ARTICLE
Journal of Cellular Biochemistry 117:2464–2472 (2016)

Journal of Cellular Biochemistry

# Glycosidases Interact Selectively With Mannose-6-Phosphate Receptors of Bull Spermatozoa

Andrea C. Aguilera,[1] Verónica Boschin,[2] Lorena Carvelli,[1] Juan C. Cavicchia,[2] and Miguel A. Sosa[1]*

[1]*Laboratorio de Biología y Fisiología Celular "Dr. Franciso Bertini," Instituto de Histología y Embriología—CONICET-FCM-UNCuyo, Facultad de Ciencias Exactas y Naturales—Universidad Nacional de Cuyo, 5500 Mendoza, Argentina*

[2]*Laboratorio de Biología y Fisiología Celular "Dr. Franciso Bertini," Instituto de Histología y Embriología—CONICET-FCM-UNCuyo, 5500 Mendoza, Argentina*

## ABSTRACT

Glycosidases may play a role in sperm maturation during epididymal transit. In this work, we describe the interaction of these enzymes with bull spermatozoa. We found that β-galactosidase associated to spermatozoa can be released under low ionic strength conditions, whereas the interaction of N-acetyl-β-D-glucosaminidase and β-glucuronidase with spermatozoa appeared to be stronger. On the other hand, α-mannosidase and α-fucosidase cannot be removed from the gametes. In addition, part of N-acetyl-β-D-glucosaminidase, β-galactosidase, and β-glucuronidase can also be released by mannose-6-phosphate. Taking into account these data, we explored the presence of cation-independent- and cation-dependent-mannose-6-phosphate receptors in the spermatozoa and found that cation-independent mannose-6-phosphate receptor is highly expressed in bull spermatozoa and cation-dependent-mannose-6-phosphate receptor is expressed at a lesser extent. In addition, by immunofluorescence, we observed that cation-independent-mannose-6-phosphate receptor is mostly located at the acrosomal zone, whereas cation-dependent-mannose-6-phosphate receptor presents a different distribution pattern on spermatozoa during the epididymal transit. N-acetyl-β-D-glucosaminidase and β-glucuronidase isolated from epididymal fluid interacted mostly with cation-independent-mannose-6-phosphate receptor, while β-galactosidase was recognized by both receptors. We concluded that glycosidases might play different roles in bull spermatozoa and that mannos-6-phosphate receptors may act as recruiters of some enzymes. J. Cell. Biochem. 117: 2464–2472, 2016. 

**KEY WORDS:** BULL EPIDIDYMIS; LYSOSOMAL ENZYMES; SPERMATOZOA; MALE REPRODUCTIVE TRACT; MANNOSE-6-PHOSPHATE RECEPTORS

The mammalian epididymis is a convoluted duct where spermatozoa acquire motility and fertilizing ability [Hinton et al., 1995]. During transit along the duct, spermatozoa interact with molecules secreted by the epididymal epithelium. These interactions result in biochemical and morphological changes in the spermatozoa. Some of these changes include adsorption of epididymal proteins on the spermatozoa surface and a molecular reorganization of the plasmalemma [Sullivan, 1999; Cuasnicu et al., 2002]. The maturation of spermatozoa in the epididymis is the result of sequential interactions with proteins secreted by the epithelium [Cooper, 1998; Robaire et al., 2006; Turner, 2008; Cornwall, 2009; Dacheux et al., 2009, 2012].

Among the proteins secreted into the lumen, glycosidases can be found at high concentrations in several mammalian species [Mayorga and Bertini, 1985; Gupta and Setty, 1995; Tulsiani et al., 1998; Belmonte et al., 2002; Hermo and Robaire, 2002; Dacheux et al., 2005; Tulsiani and Abou-Haila, 2011], although the role of these enzymes in sperm maturation is still controversial, since the intraluminal pH is not optimum for their catalytic activity [Levine and Kelly, 1978]. However, it has been demonstrated that some enzymes, such as β-galactosidase is active in the fluid of rat epididymis [Tulsiani et al., 1995].

A proteomic analysis has demonstrated that hydrolytic enzymes such as α- and β-mannosidase, β-hexosaminidase, and cathepsins A and D are found in the epididymal lumen of reproductively active bulls and they are secreted by the epithelium of the organ [Belleannee et al., 2011], and in turn, an active secretion of cathepsin D and α-L-fucosidase could be related to the reproductive

Grant sponsor: SECTyP, Universidad Nacional de Cuyo, Mendoza (Argentina); Grant number: 06/J459.

*Correspondence to: Dr. Miguel A. Sosa, Laboratorio de Biología y Fisiología Celular "Dr. Franciso Bertini," Instituto de Histología y Embriología—CONICET-FCM-UNCuyo, Facultad de Ciencias Exactas y Naturales—Universidad Nacional de Cuyo, 5500 Mendoza, Argentina. E-mail: msosa@fcm.uncu.edu.ar

Manuscript Received: 9 November 2015; Manuscript Accepted: 9 March 2016

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 March 2016

DOI 10.1002/jcb.25538 • © 2016 Wiley Periodicals, Inc.

capacity [Moura et al., 2006]. Considering the selective interaction of some soluble epididymal enzymes with epididymal spermatozoa, it may be possible that some enzymes may be transported by the spermatozoa to the site of fertilization. [Belmonte et al., 1998, 2000], In addition, it has been suggested that active glycosidases associated to spermatozoa may be important for: (i) the binding of spermatozoa to oviductal cells [Lefebvre et al., 1997]; (ii) the sperm capacitation [Taitzoglou et al., 2007]; (iii) the dispersion of the cumulus cells [Takada et al., 1994]; and (iv) the direct sperm-oocyte interactions [Miller et al., 1993; Venditti et al., 2007, 2010; Phopin et al., 2013]. However, the nature of the interaction of some glycosidases with spermatozoa remains unknown.

In most cell types, many lysosomal enzymes acquire a mannose-6-phosphate residue during its biosynthesis and they are transported selectively to lysosomes by specific receptors (mannose-6-phosphate receptors, MPRs) [Hille-Rehfeld, 1995; Dahms et al., 2008]. Two types of MPRs have been described so far, the cation-dependent (CD-MPR) and the cation-independent (CI-MPR), which co-exist in most of mammalian cell types [Dahms et al., 2008; Braulke and Bonifacino, 2009; Nadimpalli and Amancha, 2010], though the relevance of that co-existence has not been conclusively explained.

Interestingly, MPRs have also been found in the surface of epididymal spermatozoa in some mammalian species [Belmonte et al., 1998, 2002] and they could interact with enzymes secreted by the epididymal epithelium [Belmonte et al., 2002].

The aim of the present study was to determine whether secreted enzymes interact with spermatozoa in bull epididymis and to elucidate the nature of that interaction. We observed that both MPRs (CD-MPR and CI-MPR) are expressed in spermatozoa and they interact differently with the enzymes. In addition, we present evidence that CD-MPR may accompany the sperm maturation during epididymal transit.

## MATERIALS AND METHODS

### REAGENTS

The rabbit polyclonal anti-CI-MPR serum was kindly provided by Dr. Nancy Dahms (Wisconsin University, USA) and the rabbit polyclonal anti-CD-MPR serum was kindly provided by Dr. A. Hille-Rehfeld (Stuttgart, Germany). The rabbit anti-tubulin (T3526) antibody was purchased from Sigma Chemical Co. (St. Louis, MO). The biotin-conjugated anti-rabbit IgG antiserum (B-7389) the FITC-conjugated anti-rabbit IgG antiserum (F-0382) and horseradish peroxidase-conjugated avidin were also provided by Sigma. The corresponding 4-methylumbelliferyl substrates for α-mannosidase (α-MAN), N-acetyl-β-D-glucosaminidase (β-NAG), β-galactosidase (β-Gal) β-glucuronidase (β-Glu), α-fucosidase (α-Fuc) and the mannose-6-phosphate (M6P, disodium salt, cat.M6876) were also provided by Sigma. The chemiluminescent reagent was prepared with 1.25 mM luminol, and 198 μM *p*-coumaric acid in 100 mM Tris-HCl (pH 8.5) according to Mruk and Cheng [2011].

### ANIMALS

Reproductively mature bulls (*Aberdeen-Angus* ~4 years old) were used in this study. These animals had been used for reproductive purposes in farms. Bulls were fed with diets primarily composed of pasture provided by farms located in Córdoba (Argentina). All the procedures were performed according to the protocol approved by CICUAL (Commitee for Animal Care of the Universidad Nacional de Cuyo, Mendoza, Argentina).

### BIOLOGICAL SAMPLES

After slaughtering (slaughterhouse located in Corralitos, Mendoza, Argentina), epididymides were removed from the bulls and processed up to 2 h later. Testes and epididymides were transported to the laboratory on ice and processed immediately. The organs (one per animal) were carefully dissected and the caput, corpus, and cauda were processed separately. Tissues were cut into small pieces minced with a stainless steel blade. The samples were suspended (1:3 w/v) either in Hanks solution or buffer H (10 mM Tris–acetate, pH 7.2, containing 0.25 M sucrose, 1% EDTA, 1 mM PMSF, 0.02% sodium azide, and 5 mM glycerophosphate), depending on the experiment (as detailed below). Incubations were carried out, at 37°C for 30 min with gentle manual agitation to permit the release of spermatozoa. The minced tissue was settled for 10 min at 4°C and the resulting supernatant (containing fluid and spermatozoa) was centrifuged at 400*g* for 5 min and spermatozoa were separated from the fluid. The spermatozoa were washed three times with Hanks solution and finally pelleted at 1000*g* for 10 min. The epididymal fluids were stored at −20°C until use.

### RELEASE OF ACID HYDROLASES FROM SPERMATOZOA

Cauda spermatozoa were washed twice with buffer H followed by sequential incubations (30 min each one) with increasing concentrations of NaCl (0.05–0.5 M) in buffer H. After incubation on ice (with manual agitation every 10 min) samples were centrifuged for 10 min at 800*g* and the supernatants were collected and replaced with the following salt concentration. In other experiments, spermatozoa were incubated with 10 mM mannose-6-phosphate (M6P) for 2 h (with manual agitation every 10 min) on ice and centrifuged for 10 min at 800*g* as before. Under these conditions of temperature, spermatozoa remained intact, although no differences were observed when incubations were carried at 20°C. All the supernatants (containing released enzymes) and the remaining spermatozoa (resuspended in 2% Triton X-100) were stored at −20°C until use. The integrity of spermatozoa was evaluated by fluorescence microscopy using *Pisum Sativum* [Jankovicová et al., 2008] or by transmission electron microscopy [Belmonte et al., 2000].

### BINDING ASSAYS

To measure activity of MPRs on sperm surface, we performed a binding assay using β-glucuronidase (pGLU) purified from rat preputial gland [Tulsiani et al., 1975]. Briefly, $1 \times 10^6$ of cauda spermatozoa were previously washed with 1 ml of buffer H containing 0.3 M NaCl (to remove endogenous enzyme, Belmonte et al., 1998), and incubated with 250–1200 U pGLU in 250 μl buffer B (10 mM Tris–acetate, pH 7.2 containing 0.5 mM EGTA), either in the presence or absence of bivalent ions (0.5 mM $CaCl_2$ and 0.5 mM $MnCl_2$), and in the presence or absence of 10 mM mannose-6-phosphate (M6P), as described by Romano et al. [2002]. The enzyme

bound to spermatozoa was measured on the pellets after centrifugation at 1000*g*. Pellets were resuspended in 2% Triton X-100 and the activity of each enzyme was measured. Under these conditions, no interference by Triton X-100 on enzyme measurements was detected. Values of $K_D$ and the number of binding sites ($B_{max}$) were estimated from the binding curves according to Romano et al. [2002].

### BINDING OF EPIDIDYMAL ENZYMES TO SPERMATOZOA

Binding conditions were similar to those described above, except that crude epididiymal fluid was added to the binding assay instead pGLU. Briefly, $1 \times 10^6$ cells obtained from cauda were incubated with the corresponding fluid adjusted to 250–1200 U for each enzyme: α-Man, β-NAG, β-Gal, β-Glu, or α-Fuc either in the presence or in the absence of the bivalent ions, and in the presence or absence of M6P in a final volume of 250 μl of buffer B. After centrifuging at 1000*g* (for 10 min at 4°C), the pellet containing the spermatozoa was resuspended in 2% Triton X-100 and the activity of each enzyme was measured. The total binding to MPRs was that in the presence of bivalent cations and M6P. The binding to CD-MPR was deduced from the difference between the total binding and that obtained in the absence of bivalent cations.

### IMMUNOBLOTTING

Sperm proteins (from caput, corpus, or cauda) were extracted with sample buffer [Laemmli, 1970] at 95°C for 5 min and under non-reducing conditions. This extraction was repeated twice. After centrifugation at 12,000*g* for 10 min, supernatants were supplemented with 10 mM dithiothreitol, and boiled for 3 min. Protein samples were run on SDS-PAGE gels (7.5% or 10% acrylamide for CI-MPR or CD-MPR, respectively) and electrotransferred to nitrocellulose (NC) membranes (pore size 0.45 μm, GE Healthcare, Germany) for 4 h (CI-MPR) or 50 min (CD-MPR) at 250 V. CD-MPR and CI-MPR were detected on the membrane using the corresponding specific antibodies as described by Carvelli et al. [2010]. Specific bands were detected by the enhanced chemiluminescence method (ECL), and quantified by densitometric scanning on the membranes using the Image Quant LAS 4000 (GE Healthcare, Uppsala, Sweden). Detection of tubulin was used as loading control.

### IMMUNOFLUORESCENCE

Spermatozoa obtained from caput, corpus and cauda (as described above) and previously washed once with buffer H were resuspended in PBS and fixed with 2% *p*-formaldehyde (PAF, in PBS) for 10 min, then placed on slides (previously treated with 10 mM poly-lysine for 30 min) and processed for immunofluorescence according to Belmonte et al. [2000]. Briefly, the slides containing spermatozoa were blocked for 1 h at room temperature with 5% horse serum in PBS/PVP (0.1% polyvinylpyrrolidone in PBS). They were then incubated overnight at 4°C with either anti-CD-MPR or anti-CI-MPR (diluted 1:200 and 1:100, respectively) in PBS/PVP-HS (PBS/PVP containing 1% horse serum). After three washes with PBS/PVP, a FITC-conjugated anti-rabbit IgG (1:100 in PBS/PVP-HS) was added and incubated for 1 h at room temperature. Slides were washed three times with PBS and mounted on glass coverslip with UltraCruz™ mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were then examined with a Fluorescence Microscope (Nikon 80i, Japan). The percentage of stained spermatozoa was estimated by counting 200 cells with epifluorescence optics at a magnification of 600×.

### FREEZE FRACTURE

For freeze fracture, the general procedure of Cavicchia and Morales [1992] was followed. Spermatozoa from corpus and cauda epipidymis prepared as indicated above were fixed by immersion in 3% glutaraldehyde-cacodylate buffer for 20 min and placed in 30% glycerol for 2 h. One drop of each sperm sample was placed on gold freeze fracture holders, and rapidly frozen in liquid Freon 22 and stored in liquid nitrogen. The material was fractured in a Balzers BAF 301 at −105°C with no etching and shadowed with platinum followed by carbon. Replicas were cleaned with sodium hypochlorite, mounted in copper grids and observed in a electron microscope (Zeiss 900, Germany). The membrane fracture faces were labelled as either protoplasmic fracture face (P face) or exoplasmic fracture face (E face).

### OTHER PROCEDURES

The activity of α-Man, β-NAG, β-Gal, and β-Glu were measured fluorometrically, using the corresponding 4-methyl-umbellyferyl-substrate [as described by Barrett and Heath, 1977] and the activity of α-Fuc was measured using *p*-nitrophenyl substrate. One unit of enzymatic activity catalyzes the release of 1 nmol of 4-methyl-umbellyferone/h or 1 nmol of *p*-nitrophenol. Protein concentration was estimated according to Lowry et al. [1951].

### STATISTICAL ANALYSES

Data were analyzed by one-way ANOVA followed by Tukey's-Kramer multiple comparisons test, and the level of significance was set at $P < 0.05$.

## RESULTS

In this study, we first observed that glycosidases are associated to cauda epididymal spermatozoa, being mostly enriched in β-NAG (Table I). It seems that β-Gal is weakly associated to spermatozoa since it is released easily by low concentrations of sodium chloride (Fig. 1). Instead, β-Glu and β-NAG are released with higher concentrations of salts and a significant percentage still remained associated to the spermatozoa after treatments. Conversely, α-Man

TABLE I. Activity of Glycosidases Associated to Spermatozoa From the Cauda of Bull Epididymis

| Glycosidase | Units/$10^6$ spermatozoa |
|---|---|
| β-NAG | 4117.4 ± 713.2* |
| β-Gal | 637.0 ± 99.8 |
| β-Glu | 293.6 ± 22.9 |
| α-Man | 965.6 ± 91.7 |
| α-Fuc | 621.8 ± 96.9 |

Values are expressed as means ± SEM from four different animals. β-NAG, N-acetyl-β-D-glucosaminidase; β-Gal, β-galactosidase; β-Glu, β-glucuronidase; α-Man, α-mannosidase; α-Fuc, α-fucosidase.
*Significantly different from the other enzymes ($P < 0.05$).

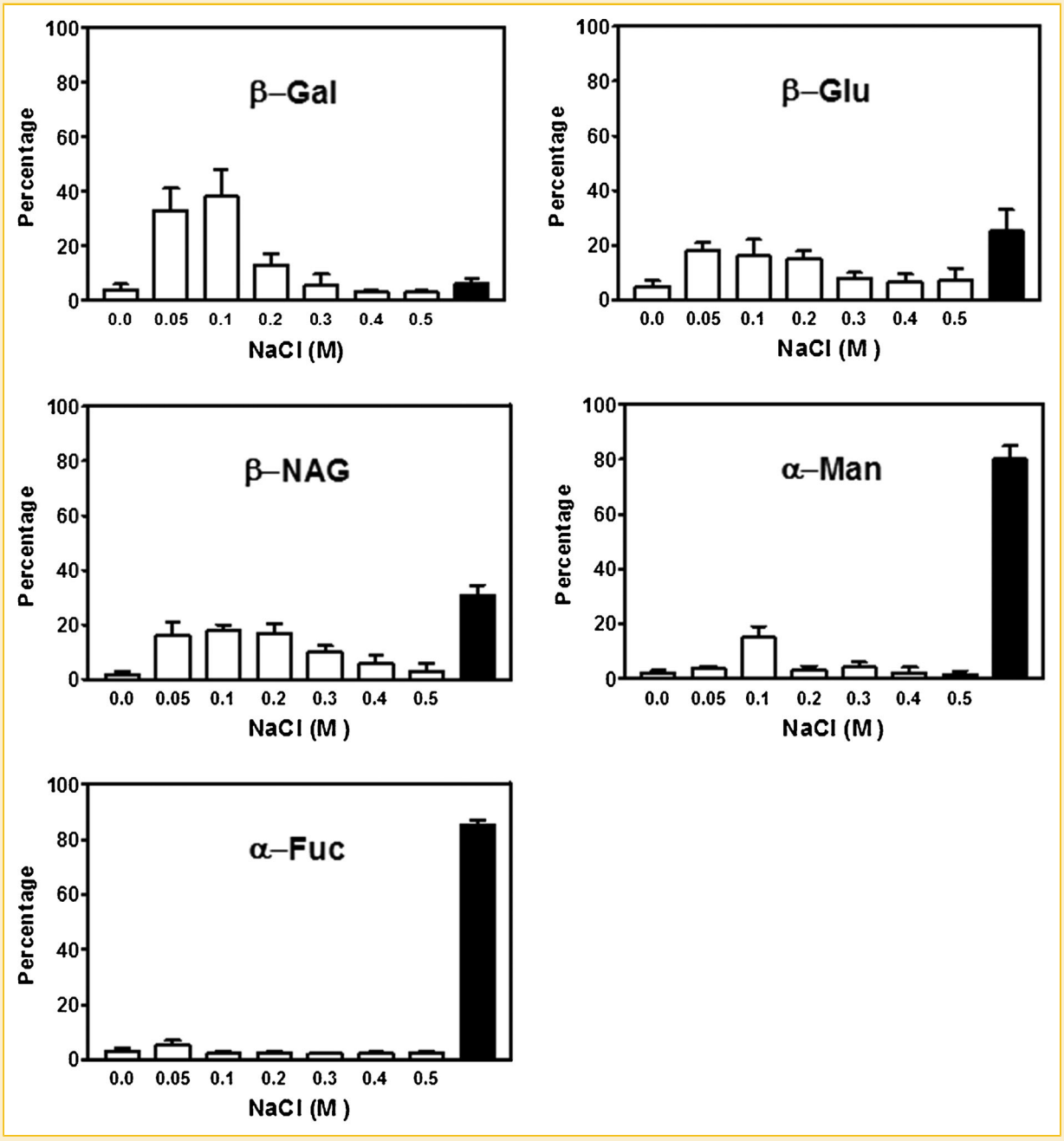



**Fig. 1.** Release of lysosomal enzymes associated to epididymal bull spermatozoa with increasing salt concentrations (NaCl). Values are expressed as percentages ± SD (n = 5) of released enzyme with the indicated salt concentration (white bars) versus remaining enzyme after salt extraction (black bars), from the total content in spermatozoa. β-NAG, N-acetyl-β-D-glucosaminidase; β-Gal, β-galactosidase; β-Glu, β-glucuronidase; α-Man, α-mannosidase; α-Fuc, α-fucosidase.

and α-Fuc are difficult to remove under high ionic strength conditions and they are mostly retained by the gamete (Fig. 1).

In most cell types, lysosomal enzymes interact with membranes via mannose-6-phosphate receptors (MPRs) for proper delivery to lysosomes. Herein, we observed that both MPRs (CD-MPR and CI-MPR) are also expressed on the surface of bull spermatozoa, and the expression does not vary between the spermatozoa of different epididymal regions (Fig. 2). The presence of glycosidases in the sperm plasma membrane may be related, at least in part, to the presence of these specific receptors. Accordingly, we have observed that a third of β-NAG is released with the phosphorylated sugar M6P and at lesser extent with β-Glu and β-Gal (Table II). On the other hand, it seems that the distribution of the CD-MPR changes during epididymal transit, from a dispersed signal in the head of epididymal spermatozoa to a concentrated post-equatorial distribution in the cauda (Fig. 3A and B). These findings are in accordance with an increased number of intramembrane particles in that zone ($11.3 \pm 0.9$ in corpus and $20.7 \pm 0.9$ particles/$\mu^2$ in cauda spermatozoa (from three areas

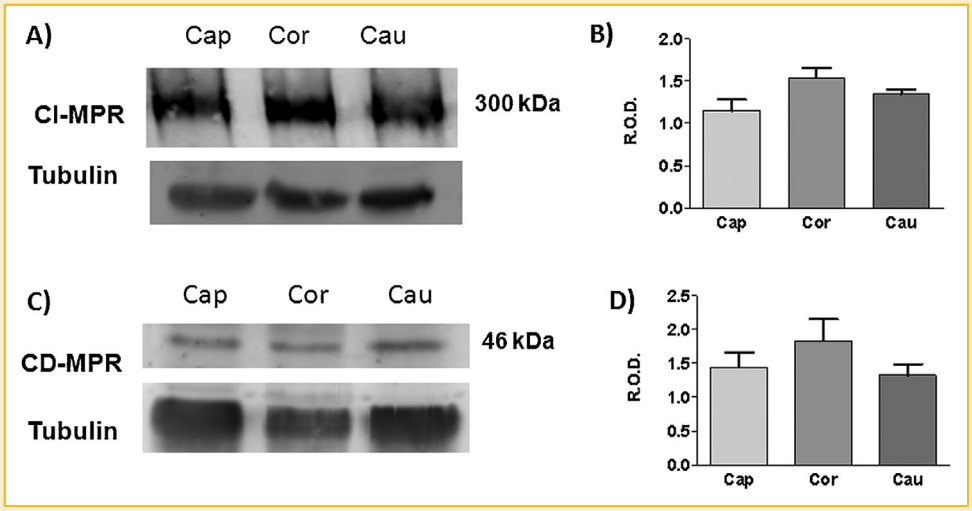



**Fig. 2.** Immunoblot for detection of mannose-6-phosphate receptors in spermatozoa from caput, corpus, or cauda of bull epididymis. A and B: detection of CI-MPR and the corresponding quantification of bands. C and D: detection of CD-MPR, and the corresponding quantitation of bands. In all cases, bars represent the means of relative optical densities (R.O.D.) ± SD (n = 3) after the substraction of tubulin detection.

measured), as observed by freeze fracture (Fig. 4). Instead, the CI-MPR showed a similar acrosomal distribution pattern in spermatozoa from the different epididymal regions (Fig. 3). By using an exogenous enzyme, such as pGlu purified from rat preputial gland (which is a polyvalent phosphomannosyl ligand), we have observed that the number of active CI-MPR ($B_{max}$) is higher than CD-MPR, consistent with the expression of both MPRs (Fig. 5). We also attempted to determine if the enzymes that are found in the epididymal fluid are ligands for MPRs of the spermatozoa and if these enzymes were able to interact either with both MPRs or with each one differently. To this end, the binding assay was performed with cauda spermatozoa and the corresponding crude fluid, either in the presence or in the absence of bivalent ions and in the presence or absence of mannose-6-phosphate (as described in materials and methods). Despite the existing competition between the enzymes, we have observed that β-NAG, and β-Glu are mostly recognized by the CI-MPR (Fig. 6B and C), whereas β-Gal showed more affinity for CD-MPR, despite its low expression (Fig. 6A).

TABLE II. Release of Glycosidases From Bull Spermatozoa With 10 mM of Mannose-6-Phosphate

| Enzyme | Percentage |
|---|---|
| β-NAG | 39.66 ± 12.0* |
| β-Gal | 13.75 ± 3.77** |
| β-Glu | 15.75 ± 5.37** |
| α-Man | 2.39 ± 0.85 |
| α-Fuc | 1.85 ± 1.25 |

Values are expressed as percentage (mean) of released enzyme from the total associated to spermatozoa ± SD from four different animals. β-NAG, N-acetyl-β-D-glucosaminidase; β-Gal, β-galactosidase; β-Glu, β-glucuronidase; α-Man, α-mannosidase; α-Fuc, α-fucosidase.

*Significantly different from all the other enzymes ($P < 0.05$).

**Significantly different from α-Man and α-Fuc ($P < 0.05$).

## DISCUSSION

In mammals, the functionality of epididymis has been studied for decades and accumulated evidence suggests an important role of this organ in sperm maturation. The epididymal epithelium synthesizes and secretes glycosidases, which would be involved in sperm maturation.

In this study, we found that several glycosidases are associated to epididymal spermatozoa in reproductively mature bulls and it appears that interaction varies between five studied enzymes. It was observed that β-Gal is associated to the spermatozoa with low affinity, as this enzyme is released easily from the spermatozoa with low concentrations of NaCl (Fig. 1). Unlike other species, it seems that β-Gal is associated to the sperm surface, rather than being acrosomal [Kuno et al., 2000], since the cells remained intact after the treatments with salts (data not shown). On the other hand, β-Glu and β-NAG are partially released with NaCl, indicating that the interaction with membrane is stronger. Unlike the other enzymes, α-Man and α-Fuc remain mostly bound to the spermatozoa, even after a strong treatment with salts, indicating that these enzymes are tighly associated to spermatozoa [Tulsiani et al., 1989; Hancock et al., 1993; Kuno et al., 2000; Khunsook et al., 2003; Venditti et al., 2010]. We also attempted to elucidate the nature of the enzyme-spermatozoa interaction. Herein, we have also observed that part of β-NAG and, at lesser extent, β-Gal and β-Glu are released from the spermatozoa by treatment with M6P, suggesting some participation of mannose-6-phosphate receptors (MPRs) in the interaction of these enzymes with spermatozoa. It is well known that MPRs play a role in the delivery of acid hydrolases, and that two distinct MPRs occur in mammalian cells, the cation dependent-(CD-MPR) and the CI-MPR receptor [Ghosh et al., 2003; Dahms et al., 2008; Braulke and Bonifacino, 2009], which co-exist in most cell types, including rat spermatozoa [Belmonte et al., 2000, 2002]. In line with this, the two MPRs are found in bovine

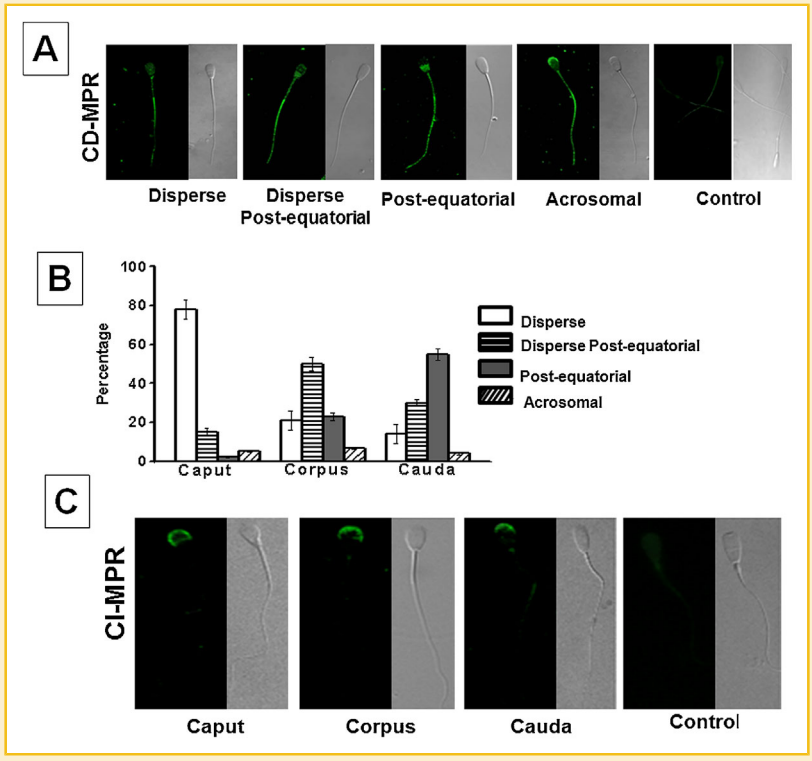
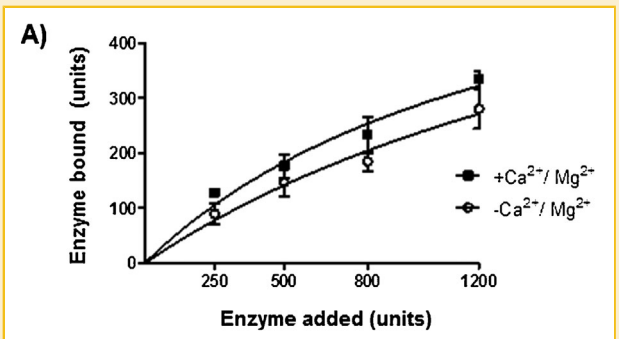



**Fig. 3.** Immunofluorescence for detection of sperm MPRs along epididymal duct. (A) Distribution patterns of CD-MPR in spermatozoa from any region; disperse, disperse with post-equatorial, exclusively post-equatorial, and acrosomal, as indicated. (B) Quantitation of cells from each epididymal region, showing the patterns described in A. Values are expressed as the means of percentages of each distribution pattern ± SD from five different animals. (C) Immunodetection of CI-MPR in bull spermatozoa, showing acrosomal location along the epididymal duct. Controls; the sperm were incubated with secondary antibody alone.


**Fig. 4.** Freeze-fracture of spermatozoa obtained from either the corpus (A) or cauda (B) of bull epididymis. The image shows the P face in the post-acrosomal region. Arrows indicate 10 nm intramembrane particles, which were quantified from the figure. *Collar-like rods of particles close to the neck region. Scale bar: 0.6 μm.




B)

Values of $B_{max}$ and $K_D$ for CI- and CD-MPR in cauda bovine spermatozoa

| | | |
|---|---|---|
| **CI-MPR** | $B_{max}$ | 21,3 |
| | $K_D$ (nM) | 0,24 |
| **CD-MPR** | $B_{max}$ | 5,02 |
| | $K_D$ (nM) | 0,4 |

Data calculated from the binding curves. Bmax is expressed as pmol/mg protein

**Fig. 5.** Binding of preputial gland β-glucuronidase to spermatozoa from bull cauda epididymis (A). Spermatozoa were incubated with 250–1200 U of enzyme in the absence (lower curve) or presence (upper curve) of bivalent cations, as described in materials and methods. The bound enzyme was settled with the spermatozoa and measured in the pellets. Non-specific binding was determined in the presence of mannose-6-phosphate and substracted from the curves. Each point represents the mean ± SD from three assays. (B) Values of $B_{max}$ (maximum of binding sites) and $K_D$ (affinity constant) for each MPR derived from the binding curves.

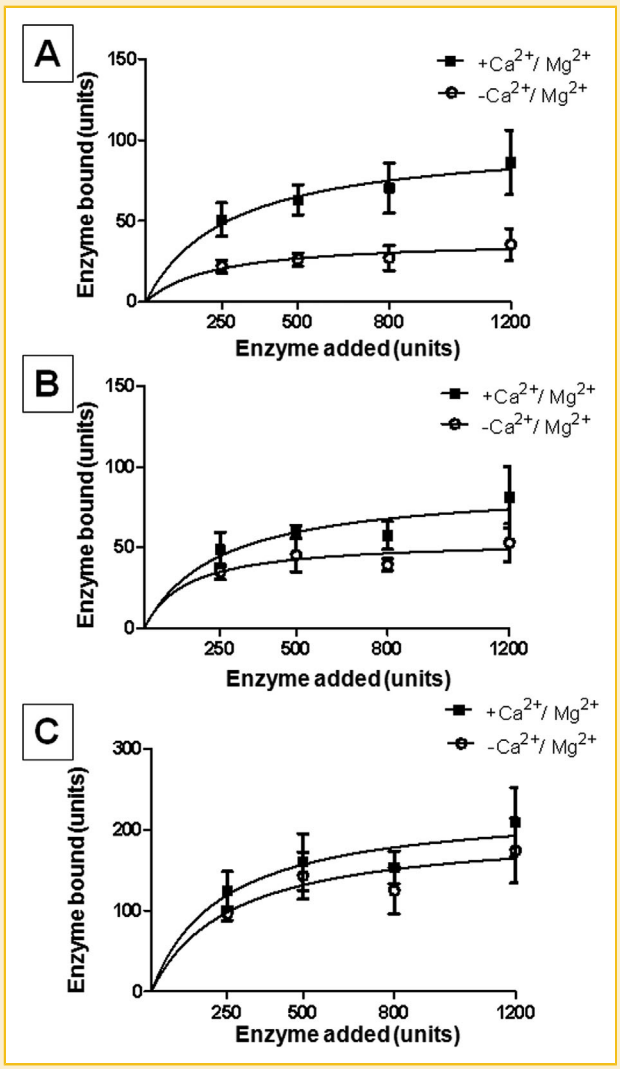



**Fig. 6.** Binding of lysosomal enzymes from epididymal fluid to spermatozoa of bull cauda epididymis. Binding was performed with $1 \times 10^6$ spermatozoa and fluid adjusted to 250–1200 U for each enzyme in a final volume of 250 μl, either in the presence (upper curve) or absence (lower curve) of bivalent ions, as described in materials and methods. The non-specific binding (in the presence of M6P) was substracted from each curve. Each point in the curves represents the mean ± SD from three independent experiments. (A) β-Gal, β-galactosidase; (B) β-Glu, β-glucuronidase; (C) β-NAG, N-acetyl-β-D-glucosaminidase.

spermatozoa, being the CI-MPR highly expressed on the spermatozoa surface (Fig. 2). Interestingly, CD-MPR exhibits different distribution patterns on spermatozoon along epididymal duct, while the CI-MPR has an unchanged acrosomal location (Fig. 3). These changes in the distribution of the CD-MPR could be due to a movement of these receptors along the sperm membrane, and may be relevant to the interaction with enzymes.

In addition, by means of binding assays [Romano et al., 2002], we demonstrated that both MPRs are active to phosphomannosyl-ligands, indicating that they could select enzymes or other proteins from the epididymal fluid. The values of $K_D$ indicate that the two MPRs have higher affinity for M6P ligands than in other tissues [Romano et al., 2002, 2005]. This could be due to a higher exposure of MPRs on the sperm surface or that other membrane proteins may enhance or potentiate their activity. It is well known that these receptors have the capacity to form dimers, which enriches interaction with phosphomannosyl ligands [York et al., 1999; Byrd et al., 2000; Ghosh et al., 2003; Dahms et al., 2008]. Therefore, changes in membrane fluidity in the spermatozoa could regulate the activity of the MPRs. Although it has been found that the epididymal spermatozoa can carry cytoplasmic remnants (droplets) [Cooper and Yeung, 2003; Tajik et al., 2007], which could contribute to the enzymatic activity measured in the spermatozoa [Garbers et al., 1970], this would not be the case, since less than 1% of cauda bull spermatozoa had droplets (data not shown). In this study we also observed that MPRs from the sperm surface can also recognize the enzymes from the epididymal fluid (Fig. 6), indicating that some enzymes secreted by the epithelium could become associated to spermatozoa via these receptors during epididymal transit. In addition, both receptors could play different functions, as they can discriminate between the secreted enzymes (Fig. 6). This selectivity can also be related to the structure of the phosphomannosyl residues on the enzymes, either bearing monovalent or polyvalent ligands [Distler et al., 1991; Chavez et al., 2007].

The reasons for the presence of these receptors in cells that lack lysosomes are unknown. One possibility is that the binding to MPRs could be a step in the activation process of the enzymes to remodelate the sperm surface, although the epididymal pH is not the optimum for this activity. It is also possible that MPRs on sperm surface are the vehicle for these enzymes to be activated in other environment, such as the female reproductive tract [Belmonte et al., 2002]. However, a role for MPRs in the interaction sperm-oocyte should not be ruled out.

In conclusion, we have demonstrated that bovine spermatozoa carry numerous glycolytic enzymes. These enzymes would have a role in sperm remodeling, or carried by the spermatozoa to another environment, or play a role in post-maturation events. According to the results obtained in this study we also suggest a new role for the MPRs, other than the transport of lysosomal enzymes to lysosomes. Thus, the recruitment of glycosidases mediated by MPRs in the epididmymal spermatozoa, may be relevant for the reproductive capacity in bulls.

## ACKNOWLEDGMENTS

Dr. Miguel Sosa is a Career Researcher of CONICET. We thank Mr. T. Sartor for his valuable technical assistance. We also thank Dr. Guillermo Nuñez for correcting this manuscript. This study was supported by SECTyP (Grant 06/J459), Cuyo University (Mendoza-Argentina).

## REFERENCES

Barrett AJ, Heath MF. 1977. Lysosomes: A laboratory handbook. In: Dingle J, editor. Amsterdam: Elsevier/North Holland and Biomedical Press. pp 118–120.

Belleannee C, Labas V, Teixeira-Gomes AP, Gatti JL, Dacheux JL, Dacheux F. 2011. Identification of luminal and secreted proteins in bull epididymis. J Proteom 74:59–78.

Belmonte SA, Challa A, Gutierrez LS, Bertini F, Sosa MA. 1998. Alpha-mannosidase from rat epididymal fluid is a ligand for phosphomannosyl receptors on the sperm surface. Int J Androl 21:277–282.

Belmonte SA, Romano PS, Fornés WM, Sosa MA. 2000. Changes in distribution of phosphomannosyl receptors during maturation of rat spermatozoa. Biol Reprod 63:1172–1178.

Belmonte SA, Romano PS, Sosa MA. 2002. Mannose-6-phosphate receptors as a molecular indicator of maturation of epididymal sperm. Arch Androl 48:53–63.

Braulke T, Bonifacino JS. 2009. Sorting of lysosomal proteins. Biochim Biophys Acta 1793:605–614.

Byrd JC, Park JH, Schaffer BS, Garmroudi F, MacDonald RG. 2000. Dimerization of the insulin-like growth factor II/mannose 6-phosphate receptor. J Biol Chem 275:18647–18656.

Carvelli LF, Bannoud N, Aguilera CA, Morales CR, Sosa MA. 2010. Castration induces changes in the cation-dependent mannose-6-phosphate receptor in rat epididymis: Possible implications in secretion of lysosomal enzymes. J Cell Biochem 110:1101–1110.

Cavicchia JC, Morales A. 1992. Characterization of nuclear pore distribution in freeze-fracture replicas of seminiferous tubules isolated by transillumination. Tissue Cell 24:75–84.

Chavez CA, Bohnsack RN, Kudo M, Gotschall RR, Canfield WM, Dahms NM. 2007. Domain 5 of the cation-independent mannose 6-phosphate receptor preferentially binds phosphodiesters (mannose 6-phosphate N-acetylglucosamine ester). Biochemistry 46:12604–12617.

Cooper TG. 1998. Interactions between epididymal secretions and spermatozoa. J Reprod Fertil Suppl 53:119–136.

Cooper TG, Yeung CH. 2003. Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. Microsc Res Tech 61:28–38.

Cornwall GA. 2009. New insights into epididymal biology and function. Hum Reprod Update 15:213–227.

Cuasnicu P, Cohen D, Elleman D, Busso D, DaRos V, Morgenfeld M. 2002. Changes in sperm proteins during epididymal maturation. In: Robaire B, Hinton B, editors. The epididymis: From molecules to clinical practice. New York: Kluwer Academic/Plenum Publishers. pp 81–102.

Dacheux JL, Castella S, Gatti JL, Dacheux F. 2005. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 63:319–341.

Dacheux JL, Belleannee C, Jones R, Labas V, Belghazi M, Guyonnet B, Druart X, Gatti JL, Dacheux F. 2009. Mammalian epididymal proteome. Mol Cell Endocrinol 306:45–50.

Dacheux JL, Belleannee C, Guyonnet B, Labas V, Teixeira-Gomes AP, Ecroyd H, Druart X, Gatti JL, Dacheux F. 2012. The contribution of proteomics to understanding epididymal maturation of mammalian spermatozoa. Syst Biol Reprod Med 58:197–210.

Dahms NM, Olson LJ, Kim JJ. 2008. Strategies for carbohydrate recognition by the mannose 6-phosphate receptors. Glycobiology 18:664–678.

Distler JJ, Guo JF, Jourdian GW, Srivastava OP, Hindsgaul O. 1991. The binding specificity of high and low molecular weight phosphomannosyl receptors from bovine testes. Inhibition studies with chemically synthesized 6-O-phosphorylated oligomannosides. J Biol Chem 266:21687–21692.

Garbers DL, Wakabayashi T, Reed PW. 1970. Enzyme profile of the cytoplasmic droplet from bovine epididymal spermatozoa. Biol Reprod 3:327–337.

Ghosh P, Dahms NM, Kornfeld S. 2003. Mannose 6-phosphate receptors: New twists in the tale. Nat Rev Mol Cell Biol 4:202–212.

Gupta G, Setty BS. 1995. Activities and androgenic regulation of lysosomal enzymes in the epididymis of rhesus monkey. Endocr Res 21:733–741.

Hancock LW, Raab LS, Aronson NN, Jr. 1993. Synthesis and processing of rat sperm-associated a-L-fucosidase. Biol Reprod 48:1228–1238.

Hermo LN, Robaire B. 2002. Epididymal cell types and their functions. In: Robaire B, Hinton B, editors. The epididymis: From molecules to clinical practice. New York: Kluwer Academic/Plenum Publishers. pp 81–102.

Hille-Rehfeld A. 1995. Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. Biochim Biophys Acta 1241:177–194.

Hinton BT, Palladino MA, Rudolph D, Labus JC. 1995. The epididymis as protector of maturing spermatozoa. Reprod Fertil Dev 7:731–745.

Jankovicová J, Simon M, Antalíková J, Horovská L. 2008. Acrosomal and viability status of bovine spermatozoa evaluated by two staining methods. Acta Vet Hung 56:133–138.

Khunsook S, Bean BS, McGowan SR, Alhadeff JA. 2003. Purification and characterization of plasma membrane-associated human sperm alpha-L-fucosidase. Biol Reprod 68:709–716.

Kuno M, Yonezawa N, Amari S, Hayashi M, Ono Y, Kiss L, Sonohara K, Nakano M. 2000. The presence of a glycosyl phosphatidylinositol-anchored alpha-mannosidase in boar sperm. IUBMB Life 49:485–489.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lefebvre R, Lo MC, Suarez SS. 1997. Bovine sperm binding to oviductal epithelium involves fucose recognition. Biol Reprod 56:1198–1204.

Levine N, Kelly H. 1978. Measurement of pH in the rat epididymis in vivo. J Reprod Fertil 52:333–335.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Mayorga LS, Bertini F. 1985. The origin of some acid hydrolases of the fluid of the rat cauda epididymidis. J Androl 6:243–245.

Miller D, Gong X, Shur B. 1993. Sperm require beta-N-acetylglucosaminidase to penetrate through the egg zona pellucida. Development 118:1279–1289.

Moura AA, Chapman DA, Koc H, Killian GJ. 2006. Proteins of the cauda epididymal fluid associated with fertility of mature dairy bulls. J Androl 27:534–541.

Mruk DD, Cheng CY. 2011. Enhanced chemiluminescence (ECL) for routine immunoblotting: An inexpensive alternative to commercially available kits. Spermatogenesis 1:121–122.

Nadimpalli SK, Amancha PK. 2010. Evolution of mannose 6-phosphate receptors (MPR300 and 46): Lysosomal enzyme sorting proteins. Curr Protein Pept Sci 11:68–90.

Phopin K, Nimlamool W, Lowe-Krentz LJ, Douglass EW, Taroni JN, Bean BS. 2013. Roles of mouse sperm-associated alpha-L-fucosidases in fertilization. Mol Reprod Dev 80:273–285.

Robaire B, Hinton BT, Orgebin-Crist MC. 2006. The epididymis. In: Knobil JN, Neil J, editors. The physiology of reproduction. New York: Elsevier. pp 1071–1148.

Romano PS, Lopez AC, Mariani ML, Sartor T, Belmonte SA, Sosa MA. 2002. Expression and binding properties of the two mannose-6-phosphate receptors differ during perinatal development in rat liver. Biochem Biophys Res Commun 295:1000–1006.

Romano PS, Carvelli L, Lopez AC, Jofre G, Sartor T, Sosa MA. 2005. Developmental differences between cation-independent and cation-dependent mannose-6-phosphate receptors in rat brain at perinatal stages. Brain Res Dev Brain Res 158:23–30.

Sullivan R. 1999. Interaction between sperm and epididymal secretory proteins. In: Cagnon C, editor. The male gamete: From basic to clinical applications. Vienna: Cache River Press. pp 130–136.

Taitzoglou IA, Kokoli AN, Killian GJ. 2007. Modifications of surface carbohydrates on bovine spermatozoa mediated by oviductal fluid: A flow cytometric study using lectins. Int J Androl. 30(2):108–114.

Tajik P, Arman A, Taktaz T. 2007. Bovine epididymal sperm morphology obtained from caput, corpus and cauda epididymides. Pak J Biol Sci 10:3936–3939.

Takada M, Yonezawa N, Yoshizawa M, Noguchi S, Hatanaka Y, Nagai T, Kikuchi K, Aoki H, Nakano M. 1994. Ph-sensitive dissociation and association of beta-n-acetylhexosaminidase from boar sperm acrosome. Biol Reprod 50:860–868.

Tulsiani DR, Keller RK, Touster O. 1975. The preparation and chemical composition of the multiple forms of beta-glucuronidase from the female rat preputial gland. J Biol Chem 250:4770–4776.

Tulsiani DR, Skudlarek MD, Orgebin-Crist MC. 1989. Novel alpha-D-mannosidase of rat sperm plasma membranes: Characterization and potential role in sperm-egg interactions. J Cell Biol 109:1257–1267.

Tulsiani DR, Skudlarek MD, Araki Y, Orgebin-Crist MC. 1995. Purification and characterization of two forms of beta-D-galactosidase from rat epididymal luminal fluid: Evidence for their role in the modification of sperm plasma membrane glycoprotein(s). Biochem J 305:41–50.

Tulsiani DR, Orgebin-Crist MC, Skudlarek MD. 1998. Role of luminal fluid glycosyltransferases and glycosidases in the modification of rat sperm plasma membrane glycoproteins during epididymal maturation. J Reprod Fertil Suppl 53:85–97.

Tulsiani DR, Abou-Haila A. 2011. Molecular events that regulate mammalian fertilization. Minerva Ginecol 63:103–118.

Turner TT. 2008. De Graaf's thread: The human epididymis. J Androl 29:237–250.

Venditti JJ, Donigan KA, Bean BS. 2007. Crypticity and functional distribution of the membrane associated alpha-L-fucosidase of human sperm. Mol Reprod Dev 74:758–766.

Venditti JJ, Swann JM, Bean BS. 2010. Hamster sperm-associated alpha-L-fucosidase functions during fertilization. Biol Reprod 82:572–579.

York SJ, Arneson LS, Gregory WT, Dahms NM, Kornfeld S. 1999. The rate of internalization of the mannose 6-phosphate/insulin-like growth factor II receptor is enhanced by multivalent ligand binding. J Biol Chem 274:1164–1171.