Removal of sialic acid from bull sperm decreases motility and mucus penetration ability but increases zona pellucida binding and polyspermic penetration *in vitro*

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Abstract

This study tested the hypothesis that sperm sialic acid (Sia) is required to reach the site of fertilization, and that successful fertilization requires recognition of Sia from both the sperm and oocyte to occur. In addition, it has recently been reported that Siglecs (Sia-binding-immunoglobulin-like lectins) are present on the sperm surface. Thus, the possibility that the recognition of oocyte Sia was sperm-Siglec-mediated was also addressed. Sperm exposed to neuraminidase (NMase) exhibited lower overall and progressive motility, which translated to a decreased ability to swim through cervical mucus from cows in oestrus. In addition, when either sperm or cumulus–oocyte complexes (COCs) were treated with NMase, a decrease in cleavage and blastocyst rate was observed. However, incubation of sperm with increasing concentrations of anti-Siglec-2, -5, -6 and -10 antibodies prior to fertilization had no effect on their fertilizing ability. Interestingly, treatment with NMase increased the number of sperm bound to the ZP but also the rate of polyspermic fertilization. Flow cytometry analysis revealed no differences in the percentage of capacitated or acrosome-reacted sperm. These results suggest that Sia are required to reach the site of fertilization but need to be removed for sperm–oocyte interaction. However, fine regulation is needed to avoid abnormal fertilization which can lead to impaired embryo development.

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Introduction

Sperm are coated with a thick (20–60 nm) glycocalyx composed of different glycolipids, glycoproteins and glycosylphosphatidylinositol (GPI)-anchored proteins (Tecle & Gagneux 2015). The oocyte is also surrounded by a heavily glycosylated matrix, the zona pellucida (ZP). Thus, glycoconjugates from both the male and female reproductive tracts are critical components of the molecular mechanisms underlying the control of the interaction between both gametes and with the female reproductive tract and its secretions. In mammals, the terminal monosaccharides of glycans are often small, negatively charged monosaccharides called sialic acids (Sia) (Tecle & Gagneux 2015). This privileged location means that Sia can potentially interact with the extracellular milieu and neighbouring cells to elicit different responses at an intracellular level.

Although all cell surfaces contain numerous Sia, the sperm sialome is especially abundant in comparison with somatic cells (Kraemer 1966, Tecle & Gagneux 2015). This sperm sialome is established during spermatogenesis, epidydmal maturation and by incorporation of sialylated seminal fluid components during ejaculation (Ma *et al*. 2016). In the epidydmis, acquisition of the sperm sialome occurs in a region-dependent matter. For example, in mouse testicular sperm, only Sia α2–3 is present, whereas Neu5Ac, which comprises more than 20% of mature mouse sperm Sia, is transferred by sialyltransferase in the caput and corpus epidydmis (Ma *et al*. 2016). The abundant sialylation found on the sperm surface highlights the importance of these sugars in sperm function. Sia have been found to mediate numerous and diverse biological roles in different cell types through their ability to act as both recognition sites for a variety of molecules such as hormones, lectins, antibodies or inorganic cations, or masking receptors located on the cell surface (Varki & Schauer 2009). In sperm, Sia likely facilitates tolerance by female innate pattern-recognition molecules, and may play a role in masking potential antigenic sperm molecules until after sperm have undergone capacitation

(Toshimori *et al.* 1988, 1991, Yudin *et al.* 2005). In addition to this immunological role, the relatively strong electronegative charge of Sia has been linked to an enhanced ability of sperm to travel through the mucus-lined female tract in macaques (Tollner *et al.* 2008*a*). In fact, reduction of Sia content in human sperm has been correlated with subfertility due to the inability of these cells to migrate through the mucus matrix in the reproductive tract (Tollner *et al.* 2011).

Apart from masking receptors, sperm surface Sia are potentially able to interact with Siglecs (sialic acid-binding-immunoglobulin-like lectins) on the female reproductive tract. Siglecs are transmembrane receptors that have distinct preferences for binding the diverse types of sialylated glycans present on cell surfaces (Macauley *et al.* 2014). These proteins have been shown to play a role as regulators of immune cell function. Binding of Siglecs to leukocyte Sia downregulates the immune response of the cell (Crocker *et al.* 2007). In mice, sperm have been found to strongly bind to Siglec-6, which is predominantly expressed by B cells (Ma *et al.* 2012). This may be the mechanism behind the lower phagocytosis rate observed when epididymal mouse sperm are coated with Sia (Ma *et al.* 2016), or the reason why removal of the highly sialylated β-defensin 126 or treatment with neuraminidase elicits female immunorecognition (Yudin *et al.* 2005). Interestingly, proteomic and transcriptional studies have identified several Siglecs in both human and bovine sperm (Lalancette *et al.* 2008, Peddinti *et al.* 2008, Amaral *et al.* 2014). In the bovine, Siglec-2, -5, -6 and -10 have been found to be preferentially expressed in the neck region and, in the case of Siglec-6 and -10, the anterior head of sperm (Alkhodair *et al.* 2018). The role of these molecules in sperm has not been elucidated to date. However, it is interesting to note that Siglec-10 was found to be differentially expressed in sperm from bulls with a high fertility index (Lalancette *et al.* 2008). Sperm-ZP binding has been shown to be Sia-mediated in both humans and cattle (Velásquez *et al.* 2007, Pang *et al.* 2011). In the cow, 85% of ZP glycoproteins contain Neu5Ac moieties (Velásquez *et al.* 2007). In this species, interaction of an unknown sperm ligand with α-2,3-linked Sia is required for ZP binding (Velásquez *et al.* 2007).

Based on this information, it seems that Sia are required for sperm to reach the site of fertilization, but removal of part of the sperm sialome is required to enable interaction with the ZP. Interestingly, 80% of the sperm Sia remain intact after capacitation (Ma *et al.* 2012). These remaining Sia could potentially interact with the oocyte vestments in order to facilitate successful fertilization. Therefore, the aim of this study was to determine how removal of bovine sperm Sia affects sperm ability to reach the site of fertilization, undergo capacitation and fertilize oocytes *in vitro*. In addition, we also tested the hypothesis that interaction with oocyte Sia occurs through sperm surface Siglecs.

Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich Chemical unless stated otherwise.

The frozen semen straws from one bull that were used in the different experiments were obtained from the National Cattle Breeding Centre (Naas, Ireland). Straws obtained from the same ejaculate of the same bull were used to carry out all experiments described in the following section.

Experiment 1: Effect of neuraminidase treatment on sperm ability to migrate through cervical mucus

The aim of Experiment 1 was to determine how treatment of sperm with *C. perfringens* type V Neuraminidase (NMase), a sialidase that cleaves sialic acid, would affect their ability to penetrate oestrus cervical mucus (CM).

Assessment of Sia loss from the sperm membrane

In order to determine the degree of sialic acid loss that occurs in the presence or absence of NMase under the conditions of this study, sperm were labelled with lectin from *Triticum vulgaris* (wheat germ agglutinin – WGA) which binds to Sia (Monsigny *et al.* 1980).

Sperm incubated in the presence or absence of NMase for 1 h or 5 h were air-dried on top of slides and then fixed in 4% paraformaldehyde/PBS for 45 min. Sperm were subsequently blocked with blocking buffer (4% BSA/PBS) for 1 h, and then incubated with biotinylated WGA (1 μg/mL) for an additional hour. After three washes in PBS, sperm were incubated for 1 h Alexa Fluor 488-streptavidin conjugate (1:500; Life Technologies). Finally, samples were washed three additional times in PBS and incubated with Hoechst 33342 for 10 min. The slides were mounted with Mowiol, and sperm were then observed under a Leica DMI6000B epifluorescence microscope equipped with a 40× oil immersion objective.

The percentage of positively stained sperm, negative sperm (no stain visible) and partially stained sperm were assessed for each treatment group. Two setups were carried out with at least 50 cells assessed per set up and treatment.

Cervical mucus penetration

CM was collected from cows observed in standing oestrus. A polyethylene catheter attached to a 20-mL syringe was inserted into the vagina and guided towards the external cervical os. Once the catheter was in position, gentle suction was applied to recover the CM. Mucus from three different cows was pooled, aliquoted and stored at −20°C. CM from the same pool was used throughout and the mucus penetration test was carried out as described by Kiernan *et al.* (2013). Motile, frozen-thawed, bull sperm were selected by washing through a 90–45% Percoll gradient. This was followed by a second wash in HEPES-buffered Tyrode medium and the assessment of sperm concentration with the use of a hemocytometer. Sperm were diluted to achieve a concentration of 20×10^6 sperm/mL, and separated into two groups. One group was incubated with 0.1 UN/mL NMase, while the other group received an equivalent volume of HEPES-buffered Tyrode medium for 1 h

or 5 h. Flattened capillary tubes (0.3 mm × 3.0 mm × 100 mm; Composite Metal Services Ltd., Shipley, UK) were marked at 10-mm intervals between 10 and 90 mm, filled with thawed mucus and put in an incubator at 38.5°C while sperm were processed. At 1 h or 5 h, a 250-µL sample from each treatment was stained with Hoechst 33342 (10 µg/mL) for 10 min at 38.5°C. Following staining, two mucus-filled capillaries were placed vertically into an Eppendorf tube containing the stained sperm dilution and left for 30 min in an incubator at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Capillary tubes were then removed and placed on a hot plate at 70°C for 3 min to immobilize the sperm. Sperm were counted across the width of the tube, one field of view wide under 40× magnification, at each 10-mm interval between 10 and 90 mm using a fluorescent microscope (Nikon Eclipse TE2000s). Four replicates were completed with two capillary tubes representing each treatment in each replicate.

Experiment 2: Effect of neuraminidase treatment of sperm or oocytes in *in vitro* fertilization

The aim of Experiment 2 was to determine how treatment of sperm or oocytes with *C. perfringens* type V Neuraminidase (NMase) would affect the outcome of *in vitro* fertilization.

In vitro fertilization

Ovaries from cows and heifers were collected at a commercial abattoir, and surface-visible follicles (>2 mm) were aspirated to recover cumulus–oocyte complexes (COCs). Good-quality COCs were matured in TCM-199 supplemented with 10% (v/v) foetal calf serum (FCS) and 10 ng/mL epidermal growth factor (n = 50 COCs per well) for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. After maturation, sperm were processed and treated with NMase for 1 h as described earlier. Matured COCs were randomly divided into two equal groups, one incubated in the presence of 0.1 UN/ mL NMase and a control group. After 1 h of incubation, sperm were washed twice by centrifugation in HEPES-buffered Tyrode medium at 500 g for 5 min, and concentration was adjusted to 2 × 10⁶ sperm/mL in fertilization media containing 113.16 mM NaCl, 3.19 mM KCl, 0.2 mM NaH_2PO_4, 2 mM $CaCl_2$, 0.487 mM $MgCl_2$, 0.5 mM pyruvate, 12.79 mM $NaHCO_3^-$, 6 mg/mL BSA and 10 µg/mL heparin (pH: 7.4). COCs were also washed three times in the same medium. Groups of 50 matured COCs were randomly allocated to the following experimental treatments: (i) NMase COCs (COCs incubated with NMase and fertilized with untreated sperm; n = 127 COCs), (ii) NMase sperm (COCs fertilized with sperm incubated with NMase; n = 124 COCs) or (iii) control (untreated COCs, fertilized with untreated sperm; n = 144 COCs). Three replicates were carried out and each treatment was equally represented in each replicate. Approximately 20 h post fertilization, cumulus cells were removed from presumptive zygotes and washed in PBS before being transferred in groups of 25 to culture drops of synthetic oviduct fluid supplemented with 5% FCS. Culture dishes were kept at 39°C under an atmosphere of 5% CO_2 and 5% O_2 in air with maximum humidity. Cleavage was assessed 48 h post fertilization and blastocyst development was recorded on Day 8 (Day 0 = day of fertilization) and expressed over the total number of oocytes fertilized.

To determine if increasing the duration of incubation with NMase could have a more dramatic effect due to more Sia being removed, COCs were fertilized with the following sperm treatments: (i) sperm NMase 1 h (sperm incubated with NMase for 1 h, n = 172 COCs), (ii) control 1 h (sperm incubated for 1 h in an equivalent volume of HEPES-buffered Tyrodes, n = 193 COCs), (iii) sperm NMase 5 h (sperm incubated in the presence of NMase for 5 h, n = 192 COCs) and (iv) control 5 h (sperm incubated for 5 h in an equivalent volume of HEPES-buffered Tyrodes, n = 181 COCs). Alternatively, COCs were incubated with or without (control) NMase for 1 or 5 h (COCs NMase 1 h, n = 118 COCs; control 1 h, n = 129 COCs; COCs NMase 5 h, n = 133 COCs; control 5 h, n = 121 COCs), and fertilized with untreated sperm. In both cases, 20 h post fertilization, presumptive zygotes were cultured and cleavage and blastocyst development were assessed as described earlier.

Assessment of sperm-ZP binding and penetration

To further explore the effect of sperm NMase treatment on sperm–oocyte interaction, the ability of treated sperm to bind to the ZP and penetrate the oocyte plasma membrane was studied. *In vitro* maturation was performed as previously described. After maturation, COCs were divided into two groups. One group was stripped free of cumulus cells by gentle pipetting in PBS and placed in fertilization media. The second group was placed in fertilization media without removing their cumulus cells. Percoll-selected motile sperm were incubated for 5 h in the presence of NMase or an equivalent volume of HEPES-buffered Tyrodes. They were then washed, as described earlier and used to fertilize both denuded and cumulus-intact oocytes. Sperm and oocytes were coincubated in an incubator with 5% CO_2, 38.5°C and maximum humidity. Three hours after incubation, cumulus-free oocytes were vortexed for 30 s to remove sperm not tightly bound to the ZP. They were then fixed in 4% paraformaldehyde/PBS for 45 min, washed three times in PBS and mounted on a slide with Mowiol mounting medium and stained with Hoechst 33342 (10 µg/mL). Oocytes were then observed with a fluorescent microscope (Olympus BX60) at 40× magnification and the number of sperm bound to their ZP was recorded. Cumulus-intact oocytes were stripped from the cumulus cells 17 h post fertilization by gentle pipetting. They were then fixed, and mounted and viewed in the same way as the previous group. Oocytes were observed under an Olympus BX60 microscope at 40× magnification, and the presence and number of pronuclei were recorded.

Experiment 3: Effect of NMase treatment on sperm motility, capacitation and the acrosome reaction

Experiment 3 aimed to determine whether release of Sia by NMase treatment would induce motility or capacitation changes and activate the acrosome reaction.

CASA analysis

Sperm exposed to NMase for 1 or 5 h, with the corresponding controls, were assessed for motility using a CASA system

(IVOS II Clinical; Hamilton Thorne, IMV Technologies, L'Aigle, France). A sample from each treatment was placed on a 20-µL 4-chamber Leja slide (Micro Optic, Wardenburg, Germany) and evaluated. Percentage of motile and progressively motile sperm were evaluated. In addition, the following motion parameters were also measured: curvilinear velocity (VCL, total distance travelled by the sperm head per unit of time; µm/s), velocity straight line (VSL, the straight line distance travelled by the sperm head per unit of time; µm/s), velocity average path (VAP, the average velocity of the sperm head; µm/s), linearity (LIN, straightness of trajectory, %), amplitude of lateral head displacement (ALH, degree of side-to-side head movement; µm), distance average path (DAP, the average path distance of the sperm head; µm), distance curved line (DCL, the averaged curved distance travelled by the sperm head; µm), distance straight line (DSL, the average straight line distance travelled by the sperm head; µm), wobble (WOB, the side-to-side movement of the sperm head measured as the ratio of velocity average path divided by the curvilinear velocity; %) and beat cross frequency (BCF the frequency at which the sperm head moves across the middle plane of the 'straightened' trajectory, Hz). Four chambers were assessed per sample with a minimum of 100 sperm cells counted in a minimum of 8 fields of view per slide. The experiment was replicated three times.

Flow cytometry

In order to analyse sperm capacitation and acrosomal status, flow cytometric analysis was performed on sperm incubated in the presence or absence of NMase. Sperm were washed as described earlier and concentration was adjusted to 3×10^6 sperm/mL. Sperm were diluted in fertilization media containing 113.16 mM NaCl, 3.19 mM KCl, 0.2 mM NaH_2PO_4, 2 mM $CaCl_2$, 0.487 mM $MgCl_2$, 0.5 mM pyruvate, 12.79 mM $NaHCO_3^-$, 6 mg/mL BSA and 10 µg/mL heparin, a medium that has been shown to support sperm capacitation. This medium was supplemented with 0.1 U/mL NMase, or with an equivalent volume of fertilization media (control). Both NMase and control sperm samples were split into two groups; in one, 5 µM Ca^{2+} ionophore was added, while in the other one, an equivalent volume of fertilization media was added. Samples were incubated in an incubator at 39°C under an atmosphere of 5% CO_2 and 5% O_2 in air with maximum humidity. At times 0 (T0), 1 h (T1), 3 h (T3) and 5 h (T5), a sample from each group was taken and 5 µM merocyanine 540 (MC; Life Technologies), a reporter probe for phospholipid scrambling (Harrison *et al*. 1996); 1 µM Yo-Pro-1 (YP; Life Technologies), a membrane-impermeable nucleic acid stain (Harrison *et al*. 1996); and 0.5 µg/mL Alexa Fluor 647-conjugated-*Arachis hypogaea* (peanut) agglutinin (PNA; Life Technologies), a lectin that binds to the outer acrosomal membrane (Flesch *et al*. 1998), were added. Samples were incubated for 10 min with the stains before flow cytometric analysis.

Flow cytometric analysis was performed with a standard bench-top BD Accuri C6 flow cytometer (Becton Dickinson Biosciences, Ann Arbor, MI, USA, with BD Accuri C6 software v. 1.0.264.21). The cytometer was calibrated daily according to the manufacturer's recommendations with 8 and 6 peak calibration beads. The cytometer was equipped

with a 488-nm and a 633-nm laser. The 488-nm laser was used for the excitation of YP and MC, and their emission was filtered using a using a 533 ± 30 and a 575 ± 25 band pass filters, respectively. The 633-nm laser was used for the excitation of PNA (675 ± 25). Filtered emissions were detected by photomultiplier tubes. A threshold of 40,000 in the forward scatter signal was applied to remove electrical noise, and very small events and samples were collected at the default low flow rate. For each sample, 15,000 single cells were recorded. Each assay was replicated three times. Titration of the dyes was carried out, and Fluorescent Minus One controls were used for an initial gating. The gating strategy used can be followed in Supplementary Fig. 1 (see section on supplementary data given at the end of this article). Further analysis was done with FCS Express 5 Flow Plus software (De Novo Software, Glendale, CA, USA).

Experiment 4: Role of sperm Siglecs in sperm–oocyte interaction

The aim of Experiment 4 was to determine whether interaction with oocyte Sia is mediated by sperm Siglecs. A motile population of frozen-thawed sperm was selected with a Percoll gradient as described earlier. Sperm concentration was then assessed and adjusted to 20×10^6 sperm/mL. A blocking assay was performed by incubating sperm for 1 h in the presence of 1:20 or 1:50 Siglec-2, -5, -6, -10 or -5/14 antibodies (Siglec-2, -6 and -10 antibodies were purchased from Santa Cruz Biotechnology; anti-Siglec-5/14, and -5 antibodies were obtained from R&D Systems). No specific anti-Siglec-14 antibody is currently available; however, a cross-reactive antibody against both Siglec-5 and 14 exists and was used to screen for Siglec-14 and compared with Siglec-5 alone. Sperm incubated with an equivalent concentration of anti-IgG antibody was used as control. After incubation, sperm were washed twice in HEPES-buffered Tyrode medium for 5 min at 500 *g*. Sperm concentration was reassessed and adjusted to 2×10^6 sperm/mL. Groups of 50 *in vitro* matured COCs were then fertilized with sperm from different treatments. *In vitro* fertilization and culture were carried out as described earlier. Cleavage and blastocyst rates were recorded. Each Siglec blocking assay was replicated three times.

Statistical analysis

Data were checked for normality of distribution and homogeneity of variance using histograms, qplots and formal statistical tests in the univariate procedure (version 9.1.3; SAS Institute, Cary, NC, USA). Data that were not normally distributed were subsequently transformed prior to analysis.

The data from IVF (cleavage rate and blastocyst rate) were subjected to arcsin transformation prior to analysis. In addition, the sperm mucus penetration data required a square root transformation to normalize the residuals, as preliminary analyses revealed that the distribution of values was positively skewed. The transformed data were used to calculate *P* values. However, the corresponding least-squares means and s.e.m. of the non-transformed data are presented in the results for clarity.

For all individual experiments, *in vitro* fertility data for the proportion of oocytes that cleaved and reached the blastocyst stage were analysed using the MIXED procedure of SAS with a model that included treatment imposed as a fixed effect and bull as a random term. Additionally, sperm mucus penetration counts along the capillary tube (10–90 mm) were cumulatively assessed and analysed thereafter using MIXED procedure of SAS (SAS Institute) with a model that included treatment imposed as a fixed effect and bull as a random term. Interaction terms if not statistically significant ($P < 0.05$) were subsequently excluded from the final model. Differences among means were determined by *F*-tests using Type III sums of squares. The PDIFF option of SAS and the Tukey test were applied to evaluate pairwise comparisons between means. The level of significance was set at $P < 0.05$.

Results

### Experiment 1: Effect of neuraminidase treatment on sperm ability to migrate through cervical mucus

To confirm whether NMase treatment induced a higher loss of Sia from the sperm membrane, sperm were labelled with WGA. Sperm were classified as positively stained (bright fluorescence observed on the acrosomal region; Fig. 1B.1), partial loss (dimmer, grainy looking

labelling; Fig. 1B.2) or negative (no fluorescence visible; Fig. 1B.3). Incubation of sperm for 1 h with this sialidase was sufficient to decrease the percentage of WGA-positive sperm by half ($80 \pm 17\%$ in the control group vs $41 \pm 2\%$ in the NMase group; $P < 0.05$; Fig. 1A). In both control and NMase groups, a loss of Sia was observed between 1 h and 5 h of incubation (percentage of positive cells: $80 \pm 17\%$ at 1 h vs $28 \pm 10\%$ at 5 h in the control group; $41 \pm 2\%$ at 1 h vs $8 \pm 6\%$ at 5 h in the NMase group; $P < 0.05$). However, the percentage of WGA-positive cells remained higher in the control in comparison with the NMase treatment group at 5 h ($28 \pm 10\%$ in the control group vs $8 \pm 6\%$ in the NMase group; $P < 0.05$).
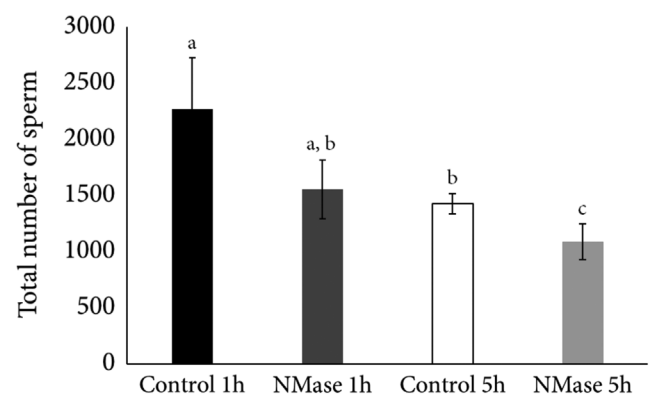
Incubation of sperm for 1 h in the presence of NMase had no effect on the ability of sperm to migrate through CM. Fewer control sperm were observed in the capillary tube after 5 h of incubation in comparison with 1 h of incubation ($P < 0.05$; Fig. 2), suggesting a time-dependent decrease in mucus penetration ability. In addition, sperm incubated in the presence of NMase for 5 h had a reduced penetration ability in comparison with the control ($P < 0.05$; Fig. 2).

### Experiment 2: Effect of neuraminidase treatment of sperm or oocytes in *in vitro fertilization*

Treatment of COCs with NMase decreased the percentage of cleaved oocytes from $93 \pm 2\%$ in the control group to $35 \pm 4\%$ ($P < 0.001$). This translated into a decrease in the percentage of blastocysts from $44 \pm 4\%$ in the control to $5 \pm 2\%$ in the NMase-treated group ($P < 0.001$; Fig. 3). Although not as dramatic, the same trend was also evident when sperm were treated with NMase. Cleavage rate was reduced from $93 \pm 2\%$ to $66 \pm 4\%$, and the proportion of oocytes forming blastocysts at Day 8 was $27 \pm 4\%$ vs $44 \pm 4\%$ in the control ($P < 0.001$; Fig. 3).
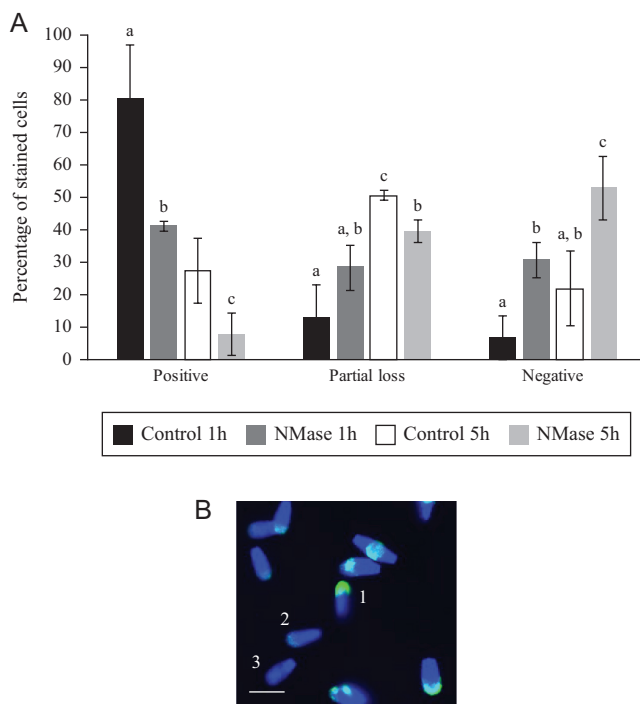
Figure 1 (A) Percentage of sperm of WGA-positive, WGA-negative or exhibiting dimmer fluorescence (partial loss of WGA labelling), after incubation for 1 h or 5 h in the presence or absence of NMase. Data reported as mean ± s.e.m. Different superscripts between treatment groups within positive, partial loss or negative indicate a difference ($P < 0.05$). (B) Representative images of sperm classified as positive (1), partial loss of fluorescence (2) and negative (3). Bar equals 10 µm. Experiment 1.


Figure 2 Total number of sperm found in the mucus-filled capillary tube after incubation for 1 h or 5 h in the presence or absence (control) of NMase. Data reported as least-squares means ± s.e.m. Different superscripts indicate a difference ($P < 0.05$). Experiment 1.
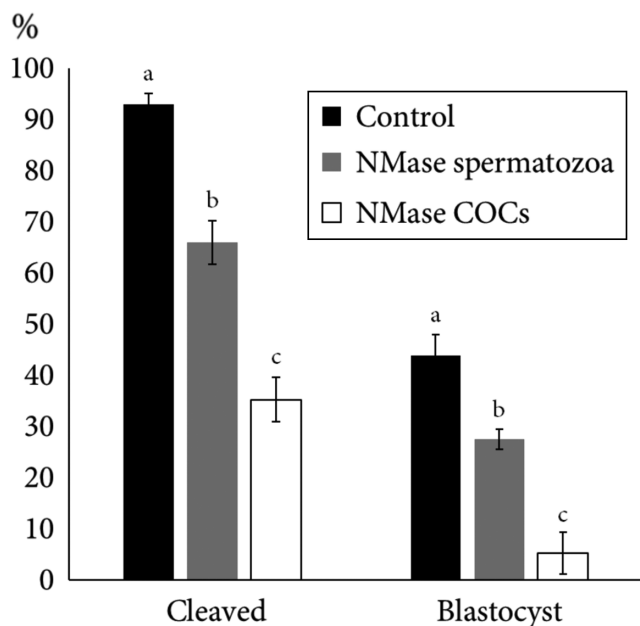
Figure 3 Percentage of cleaved zygotes observed on Day 2 (Day 0 = day of fertilization) and blastocysts on Day 8 after treatment of cumulus–oocyte complexes (COCs) or sperm with neuraminidase (NMase; $n = 50$ COCs per treatment, $n = 3$ replicates). Untreated COCs fertilized with untreated sperm were used as control. Data reported as least-squares mean ± s.e.m. Different superscripts between treatment groups within cleavage or blastocyst rate indicate a difference ($P < 0.001$). Experiment 2.

In order to determine whether the results observed in the sperm treatment group were really due to a removal of sialic acid from these cells, rather than a carryover effect of NMase on the oocytes, sperm or oocytes were incubated for 1 or 5 h in the presence of the enzyme. As expected, increasing COC incubation with NMase to 5 h lowered cleavage rate from 75 ± 4% in the control to 9 ± 2% ($P < 0.001$; Fig. 4A). This also led to a decrease in blastocyst rate (1 ± 0.7% in the NMase-treated group vs 31 ± 4% in the control group; $P < 0.01$). The same tendency was observed when time of incubation of sperm with NMase was increased. In this case, cleavage rate decreased to 44 ± 4% from the 81 ± 3% observed in the 5-h control group ($P < 0.05$; Fig. 4B). Blastocyst rate decreased from 43 ± 4%, in the control group, to 13 ± 3% when sperm were treated with NMase for 5 h.

Treating sperm with NMase for 5 h increased their ability to bind to the ZP. This was evidenced by a higher average number of NMase-treated sperm found on the ZP in comparison with the control group (17 ± 3.0 vs 8 ± 2.0, respectively; $P < 0.05$; Fig. 5A). Surprisingly, although no differences were observed in overall penetration rate when both monospermic and polyspermic fertilization were taken into account, a higher rate of polyspermic penetration was observed in the NMase group when compared to the control (12% vs 2%, respectively; $P < 0.05$; Fig. 5B and C).
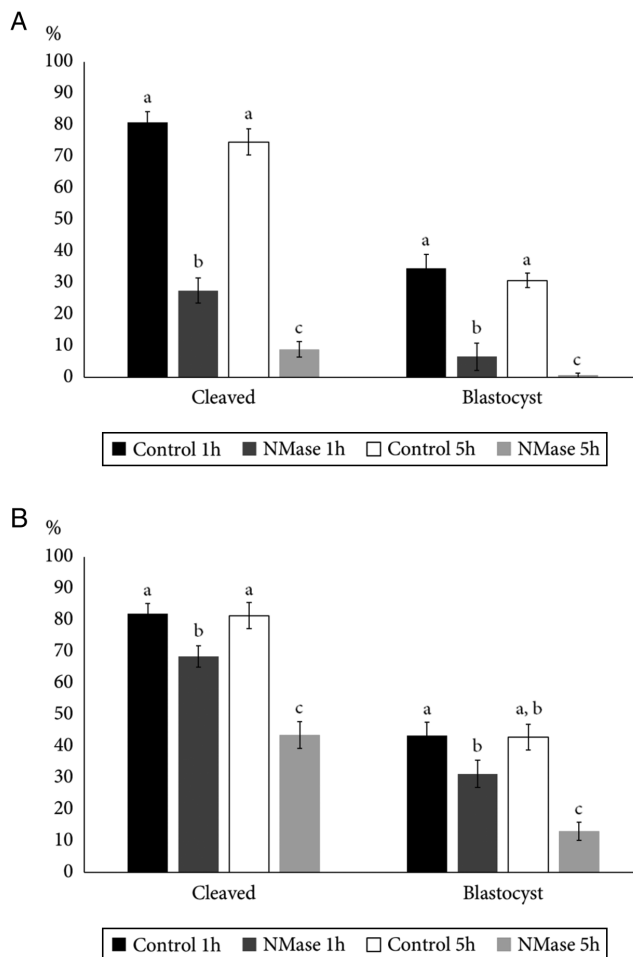
Figure 4 (A) Percentage of cleaved zygotes observed on Day 2 (Day 0 = day of fertilization) and blastocysts on Day 8 after treatment of cumulus–oocyte complexes (COCs) for 1 or 5 h with neuraminidase (NMase; $n = 50$ COCs per treatment, 3 replicates). Untreated COCs were used as control. Data reported as means ± s.e.m. Different superscripts between treatment groups within cleavage or blastocyst rate indicate a difference ($P < 0.001$). (B) Cleavage and blastocyst rate after incubation of sperm for 1 or 5 h in the presence of NMase. COCs fertilized with untreated sperm were used as controls. Data reported as least-squares means ± s.e.m. Different superscripts between treatment groups within cleavage or blastocyst rate indicate a difference ($P < 0.01$). Experiment 2.

Experiment 3: Effect of NMase treatment on sperm motility, capacitation and acrosome reaction

In order to determine whether the results observed in the mucus penetration and IVF experiments were due to an effect of NMase treatment on sperm motility, CASA was performed (Table 1). As expected, motility and progressive motility dramatically decreased from the 1-h to the 5-h incubation time points in both NM treatment (overall motility: 62 ± 4.8% vs 14 ± 4.6%; progressive motility: 57 ± 4.3% vs 12 ± 4.3%; $P < 0.001$) and control (overall motility: 78 ± 4.6% vs 21 ± 4.6%; progressive motility: 72 ± 4.3% vs 18 ± 4.3%: $P < 0.001$) groups.
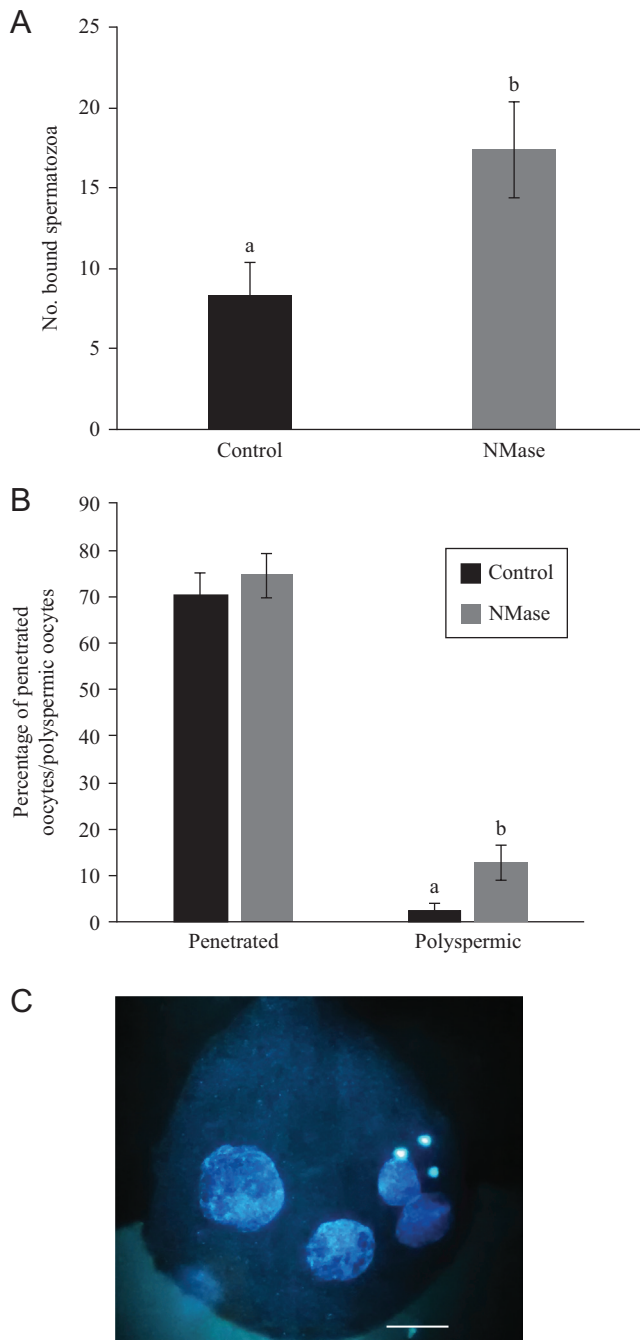
Figure 5 (A) Average number of sperm bound to the ZP after treatment with neuraminidase (NMase). (B) Overall penetration, and polyspermy rates in cumulus–oocyte complexes (COCs) fertilized with treated sperm. (C) Representative image of a polyspermic fertilization found in the NMase treatment group where 4 pronuclei are clearly visible in an oocyte. Bar represents 40 μm. Different superscripts between treatment groups indicate a difference ($P < 0.01$). Data reported as least-squares means ± S.E.M. Experiment 2.

Incubation of sperm with NMase for 1 h decreased both overall motility and progressive motility in comparison with the untreated control ($62 \pm 4.8\%$ vs $78 \pm 4.6\%$ and $57 \pm 4.3\%$ vs $72 \pm 4.3\%$, respectively: $P < 0.001$). After 5 h

of incubation, overall motility of NMase-treated sperm did not differ from control sperm, but the percentage of progressive cells was lower ($12 \pm 4.3\%$ vs $18 \pm 4.3\%$: $P < 0.001$). Incubation with NMase had no effect on the additional motion parameters studied (VCL, VSL, VAP, LIN, ALH, DAP, DCL and DSL), except for a higher rate of WOB and higher BCF observed in this group after 5 h of incubation in comparison with the control ($66 \pm 2.9\%$ vs $63 \pm 2.9\%$ and 37 ± 1.9 vs 33 ± 1.9 Hz, respectively; $P < 0.05$).

Flow cytometric analysis was performed in NMase-treated and control sperm to determine their ability to undergo capacitation and the acrosome reaction. Samples were labelled with MC, PNA and YP. Non-viable sperm exhibited YP staining and were excluded from the analysis by gating them out. The analysis of the data was performed in single viable sperm as described in Supplementary Fig. 1.

The highest percentages of MC-labelled sperm in both control and NM treatment were observed at T1. Supplementation of the media with Ca^{2+} ionophore did not increase the percentage of capacitated sperm (Table 2). In addition, no differences were observed in MC-labelling between control and NMase-treated sperm at any of the time points studied ($P > 0.05$).

When PNA labelling was analysed, three population of sperm could be observed from T3 which we referred to as 'low', 'medium' and 'high' PNA-labelled sperm. Medium and high PNA-labelled sperm were considered acrosome-reacted. As expected, the percentage of PNA-labelled cells, as well as fluorescence intensity, increased over time (Table 2). As expected, the presence of Ca^{2+} ionophore induced an increase in the percentage of acrosome-reacted sperm from T1 onwards, reaching the highest value at T3 (Table 2). Again, no differences were observed between treatments.

Experiment 4: Role of sperm Siglecs in sperm–oocyte interaction

Because bull sperm express Siglecs on the anterior head and neck region (Alkhodair *et al.* 2018), we hypothesized that interaction with the oocyte Sia is mediated by sperm Siglecs. However, 1-h incubation of sperm with anti-Siglec-2, -5, -6, -10 or -5/14 antibodies had no effect on their ability to fertilize *in-vitro*-matured oocytes (Table 3).

Discussion

The main findings of this study were: (1) Incubation of sperm in fertilization media led to a progressive loss of Sia which was more dramatic when NMase was added to the media. (2) Treatment of sperm with NMase led to a reduced mucus penetration ability, as well as a decrease in overall and progressive motility. (3) Removal of Sia from the COCs or sperm using NMase

Table 1 Motility parameters assessed in sperm incubated for 1 h or 5 h in the presence or absence of NMase.

	Control 1 h	NMase 1 h	Control 5 h	NMase 5 h
Motility (%)	78 ± 4.6[a]	62 ± 4.8[b]	21 ± 4.6[c]	14 ± 4.6[c]
Progressive motility (%)	72 ± 4.3[a]	57 ± 4.3[b]	18 ± 4.3[c]	12 ± 4.3[d]
VCL	183 ± 8.4[a]	193 ± 8.8[a]	150 ± 8.4[b]	143 ± 8.4[b]
VSL	91 ± 9.51[a,c]	100 ± 9.7[c]	87 ± 9.7[a,b]	90 ± 9.5[a,b]
VAP	104 ± 8.7[a,c]	112 ± 8.9[a]	92 ± 8.7[b]	96 ± 8.7[b,c]
LIN (%)	49 ± 3.5[a]	51 ± 3.6[a]	58 ± 3.5[b]	62 ± 3.5[b]
ALH	8 ± 0.3[a]	8 ± 0.3[a]	6 ± 0.3[b]	5 ± 0.3[b]
DAP	46 ± 3.9	51 ± 4.1	42 ± 3.9	44 ± 3.9
DCL	82 ± 4.3[a]	88 ± 4.6[a]	67 ± 4.3[b]	63 ± 4.3[b]
DSL	50 ± 3.2[a]	44 ± 3.7[a,b,c]	38 ± 3.2[b]	41 ± 3.2[b,c]
WOB (%)	57 ± 2.9[a]	57 ± 2.9[a]	63 ± 2.9[b]	66 ± 2.9[c]
BCF	28 ± 1.9[a]	31 ± 2[a]	33 ± 1.9[b]	37 ± 1.9[c]

Data reported as least-square means ± s.e.m. Different superscripts between treatment groups indicate a difference ($P < 0.01$). Experiment 3. ALH, amplitude of lateral head displacement; BCF, beat cross frequency; DAP, distance average path; DCL, distance curved line; DSL, distance straight line; LIN, linearity; VAP, velocity average path; VCL, curvilinear velocity; VSL, velocity straight line; WOB, wobble.

had a negative effect on fertilization rate and subsequent embryo development. (4) Incubation of sperm with anti-Siglec-2, -5, -6, -10 or -5/14 antibodies had no effect on their ability to fertilize *in-vitro*-matured oocytes. (5) Treatment of sperm with NMase led to an increased ability to bind to the ZP, and an increased rate of polyspermic fertilization.

The primary biological role of sperm is to transmit the paternal genetic message encoded in the DNA to the next generation. In order to fulfil this goal, the male gamete must be able to transverse the mucus-lined female reproductive tract, evade the immune surveillance of the female, interact with the cumulus cells and selectively bind the ZP and the plasma membrane of the oocyte. A growing body of evidence links glycans and glycan-binding molecules to most of these sperm functional roles (Yudin *et al.* 2005, Tollner *et al.* 2008a,b, Kadirvel *et al.* 2012, Roy *et al.* 2014, Ma *et al.* 2016). Changes in the sperm glycome occur due to proteolysis, shedding or incorporation of glycoproteins that takes place during the epididymal transit (Toshimori *et al.* 1988, 1991, Tollner *et al.* 2008a,b, Yudin *et al.* 2005, Villaverde

Table 2 Percentage of merocyanine 540 (MC) and Alexa Fluor 647-conjugated-peanut agglutinin (PNA) labelled control and NMase-treated bull sperm.

Antibody dilution	Oocytes fertilized (*n*)	% Cleavage rate (mean ± s.e.m.)	*n* cleaved/*n* oocytes fertilized	% Blastocyst rate (mean ± s.e.m.)	*n* blastocyst/*n* oocytes fertilized
Siglec-2					
1:50	142	85 ± 4.1	118/142	32 ± 6.4	45/142
1:20	132	75 ± 3.9	99/132	35 ± 4.9	46/132
IgG					
1:20	143	81 ± 3.7	116/143	40 ± 2.0	57/143
Siglec-5					
1:50	113	82 ± 2.6	93/113	40 ± 3.9	45/113
1:20	111	84 ± 6.3	93/111	50 ± 6.1	55/111
IgG					
1:20	107	72 ± 5.3	77/107	36 ± 3.5	39/107
Siglec-6					
1:50	116	75 ± 7.9	87/116	29 ± 16.3	34/116
1:20	111	77 ± 6.2	85/111	23 ± 5.1	25/111
IgG					
1:20	109	67 ± 5.8	73/109	19 ± 8.5	21/109
Siglec-10					
1:50	130	71 ± 11.6	92/130	33 ± 7.4	43/130
1:20	127	77 ± 6.7	98/127	28 ± 7.3	35/127
IgG					
1:20	125	66 ± 5.7	83/125	11 ± 7.5	14/125
Siglec-5/14					
1:50	131	84 ± 2.7	110/131	37 ± 7.4	48/131
1:20	120	73 ± 7.5	88/120	38 ± 6.9	45/120
IgG					
1:20	130	74 ± 6.3	96/130	25 ± 10.1	32/130

Both treatments were incubated in the presence (activated) or absence (non-activated) of Ca^{2+} ionophore. Data reported as least-squares means ± s.e.m. Experiment 3.

Table 3 Effect of incubation of bull sperm with different Siglec antibodies on cleavage and blastocyst rate.

| | % MC labelled sperm (T1) | | % High and Medium PNA labelled sperm | | | | | | | |
| | | | Activated | | | | Non-activated | | | |
Treatment	Activated	Non-activated	T0	T1	T3	T5	T0	T1	T3	T5
Control	56±4.0	55±4.1	0.2±5.2	36±6.5	50±5.2	46±5.2	0.1±5.24	2±6.5	3±5.2	5±5.2
NMase	49±4.0	38±5.0	0.2±5.2	36±6.5	50±5.2	55±5.2	0.2±5.24	2±6.5	4±5.2	7±5.2

Sperm incubated with IgG prior to fertilization were used as control in all cases. Experiment 4.

et al. 2016). In addition, glycan-modifying enzymes (such as glycosyltransferases and glycohydrolases) in the epididymal fluid also induce rearrangement of glycan moieties at this time (Nicolson *et al.* 1977, Tulsiani *et al.* 1995*a*,*b*, Ma *et al.* 2012, Pini *et al.* 2017). As a result, at the time of ejaculation, the surface of sperm is composed of 40% Sia, 30% fucose and 27% hexosamines (Calzada *et al.* 1994). Interestingly, this surface glycome can also be altered following cryopreservation techniques (Pini *et al.* 2017).

In order to study the role of Sia on sperm function and gamete interaction, sperm or oocytes were incubated in the presence of 0.1 UN/mL NMase from *C. perfringens* type V, an enzyme that cleaves terminal sialic acid residues which are α-2,3-, α-2,6- or α-2,8-linked to Gal, GlcNac, GalNAc, AcNeu, GlcNeu, oligosaccharides, glycolipids or glycoproteins. Although it affects all linkages, this type of NMase is most efficient against α-2,3 linked Sia. It can be argued that the results reported in this study can be due to the presence of NMase enzyme in the fertilization media rather than to the activity of the enzyme cleaving terminal Sia. However, the percentage of WGA-positive cells was reduced in half in the NMase group in comparison with the control at 1 h, and was more than 3 times lower at 5 h of incubation. This indicates a NMase-dependent loss of Sia, which is in agreement with the work of Velásquez *et al.* (2007). Velásquez *et al.* used NMase under the same conditions that were used in this study, and were able to block its effect by using an inhibitor, which would suggest that the results observed were due to the action of the enzyme as well.

Treatment of sperm with NMase for 1 h had no effect on the ability of sperm to migrate through CM collected from cows in oestrus. This was surprising, as this treatment induced a decrease in overall and progressive motility in comparison with the control. However, increasing the time of incubation to 5 h reduced the ability of NMase-treated sperm to migrate through this matrix. Similar results have been observed in primates, where treatment with NMase or removal of the highly sialylated DEFB126 protein affected mucus penetration ability (Tollner *et al.* 2008*a*). In our study, incubation of sperm with NMase for 5 h decreased the rate of progressive motility, which could help explain the results observed in the mucus penetration assay. In addition, it has been proposed that Sia confers sperm

with an electronegative surface charge that facilitates migration through mucus by minimizing the interaction of sperm with the negatively charged CM (Tollner *et al.* 2012). The importance of Sia in this particular sperm characteristic is highlighted by the fact that men carrying a mutation in the *DEFB126* gene are subfertile due apparently to a reduced ability of their sperm to penetrate CM (Tollner *et al.* 2011).

Sia have also been implicated in the recognition and binding of the ZP by sperm (Velásquez *et al.* 2007, Pang *et al.* 2011). In the present study, exposing oocytes to NMase led to a decrease in both cleavage and blastocyst rate in comparison with untreated control oocytes. Increasing time of incubation with NMase from 1 h to 5 h had a more dramatic effect, as evidenced by a drop of 65 percentage points in cleavage in comparison with the control group. This supports the theory that oocyte Sia are required for sperm-ZP interaction in the bovine. Velásquez *et al.* (2007) reported that a α-2,3 linked Sia is required for ZP binding in this species. In their study, selective cleavage by specific sialidases or selective blockage of this site by specific lectins led to a decrease in the number of sperm bound to the ZP (Velásquez *et al.* 2007). The results presented in this study take this one step further and show that, as expected, fertilization and embryo development rates decrease by removing these Sia from the oocyte. The sperm receptor that binds to this oocyte Sia remains unknown. However, a recent study has characterized Siglecs-2, -5, -6, -10 and -14 on the sperm surface (Alkhodair *et al.* 2018). Siglecs-6 and -10 are particularly interesting with regard to our study as they are present on the anterior head of sperm (Alkhodair *et al.* 2018), making them susceptible to interact with the female gamete. In addition, Siglec-10 was found to be differentially expressed in sperm from bulls with a high fertility index (Lalancette *et al.* 2008). Thus, anti-Siglec-2, -5, -6, -10 and -5/14 were added to sperm before fertilization in an attempt to block sperm–oocyte interaction. However, no differences were observed between any of these treatments and the control, indicating that these Siglecs are not directly involved in oocyte recognition.

During capacitation, mouse sperm release sialidases that lead to the loss of 20% of the sperm sialome (Ma *et al.* 2012). Removal of sperm Sia is probably also aided by sialidases in the oocyte and in the female reproductive tract (Ganguly *et al.* 1976, Velásquez *et al.*

2007). The importance of this event is highlighted by the fact that men who lack one of the two sperm sialidases exhibit subfertility (Ma *et al*. 2012). However, this also means that 80% of the sperm Sia content remains intact in the surface. This led us to hypothesize that sperm Sia might also be involved with the recognition of the oocyte vestments. Subjecting sperm to the same NMase treatment as that used on the oocytes resulted in a decrease in cleavage and blastocyst rates, albeit not as dramatic as when oocytes were exposed to this enzyme. A 5-h incubation of sperm with NMase decreased cleavage rate by 38% and blastocyst rate by 30%. To further investigate these observations, oocytes fertilized with NMase-treated sperm were fixed at 3 h and 17 h post fertilization which allowed us to study sperm-ZP binding and penetration. Surprisingly, treatment with NMase increased the ability of sperm to bind to the ZP. This is consistent with observations in humans, where treatment of sperm with NMase from *A. ureafaciens* led to a dose-dependent enhancement of sperm attachment to the ZP (Lassalle & Testart 1994). In addition, a recent study has also shown that biantennary complex types N-glycans terminating with α2,6 sialic acids are lower in sperm recovered from buffalos during the breeding season than during the non-breeding season (Accogli *et al*. 2017). However, the increase in sperm-ZP binding made it difficult to explain the decrease in cleavage rate observed in the *in vitro* fertility experiments in this study. When sperm penetration and polyspermic rate were assessed 17 h post fertilization, a higher rate of polyspermic penetration was observed in the NMase group when compared to the control. This higher rate of abnormal fertilization could explain in part the decreased cleavage rate observed in the initial experiments.

Sperm capacitation refers to the set of physiological changes that confer on sperm the ability to fertilize the oocyte (Yanagimachi 1994). These changes include hyperactivation of flagellar motility, regulation of signal transduction pathways that allow a response to chemoattractants, and expression of the ability to acrosome react and to interact with the oocyte (Florman & Fissore 2014). This process takes place in the female reproductive tract but does not occur at the same time in the whole sperm population (Buffone *et al*. 2012). The oviduct appears to regulate sperm capacitation to avoid over-capacitation and premature acrosome reaction, ensuring a constant supply of fertile sperm to the site of fertilization (Boilard *et al*. 2002, Fazeli *et al*. 2003). Treatment with NMase had no effect either on different motility parameters that have been correlated with hyperactivation (VCL, VSL, LIN, VAP, ALH, DAP, DCL, and DSL; Cancel *et al*. 2000), or in M540 labelling, a marker that has been correlated with early-capacitation-dependent changes in membrane fluidity (Harrison *et al*. 1996, Rathi *et al*. 2001). In addition, no differences were observed in the ability of sperm to acrosome react, as

evidenced by the same percentage of PNA-labelled sperm in both NMase and control groups observed over time. This would suggest that the sialidase-induced increase in sperm-ZP binding is independent of capacitation or acrosomal status.

Sialidases secreted by the sperm during capacitation, as well as sialidases present in the oviduct, remove sperm surface Sia, which probably uncovers underlying molecules that can interact with the ZP. Indeed, in Experiment 1, a progressive loss of Sia was observed in sperm incubated in fertilization media, but control sperm presented more Sia on their surface than NMase-treated sperm. Thus, treatment of the whole sperm population with NMase would synchronize the acquisition of oocyte-binding ability, increasing the number of sperm binding to the ZP and, therefore, increasing the chances of polyspermy. This higher polyspermic rate likely alters early embryo development, which results in low cleavage and blastocyst rates. The results presented in this study, together with the literature available, suggest that sperm require Sia to reach the fertilization site but, once there, they need to be removed in order for bovine sperm to interact, bind and fertilize the oocyte. However, fine regulation of this process is required to avoid abnormal fertilization and embryo development.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-17-0429.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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