

# Role of Sialic Acid in Bovine Sperm–Zona Pellucida Binding

JOSÉ GUILLERMO VELÁSQUEZ,<sup>1,4</sup> SEBASTIÁN CANOVAS,<sup>1</sup> PATRICIA BARAJAS,<sup>1</sup> JULIÁN MARCOS,<sup>2</sup> MARÍA JIMÉNEZ-MOVILLA,<sup>2</sup> RICARDO GUTIÉRREZ GALLEGU,<sup>3</sup> JOSÉ BALLESTA,<sup>2</sup> MANUEL AVILÉS,<sup>2\*\*</sup> AND PILAR COY<sup>1\*</sup>

<sup>1</sup>Department of Physiology, Veterinary Faculty, University of Murcia, Murcia, Spain

<sup>2</sup>Department of Cell Biology, Medicine Faculty, University of Murcia, Murcia, Spain

<sup>3</sup>Pharmacology Research Unit, Municipal Institute of Medical Research,

Department of Experimental and Health Sciences, University Pompeu Fabra, Barcelona, Spain

<sup>4</sup>Colombian Corporation for Agricultural Research and University of La Salle, Villavicencio, Meta, Colombia

**ABSTRACT** Sperm binding activity has been detected in zona pellucida (ZP) glycoproteins and it is generally accepted that this activity resides in the carbohydrate moieties. In the present study we aim to identify some of the specific carbohydrate molecules involved in the bovine sperm–ZP interaction. We performed sperm binding competition assays, in vitro fecundation (IVF) in combination with different lectins, antibodies and neuraminidase digestion, and chemical and cytochemical analysis of the bovine ZP. Both MAA lectin recognising  $\alpha$ -2,3-linked sialic acid and neuraminidase from *Salmonella typhimurium* with catalytic activity for  $\alpha$ -2,3-linked sialic acid, demonstrated a high inhibitory effect on the sperm–ZP binding and oocyte penetration. These results suggest that bovine sperm–ZP binding is mediated by  $\alpha$ -2,3-linked sialic acid. Experiments with trisaccharides (sialyllactose, 3'-sialyllactosamine and 6'-sialyllactosamine) and glycoproteins (fetuin and asialofetuin) corroborated this and suggest that at least the sequence Neu5A-c( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc is involved in the sperm–ZP interaction. Moreover, these results indicate the presence of a sperm plasma membrane specific protein for the sialic acid. Chemical analysis revealed that bovine ZP glycoproteins contain mainly Neu5Ac (84.5%) and Neu5GC (15.5%). These two types of sialic acid residues are probably linked to Gal $\beta$ 1,4GlcNAc and GalNAc by  $\alpha$ -2,3- and  $\alpha$ -2,6-linkages, respectively, as demonstrated by lectin cytochemical analysis. The use of a neuraminidase inhibitor resulted in an increased number of spermatozoa bound to the ZP and penetrating the oocyte. From this last result we hypothesize that a neuraminidase from cortical granules would probably participate in the block to polyspermy by removing sialic acid from the ZP. *Mol. Reprod. Dev.* 74: 617–628, 2007.

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## INTRODUCTION

The zona pellucida (ZP), a transparent envelope surrounding the plasma membrane of mammalian oocytes, is a highly glycosylated extracellular matrix. In mammals, this matrix is constituted by three or four glycoproteins named ZP1, ZP2 (ZPA), ZP3 (ZPC) and ZP4 (ZPB) (Harris et al., 1994; Yonezawa et al., 2001; Lefievre et al., 2004). Sperm binding activity has been detected in ZP glycoproteins and it is generally accepted that this activity resides in the carbohydrate moieties. However, identification of the precise molecular mechanism, which could play a key role during the sperm–ZP interaction, is still under debate (Benoff, 1997; Dell et al., 1999; Dean, 2004; Shur et al., 2004; Wassarman, 2005). In vitro inhibition or competition assays using different lectins, antibodies, carbohydrates and glycosidases are useful tools to identify the molecules involved in the gamete interaction. In bovine species, evidence exists to support that mannose could be a specific residue involved in the bovine sperm–ZP binding (Amari et al., 2001; Yonezawa et al., 2005). Whereas uncapacitated bull spermatozoa bind the fucose residues, this fucose binding property was lost after capacitation, and the spermatozoa showed affinity to bind mannose–BSA (Suarez et al., 1998; Revah et al., 2000). Similarly, it was observed that the sperm surface hyaluronan binding protein (HABP1) present in rat,

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\*Correspondence to: Dr. Pilar Coy, Departamento de Fisiología, Facultad de Veterinaria, Campus de Espinardo, Universidad de Murcia, Murcia 30071, Spain. E-mail: pcoy@um.es

\*\*Correspondence to: Dr. Manuel Avilés, Departamento de Biología Celular, Facultad de Medicina, Campus de Espinardo, Universidad de Murcia, Murcia 30071, Spain. E-mail: maviles@um.es

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mice, bull and human sperm plasma membrane is involved in the sperm–ZP interaction (Ranganathan et al., 1994) and interacts with the clustered mannose residues of the ZP glycoproteins (Ghosh and Datta, 2003). Moreover, it has been reported (Amari et al., 2001) that mannose residues associated to the neutral N-linked oligosaccharides in the bovine ZP glycoproteins play a key role in the sperm–ZP binding. However, 77% of the carbohydrate chains from bovine ZP glycoproteins are acidic chains containing sialic acid (Katsumata et al., 1996). The number of such acidic chains decreases significantly in the fertilised oocytes which suggests a role for the sialic acid at some level of the fertilisation process. To date, no references demonstrating the involvement of the sialic acid in the bovine spermatozoon–ZP binding are available.

Fertilisation results in oocyte cortical granule exocytosis, a reaction which produces biochemical and biological modifications of the ZP responsible for the blocking of polyspermy (Hoodbhoy and Talbot, 1994; Sun, 2003; Dean, 2004). These modifications include proteolysis of the ZP2 (ZPA), hardening of the ZP, formation of disulfide bonds by oxidation of cysteine residues in ZP, and/or removal of carbohydrates by specific cortical granule glycosidases (Moller and Wasserman, 1989; Miller et al., 1992, 1993; Iwamoto et al., 1999; Rath et al., 2005). Since one of the main changes demonstrated in the bovine ZP after fertilisation is the loss of sialic acid residues (Katsumata et al., 1996), we hypothesise that this residue may be involved in the sperm–ZP interaction in bovine and that a neuraminidase (sialidase) from cortical granules could take part in the mechanism of blocking to polyspermy by digesting such sialic acid following oocyte activation (cortical granule exocytosis). In order to investigate the veracity of this hypothesis, we performed sperm binding competition assays, in vitro fecundation (IVF) in combination with different lectins, antibodies and neuraminidase digestion, and chemical and cytochemical analyses of the bovine ZP.

## MATERIALS AND METHODS

### Chemicals and Media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical (Madrid, Spain). (DIG)-labelled lectins (MAA, PNA and SNA) and mouse monoclonal IgG anti-DIG anti-

bodies were purchased from Roche (Barcelona, Spain). HRP-labelled LFA lectin and mouse monoclonal antibody against sialyl-Lewis<sup>a</sup> tetrasaccharide (Neu5Ac(α2-3)Gal(β1,3)(αFuc 1-4)GlcNAc) were from Calbiochem (Madrid, Spain). The taxonomic names and specificity of the lectins used in the present study are shown in Table 1. Mouse monoclonal antibody against sialyl-Lewis<sup>x</sup> tetrasaccharide (Neu5Ac(α2-3)Gal(β1,4)(αFuc 1-3)GlcNAc) was from Kamy Company (Seattle, WA). 3'-Sialyllactosamine (Neu5Ac(α2-3)Gal(β1-4)GlcNAc) and 6'-sialyllactosamine (Neu5Ac(α2-6)Gal(β1-4)GlcNAc) were from Dextra laboratories (London, United Kingdom). N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (neuraminidase inhibitor) was from Toronto Research Chemicals (Downsview, Ontario, Canada). Recombinant erythropoietin was from the European Pharmacopoea (Strasbourg, France). Goat anti-mouse IgG + IgM-gold complex (15 nm) was purchased from Biocell Research Ltd. (Cardiff, UK). Unconjugated rabbit anti-mouse immunoglobulin was from Dako (Madrid, Spain). Protein A-colloidal gold conjugate (15 nm) was from the Department of Cell Biology (Utrecht University, Utrecht, The Netherlands).

The washing medium for ZP and oocytes was Dulbecco's phosphate buffered saline (DPBS). The oocyte collection medium (OCM) consisted of modified TCM-199 supplemented with Hanks salts and L-glutamine, 4.20 mM sodium bicarbonate, 10.0 mM HEPES, 2% foetal calf serum (FCS, Biological Industries, Beitz Haemek, Israel), 2.0 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. The oocyte maturation medium (OMM) was TCM-199 supplemented as described previously (Edwards and Hansen, 1996). Sperm washing was performed with Sperm-TALP medium and the incubation medium used for the ZP–sperm binding and IVF assays was IVF-TALP, both media being prepared as described in Parrish et al. (1998).

### Sperm–ZP Binding Assay

Oocytes surrounded by four or more layers of cumulus cells were obtained from bovine ovarian follicles (3–8 mm diameter) as described previously (Payton et al., 2004; Coy et al., 2005a). Isolated ZP were obtained by passing the cumulus-free oocytes through a glass pipette of approximately 80 µm diameter. ZP were then twice rinsed in DPBS and stored for up to 1 week at 4°C

TABLE 1. Lectins and Antibodies Used

Lectins/antibodies	Carbohydrate-binding specificity	References
<i>Arachis hypogaea</i> (PNA)	Galβ1,3GalNAc	Lotan et al., 1975
<i>Helix pomatia</i> (HPA)	Terminal α- and β-linked GalNAc residues	Piller et al., 1990; Wu and Sugii, 1991
<i>Dolichos biflorus</i> (DBA)	βGalNAc	Piller et al., 1990; Wu and Sugii, 1991
<i>Glycine max</i> (SBA)	GalNAc	Piller et al., 1990; Wu and Sugii, 1991
<i>Maackia amurensis</i> (MAA)	Neu5Ac/Neu5Gcα2,3Gal β1,4GlcNAc	Wang and Cummings, 1988; Knibbs et al., 1991; Brinkman-Van der linden et al., 2002
<i>Sambucus nigra</i> (SNA)	Neu5Ac/Neu5Gcα2,6 Gal/GalNAc	Shibuya et al., 1987; Mandal and Mandal, 1990; Brinkman-Van der linden et al., 2002
<i>Limax flavus</i> (LFA)	NeuGC, Neu5Ac	Knibbs et al., 1993

until use. Batches of 50 ZP were rinsed in DPBS after the different treatments described below and transferred to Nunc wells containing 500  $\mu$ l of pre-equilibrated IVF-TALP (Parrish et al., 1998).

Frozen bull semen was processed in our laboratory by a Percoll gradient as described by Parrish et al. (1998). Sperm were added to the isolated ZP at a concentration of  $1 \times 10^5$  cells/ml and cultured for 3 hr in IVF-TALP at 38.5°C in 5% CO<sub>2</sub> atmosphere. At the end of the coincubation period, ZP were washed in DPBS four times and then fixed in 0.5% glutaraldehyde (Serva, Heidelberg, Germany) in DPBS for 30 min at room temperature (Coy et al., 2005b). The samples were washed again in DPBS and stained with Hoechst 33342 DNA staining solution (1 mg/ml in DPBS) for 15 min. Finally, the ZP were aspirated into a pipette containing the mounting medium (DPBS/glycerol, 1:1), smeared onto a slide and assessed under an epifluorescence microscope (Leica) at 200  $\times$  magnification to determine the number of spermatozoa attached to each ZP.

In order to find out if the sialic acid residues contained in the bovine ZP glycoproteins are involved in the sperm-ZP interaction, isolated ZP were incubated with sialidases, lectins, or monoclonal antibodies with known specificity for different sialic acid moieties. The spermatozoa, however, were incubated with carbohydrate residues or glycoproteins either containing or devoid (controls) of sialic acid. The different series of incubation experiments for ZP and spermatozoa were carried out separately.

**Incubation of ZP with neuraminidase and neuraminidase inhibitor.** A total of 182 isolated ZP in three replicates were incubated in 500  $\mu$ L of pH 4.5 acetate buffer with a *Clostridium perfringens* (CP) neuraminidase (type V, Sigma), which has a broad specific activity showing catalytic activity with  $\alpha$ -2,3-,  $\alpha$ -2,6- and  $\alpha$ -2,8-linkages (Cassidy et al., 1965; Nees and Schauer, 1975). Another 298 isolated ZP in three replicates were incubated in pH 6 OMM with *Salmonella typhimurium* (ST) neuraminidase (Takara Bio, Inc., Otsu, Shiga, Japan), that shows a preference for the  $\alpha$ -2,3-linkage (Hoyer et al., 1991). Both enzymes were tested at two different concentrations (1 and 10 IU/ml) at 38.5°C and the incubation time was 17 hr. Isolated ZP incubated under the same conditions but without neuraminidase were used as control. Sperm-ZP binding assays were performed.

**Incubation of ZP with lectins and antibodies.** Three sialic acid specific lectins (LFA, MAA and SNA) and two antibodies against sialyl-Lewis<sup>a</sup> and sialyl-Lewis<sup>x</sup> were tested using the sperm-ZP binding assay to quantify their inhibitory effect on the number of bound sperms. A total of 538 isolated ZP were incubated with 5, 10, 25, 100 and 200  $\mu$ g/ml of MAA and LFA for 1 hr at 38.5°C in OMM medium before the sperm-ZP assay. A total of 97 isolated ZP were incubated with SNA for 1 hr at 38.5°C at concentrations of 25, 50, 100 and 200  $\mu$ g/ml before the assay. Doing so, the effect of the different lectins and their concentrations could be evaluated in three replicates for each experiment. Isolated ZP

incubated under the same conditions but without the lectins were used as control in all the experiments. A total of 504 isolated ZP were incubated with 40  $\mu$ g/ml of either of the two antibodies for 1 hr at 38.5°C in OMM medium before the sperm-ZP binding assay. ZP were washed, fixed and Hoechst stained as described above. Isolated ZP incubated under the same conditions but without the antibodies were used as control. This experiment was performed in eight replicates.

**Incubation of spermatozoa with carbohydrate residues and glycoproteins.** This experiment was performed to study their possible effect on blocking the ZP sperm receptor. Frozen semen processed by a Percoll gradient was incubated for 30 min with one among 5 mM methyl  $\alpha$ -mannopyranoside, 5 mM sialyllactose, 5 mM 3'-sialyllactosamine, 5 mM 6'-sialyllactosamine, fetuin (100, 200, or 300  $\mu$ g/ml), and asialofetuin (200  $\mu$ g/ml). Isolated ZP (N = 854 and N = 632 for carbohydrates and glycoproteins experiments, respectively, in six replicates) were then added to the IVF-TALP medium for the sperm-ZP binding assay. A control was performed to analyse the effect of the 3'-sialyllactosamine on the sperm motility and acrosome reaction. Motility of sperm was determined using a CASA system (Sperm Class Analyzer, Microptic, Barcelona, Spain). Images were obtained at 200  $\times$  magnification in a contrast phase microscope. Spermatozoa with an average path velocity (VAP) less than 20  $\mu$ m/sec were considered immotile. A minimum of five fields per sample was evaluated.

To evaluate the acrosome reaction, fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA) was used. The spermatozoa were examined under an epifluorescence microscope and sperm with FITC-PNA acrosome staining were considered acrosome reacted.

### In Vitro Fertilisation Assays

Cumulus oocyte complexes (COCs) were washed twice in OMM after collection and then cultured for 24 hr (30–40 COCs/500  $\mu$ l OMM) at 38.5°C in 5% CO<sub>2</sub> atmosphere and humidified air (Edwards and Hansen, 1996). The oocytes presumed to be mature were stripped of cumulus cells by pipetting and then fertilised with Percoll-prepared frozen-thawed semen (Parrish et al., 1998). The excess of ZP-bound spermatozoa was removed 18–20 hr later by vortexing (4 min) and the putative zygotes were subsequently fixed and stained as described above to demonstrate the presence of sperm head in the zygote cytoplasm.

As a preliminary test to evaluate whether the effect of *C. perfringens* neuraminidase was really due to its enzymatic action, 268 oocytes in three replicates were incubated with one among (i) the neuraminidase previously heat-inactivated by boiling for 15 min (1 IU/ml), (ii) the active neuraminidase (1 IU/ml), and (iii) no enzyme (control). Immediately afterwards, they were in vitro fertilised. The sperm concentration used was of 750,000 cells/ml and penetration percentages were assessed.

In order to find out if neuraminidase plays some role in the sperm-oocyte interaction and in the subsequent

blocking to polyspermy, 467 oocytes in three replicates were allocated in four groups and in vitro fertilised: two groups were incubated for 1 hr with either 1 or 10 IU/ml *C. perfringens* neuraminidase; another group was incubated with 10 mM *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (neuraminidase inhibitor) and the fourth group was used as a control. The sperm concentration was increased to 1,500,000 cells/ml for better evaluation of the penetration percentages, monospermy and the number of sperm/oocyte. These data were assessed under an epifluorescence microscope as described above. We performed an additional experiment, on 472 oocytes, with and without the presence of inhibitor in the culture, in order to evaluate the number of spermatozoa bound to the ZP after the in vitro fertilisation under the same conditions. A further control was performed consisting the incubation of neuraminidase from *C. perfringens* with 1 mM *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid showing 96.7% inhibition compared to the group without inhibitor. The glycosidase assay was performed as previously described (Aviles et al., 1996). Briefly, neuraminidase activity was assayed (0.1 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ) using 0.2 mM 4-methyl-umbelliferyl-*N*-acetylneuraminic acid (4-MU-Neu5Ac) as substrate at 37°C, pH 7.0 for 30 min. The total assay volume was 100  $\mu\text{l}$ , including 20  $\mu\text{l}$  of 1 mM 4-MU-Neu5Ac, 1  $\mu\text{l}$  of *C. perfringens* neuraminidase (1 IU/ml) and PBS. The reaction was stopped by placing the different samples at 4°C and by adding 500  $\mu\text{l}$  of 0.1 M (pH 10) glycine-carbonate buffer. After that, the reaction was measured using a Hitachi f4500 fluorimeter at wavelengths of 360 nm (excitation) and 460 nm (emission).

#### Chemical Analysis of the Sialic Acid in the Bovine Zona Pellucida

Trifluoro acetic acid (TFA; 10  $\mu\text{l}$ , 2 M) was added to 20 bovine ZP, the mixture vortexed thoroughly and subsequently incubated for 1 hr at 50°C in a heating block. The hydrolysate was left to cool to room temperature and the liberated sialic acid residues were derivatised with 1,2-diamino-4,5-methylene dioxybenzene (DMB) using a modified protocol previously described (Stanton et al., 1995). The derivatisation reagent was prepared by dissolving 0.42 mg of DMB in 49  $\mu\text{l}$  of demineralised water and adding 133.5  $\mu\text{l}$  of 10 mM TFA, 17.5  $\mu\text{l}$  of 2-mercaptoethanol and 67  $\mu\text{l}$  of 72 mM sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ). From this solution (final concentrations: 7 mM DMB, 18 mM  $\text{Na}_2\text{S}_2\text{O}_4$ , 0.94 M 2-mercaptoethanol and 5 mM TFA), 50  $\mu\text{l}$  was added to each hydrolysate and the mixture incubated for 2 hr at 50°C in the dark. Samples were analysed by reverse phase-HPLC immediately after derivatisation. In parallel to the ZP material, we processed a blank sample, a reference glycoprotein (recombinant erythropoietin), and sialyllactose. HPLC analyses were carried out on a Hewlett Packard 1090 Series II chromatograph equipped with a Hewlett Packard 1100 series fluorescence detector. Chromatographic separations were carried out on a Waters Nova-Pak  $\text{C}_{18}$  (3.9  $\times$  150 mm) column. DMB

derivatised sialic acids were eluted under isocratic conditions (acetonitrile/methanol/water, 9:7:84) at a flow rate of 1 ml/min for 15 min. Subsequently, the system was washed with a fast gradient (acetonitrile/methanol/water, 25:7:68) for 1 min, kept at these conditions for 2 min and re-equilibrated at the initial conditions for the next run. Fluorescence detection was performed by monitoring at excitation and emission wavelengths of 373 and 448 nm, respectively.

#### Cytochemical Analysis of the Sialic Acid in the Bovine Zona Pellucida

Different lectins and antibodies (see Table 1) in combination with neuraminidase treatment were used to investigate the different sialic acid residues present in the bovine ZP from immature oocytes. Cumulus enclosed oocytes were fixed in 0.5% glutaraldehyde buffered in pH 7.4 sodium cacodylate for 2 hr at 4°C. After extensive washing in the cacodylate buffer, the oocytes were embedded in 2% agarose. Then the samples were processed for embedding in LR white resin (hard grade) according to the procedure previously described (Newman, 1989). Neuraminidase treatment was used to remove terminal sialic acid residues (Aviles et al., 1997), thus exposing potential ligands for lectins marked by these acidic sugars. Grids carrying the sections were treated with type V *C. perfringens* neuraminidase (1 U/ml) in pH 5.0 acetate buffer at 37°C for 3 hr. Colloidal gold particles were used as marker for cytochemistry at the ultrastructural level. For DIG- and HRP-labelled lectins, a three step-method was used (Aviles et al., 1997; Jimenez-Movilla et al., 2004).

For the mouse monoclonal antibodies against sialyl-Lewis tetrasaccharides a two-step method was used as described previously (Aviles et al., 2000a). The ultrathin sections were analysed in a Phillips Tecnai 12 electron microscope (Eindhoven, The Netherlands).

The following controls were used. Negative controls: (1) pre-incubation of the lectins with the corresponding hapten-sugar inhibitor; (2) Pre-incubation of the grids with neuraminidase for LFA, MAA and SNA lectins.

Positive controls: Mouse ZP for DBA, HPA, PNA, and SBA lectins were used as previously described (Aviles et al., 1997, 2000a,b). Human ZP was used for anti-sialyl-Lewis<sup>a</sup> and sialyl-Lewis<sup>x</sup> antibody binding as previously described (Jimenez-Movilla et al., 2004).

#### Statistical Analysis

The data were presented as the mean  $\pm$  SEM, and all rates were modelled according to the binomial model of variables. The variables: percentage of oocyte penetration, number of sperm cells per ZP or per penetrated oocyte and monospermy percentage were analysed by one-way ANOVA (SPSS 11.0). When ANOVA revealed a significant effect, values were compared by the Tukey test. A *P*-value <0.05 was taken to denote statistical significance.

## RESULTS

**Sialic Acid and Neuraminidase Are Involved in the Bovine Sperm–Oocyte Interaction**

Sperm binding to the ZP is considered a process mediated by carbohydrates (Benoff, 1997; Dell et al., 1999). Consequently, a decrease in the number of sperm bound to the ZP after neuraminidase treatment would indicate a role of the sialic acid in the sperm–ZP binding. In this study, neuraminidase treatment with enzymes from both *C. perfringens* and *S. typhimurium* decreased the number of sperms bound to the ZP. This decrease was dose dependent as shown by the higher number of sperm bound to the ZP at 1 IU/ml compared with the 10 IU/ml dilution (Table 2). Moreover, the percentages of inhibition reached in these experiments were higher than 75%. When in vitro fertilisation assays were performed, the results show that neuraminidase strongly decreased the penetration percentages from 80% in the control group to 23% in the 10 IU/ml neuraminidase group (Table 3). This effect should be due to the enzymatic activity of the neuraminidase since the heat inactivated enzyme molecule did not show any effect (data not shown). Sialic acid content is reduced after fertilisation (Amari et al., 2001), and we think that its reduction might be produced by the release of a neuraminidase contained in the oocyte's cortical granules. To test this hypothesis, a neuraminidase inhibitor, *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid, was used. The results obtained showed that the number of spermatozoa bound to the ZP in the group of oocytes cultured in the presence of the inhibitor increased by more than 100% with respect to the control group ( $38.3 \pm 1.4$  and  $18.4 \pm 0.9$ , respectively; Fig. 1A,B). In addition to this, we observed that the percentages of monospermy decreased by >17% compared to the control or neuraminidase-treated groups (Table 3). This result is consistent with a higher number of sperm bound to the ZP thus increasing the probability to penetrate the ZP and the oocyte.

**Bovine Sperm–ZP Binding Is Mediated by  $\alpha$ -2,3-Linked Sialic Acid**

The neuraminidase from *C. perfringens* used in this study shows catalytic activity on  $\alpha$ -2,3-,  $\alpha$ -2,6- and  $\alpha$ -2,8-linkages; however, neuraminidase from *S. typhimurium* shows a preference for the  $\alpha$ -2,3-linkage (Hoyer

**TABLE 3. Effect of *C. perfringens* Neuraminidase and Neuraminidase Inhibitor (*N*-acetyl-2,3-dehydro-2-Deoxyneuraminic acid) on IVF**

	N	Penetration (%) <sup>*</sup>	Monospermy (%) <sup>*</sup>	Number of sperm/oocyte <sup>*</sup>
Control	121	$80.2 \pm 0.4^a$	$92.8 \pm 0.3^a$	$1.1 \pm 0.3^b$
Inhibitor	124	$68.5 \pm 0.5^a$	$75.3 \pm 0.4^b$	$1.3 \pm 0.6^{a,b}$
1IU/ml	114	$33.3 \pm 0.5^b$	$92.1 \pm 0.3^a$	$1.1 \pm 0.3^b$
10IU/ml	108	$23.1 \pm 0.4^b$	$92.0 \pm 0.3^a$	$1.1 \pm 0.3^b$

<sup>\*</sup>Mean  $\pm$  s.e.m.

<sup>a,b</sup>Different superscripts in the same column indicate significantly different values ( $P < 0.005$ ).

et al., 1991). Thus, from the results in Table 2, where it is shown that each enzyme at a concentration of 10 IU/ml caused a 76–77% inhibition on sperm–ZP binding, we can infer that the  $\alpha$ -2,3-linked sialic acid is involved in such binding. The results from the inhibition assays with lectins in this study corroborated this finding. Incubation of the ZP with MAA and LFA lectins resulted in a significant decrease in the mean number of sperms bound to the ZP (Fig. 2), ranging from 40 to 92% inhibition of sperm–ZP binding. Moreover, this effect depended on the lectin concentration. In contrast, SNA lectin did not show any inhibitory effect on the number of sperm bound to the ZP at any of the concentrations used ( $20.1 \pm 1.5$ ,  $18.6 \pm 1.8$ ,  $15.0 \pm 2.1$ ,  $15.0 \pm 1.4$  and  $22.5 \pm 2.3$  spermatozoa/ZP for control, 25  $\mu$ g/ml, 50  $\mu$ g/ml, 100  $\mu$ g/ml and 200  $\mu$ g/ml of SNA, respectively). As is known, MAA lectin recognises sialic acid linked to the penultimate carbohydrate of the oligosaccharide chain by an  $\alpha$ -2,3-linkage and SNA recognises  $\alpha$ -2,6 sialic acid linked to the penultimate carbohydrate of the oligosaccharide chain, whereas LFA has broader activity recognising  $\alpha$ -2,3-,  $\alpha$ -2,6- and  $\alpha$ -2,8-linkages.

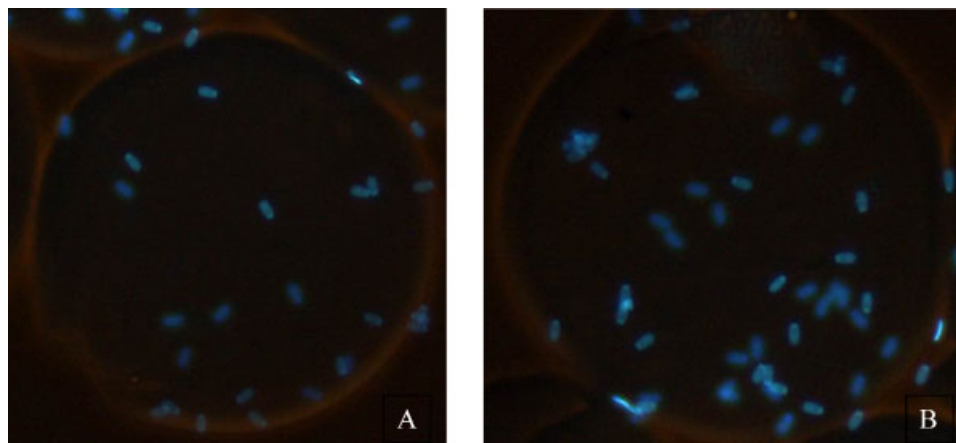
The sialyl-Lewis antigens are tetrasaccharides that contain a Neu5Ac residue  $\alpha$ -2,3-linked and an  $\alpha$ -1,3/4-linked fucose residue. Some of these antigens have been previously detected in the human ZP (Jimenez-Movilla et al., 2004). We investigated if the sialic acid present in the context of the Lewis antigens is involved in the sperm–ZP interaction. For this, ZP were incubated for 1 hr with antibodies against the sialyl-Lewis<sup>a</sup> and sialyl-Lewis<sup>x</sup> tetrasaccharides, but no effect on the number of spermatozoa bound to the ZP was observed. This

**TABLE 2. Effect of the Neuraminidases on the Number of Spermatozoa Bound to the ZP After a Sperm–ZP Binding Assay**

	<i>C. perfringens</i>			<i>S. typhimurium</i>		
	N	Number of sperm/ZP <sup>*</sup>	% inhibition	N	Number of sperm/ZP <sup>*</sup>	% inhibition
CONTROL	59	$15.42 \pm 1.10^a$		108	$23.46 \pm 0.94^a$	
1 IU/ml	61	$8.70 \pm 1.51^b$	43.58	88	$15.80 \pm 1.00^b$	32.65
10 IU/ml	62	$3.65 \pm 1.01^c$	76.32	102	$5.36 \pm 0.94^c$	77.16

<sup>\*</sup>Mean  $\pm$  s.e.m. of spermatozoa bound to ZPs.

<sup>a,b,c</sup>Different superscripts in the same column indicate significantly different values.



**Fig. 1.** Effect of the neuraminidase inhibitor *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid on the number of spermatozoa bound to the ZP. **A:** A oocyte from the control group. **B:** A oocyte from the group cultured in the presence of the neuraminidase inhibitor. The number of spermatozoa bound to the ZP in the last group was 100% higher than that in the control.

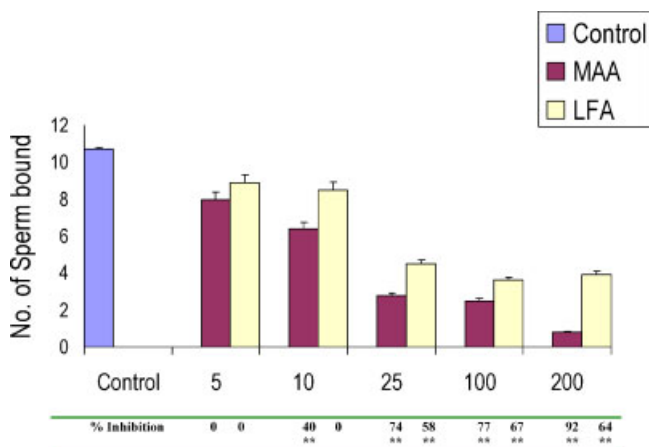
number had a mean value of  $22.6 \pm 0.6$  spermatozoa/ZP for the control group and  $21.3 \pm 0.7$  and  $20.2 \pm 1.0$  for the sialyl-Lewis<sup>x</sup> and sialyl-Lewis<sup>a</sup> antibody groups, respectively.

### Bovine ZP Glycoproteins Contain Two Main Types of Sialic Acid Residues With Different Linkage to the Penultimate Carbohydrate Residues

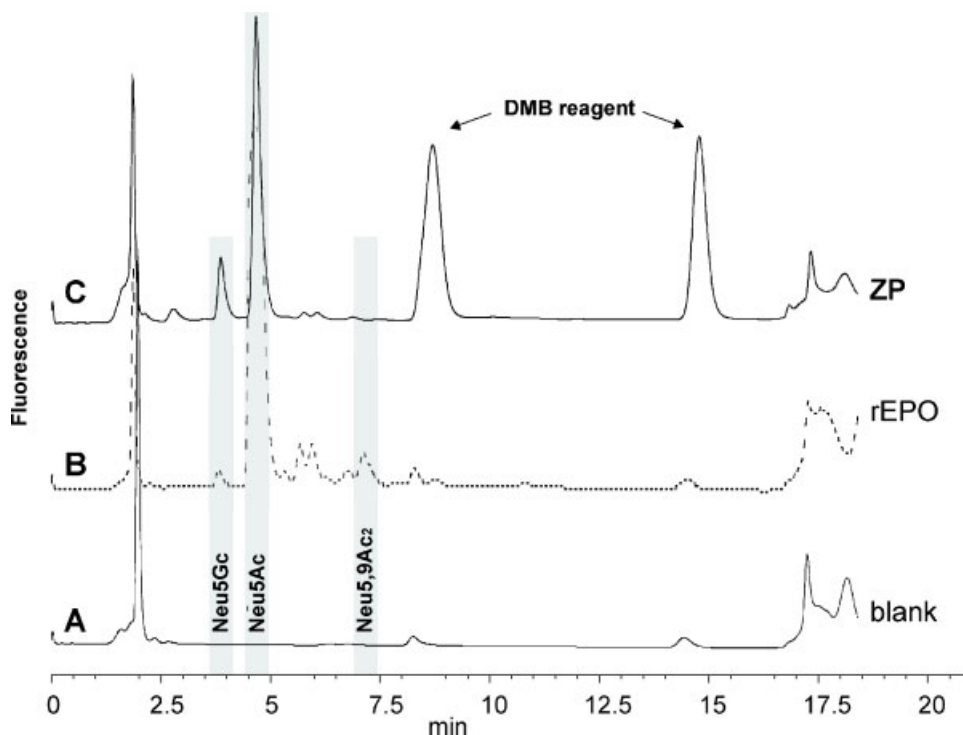
**Chemical analysis.** Sialic acid residues, released from any type of precursor (e.g. *O*-glycans, *N*-glycans, etc.) are  $\alpha$ -keto acids that react specifically with DMB and their three-substituted 6,7-methylene dioxy-2(1H)-quinosalinone derivatives (Hara et al., 1989). No other monosaccharide released during the acid hydrolysis reacts with DMB. Most of the frequently occurring sialic acid residues can be separated by reverse-phase HPLC (Klein et al., 1997) while fluorescence detection allows

analysis of sub-picomole levels of these derivatives (Ito et al., 2002). The blank sample rendered an HPLC profile (Fig. 3A) from which the reagent peaks could be identified and the standard samples yielded the specific retention times of Neu5Ac-Q (from sialyl lactose) and Neu5Ac-Q, Neu5Gc-Q and Neu5,9Ac<sub>2</sub>-Q (from recombinant erythropoietin; Fig. 3B). The HPLC profile from ZP derived DMB-derivatised sialic acids (Fig. 3C) displayed two major peaks, in addition to the peaks corresponding to the reagent, at retention times coinciding with those of Neu5Gc-Q and Neu5Ac-Q. Three minor peaks coincided with equivalent peaks present in sialic acid-containing samples (including ultra-pure Neu5Ac-2Ome) but were absent in the blank and therefore they were not considered as genuine residues. No other sialic acid species were identified on bovine ZP. From the peak areas, a relative quantification showed that Neu5Gc represents ca. 15.5% of the sialic acid content of bovine ZP whereas Neu5Ac represents ca. 84.5%.

**Cytochemical analysis.** The chemical analysis demonstrated the presence of two types of sialic acid residues contained in the bovine ZP glycoproteins. However, it is well known that these sialic acid residues could be present in different forms depending on the linkage and the penultimate carbohydrate residue (Angata and Varki, 2002). To investigate this a cytochemical analysis was performed using different lectins and antibodies in combination with neuraminidase treatment. Strong labelling was observed in the entire thickness of the ZP with the LFA, MAA (Fig. 4A) and SNA sialic specific lectins, and scarce labelling was detected with the SBA lectin (Fig. 4C). No labelling whatsoever was observed with the HPA, DBA and PNA lectins, nor the anti-sialyl-Lewis<sup>a</sup> and sialyl-Lewis<sup>x</sup> antibodies. After neuraminidase digestion, the specific labelling observed previously with the sialic-acid specific lectins disappeared (Fig. 4B) and only the SBA lectin still showed a strong reactivity after this enzymatic treatment (Fig. 4D).



**Fig. 2.** Effect of different concentrations (5, 10, 25, 100 and 200 µg/ml) of MAA and LFA on the ZP–sperm binding. \*\* Indicates significantly different values ( $P < 0.001$ ) in the percentages of inhibition.



**Fig. 3.** Overlaid HPLC-FLD (excitation 373 nm, emission 448 nm) chromatograms of the sialic acid content of: (A) a blank sample, (B) recombinant erythropoietin and (C) bovine ZP. Indicated (grey shaded areas) are the elution positions of Neu5Ac, Neu5, 9Ac2, Neu5Gc and DMB reagent peaks.

### Bovine Sperm Possess a Specific Receptor for $\alpha$ -2,3-linked Sialic Acid

When the spermatozoa were incubated with either carbohydrate residues or glycoproteins in an attempt to block the sites responsible for the binding to the ZP, the results again corroborated our hypothesis regarding the involvement of the sialic acid in the sperm–ZP interaction. A 23% decrease in the number of spermatozoa bound to the ZP was observed when sialyllactose trisaccharides, containing equimolar amount of 3'- and 6'-sialyllactose, were used ( $P=0.002$ ). However, 3'-sialyllactosamine alone produced 39.6% inhibition in the sperm binding to the ZP, whereas 6'-sialyllactosamine had no effect on binding. No effect was observed in the acrosome reaction and sperm motility using 3'-sialyllactosamine. These data strongly support our previous findings indicating that  $\alpha$ -2,3-linked sialic acid plays a key role in the sperm–ZP binding. We showed evidence for the inhibitory effect of Neu5Ac, however, we do not have data on the role played by Neu5Gc. The methyl- $\alpha$ -mannopyranoside carbohydrate residue did not affect the results of the sperm–ZP binding assays ( $P=0.264$ ). Regarding the fetuin and asialofetuin glycoproteins, a significant decrease in the number of spermatozoa bound to the ZP was observed when concentrations of 100, 200 or 300  $\mu\text{g}/\text{ml}$  of fetuin were used (21.9, 23.8 and 26.2, respectively,  $P < 0.05$ ). In contrast, no differences were found between the control group and the group treated with asialofetuin. As is

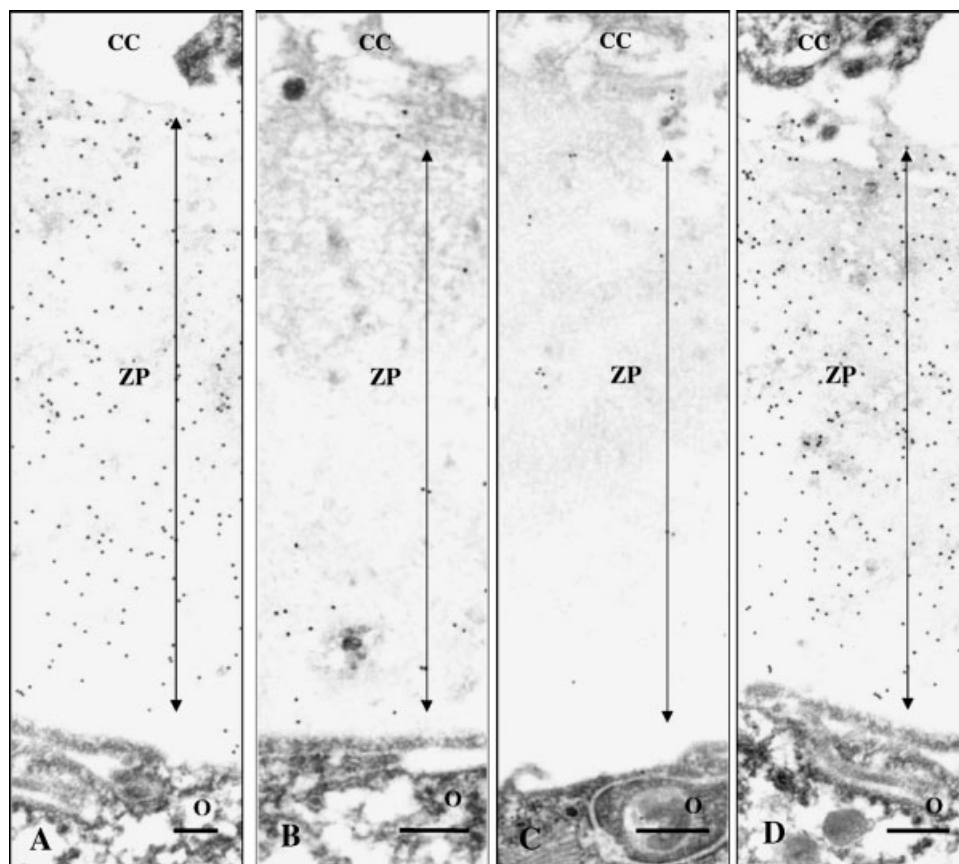
known, fetuin is a glycoprotein that contains sialylated core-1 *O*-glycans and sialylated *N*-glycans with triantennary (major) and biantennary (minor) structures (Spiro and Bhoyroo, 1974; Van Halbeek et al., 1980), whereas asialofetuin differs only in the absence of sialic acid.

### DISCUSSION

#### Neu5Ac( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc Oligosaccharide Is Involved in the Sperm Binding to the Zona Pellucida

It was previously reported that bull sperm have affinity for different carbohydrate residues, possibly through the tempo-spatial surface expression of distinct lectins. Thus, uncapacitated sperm bind the fucose residues, whereas these fucose-binding properties were lost after capacitation and, in turn, sperm showed affinity to mannose–BSA (Suarez et al., 1998; Revah et al., 2000). Recently, it was observed that sperm interact with the clustered mannose residues of the ZP glycoproteins by a HABP1 present in their plasma membrane (Ranganathan et al., 1994). Moreover, using different binding assays (Amari et al., 2001), it was reported that mannose residues associated to the neutral *N*-linked oligosaccharides present in the bovine ZP glycoproteins played a key role in the sperm–ZP interaction.

However, in the different studies previously reported, the involvement of the sialic acid in the sperm–ZP



**Fig. 4.** Immature oocyte. **A,B:** MAA lectin. Uniform and strong labelling is observed in ZP. **B:** After neuraminidase treatment, no reactivity is observed throughout the ZP. **C,D:** SBA lectin. ZP showed scarce labelling. **D:** After neuraminidase digestion, strong and uniform reactivity is observed in ZP. O, oocyte; ZP, zona pellucida; CC, cumulus cell. Bar: 0.5  $\mu$ m.

binding was not tested despite the important modification of its content after fertilisation (Katsumata et al., 1996). To address this point, we performed different sperm–ZP binding competition assays using different lectins with well-known specificities, antibodies and enzyme digestion. We have used two lectins, MAA and SNA, that recognise sialic acid linked to the penultimate carbohydrate of the oligosaccharide chain by an  $\alpha$ -2,3- and  $\alpha$ -2,6-linkage, respectively. Furthermore, a broad specificity sialic acid lectin, LFA, was also used. The results obtained strongly suggest that the  $\alpha$ -2,3-linked form is necessary for the specific binding between gametes. Inhibition of sperm–ZP binding by LFA and MAA lectins is specific and is not due to steric hindrance as demonstrated by the removal of the sialic acid through neuraminidase treatment. Moreover, both MAA and SNA lectins have a similar molecular weight, 130 and 140 kDa, respectively (Kawaguchi et al., 1974; Broekaert et al., 1984); but specific inhibition was detected only with MAA lectin.

The sequence Neu5Ac/Neu5Gc( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc identified by MAA lectin is a usual terminal epitope of complex type *N*-linked oligosaccharides in mouse and pig ZP glycoproteins (Noguchi and Nakano, 1992;

Easton et al., 2000). However, this trisaccharide was also described in *O*-linked oligosaccharides of mouse and pig ZP glycoproteins (Hokke et al., 1993, 1994; Easton et al., 2000; Dell et al., 2003). We have no indications regarding which types of oligosaccharides are involved in the bovine sperm–ZP binding (*N*- vs. *O*-linked Neu5Ac/Neu5Gc( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc trisaccharide). A previous study (Amari et al., 2001) as well as our unpublished observations showed that the solubilised bovine ZP is resistant to the *N*-glycosidase *F* treatment. Further studies are necessary to address this point.

Cell–cell or cell–matrix interactions are much more specific in the presence of an appropriate receptor for the ligand. This is also true for the gamete interaction. In this study, we present supporting data for the important role played by the sialic acid contained in the ZP on the binding of the sperm to the extracellular matrix. Thus, it is necessary to demonstrate that there is a specific sialic acid receptor in the bovine sperm plasma membrane. To prove the existence of this receptor, we used the indirect method of sperm binding competition assay. In these assays, different trisaccharides and glycoproteins that contained sialic acid residues were used. This

methodological approach is widely accepted by the scientific community and has been previously used in different species (Litscher et al., 1995; Johnston et al., 1998; Mori et al., 2000).

In this study, we observed a significant inhibition of the sperm binding to the ZP in the presence of fetuin. However, no inhibition was observed with asialofetuin, the asialo form of this glycoprotein. A similar result, 23% inhibition, was obtained with the sialyllactose trisaccharide. This compound contains equivalent molar amounts of the 3'-sialyllactose and 6'-sialyllactose. To test if this inhibition was specific to the  $\alpha$ -2,3-sialyl or  $\alpha$ -2,6-sialyl trisaccharide, a competition assay was performed with each one of these compounds. The results obtained clearly demonstrated that the sperm binding was specifically inhibited by the 3'-sialyllactosamine suggesting that a specific receptor for  $\alpha$ -2,3-sialic acid is present in the sperm plasma membrane. Moreover, the  $\alpha$ -2,6-sialic acid has no role in this process.

Our results show that sialic acid plays a key role. Although the competition inhibition assays using trisaccharides and glycoproteins are statistically significant, they are not very efficient compared to the results obtained using the natural ZP treated with lectins and glycosidases. This suggests that an appropriate structure is necessary for optimal biological activity. Thus, monosaccharides have no inhibitory function. However, when monosaccharides are clustered (e.g. mannose) they may have an effect (Amari et al., 2001; Yonezawa et al., 2005). Finally, these results are in concordance with those previously described in other systems indicating that the availability of an oligosaccharide for binding could be markedly influenced by the polypeptide on which it is presented (Solis et al., 1994), by the supramolecular structure of the ZP matrix (Dean, 2004) or by the branching pattern of the oligosaccharide (Litscher et al., 1995). In the latter case, the number of poly-lactosamine repeating units could also play an important role in the accessibility for the specific sperm membrane protein.

Our evidence suggesting the involvement of the sialic acid and previous results which suggest the implication of mannose residues and a mannose sperm binding protein, indicate that the interaction between the sperm (acrosome-intact sperm) and the ZP is mediated by a multiple complex involving several sperm plasma membrane proteins and several carbohydrates contained in the ZP (Storey, 1995; Shur, 1998; Thaler and Cardullo, 2002; Rodeheffer and Shur, 2004). Our data show that bovine sperm has a high affinity for binding to the sialic acid residues whereas the sperm interaction with mannose residues previously observed may correspond to a lower affinity (Amari et al., 2001). The existence of two independent binding sites on the sperm with different binding specificities has been previously reported (Thaler and Cardullo, 1996; Johnston et al., 1998; Mori et al., 2000). Identification of the specific ZP glycoprotein (ZPA, ZPB or ZPC) involved and the *N*-linked versus *O*-linked oligosaccharides that contain the sialic acid residues responsible for the sperm-ZP

binding in bovine is currently in progress in our laboratories.

#### Composition of Bovine Zona Pellucida Sialic Acid

For the sperm-ZP interaction, it is important (i) that the egg binding proteins are present in the acrosome-intact capacitated sperm plasma membrane and (ii) the presence of the appropriate sugar residue in the ZP. For this reason, it is necessary to prove not only that sialic acid is present in the bovine ZP but also that it is present in the outer region of the ZP. This region of the ZP is where the first contact between the capacitated sperm and the ZP occurs. Previous studies have suggested that in different species the composition of the ZP varies across its thickness. Some of the carbohydrates analysed were previously suggested to be the primary receptor but they were not detected in the outer region of the ZP (Aviles et al., 1997, 2000a).

Bovine ZP glycoproteins have been partially characterised by biochemical and biophysical methods. The bovine ZP glycoproteins contain neutral and acidic *N*-linked oligosaccharides. The acidic *N*-linked oligosaccharides are the main component and almost all the acidic chains are neutralised by treatment with neuraminidase (Katsumata et al., 1996). Sugar mapping analysis of pyridylaminated *N*-linked chains by reverse-phase and normal-phase HPLC and 500-MHz 1H-NMR spectroscopy revealed that the acidic chains are di-, tri- and tetra-antennary, fucosylated complex-type chains that have *N*-acetylglucosamine repeats in the nonreducing regions.

After *in vitro* fertilisation, a clear decrease in the amount of the acidic chains present in the *N*-linked oligosaccharides of the bovine ZP was observed (Katsumata et al., 1996). Moreover, our data described in the present study clearly show that sperm binding and *in vitro* fertilisation proportions are modified by lectins and neuraminidase treatment which, respectively, block or remove the sialic acid residues. For this reason, we considered a better characterisation of the different sialic acid residues contained in the bovine ZP is necessary. Two types of analyses were performed: cytochemical and chemical. The use of different specific lectins and antibodies against different sialic acid residues showed the presence of the following structures: Neu5Ac/Neu5Gc( $\alpha$ 2-3)Gal( $\beta$ 1-4)GlcNAc (MAA) and Neu5Ac/Neu5Gc( $\alpha$ 2-6)Gal( $\beta$ 1-4)GlcNAc (SNA). The existence of the glycolyneuraminic acid variant of the structures described above cannot be discarded because it has been recently described that both MAA and SNA lectins also recognise the NeuGc (Brinkman-Van der Linden et al., 2002). This issue was addressed through the chemical analysis of the sialic acid content, including identification of the different sialic acid species. This proved the presence of both Neu5Ac and Neu5Gc forms of sialic acids in the bovine ZP glycoproteins. Neu5Ac accounted for approximately 85% of the sialic acid content whereas Neu5Gc accounted for the remaining 15%. From the sialic acid analysis, no

evidence was found for the presence of *O*-acetylated sialic acid residues in bovine ZP, even though other bovine glycoproteins are known to contain both Neu5Gc and/or *O*-Ac-NeuAc residues (Rohrer et al., 1998; Che et al., 1999; Toba et al., 2000). These results are in disagreement with a previous study that indicated that the majority of the sialic acid present in bovine ZP is the acetylated form (Parillo et al., 2000). In our study, different approaches have been used to investigate the nature of the sialic acid present. First, a very sensitive method using HPLC analysis with a known protein (recombinant erythropoietin) which contains *O*-acetylated sialic acid demonstrated that our system works and is very sensitive. Second, the use of specific sialic acid binding lectins in combination with neuraminidase digestion showed that all the labelling obtained with the different lectins disappeared indicating that these sialic acid residues are sensitive to the neuraminidase treatment, in accordance with the results from Katsumata et al. (1996) and in contrast with previously reported data (Parillo et al., 2000).

### Block to Polyspermy

After fertilisation, the ZP is modified by the cortical granule content producing a change in its biological and biochemical properties called the zona reaction. Such change is involved in the block to polyspermy (Yanagimachi, 1994; Sun, 2003). The molecular mechanisms responsible for these changes are not well understood, and could perhaps be addressed if the contents of the cortical granules were better known. The changes affect different steps of the fertilisation process such as the sperm binding properties of the ZP, induction of the acrosome reaction by the ZP, sperm–egg fusion, partial proteolysis of the ZPA (ZP2) and the resistance to disulfide bond reducing agents of the ZP (Iwamoto et al., 1999).

In relation to the sperm–ZP binding properties, in mice there is almost no binding of sperm to the ZP after fertilisation (Wassarman, 2005). Thus, it is generally accepted, at least in the murine model, that the primary sperm receptor might be removed or become inaccessible for the spermatozoon after fertilisation (Dean, 2004). However, this block to polyspermy at the level of the sperm binding to the ZP seems to be not so efficient or necessary in other animal models. Thus, the block to polyspermy seems to be associated to the block at the level of the viteline envelope in the rabbit model (Yanagimachi, 1994). In the cow and pig, the ZP of in vitro fertilised oocytes is still recognised and bound by free swimming sperm, even when the number of sperm is approximately reduced to half (Hatanaka et al., 1992; Katsumata et al., 1996). The binding of the sperm to the ZP after fertilisation does not necessarily indicate that the blockage to polyspermy has not been efficient. A recent genetic study using “humanised mouse” ZP demonstrated a complete blockage to polyspermy despite the high affinity binding properties of the ZP (Rankin et al., 2003; Dean, 2004; Hoodbhoy and Dean, 2004).

From the findings of the present study, we can hypothesise that the removal of sialic acid residues in the bovine ZP after fertilisation (Katsumata et al., 1996) could be due to the releasing of neuraminidase from the cortical granules. In the mouse model, hexosaminidase has been detected in cortical granules and competitive inhibition of the enzyme activity during fertilisation resulted in a loss of the zona block to polyspermy (Miller et al., 1993). Similarly, the model proposed from our results is that the neuraminidase cleaves the terminal sialic acid from ZP which would otherwise serve as a substrate for sperm binding. This hypothesis is consistent with the reduction in the sperm–ZP binding and the reduction in the fertilisation percentages observed when the neuraminidase was used in the IVF system, as well as with the increase in the polyspermy percentages and in the mean number of sperm per oocyte observed when the neuraminidase inhibitor was employed.

In conclusion, results from this paper show that different sialic acids are present in the bovine ZP, and that the  $\alpha$ -2,3-sialic acid linked to *N*-acetylglucosamine plays an important role in the sperm–ZP binding. Moreover, a neuraminidase from cortical granules probably participates in the mechanism of blocking to polyspermy by removing sialic acid from the ZP.

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