Analysis of the participation of N-acetylglucosamine in the different steps of sperm–zona pellucida interaction in hamster

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Introduction

Sperm–oocyte interaction is a complex process comprising several steps. Given that the oocyte extracellular matrix (the zona pellucida or its homologue in non-mammals) is composed of glycoproteins, the participation of carbohydrates in sperm–oocyte interaction has been extensively studied (Miller and Ax, 1990; Benoff, 1997; Tulsiani et al., 1997; Töpfer-Petersen, 1999). Carbohydrates are assumed to be involved in the first reversible recognition process between the sperm and the oocyte, known as attachment; the tight binding that occurs thereafter, which can be experimentally evaluated given its resistance to pipetting; the acrosome reaction (AR), which takes place as a consequence of ‘primary binding’; the zona pellucida penetration, a dynamic process of association–dissociation due to the ‘secondary binding’ between acrosome-reacted sperm and the interruption of this connection by the hydrolytic activity of acrosomal enzymes; and finally, sperm fusion with the oolema (Ahuja, 1982; Huang et al., 1982; Jones et al., 1988; Boldt et al., 1989; Kumar et al., 1990; Brandelli et al., 1994; Keller and Vacquier, 1994; Ponce et al., 1994; Litscher et al., 1995; Thaler and Cardullo, 1996; Miranda et al., 1997; Tulsiani et al., 1997; Gougoulidis et al., 1999).

There are few reports in this field for the hamster. The first study that recognized the zona pellucida glycoproteins. A study in hamster indicated that pre-incubation of sperm with N-acetylglucosamine (GlcNAc) affected the interaction with the zona pellucida, but only gave a qualitative analysis of the GlcNAc residues of the zona pellucida in the early steps of the interaction with sperm.

Key words: acrosome reaction/carbohydrates/fertilization/N-acetylglucosamine/sperm–zona pellucida interaction

Materials and methods

Materials

All reagents used were of the highest purity or analytical grade. Reagents were purchased from Sigma, Fisher, Merck or J.T. Baker. Monosaccharides

et al., 1982). In the same year, Ahuja (1982) reported that in addition to fucose, amino-acetylated sugars had a strong inhibitory effect on fertilization, but localized the terminal sugar residues involved in this process to the sperm. Later reports supported the, at present, assumed hypothesis that sperm carry the lectin-like proteins that recognize the sugar residues located on the zona pellucida glycoproteins. A study in hamster indicated that pre-incubation of sperm with N-acetylglucosamine (GlcNAc) affected the interaction with the zona pellucida, but only gave a qualitative analysis of sperm–oocyte attachment (Kumar et al., 1990).

In recent years, our laboratory has been studying the participation of GlcNAc in human sperm function, with results suggesting its participation in zona pellucida binding and induction of the AR (Brandelli et al., 1994; Miranda et al., 1997; Miranda et al., 2000). A deeper study of the different steps comprised in sperm–zona pellucida interaction, however, required a change to an animal model. Consequently, the present study analyses the role of sugar residues in hamster sperm interaction with the zona pellucida.
were from Sigma and at least 98% pure. Fatty-acid free bovine serum albumin (BSA) was from Serological Corporation and pregnant mare’s serum gonadotrophin (PMSG) was from Sintex (Novormon).

**Sperm**

Hamster sperm morphology and acrosome reaction. Hamster sperm acrosome status was evaluated by light microscopy (dark field), according to the classification of Yanagimachi and Phillips (1984). Cells with an intact (a), modified (b), and completely lost acrosome (c), are shown.

were from Nikon and at least 98% pure. Fatty-acid free bovine serum albumin (BSA) was from Serological Corporation and pregnant mare’s serum gonadotrophin (PMSG) was from Sintex (Novormon).

**Sperm**

Animals were housed and manipulated following NIH Guidelines (Guiding Principles in the Care and Use of Animals, DHEW Publication, 80–23). Hamster sperm were obtained from adult male epididymides by swim-out in sucrose-phosphate-buffered medium and purified through a glass bead column as described (Bavister, 1989). Sperm were incubated under capacitating conditions using TL medium (101.02 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 35.7 mM NaHCO₃, 0.35 mM NaH₂PO₄, 9 mM sodium lactate, 4.5 mM glucose and 1 mg/ml PVA), supplemented with 0.25 mM sodium pyruvate, 3 mg/ml fatty-acid free BSA and motility factors (2 mM n-penicillamine, 10 mM hypotaurine and 100 μM epinephrine) [Yanagimachi’s albumin lactate pyruvate medium (TALP)] (Bavister, 1989). Alternatively, a modified TALP was prepared replacing CaCl₂ with SrCl₂ (TALP–Sr). Medium (0.5 ml/well) was placed in 24-well dishes (Costar, Corning), covered with mineral oil and equilibrated for 1 h in 5% CO₂ in air, at 37°C. Aliquots of the purified sperm suspension were added to each well (5 × 10⁵ cells/ml) and incubated for a total period of 6 h. At different times, aliquots were taken from the wells, placed on pre-warmed slides and the AR evaluated by light microscopy (dark field, 200×) on at least 100 motile cells, using the classification of Yanagimachi and Phillips (1984). Sperm showing no changes in the acrosomal cap (Figure 1, panel A) were considered intact, while those with any alteration (Figure 1, panel B) or no visible acrosome (Figure 1, panel C) were classified as reacted. Images were obtained with a Nikon Optiphot microscope, coupled to an image analyser (IPLab; Scanalytics, Inc., Fairfax, VA, USA). Aliquots were also taken for objective-motility evaluation by light microscopy (200×), though different patterns of movement were not evaluated. To analyse the effect of sugars on the AR, monosaccharides were added to the capacitation wells at the end of the pre-incubation period (3 h). For those experiments requiring the presence of GlcNAc after primary binding, the monosaccharide (1 mM) was added after the first 45 min of co-incubation (addition) or, alternatively, the oocytes were transferred to sperm-free sugar-containing droplets (transfer) after an initial 45 min under control conditions. The control assay for this last procedure consisted of the transfer of the sperm–oocyte complexes to a droplet, which is both sperm and sugar free.

**IVF assays**

Fresh oocytes (10–15/droplet) were placed in 50 μl droplets of TALP medium, and an aliquot of sperm previously capacitated for 3 h was added to a final concentration of 100 000 cells/ml (Bavister, 1989). After 3 h co-incubation at 37°C in 5% CO₂, oocytes were washed and fixed as previously described. In order to facilitate the visualization of sperm, oocytes were stained for 6–8 min with Hoechst 33342 (30 μg/ml in 2.3% sodium citrate:ethanol, 3:1), washed in citrate:ethanol and mounted under 25× and observed for 6–8 min with an upright microscope equipped with epifluorescent illumination. The number of penetrating sperm per oocyte and the percentage of oocytes with penetrating sperm were quantified. Since the event under evaluation was the ability to pass through the zona pellucida, penetrated sperm were considered as those located in the perivitelline space or the ooplasm. As a measure of the binding event in these oocytes, the total (penetrated plus bound) number of sperm per oocyte as well as the number of oocytes with sperm (in any of these locations) were recorded. In order to compare the effects of GlcNAc on both binding and penetration, the values obtained when considering total or penetrated sperm were used as the measures for binding and penetration, respectively. In addition, to determine the effect of monosaccharides during IVF, different incubation procedures were used. Unless otherwise stated, carbohydrates (1 mM) were present during the entire co-incubation period (3 h). For those experiments requiring the presence of GlcNAc after primary binding, the monosaccharide (1 mM) was added after the first 45 min of co-incubation (addition) or, alternatively, the oocytes were transferred to sperm-free sugar-containing droplets (transfer) after an initial 45 min under control conditions. The control assay for this last procedure consisted of the transfer of the sperm–oocyte complexes to a droplet, which is both sperm and sugar free.

**Statistical analysis**

Results were expressed as the percentage of acrosome-reacted sperm (%AR), the percentage of oocytes with bound sperm, the number of sperm bound/oocyte, the percentage of penetrated oocytes, and the number of penetrating sperm, and fixed in 0.1% formaldehyde in TL for 5 min. The percentage of oocytes with bound sperm and the number of bound sperm per oocyte were determined by microscopic examination (200×). In order to analyse the effect of sugars on sperm–zona pellucida binding, the different monosaccharides were added to the medium used for sperm–oocyte co-incubation. Droplets with no sugars were used as controls. For the pre-incubation experiments, 10 mM GlcNAc was added to the capacitation well 2.25 h after the beginning of the sperm incubation. After a further 30 min, 2.5 ml of sperm suspension was centrifuged for 5 min at 750 g. The sperm pellet was resuspended in 10 ml of fresh medium and centrifuged again, to ensure that the GlcNAc concentration during co-incubation would be below 0.03 mM. The final pellet was then resuspended in 1 ml of medium and used for insemination. For the control assays, the sperm suspension was subjected to the same manipulation, with no addition of sugar. For oocyte pre-treatment, they were incubated for 30 min with GlcNAc (10 mM), washed by pipetting through 5 droplets of medium, and placed for 15 min in a final fresh droplet before the addition of sperm.
sperm per oocyte. In some cases, results were normalized to the control values, which were considered as 100%. Average inhibition was calculated as (control – treated) × 100% control. Graphics represent averages ± standard error SEM of 3–30 different experiments. In order to assume normal distribution, percentages were converted to ratios and all data subjected to the arcsine square root transformation. Statistical analysis was performed using the parametric t-test for simple comparisons and the Tukey test for multiple comparisons, with the aid of Graph Pad software.

Results

Effect of monosaccharides on sperm–zona pellucida binding

In order to determine which sugar residues might play a role in sperm–zona pellucida interaction in hamster, the effect of the presence of different monosaccharides on sperm–zona pellucida binding assays was evaluated. Of the seven monosaccharides assayed, only GlcNAc was able to significantly affect sperm–zona pellucida binding when used at a 1 mM concentration, with inhibition values of 38 ± 6% and 67 ± 5% for oocytes with bound sperm and the number of sperm bound/oocyte, respectively (Figure 2). Fucose was the only other monosaccharide that showed an inhibitory trend, which became significant when a higher sugar concentration (50 mM) was tested (Table I).

In order to verify the specificity of the effect on the binding event, sperm functional parameters were checked. Under these conditions, motility was not affected even at the highest concentration of monosaccharides tested (see Table I), indicating that the inhibition obtained in binding assays would not be due to a toxic effect. Only mannose and N-acetylgalactosamine reduced motility to 30–40% when present at 50 mM and for 3 h (data not shown).

With regard to the AR, hamster has the peculiarity that, once capacitation is complete, almost all sperm undergo this exocytotic event spontaneously, and it can be visualized by light microscopy (Yanagimachi and Phillips, 1984; Cross and Meizel, 1989), for which reason it can be determined in motile cells. When the AR was evaluated under conditions similar to those used in the binding experiments (addition of 1 mM sugar after capacitation), GlcNAc specifically inhibited the spontaneous AR after both 30 min and 3 h of treatment (Table II). No effect was seen for the other monosaccharides, even when tested at a higher concentration (50 mM, data not shown).

Given the significant effects produced by GlcNAc, and the previous studies involving this sugar carried out by our laboratory, this monosaccharide was chosen to continue our research.

Replacement of Ca in capacitation medium

Taking into account the fact that the zona pellucida binding assays would be affected by the acrosomal status of the sperm, it was necessary to work under AR-inhibiting conditions to determine the

**Table I.** Effect of 50 mM monosaccharides in sperm functional parameters

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<tr>
<th>Monosaccharide</th>
<th>Functional parameter (% of control)</th>
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<tr>
<td></td>
<td>Binding</td>
</tr>
<tr>
<td>Galactose</td>
<td>112 ± 10</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>82 ± 26</td>
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<tr>
<td>D-Fucose</td>
<td>52 ± 8*</td>
</tr>
<tr>
<td>Glucose</td>
<td>122 ± 18</td>
</tr>
<tr>
<td>Mannose</td>
<td>92 ± 11</td>
</tr>
<tr>
<td>GalNAc</td>
<td>114 ± 18</td>
</tr>
<tr>
<td>GlcNAc (1 mM)</td>
<td>44 ± 5*</td>
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</table>

After sperm incubation under capacitating conditions for 3 h, sperm were incubated with salt-stored oocytes in the presence of different monosaccharides. After 45 min, binding to zona pellucida and motility were quantified. Results were normalized against controls (taken as 100%), and represent the average of 4–6 experiments.

| *P < 0.05 vs. 100% (control). |

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Acrosome reaction (% of control)</th>
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<tr>
<td></td>
<td>30 min</td>
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<tr>
<td>Galactose</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>Glucose</td>
<td>105 ± 7</td>
</tr>
<tr>
<td>d-Fucose</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>96 ± 14</td>
</tr>
<tr>
<td>Mannose</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>GalNAc</td>
<td>92 ± 9</td>
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<tr>
<td>GlcNAc</td>
<td>35 ± 4*</td>
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</table>

After sperm incubation under capacitating conditions for 3 h, different monosaccharides (final concentration 1 mM) were added. Acrosome reaction was quantified after both 30 min and 3 h of incubation in the presence of the sugar. Results were normalized against controls (taken as 100%), and represent the average of 3–11 experiments. *P < 0.05 versus 100% (control).

**Table II.** Effect of different monosaccharides on the spontaneous AR

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Acrosome reaction (% of control)</th>
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<tr>
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After sperm incubation under capacitating conditions for 3 h, different monosaccharides (final concentration 1 mM) were added. Acrosome reaction was quantified after both 30 min and 3 h of incubation in the presence of the sugar. Results were normalized against controls (taken as 100%), and represent the average of 3–11 experiments. *P < 0.05 versus 100% (control).
effects of the monosaccharide on the binding process itself. For this purpose, a modified medium was used in which Ca, known to be an absolute requirement for the occurrence of AR, was replaced by Sr. This system had been previously reported by our laboratory as being capable of supporting sperm capacitation and zona pellucida binding but not AR, in humans (Marín-Briggiler et al., 1999).

In order to analyse the ability of Sr as a Ca replacement for hamster sperm capacitation, spontaneous AR was measured in sperm incubated under different conditions. Under control conditions, AR began to increase at approximately 2.5 h of incubation, when capacitation was completed, and almost all sperm were reacted 3 h later (Figure 3). In contrast, when sperm were incubated in Sr-containing medium, the spontaneous AR did not increase beyond basal (control) levels. In order to determine whether these cells were ready to undergo AR (fully capacitated), Ca was added to the Sr-incubated sperm after the capacitation period (3 h). Results indicated that following Ca addition, Sr-incubated sperms could undergo AR with similar kinetics and similar maximum values as those capacitated in the presence of Ca. This effect was not due to the higher ion concentration, since neither the addition of Sr instead of Ca to Sr-incubated sperm nor the addition of either of these two cations to Ca-incubated sperm, resulted in any changes in AR levels.

**Sperm–zona pellucida binding in Sr medium**

The ability of sperm capacitated in Sr medium to bind to the zona pellucida and the capacity of this ion to sustain binding was analysed using Sr-modified medium throughout the assays. Results indicated that sperm were capable of binding to the zona pellucida in quantities indistinguishable from those obtained when Ca was used for all procedures (Figure 4), indicating that Sr effectively replaced Ca in these processes, i.e. in sperm capacitation and binding to the zona pellucida.

**AR on the zona pellucida**

Once the ability of Sr to replace Ca for the sperm binding to zona pellucida was verified, the occurrence of AR on the zona pellucida was analysed. While the AR takes place approximately 1 h after sperm–zona pellucida co-incubation in Ca-containing medium (Uto et al., 1988), no AR could be detected in Sr-modified medium, even after 2 h of incubation (Figure 5). In addition, when Sr-incubated sperm were placed in Ca-containing medium for co-incubation, the occurrence of the AR on the zona pellucida reached the same values as those obtained with Ca-capacitated sperm (Figure 5, grey bar).

**Effect of GlcNAc on sperm–zona pellucida binding in the absence of AR**

Once the conditions for sperm binding to zona pellucida without AR were attained, all subsequent binding assays were carried out using Sr medium, unless indicated. When the effect of GlcNAc on sperm binding to the zona pellucida was re-analysed using this medium and Sr-incubated sperm, the presence of the monosaccharide was able to repeat the inhibitory effect (Figure 6), suggesting that it would be affecting primary binding.
Pre-incubation of gametes with GlcNAc

In order to verify which gamete was affected by the monosaccharide, both sperm and oocytes were pre-incubated with GlcNAc and then co-incubated under control conditions. Only the pre-incubation of sperm with GlcNAc was able to reproduce the inhibitory effect (Figure 7). Interestingly, a higher concentration of the sugar (10 mM) had to be used in order to obtain a significant effect. The washing procedure (described in Materials and methods) guaranteed a GlcNAc remnant below 0.03 mM during co-incubation, which is within the non-inhibitory region of the dose–response curve (Figure 8).

Effect of GlcNAc on hamster IVF

To further analyse which of the steps comprised in the sperm–zona pellucida interaction process was affected by GlcNAc, IVF was performed in order to study both the binding and the penetration of the zona pellucida. Those sperm that had traversed the zona pellucida and were located either in the perivitelline space or in the oolema, were considered as penetrating. When GlcNAc (1 mM) was present during the entire assay, a specific and significant inhibition in the proportion of penetrated oocytes (92 ± 8%) was found, as expected (Figure 9). Since the GlcNAc effect on IVF seemed to be greater than that previously observed in the binding assays, the degree of inhibition on the two events, i.e. binding and penetration, was analysed in the same oocytes (as detailed in Materials and methods). Results indicated that GlcNAc inhibition was significantly greater for penetration than for binding (Table III).

The different degrees of inhibition by GlcNAc in the two assays suggest the possible participation of this sugar in several steps during fertilization. Considering that this sugar does not affect sperm–oocyte fusion (Ahuja, 1982; Ponce et al., 1994), it could be implicated in more than one event during sperm interaction with the zona pellucida. In order to determine whether GlcNAc was involved in any step of fertilization after primary binding, the monosaccharide was added to the assay medium after 45 min of gametes co-incubation, when sperm were already bound to the zona pellucida. Two experimental designs were attempted for this purpose. In the first (addition), the sugar was added to the same droplet where sperm–oocyte fusion (Ahuja, 1982; Ponce et al., 1994), it could be implicated in more than one event during sperm interaction with the zona pellucida. In order to determine whether GlcNAc was involved in any step of fertilization after primary binding, the monosaccharide was added to the assay medium after 45 min of gametes co-incubation, when sperm were already bound to the zona pellucida. Two experimental designs were attempted for this purpose. In the first (addition), the sugar was added to the same droplet where
co-incubation was started; in the other (transfer), oocytes were washed and placed in a fresh sugar-containing but sperm-free droplet. Only the presence of GlcNAc during the entire fertilization assay produced a significant effect, while no differences were observed for the other treatments (Figure 10).

With the purpose of establishing whether those sperm already bound to the zona pellucida might not react and consequently would not penetrate the zona pellucida in the presence of GlcNAc, AR on the zona pellucida was determined in the presence of this monosaccharide. Results showed that those sperm that could bind to the zona pellucida were not able to undergo the AR (Figure 11).

Discussion

The participation of glycosidic residues in the sperm–oocyte interaction has been widely studied in many species. Nevertheless, the limitations of the system can sometimes make it difficult to discriminate in which of the several steps involved in this process the sugars are implicated. A number of related studies have been carried out by our laboratory and reported that GlcNAc is involved in the human sperm AR (Brandelli et al., 1994; Brandelli et al., 1995; Brandelli et al., 1996). In addition, our studies indicated that this carbohydrate participates in human sperm binding to the zona pellucida, though it was not possible to distinguish whether GlcNAc was involved in primary binding and/or penetration (Miranda et al., 1997; Miranda et al., 2000). For this reason, it was decided to move from the human to an animal model where the availability of gametes and the possibility of designing different experimental procedures were greater.

As a first step, those sugars involved in the sperm–zona pellucida interaction in hamster were determined. These assays already indicated a noticeable difference between the effects of GlcNAc and the other monosaccharides tested. GlcNAc was the only sugar capable of inhibiting sperm–zona pellucida binding at 1 mM. A higher concentration of monosaccharides was also assayed, and fucose was found to have an inhibitory effect at this high dosage.

Although the usefulness of salt-stored oocytes for this kind of assay has been well proven (Yanagimachi et al., 1979; Boatman et al., 1988; Yoshimatsu et al., 1988), the procedure was repeated using fresh oocytes and a similar inhibition by GlcNAc was obtained (data not shown).
Despite the fact that other groups had previously reported the participation of GlcNAc in hamster fertilization (Ahuja, 1982; Kumar et al., 1990), the specific step involved was still unknown. Intriguingly, this sugar seems to be implicated in the sperm–oocyte interaction in Ascidia, Xenopus, Drosophila, mouse and human, among others (Oikawa et al., 1973; Shur and Hall, 1982; Godknecht and Honegger, 1995; Matsuura et al., 1995; Miranda et al., 1997; Perotti et al., 2001; Vo et al., 2003). Although this may raise the issue of non-specificity, it could also be considered a basic requirement during gamete interaction in many systems, and would also support the ubiquitous presence of this terminal residue in the extracellular coat of oocytes from different species (Skutelsky et al., 1994; Matsuura et al., 1995; Mozingo and Hedrick, 1999). The species-specificity would not reside on a single sugar residue, but on more complex systems involving not only the micro (molecular), but also the macro environment (O’Rand, 1988).

Surprisingly, GlcNAc was found to inhibit the spontaneous AR in hamster. To the extent of our knowledge, there is no previous report of a monosaccharide producing this kind of effect. Its possible mechanism of action is at present under study in our laboratory. Although this difference was unlikely to be the cause of the inhibition observed in sperm–zona pellucida binding, it produced a non-equivalent situation for the control and treated conditions that could unmask or alter the result. For this reason, we decided to find experimental conditions that would allow us to analyse binding in the absence of AR. Since it is well known that Ca is an absolute requirement for the AR to take place, the use of Sr as a replacement was analysed. Our laboratory had previously reported Sr to be capable of supporting capacitation in humans (Marín-Briggiler et al., 1999). The present results indicate that hamster sperm capacitated in Sr were not capable of undergoing either spontaneous or zona pellucida-induced AR, but are ready to undergo AR once the adequate ion requirements are provided, suggesting that capacitation events related to the ability to bind to the zona pellucida and AR would be taking place in a Sr medium. It is important to state that the conditions used in this study are not likely to be Ca-free, since the presence of some Ca in the media as a contaminant coming from other components cannot be excluded. Consequently, although the putative Ca contamination would surely be below the concentration required for AR (Fraser, 1982; Yanagimachi, 1982; Fraser, 1987a; Stock and Fraser, 1989; Marín-Briggiler et al., 1999), its contribution to the capacitation process cannot be disregarded. Moreover, the completion of some of the capacitation-related events after Ca addition cannot be ruled out, especially considering the 30 min delay observed in the time-course of spontaneous AR. This possibility is also currently being analysed in our laboratory.

These results seem to agree with previous reports suggesting that Sr was capable of supporting capacitation (Mortimer, 1986a; Mortimer et al., 1986b; Fraser, 1987b), but disagree concerning the ability of Sr to sustain the AR (Yanagimachi and Usui, 1974; Fraser, 1987b; Mortimer et al., 1988; Stock and Fraser, 1989). Hamster has the distinctive characteristic that almost all sperm undergo the AR spontaneously after capacitation has been completed, and it can be easily visualized in motile (live) sperm under light microscopy. This makes the system unique, but at the same time the possible existence of some differences with other species should be kept in mind when projecting the results. In the species used in the studies mentioned above (mouse, human and guinea pig), the non-physiological feature of the spontaneous AR might explain the differences observed. In accordance with this possibility, a previous study by this laboratory reported that even when spontaneous AR in human sperm was similar in Ca and Sr media, the physiological induction by follicular fluid could not be sustained by Sr (Marín-Briggiler et al., 1999). Another possibility, presently under study in our laboratory, is that species differ in their ionic requirements as previously suggested (Stock and Fraser, 1989). Finally, although the ability of Sr to replace Ca in mouse sperm AR has been reported, a significantly reduced fertilization was obtained using this ion (Yanagimachi, 1978; Fraser, 1987b), but this could be reversed when Sr-capacitated sperm were used for fertilization in Ca medium (Fraser, 1987b).

When Sr-capacitated sperm were co-incubated with oocytes in the same medium, we found that the binding to the zona pellucida was indistinguishable from that taking place in Ca, suggesting that Sr is also able to support sperm–zona pellucida binding. Although this contradicts previous studies, experimental differences could be the reason for these discrepancies. An initial study reported a reduced sperm–zona pellucida binding when Sr was replacing Ca (Heffner et al., 1980). However, the use of a minimal media and oocyte activation by Sr in this study could be the cause for its failure, as previously suggested by another group which sustained that binding to zona pellucida was taking place in a Sr medium although they did not quantify it (Fraser, 1987b). Another study by Mortimer et al., (1988) reported that Sr had a reduced ability to sustain binding to zona pellucida in humans. As discussed in a previous report by this laboratory, where we found human sperm–zona pellucida binding to be similar in Sr or Ca medium, we attributed this discrepancy to the different experimental conditions used in both cases (Marín-Briggiler et al., 1999). While Mortimer et al. incubated whole oocytes with 20h capacitated sperm (5 x 10⁶/ml), which were reported to have a diminished ability to bind to zona pellucida (Singert et al., 1985), we carried out hemizona assays (which render a more precise control) with 5h incubated sperm (3 x 10⁶/ml) (Marín-Briggiler et al., 1999).

When sperm–zona pellucida binding assays were performed in Sr-modified medium and the effect of GlcNAc was tested, the inhibition by GlcNAc was reproducible, supporting a specific effect of this sugar on binding, independent of its effect on the AR. Taken together, the above presented results show: (a) sperm binding to the zona pellucida in either Ca- or Sr-modified medium is indistinguishable, (b) AR takes place on the zona pellucida in the presence of Ca but not Sr, and (c) GlcNAc inhibits sperm binding to zona pellucida under both conditions. It is reasonable to conclude that GlcNAc is specifically affecting the primary binding of sperm to the zona pellucida.

As mentioned previously, preliminary studies had reported the effect of GlcNAc on sperm–zona pellucida interaction (Ahuja, 1982; Kumar et al., 1990). Two of these reports attempted to analyse which gamete was being affected by the sugar, and while one suggested that it was the oocyte (Ahuja, 1982), another proposed a sperm-mediated effect (Kumar et al., 1990). In order to verify that the currently accepted theory of lectin-like proteins located on the sperm and their complementary zona pellucida-glycosidic residues located on the oocyte were behind our results, pre-incubation experiments were carried out in the presence of GlcNAc. Interestingly, when 1 mM GlcNAc was used, no differences were found (data not shown). When ten times more sugar was assayed, only sperm pre-treatment with GlcNAc reproduced the inhibitory effect. The lack of effect of sperm pre-incubated with 1 mM GlcNAc suggests a low affinity association of GlcNAc to sperm. In accordance with this result, AR inhibition by GlcNAc could be reversed after sperm washing and incubation in control medium, even when 10 mM was used (data not shown). These results indicated that a toxic effect of the sugar on sperm is unlikely. However, a specific and reversible perturbation of some metabolic process cannot be ruled out and is currently under investigation. Other studies also used similar or
higher concentrations of monosaccharides and/or longer exposure time (up to 3 h), reporting no deleterious effects on sperm or oocytes (Ahuja, 1982; Huang et al., 1982; Ponce et al., 1994). The present study also involved testing higher concentrations of monosaccharides with exposure times of up to 3 h, with no deleterious effect on either gamete (data not shown).

The presence of GlcNAc during IVF assays resulted in a greater inhibition of zona pellucida penetration than of zona pellucida binding. Since previous studies reported that GlcNAc does not affect fusion (Ahuja, 1982; Ponce et al., 1994) GlcNAc could be involved in more than one step during sperm interaction with the zona pellucida. The lack of effect observed when the sugar was added after primary binding had taken place would suggest that GlcNAc is not involved in zona pellucida penetration in hamster, unlike that observed in the mouse (Miller et al., 1993). However, a preferential binding of sperm to the zona pellucida due to a lower affinity for the monosaccharide, cannot be discarded. Given that the zona pellucida-induced AR was found to be blocked in the presence of GlcNAc, the overall inhibition observed in the IVF assays is, at least, a consequence of the addition of its effect on both binding and AR.

In summary, the present results indicate that the presence of GlcNAc is able to affect sperm binding and AR on the zona pellucida. The persistence of GlcNAc inhibition under conditions where AR could not take place sustains the participation of this sugar in primary binding. The ability of the monosaccharide to inhibit AR on the zona pellucida would suggest its involvement in the zona pellucida-induced AR, although a downstream blockage of a signalling cascade cannot be ruled out given that spontaneous AR was also affected. Since sperm pre-incubation with GlcNAc was able to inhibit the subsequent binding to the zona pellucida, sugar-binding sites located on sperm and GlcNAc residues of the zona pellucida could be mediating this event.

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