mixed with 5 μl of CTC solution on a warmed slide (37º C). After 30 sec, 5 μl of 0.2% glutaraldehyde in 0.5 M Tris pH 7.4, were added. Finally, 5μl of 0.22 M 1, 4-diaza-byciclo (2.2.2) octane *(DABCO, Sigma, USA)* in 50% glycerol were added to retard fluorescence fading. After adding a coverslip, slides were gently compressed using a tissue paper. The slides were then sealed along the edges with colourless nail varnish and examined with an Olympus BH2 microscope equipped with epifluorescence optics (excitation at 405 nm BP filter and CTC fluorescence emission at 455 DM).

Three fluorescent patterns were observed: F pattern (non capacitated sperm with bright uniform fluorescence over the head), B pattern (capacitated sperm with bright anterior head and faint fluorescence in the post-acrosomal region) and acrosomal reaction pattern (reacted sperm with poor fluorescent head and a thin bright band along the equatorial segment).

Calcium ionophore A23187-induced AR

Sperm capacitation was determined by the ability of sperm to undergo AR in the presence of calcium ionophore A23187 (Tardif et al., 1999).

After 120 min of incubation in different media, sperm samples were mixed with calcium ionophore A23187 at a final concentration of 10 μM and incubated for 15 min at 39º C in 5% CO_2 atmosphere. Two mM $CaCl_2$ with the ionophore were added to calcium-free media, to promote its maximal uptake. The number of AR sperm was estimated before and after treatment, by Wells-Awa stain.

Wells & Awa staining assay

Dye solutions, prepared according to Berger et al.(1989), contained 2.9% sodium citrate, 95% ethanol, 1% Eosin B and 1% Fast green FCF. Ten min before use, 1.7 parts ethanol, 1.4 parts fast green FCF and 0.7 parts Eosin B were mixed.

Sperm suspensions were fixed for 15 min in 0.3% formol solution and centrifuged for 5 min at 8700 x g. The supernatant was removed and sperm were resuspended in 1 ml 0.9% NaCl. Sperm suspension was centrifuged again at 8700 x g for 5 min. Pellets were resuspended in 25 μl of sodium citrate and 50 μl aliquots of stain were added to each sample. After 10 min, a 10 μl aliquot of dye was smeared onto a slide and dried. Percentage of AR sperm was determined at magnification of 40 x (a total of 200 cell/slide were counted) by bright field microscopy. Acrosome-intact sperm were considered to be those having a thickened blue-green region at the apex of the head, a blue-green cap covering the anterior two-thirds of the head and a pink colour in the posterior one-third of the head. A sperm was considered to have lost its acrosome when the apical thickened or cap were lost or lifted.

Eosin - nigrosin staining assay

Viability was estimated using eosin–nigrosin exclusion technique (Althouse, 1997). Sperm suspensions were mixed with an equal volume of 0.5% eosin-nigrosin solution and smeared onto a warm slide (37º C). After drying the sample, sperm were scored (at least 200 cell/slide) under light microscope (100x). Live sperm showed no staining and dead cells showed pink coloration. The presence of spermatozoa with a partial coloration was considered as dead cell.

Statistical analysis of data

The mean and SD were calculated in all cases. To test the differences between the different incubation time within each concentration, data were analysed by Student's t-test. One - way Analysis of Variance and multiple comparison (Duncan Test) was used for the statistical analysis of data to observe differences in percentage of capacitated sperm in different media and heparin concentration. The model included presence or absence of heparin, incubation media and their interaction. The level of significance was considered $p \leq 0.05$.

RESULTS

Effect of the concentration of heparin on sperm CTC patterns in CM media

In order to evaluate the effect of heparin on sperm physiological status, sperm suspensions were incubated in CM with different heparin concentration. Chlortetracycline and eosin-nigrosin staining patterns were assayed every 30 min up to 120 min. Significant differences were observed in the distribution of CTC-patterns in cells incubated with and without heparin in CM. The number of B pattern without heparin was comparable to the usually observed for pig sperm, and was higher in the presence of heparin, regardless of its concentration at every incubation time tested (Table I) ($p \leq 0.05$).

The rate of viable cells after 120 min of incubation was significantly lower when 100 mg/ml heparin was used than in the presence of 10 mg/ml heparin (71±1.3 against 39± 2.3). Considering this, 10mg/ml heparin and 120 min of incubation were chosen as parameters for next experiments.

Effect of heparin on sperm under capacitating and non capacitating conditions

Capacitation, assessed by CTC and calcium ionophore A23187 as inductor of AR was used to investigate the effect of heparin in different incubation media.

As shown in Fig. I-A, the CTC patterns of cells incubated in NC media, regardless the presence of Ca^{2+} and HCO_3^- , did not vary significantly after 120 min incubation ($p > 0.05$). The presence of heparin in the media did not affect the CTC pattern in any case, and the percentage of B pattern was similar to that without it ($p>0.05$).

The CTC patterns of cells incubated in CM and NC media containing both Ca^{2+} and HCO_3^- (without BSA), were similar (Fig. 1-A), and presented significantly higher proportion of B pattern than all the other media tested ($p<0.001$). In both of this media, the proportion of B pattern was significantly higher in the presence of heparin ($p < 0.001$) (Fig. 1-A).

Capacitated sperm ability to undergo AR upon exposure to calcium ionophore A23187 is shown in figure 1-B. AR values assessed before and after treatment, were similar when Ca^{2+} or HCO_3^- were absent, regardless the presence of heparin. In CM and NCCB, AR was significantly higher after incubation with calcium ionophore ($p<0.001$), and consistent with values usually found for pig sperm capacitation. It showed a significant increment in the presence of heparin ($p<0.05$). Additionally, in CM with heparin, the percentage of AR sperm was significantly higher than in all of the other treatments ($p< 0.05$). The viability assessed by eosin-nigrosin staining was around 70 % throughout the whole experiment.

TABLE I

Effect of heparin on sperm capacitation

incubation period (minutes)	heparin concentration (μg/ml) 0	10	100
0	5.0±1.8	6.0± 1.8	5.2±1.2
30	6.5±2.6	15.0±1.8	18.5±1.9
60	15.5±4.0	31.5±3.1	29.7±2.5
90	27.0±1.6	38.0±2.4 (*)	41.7±2.2 (*)
120	28.7±2.6	46.5±3.4 (*)	45.7±4.6 (*)

Rate of capacitated sperm (percentage of B pattern) after incubation in CM at the indicated time and heparin concentrations. Each time value represents the mean of 4 replicates from different boars ± SD (200 total spermatozoa were scored in each experiment). (*) denotes significant differences ($p \leq 0,05$) in the number of B pattern in relation to control.

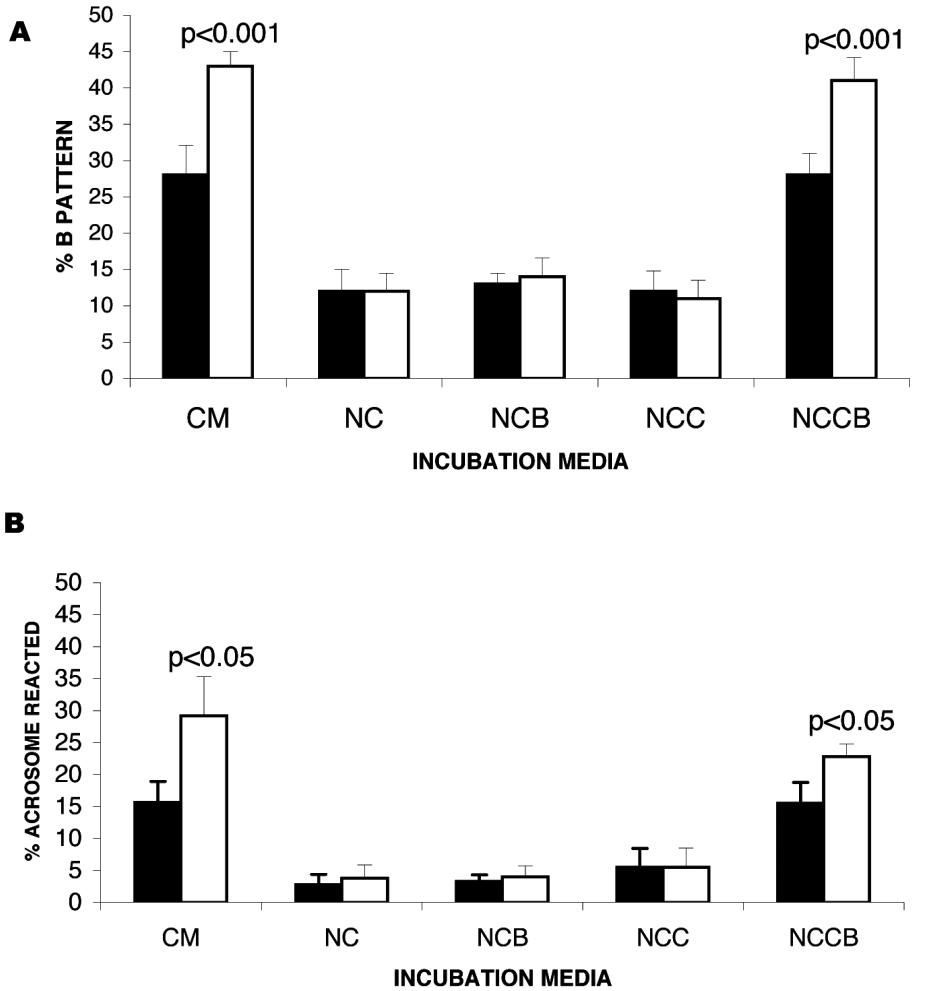

Figure 1: Effect of heparin on sperm capacitation after 120 min of incubation (39ºC) under different culture conditions: **(CM)** capacitating medium, **(NC)** (Non-capacitating medium) CM without $CaCl_2$, $NaHCO_3$ and BSA , **(NCC)** NC + $CaCl_2$, **(NCB)** NC + $NaHCO_3$, **(NCCB)** NC +$CaCl_2$ + $NaHCO_3$. Without heparin **(black bars)**, with 10 μg/ml heparin **(white bars)**. Each point represents the mean ± SD (n=6), 200 total cells were scored per animal. (A) Percentage of capacitated sperm (B pattern) according to chlortetracycline staining. (B) Percentage of capacitated sperm assessed by the ability to undergo the ionophore-induced acrosome reaction detected by Wells & Awa staining.

Localization of heparin-binding sites on sperm plasma membrane

Fluorescence microscopy of spermatozoa incubated with biotinylated heparin was used to evidence the heparin-binding sites. Figure 2 A, B shows that they were located mostly on the acrosomal region of the sperm head (approximately 50% of total cells) and that the binding of biotinylated heparin was inhibited in the presence of unlabeled heparin (figure 2 C,D).

DISCUSSION

Heparin is essential for *in vitro* capacitation of cattle spermatozoa (Chamberland, 2001). In pigs, we observed an increment in the number of capacitated sperm (B pattern) for each time of incubation when heparin is present in CM, probably due to its accelerating effect on spermatozoa metabolism, as in cattle (Chamberland et al., 2001).

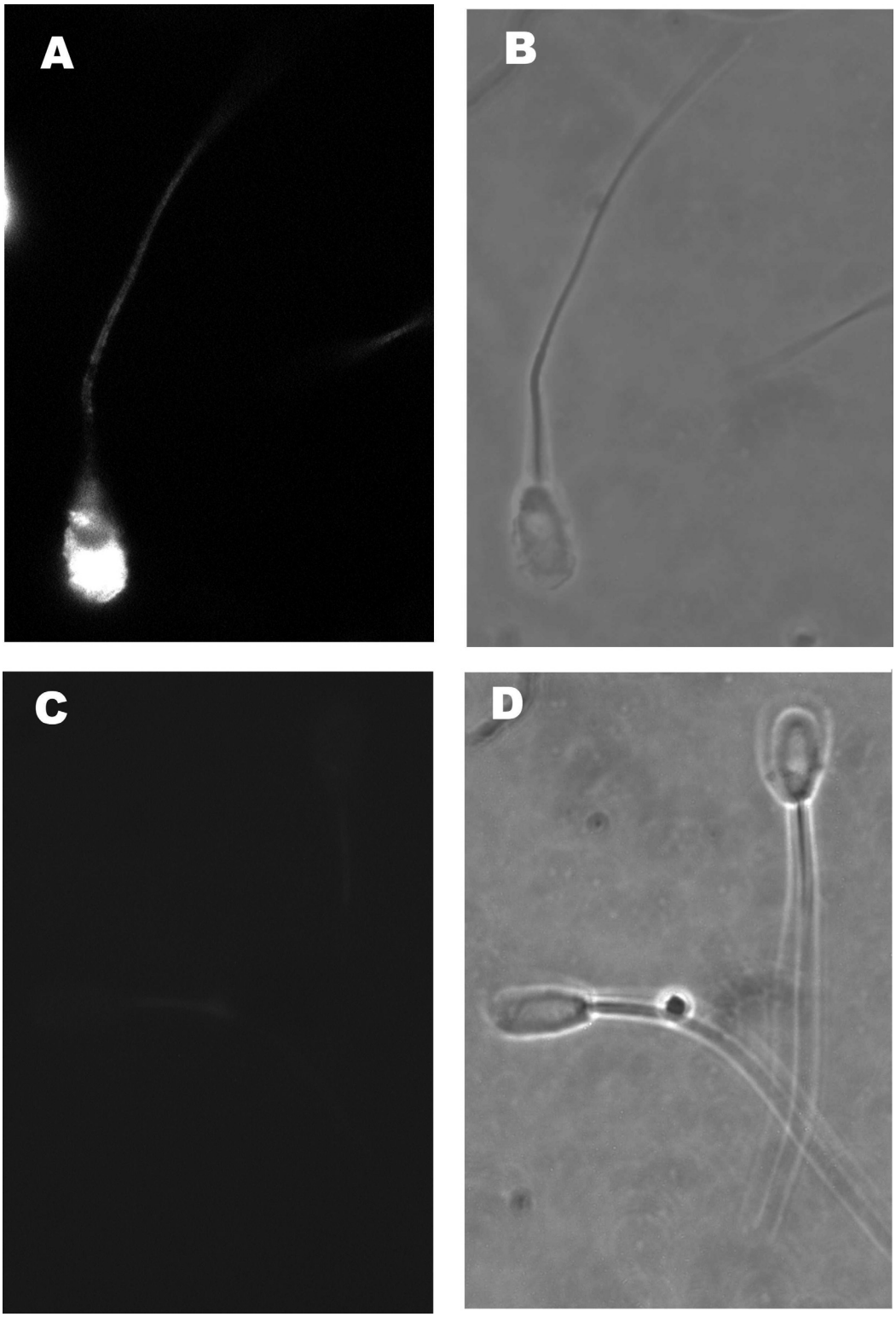

Figure 2: Heparin binding to spermatozoa. A, B: spermatozoa where incubated with biotinylated heparin (100mg/ml) and the binding sites where revealed using FITC-streptavidin. C, D: The same experiment was performed in the presence of 100 μg/ml unlabelled heparin. A, C: Fluorescence images. B, D: Visible light micrographs. (1000 x magnification).

We observed that the optimal conditions to promote *in vitro* sperm capacitation were the incubation in capacitating medium supplemented with 10 mM heparin for up to 120 min, conditions that showed an increase in the number of capacitated sperm and acrosome reacted sperm, without affecting the viability of the sperm cells.

In vitro capacitation has been accomplished under several conditions in defined media that mimic the composition of the oviductal fluid. Certain components such as serum albumin, calcium and bicarbonate play important regulatory roles in promoting capacitation (Visconti and Kopf, 1998; Harrison et al., 1996; Tardif et al., 2003). Ca^{2+} influx is involved in signalling events; however it is not sufficient for capacitation triggering in the absence of bicarbonate. The increase in cAMP and posterior phosphorylation of a set of proteins during sperm capacitation requires the presence of both calcium and bicarbonate in the media (Visconti and Kopf, 1998), suggesting a cooperative effect between these compounds.

We show that heparin enhances in vitro capacitation of porcine sperm as determined by CTC staining and by the ability of spermatozoa to undergo AR after exposure to calcium ionophore. Additionally, to enhance capacitation by heparin, the culture media must contain bicarbonate and calcium, indicating that heparin acts only under capacitating conditions.

No significant differences were observed between the percentage of capacitated sperm in CM and NCCB without heparin, and in both cases, this values augment in presence of heparin. These data support previous indications that BSA is not essential for porcine sperm capacitation *in vitro* (Tardif et al., 2003). Albumin is believed to facilitate the cholesterol efflux from sperm plasma membrane. However, this albumin-mediated cholesterol efflux has been demonstrated only in rodents and human (Cross, 1998).

Studies in bovine sperm capacitation have shown that capacitation *in vitro* can be accomplished in media containing heparin or oviductal fluid in which the active capacitating agents are thought to be heparin like glycosaminoglycans. Glycosaminoglycans may promote capacitation by removing seminal plasma protein (spermadhesins) involved in the formation of a coating layer on spermatozoa surface and are normally thought to function to inhibit capacitation (Visconti and Kopf, 1998).

Taking into account that heparin-like glycosaminoglycans, are components of the oviductal fluid in the sow (Thientai et al., 2000), we propose that heparin can optimize capacitation, by release of the peripheral decapacitation factors, as occurs *in vivo*.

Several studies have demonstrated that bicarbonate is a key effector of *in vitro* capacitation (Harrison et al., 1996; Tardif et al., 2003). Recently Gadella et al., (2004) have established that only those spermatozoa that have functionally completed maturation in the epididymus are sensitive to bicarbonate whereas immature sperm (containing cytoplasmatic droplets) failed to respond to this physiological challenge. In boars of proven fertility, the presence of 14 to 20% of cytoplasmatic droplets is considered as normal. On the other hand, some sperm undergo spontaneous acrosome reaction when they are incubated under capacitation conditions without any specific AR inductor. This response is generally attributed to a degenerative process of sperm that are fully capacitated.

In the presence of heparin, an increment in the rate of AR sperm is observed. To evaluate the possibility that heparin could directly affect the number of AR sperm, this glycosaminoglycan was added to sperm suspensions at the end of incubation in CM. The rate of AR did not vary significantly after 15 min of incubation in the presence of heparin (unpublished data). At least under these experimental conditions, heparin does not affect the proportion of AR sperm. Perhaps, the AR increase corresponds to a subpopulation of unstable, fully capacitated sperm.

The capacitating effect of heparin is observed using either CTC or the AR inductor calcium ionophore. However, the absolute values obtained in each case were

different, being higher for CTC. This is in accordance with the fact that CTC shows increased membrane fluidity which occurs well before sperm are able to undergo AR (Tardif et al., 1999).

When sperm were incubated with biotinylated heparin, we observed that heparin binds to some sperm in a specific and saturable manner, and that the binding sites were located mostly on the acrosomal region. The possibility that heparin binds to boar sperm head in a different fashion according to the physiological state of spermatozoa is presently being investigated. This may represent a useful tool to develop a new staining assay for boar sperm capacitation and for the study of the specific role of heparin-like glycosaminoglycans in the capacitation process.

In vivo, boar spermatozoa accumulate in the uterotubal junction-isthmus of the oviduct (sperm reservoir) by binding to the apical membrane of the epithelia (Suarez, 2002; Thienthai et al., 2004), with a preferential binding of the uncapacitated forms. These cells can be released from this site when they are capacitated (Fazeli et al., 1999; Rodríguez-Martínez et al., 2001). In cattle, heparin and other sulfated GAGs are powerful modulators of sperm binding to oviductal cells *in vitro* and are able to cause an increase of flagellar beating, followed by the release of sperm with high linear motility, probably through early capacitating effects (Talevi and Gualtieri, 2001).

There is evidence of the presence of sulfated and non-sulfated GAGs in the porcine oviduct and of an increase of these compounds in the fluid collected from isthmus and ampulla during standing oestrous (Thientai et al., 2000). The non-sulfated GAG Hialuronan was recently detected in sperm reservoir performing different roles on sperm physiology depending on the moment of the sexual cycle of the sow (Rodríguez-Martínez et al., 2005). However, the effect of the sulfated GAGs like heparin in these sites remains uncertain. These findings suggest that sulfated GAGs may play a role in modulating sperm viability and capacitation during sperm transport in the pig oviduct. The *in vitro* capacitating effect of heparin shown in this work supports this hypothesis.

Further studies will be necessary to elucidate the specific morphological and biochemical changes which result from the action of heparin in the capacitation process of pig sperm.

ACKNOWLEDGMENTS

We thank Fernando Cane of Instituto de Porcinotecnia for provision of semen, María Inés Oyarzábal for statistical assistance and Facultad de Ciencias Veterinarias, ANPCYT and Fundación Antorchas for financing.

REFERENCES

ALTHOUSE GC (1997) Evaluating porcine semen for artificial insemination: standard tests. Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois. Compendium on continuing education for the practicing veterinarian 19 (suppl.): 30-35

BERGER T, TURNER K, MEIZEL S, HEDRICK J (1989) Zona pellucida-induced acrosome reaction in boar sperm. Biol Reprod 40: 525-530

CHAMBERLAND A, FOURNIER V, TARDIF S, SIRARD M, SULLIVAN R, BAILEY J (2001). The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. Theriogenology 55: 823-835

CROSS N (1998) Role of cholesterol in sperm capacitation. Biol Reprod 59: 7-11

DEBBAGE PL, LANGE W, HELLMANN T, GABIUS HJ (1988). Receptors for sulfated polysaccharides in human placenta. J Histochem Cytochem 36: 1097-1102

FAZELI A, DUNCAN A, WATSON P, HOLT W (1999) Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod 60(4): 879-886

GADELLA BM, VAN GESTEL RA (2004) Bicarbonate and its role in mammalian sperm function. Anim Reprod Sc 82-83: 307-329

HARRISON R, ASHWORTH P, MILLER N (1996) Bicarbonate/CO_2 an effector of capacitation induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Mol Reprod Dev 45: 378-91

MILLER D, WINER M, AX R (1990) Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol Reprod 42: 899-915

PARRISH J, SUSKO-PARRISH J, WINER M, FIRST N (1988) Capacitation of bovine sperm by heparin. Biol Reprod 38: 1171-1180

PARRISH J, SUSKO-PARRISH J, HANDROW R, FIRST N (1989) Effect of sulfated glycoconjugates on

capacitation and the acrosome reaction of bovine end hamster spermatozoa. Gamete Res 24:403-413

RODRÍGUEZ-MARTÍNEZ H, THIENTHAI P, SUZUKI K, FUNAHASHI H, EKWALL H, JOHANNISSON A (2001) Oviduct involvement in sperm capacitation and oocyte development. Reprod Suppl 58: 129-145

RODRÍGUEZ-MARTÍNEZ H, SARAVIA, F, WALLGREN M, THIENTHAI P, JOHANNISSON A; VÁZQUEZ J, MARTÍNEZ E, ROCA J, SANZ L, CALVETE J (2005) Boar spermatozoa in the oviduct. Theriogenology 63: 514-535

SUAREZ S (2002) Formation of a reservoir of sperm in the oviduct. Reprod Dom Anim 37: 140-143

TALEVI R, GUALTIERI R (2001) Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium in vitro. Biol Reprod 64: 491-498

TARDIF S, SIRARD MA, SULLIVAN R, BAILEY J (1999) Identification of capacitation-associated phosphoproteins in porcine sperm electroportated with ATP-γ-^{32}P. Mol Reprod Dev 54: 292-302

TARDIF S, DUBÉ C, BAILEY J (2003) Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. Biol Reprod 68: 207-213

THIENTHAI P, KJELLÉN L, PERTOFT H, SUZUKI K, RODRÍGUEZ-MARTÍNEZ H (2000) Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. Reprod Fertil Dev 12: 173-182

THIENTHAI P, JOHANNISSON A, RODRÍGUEZ-MARTÍNEZ H (2004) Sperm capacitation in the porcine oviduct. Anim Reprod Sc 80: 131-146

VALENCIA A, WENS M, MERCHANT H, REYES R, DELGADO N (1984) Capacitation of human spermatozoa by heparin. Arch Androl 12: 109-113

VISCONTI P, KOPF G (1998) Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 59: 1-6

WANG W, ABEYDEERA L, FRASER L, NIWA K (1995) Functional analysis using chlortetracycline fluorescence and in vitro fertilization of frozen-thawed ejaculated spermatozoa incubated in a protein free chemically defined medium. J Reprod Fertil 104: 305-3