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Sperm and Egg Jelly Coat from Sea Urchin *Lytechinus variegatus* Collected in Rio de Janeiro Contain Distinct Sialic Acid-Rich Polysaccharides

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ABSTRACT

This work found the occurrence of a distinct sialic acid-rich polysaccharide in the sperm surface of the sea urchin Lytechinus variegatus*, which differed significantly from a similar molecule found in the egg jelly. The sperm polysaccharide extracted by protease digestion was purified using anion exchange chromatography and characterized using agarose gel electrophoresis, gas chromatography/mass spectrometry and NMR spectroscopy. This polysaccharide was highly sulfated and composed almost exclusively of N-acetylneuraminic acid. In contrast, the sialic acid-rich polysaccharide from the egg jelly was composed of N-glycolylneuraminic acid and contains several other hexoses in its structure. This new molecule could help to characterize in further detail the mechanism of fertilization in the sea urchin model system. Sulfated polysaccharides from the jelly coat of sea urchins showed species-specificity in inducing the sperm acrosome reaction, providing an example of a signal transduction event regulated by the sulfated polysaccharide. The new sialic acid-rich polysaccharide found in the sperm head could represent a new molecule involved in the biology of the sea urchin fertilization.*

Key words: sea urchin, sperm, egg jelly coat, fertilization, sialic acid, sulfated polysaccharide

INTRODUCTION

Fertilization is the result of a series of interactions between the egg and sperm surface molecules (Lennarz et al. 1993). These processes require the presence of species-specific molecules, especially in free spawning marine invertebrates, in order to avoid polyspermy and inter-specific hybridization (Mah et al. 2005). Studies on the structural characterization of polysaccharides from sea urchins led to the discovery of unique polymers (Alves et al. 1997; Vilela-Silva et al. 1999, 2002, 2008). These polysaccharides have simple, linear structures, composed of repeating units of oligosaccharides. The sulfation patterns, glycosidic linkage and repeating monosaccharide units differ among various species of sea urchins. They show species-specificity in inducing the acrosome reaction, providing a clear example of a signal transduction event mediated by the sulfated

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polysaccharide. This distinct cell-cell recognition mechanism mediated by the sulfated polysaccharide co-exists with the sperm bindin recognition of its egg receptor. The bindin protein exposed by the acrosomal process, reacts with a matching egg membrane receptor (Glabe and Vacquier 1977).

Another important egg jelly molecule is the sialic acid-rich polysaccharide. It potentiates the sperm acrosome reaction induced by the egg´s sulfated polysaccharide (Hirohashi and Vacquier 2002). Little information is available concerning the structure of this polysaccharide. It contains polysialic acid chains linked to a core composed of several hexoses. In *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus*, this structure terminates with non-reducing end sulfated *N*-glycolylneuraminic acid (Neu5Gc) residues (Kitazume et al. 1994, 1996). Miyata et al. (2004, 2006) reported a polysialic acid on the flagella region of the sea urchins *H. purcherrimus*, *S. purpuratus* and *S. franciscanus*. This 8-*O*-sulfated Neu5Ac, capped α-2→9-linked polyNeu5Ac-containing molecule, called "flagellasialin", seemed to be related with sperm motility, required for the fertilization. The involvement of flagellasialin in the motility of sea urchin sperm through the intracellular calcium ion regulation has been reported (Kambara et al. 2011). The presence of α-2→8-linked di-, tri-, tetra and polyNeu5Ac unique gangliosides has also been documented in these cells (Ijuin et al. 1996; Miyata et al. 2011). Most sialic acids on the vertebrate cell surface participate in recognition and interaction events during the growth, development and immune response (Nasirikenari et al. 2006; Nacher et al. 2010). Sialic acids are rarely linked to each other to form a polymerized structure (Miyata et al. 2006). Polysialic acids with the degree of polymerization ranging from 8 to 200 sialyl acid residues are found in few animals and some pathogenic bacteria capsules (Sato and Kitajima 2013).

Highly specific interactions assure not only high rates of fertilization but also avoid inter-specific crosses. These aspects are particularly relevant for free spawning marine species, such as sea urchins. Sulfated polysaccharides from the egg jelly coat that induce the sperm acrosome reaction in a specie-specific way, raises an important question: what is the sperm molecule, which interacts with the egg jelly sulfated polysaccharide? An apparent response to this question came when a group of proteins named "receptors for the egg jelly" was described in *S. Purpuratus* species (Vacquier and Moy 1997). The present work looks for acidic carbohydrate on the surface of sea urchin sperm, which could be regulating the interaction of the egg jelly sulfated polysaccharide with the sperm, through a carbohydrate-carbohydrate interaction, as reported for sponge cell-cell adhesion (Vilanova et al. 2009) and also for some mammalian cell interaction (Hakomori 2004a, 2004b). This work aimed to study the sialic acid-rich polysaccharides found in sperm head of sea urchin and to compare this molecule with a similar one found in the female egg jelly.

MATERIAL AND METHODS

Extraction of Acidic Polysaccharides from the Sea Urchin Gametes

Mature specimens of the sea urchin *L. variegatus* were collected from the Guanabara Bay (Rio de Janeiro, Brazil) and given intracelomic injection of 0.5 M KCl (~2.0 mL per specimen) to obtain the gametes (Cinelli et al. 2007). Eggs were recognized by an orange color and collected in a solution containing 450 mM NaCl, 9.0 mM KCl, 48 mM $MgCl_2$, 10 mM $CaCl_2$, and 6.0 mM $NaHCO_3$ (pH 8.0). Sperms were recognized by a whitish color and collected in the same solution, except replacing $CaCl_2$ by 20 mM Tris-HCl. The jelly coat was detached from the eggs by pH shock as previously reported and subjected to centrifugation (10.000 *x* g for 30 min). The supernatant containing the jelly coat was dialyzed against the distilled water, lyophilized and stored at -20°C (Cinelli et al. 2009). The undiluted sperm were immediately centrifuged (3,000 × *g* for 15 min). The supernatant, which corresponded to the seminal fluid, was discarded and the pellet containing the sperms was dialyzed against distilled water, lyophilized and stored at -20°C (Cinelli et al. 2009). The crude polysaccharides were extracted from the egg jellies and sperm by papain digestion and purified by ethanol precipitation, as previously described (Albano and Mourão 1986).

Purification of the Acidic Polysaccharides from Sea Urchin Gametes

The crude polysaccharides from the sperm and egg jelly (~20 mg of each) were applied to a Mono Q-HPLC column (HR 5/5; Pharmacia Biotech Inc., Uppsala, Sweden) and equilibrated

with 20 mM Tris-HCl (pH 8.0). The column was washed exhaustively with this solution and then eluted with a linear gradient of NaCl (0→2 M) in the same buffer. The flow rate of the column was 0.5 mL min^{-1}. Fractions of 0.5 mL were collected and the eluents were analyzed by their metachromasia using 1,9-dimethylmethylene blue (Farndale et al. 1986). The NaCl concentration was estimated by conductivity. Fractions containing the acidic polysaccharides were pooled, dialyzed against distilled water, and lyophilized.

Agarose Gel Electrophoresis

Acidic polysaccharides purified from the sea urchin gametes were analyzed by agarose gel electrophoresis as previously described (Dietrich and Dietrich 1976). Briefly, the samples (approximately 15 µg) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane-acetate (pH 9.0). The sulfated polysaccharides in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v) (Salgado et al. 2009).

Polyacrylamide Gel Electrophoresis

The molecular masses of the acidic polysaccharides from the gametes were estimated by polyacrylamide gel electrophoresis. Samples (10 µg of each) were applied to a 6%, 1-mm-thick polyacrylamide gel slab in 0.02 M Tris-HCl (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% acetic acid, then washed in 1% acetic acid. The molecular mass markers were standard glycosaminoglycans, the same as those used previously (Tovar et al. 1998).

Carbohydrate Content of the Sea Urchin Polysaccharide

Total hexose was estimated by the method of Dubois et al. (1956) using galactose as standard. For the identification of the type of hexose, the polysaccharide (~5 mg) was hydrolyzed with 6 M trifluoracetic acid at 100°C for 5h, reduced with sodium borohydride and the alditols obtained were acetylated with acetic anhydride: pyridine (1:1, v / v) (Kircher 1960). The alditols acetates were dissolved in chloroform and analyzed on GC-MS. The column used was ZB-5ms. The initial temperature of the run was 110°C and the final 250°C with an increase of 2°C/min (Ahmed et al. 1997).

Identification of the Sialic Acid

Total sialic acid content was estimated by the Ehrlich assay (Kabat and Mayer 1971), using *N*-glycolylneuraminic acid as standard. Identification of the sialic acid was based on two methods: electrospray ionization tanden mass spectrometry (ESI-MS) or methanolysis followed by the analysis of the trimethylsilylated derivatives on a mass spectrometry/gas liquid chromatography (MS-GC) unit.

For the acidic polysaccharide from the egg jelly, ESI-MS was employed. Sialic acid from the egg jelly polysaccharide and related standards were analyzed by ESI-MS using the Finnigan LCQ ion trap device-Duo (Thermo Electron, San Jose, CA). The samples were introduced into the mass spectrometer under a flow of 5 - 10 µL min^{-1} with the aid of a micro pump infuser (Harvard Apparatus, Cambridge, MA, USA). The analysis was in positive mode (ESI^{+}). The source and the capillary voltage were 4.5 kV and 3 V (ESI +). The temperature of the capillary was maintained at 200°C and the spectra were collected in the range of 200 to 1000 m/z. The source-induced dissociation (SID) was 25 V. The experimental collision-induced dissociation of the ion captured (ESI-MS/MS or ESI-MSn) was made 20-60% (1-3 eV) and normalized by the collision energy relative. The spectrum was processed using the X caliber program (Thermo Electron).

Identification of the sialic acid of the sperm polysaccharide was based on a different methodology. The polysaccharide was methanolized (0.5 M HCl in methanol at 80°C for 18h), neutralized in silver carbonate and re-*N*-acetylated with acetic anhydride. The dried residue was trimethylsilylated by the addition of bis (trimethylsilyl)-trifluoro-acetamide/pyridine (1:1, v/v) (Sweeley et al. 1963). The products were analyzed on a MS-GC unit on a DB-1 fused silica column (30 m × 0.25 mm i.d.), using helium as the carrier gas. The column temperature was programmed from 120 to 240°C at 2°C min^{-1}.

NMR Spectroscopy

NMR spectra were obtained on a Bruker DRX 600 with a 5 mm triple resonance probe. The samples for NMR spectroscopy were deuterium exchanged by repeated lyophilization from deuterium oxide

and dissolved in 0.5 mL of D_2O before the analysis. Heteronuclear single quantum coherence spectroscopy (HSQC) 2D ^{1}H-^{13}C spectra with multiplicity editing during selection step was recorded using the Broker program hsqcedtetgp (Willker et al. 1993). ^{1}H protons were referenced to internal trimethylsilyl propionic acid anion at 10 ppm and ^{13}C spectra were referenced to external methanol at 50 ppm.

Light Microscopy

Gonads, eggs and sperms were fixed in 2.5% glutaraldehyde in filtered seawater and processed according to standard histological techniques for paraffin embedding. Five micrometer serial slices were stained with toluidine blue at pH 7.2 (Kiernan 1990).

Lectin Staining

The de-paraffinized and hydrated sections were washed in 3% H_2O_2 for 10 min to block the endogenous peroxidase activity and then washed in phosphate buffer saline (PBS) 0.05 M (pH 7.4). Thereafter, they were incubated in a solution of biotinylated lectins in appropriate dilutions (1:4,000) at room temperature for 1h. After three washes in PBS, sections were incubated in a solution containing 0.05% 3,3 '-diaminobenzidine (DAB) and 0.005% H_2O_2 in 0.05 M PBS (pH 7.4) at room temperature for 1 min before dehydration and mounting blade. The following biotinylated lectins were used: LCA (*Lens culinaris*, α-mannose), PNA (*Arachis hypogaea*; β-galactose) and AAA (*Aleuria aurantia*, α-fucose), which were purchased from Vector (Burlingame CA, USA).

RESULTS

Sea Urchin Sperm and Egg Jelly Coat Contain Distinct Sialic Acid-Rich Polysaccharides

Polysaccharides from the egg jelly and sperm of sea urchin were purified on an anionic exchange chromatography. The columns followed using metachromatic assay (Fig.1). The acidic polysaccharides from the egg jelly eluted from the column as two distinct fractions (Fig. 1, Panel A). The one eluted at ~0.8 M NaCl corresponded to the sialic acid-rich polysaccharide (egg-SP) as demonstrated previously (Alves et al. 1997; Vilela-Silva et al. 1999, 2002). The second acidic polysaccharide fraction from the sea urchin jelly coat eluted at ~1.0 M NaCl contained a sulfated fucan (SF), with a peculiar structure as characterized previously (Alves et al. 1998; Vilela-Silva et al. 1999, 2002). It was interesting to observe that the sperm also contained a sialic acid-rich polysaccharide (sperm-SP). No sulfated fucan was detected (Fig. 1, Panel B).

The presence of sialic acid on the sperm polysaccharide was confirmed by the positive Ehrlich assay (not shown in the panel). Metachromatic negative fractions corresponding to nucleic acid (NA) that were absent on the extracts from the sea urchins egg jelly were also detected. No hexuronic acid or hexosamine were detected in the sialic acid-rich polysaccharide using carbozole (Bitter and Muir 1962) and Elson-Morgan (Rondle and Morgan 1955) reactions, respectively.

The acidic polysaccharides from the sea urchin gametes differed significantly on their mobility on agarose gel electrophoresis (Fig. 1, Panel C), suggesting they had different structures. They also differed in their molecular masses, as indicated by polyacrylamide gel electrophoresis (Fig. 1, Panel D). Sulfated fucan stayed at the origin of the gel, denoting its high molecular mass. The two sialic acid-rich polysaccharides migrated as a wide dispersed polymer but with significantly lower molecular masses when compared with the sulfated fucan. These acidic polysaccharides from the sea urchin gametes had distinct electrophoretic mobilities when compared with the standard glycosaminoglycans (left lane on the electrophoresis). They also resisted digestion with chondroitin ABC lyase and to nitrous acid deamination (not shown in the panel).

The chemical analysis showed that the sialic acid-rich polysaccharide from the egg jelly coat had a complex sugar composition (Table 1). It contained mannose, glucose, galactose and fucose, besides sialic acid. No significant amount of hexose was found in the sialic acid-rich polysaccharide from the sea urchin sperm, as was also confirmed by GC/MS analysis and NMR spectroscopy (see below).

No signals assigned to amino acids were observed in the NMR spectra, such as signals from the aromatic amino acids at 6.0 - 7.0 ppm. The sulfate content of these polysaccharides was not determined due to small amount of the material. Alternatively, the high sulfate content was estimated by their metachromatic property comparing to that obtained with a standard chondroitin 6-sulfate (Table 1).

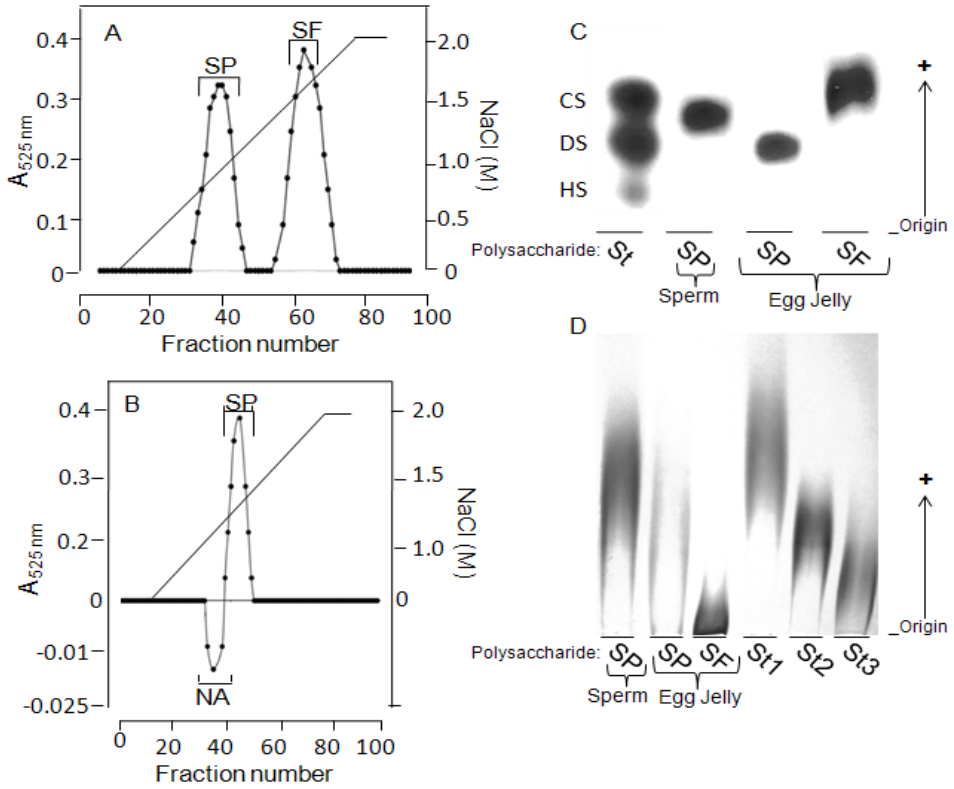

Figure 1 - A, B: Purification of the acidic polysaccharides from the egg jelly (Panel A) and sperm (Panel B) of the sea urchin *L. variegatus*. The crude polysaccharides from egg jellies or from the sperm were applied to a Mono Q-HPLC column and eluted with a linear gradient of 0→2 M NaCl (---). Fractions of 0.5 mL were collected and analyzed by their metachromasia using 1,9-dimethylmethylene blue (●). The NaCl concentration was estimated by conductivity. Fractions containing acidic polysaccharides were pooled, as indicated by the horizontal bars. NA in Panel B indicates nucleic acid, which yield a negative absorbance on the metachromasia assay. C, D: Approximately 15 µg of each of the acidic polysaccharides from the sea urchin gametes and a standard mixture of glycosaminoglycans were applied to a 0.5% agarose gel (Panel C) or to a 6% polyacrylamide gel (Panel D) for electrophoresis. SF and SP indicate sulfated fucan and sialic acid-rich polysaccharide. CS, DS and HS indicate standards of chondroitin 4-sulfate, dermatan sulfate and heparan sulfate, respectively. The standards of molecular mass used were: low-molecular-weight heparin (St1, ~5 kDa), unfractionated heparin (St2, ~18 kDa) and chondroitin 6-sulfate (St3, ~60 kDa).

Table 1 - Sugar composition of the sulfated polysaccharides from the sea urchin gametes.

		Egg jelly		Sperm
		Sulfated fucan	**Sialic acid-rich polysaccharide**	**Sialic acid-rich polysaccharide**
Sialic acid	total	<0.10	0.49	1.00
	Neu5Glc		0.49	<0.10
	Neu5Ac		<0.10	1.00
Hexose	total	1.00	0.51	<0.10[a]
	Man	<0.10	0.17	
	Glc	<0.10	0.16	
	Gal	<0.10	0.09	
	Fuc	1.00	0.09	
Metachromasia ($\Delta A_{525\,nm}$ per 2 µg of sugar)[b]		0.410	0.327	0.342

[a]The GS/MS analysis (Fig. 2, Panels A-D) and NMR spectroscopy (Fig. 2, Panels I,J) of this polysaccharide confirm the preponderant occurrence of sialic acid.

[b]The metachromasia obtained with the acidic polysaccharides from the sea urchin gametes are of the same magnitude as obtained with an standard of chondroitin 6-sulfate, chondroitin 4-sulfate and dermatan sulfate ($A_{525\,nm}$ = 0.340 per 2 µg of sugar). Hyaluronic acid and desulfated chondroitin showed no effect.

The GS/MS analysis of the trimethylsilylated derivatives from the sperm polysaccharide (Fig. 2, Panels A-D) showed that Neu5Ac was the preponderant sialic acid in the sperm polysaccharide, as indicated by its retention time (Panels A *vs.* C) and fragmentation pattern (Panels B *vs.* D), compared with the standard Neu5Ac.

An alternative methodology was used to identify sialic acid found in the egg jelly sialic-rich polysaccharide. Electrospray ionization tandem mass spectrometry (ESI-MS) was employed, since it showed more clear results in the case of this polysaccharide enriched with other types of hexoses. The spectrum of the sea urchin polysaccharide showed that the molecular ion of the sialic acid (Fig. 2, Panels E, F) had the same mass and the same fragmentation as that of standard Neu5Gc (Fig. 2, Panels G, H).

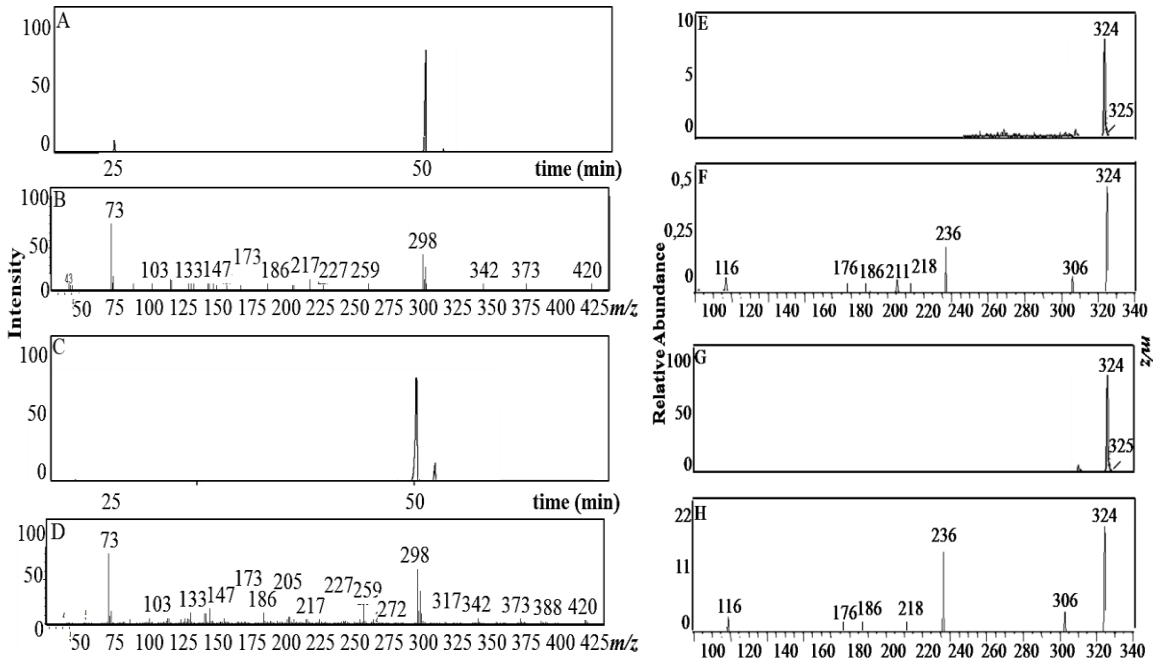

Figure 2 - A-D- Characterization of the sialic acid found in the sperm polysaccharide from sea urchin. Panels A and C show the elution profile of TMS ether derivatives from the acidic polysaccharide from the sperm and a standard of Neu5Ac, respectively, while Panels B and D show the fragmentation patterns of these two derivatives. E-H: Molecular mass and positive EI-spectrum of GC/MS of sialic acid from the egg jelly polysaccharide. Panels E and F show the molecular mass and fragmentation pattern of the sialic acid obtained from egg jelly polysaccharide, respectively. Panels G and H show the same result for a standard of Neu5Gc.

The sialic acid glycoconjugate from the egg jelly coat was analyzed using one and two-dimensional 800 MHz NMR spectra, which showed a mixture of poorly resolved signals (not shown here), indicating clearly a complex chemical structure as predicted from its chemical composition (Table 1). In contrast, the ^{1}H NMR spectrum of the sperm polysaccharide showed well-resolved signals. It was not possible to trace clear spin systems in the ^{1}H-^{1}H TOCSY and COSY spectra. Therefore, it was not possible to assign precisely the NMR signals. However, the edited ^{1}H-^{13}C HSQC spectrum of the sialic acid-rich polysaccharide from the sperm provided essential information (Fig. 2, Panels I, J). In this experiment, the signals of carbons with odd (red) number of protons were opposite to those with even number (blue) (Willker et al. 1993). This spectrum allowed discriminating glycerol carbons from deoxy carbons at C3 (red) from the other carbons of the pyranose ring (blue). The high number of carbons resonating between 55 and 75 ppm (Fig. 2, Panel J) showed that the polysaccharide contained sialic acids at different chemical environments. The absence of low field signals corroborated the absence of other sugars in this polysaccharide. The presence of high field signal at 23.1 ppm, assigned to the acetyl group (Barb et al. 2013), in conjunction with the C3 signal at 39.5 ppm C3, correlating with the ^{1}H resonances at 2.87 and 1.98 ppm (Fig. 2, Panel I), characteristic of the H3eq and H3ax signals, confirmed that Neu5Ac was the

major component of the sperm polysaccharide. Besides, the 50.2 ppm signal, which correlated with a proton at 3.85 ppm, was typical of a C5 substituted by an acetamido group at C5. In addition, signal of C4 could be attributed to the resonance at 71.1 and 3.58 ppm. The occurrence of a strongly down-fielded signal at 77.4 ppm corroborated the substitution by a $(OSO_3)^-$ group (Langeslay et al. 2013) in one of the carbons of the glycerol moiety.

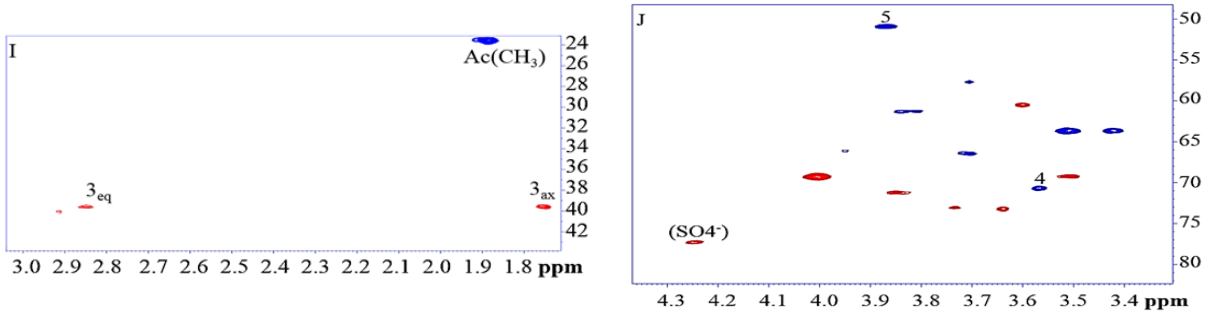

Figure 2 - I, J: 600 MHz 2D ^{1}H - ^{13}C edited HSQC spectrum of the acidic polysaccharide from the sperm of sea urchin. Red signals are the carbons with odd number of protons and blue are the carbons with even number of protons. Panel I shows the deoxy carbon C3 and the CH_3 from the acetoamide moiety of Neu5Ac. Panel J shows the carbons from the glycerol chain (in red) and C4, C5 from Neu5Ac ring (in blue).

Localization of the Acidic Polysaccharides in the Male and Female Gonads of Sea Urchins

The male and female gonads of sea urchins were stained with toluidine blue in order to identify the distribution of the acidic polysaccharides (Fig. 3, Panels A-D). The gonads showed intense metachromasia in the eggs and sperm, denoting the presence of the acidic polysaccharides. The female gonad lumen (Fig. 3, Panel B) showed an intense metachromasia around the eggs (that was in their jelly coat), whereas sperm were mostly stained at their head (arrows in Panel C of Fig. 3). The use of biotinylated lectins also allowed to detect the specific sugars on the egg jelly coat (Fig 3, Panels E-G).

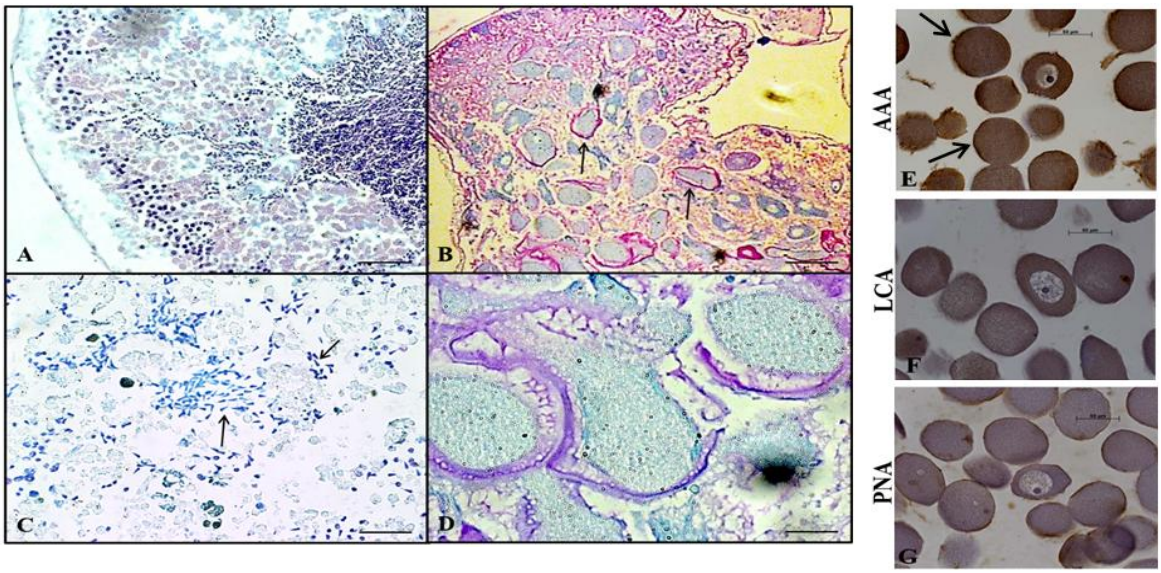

Figure 3 - A-D: Light micrographs of the male (Panel A, x40) and female (Panel B, x40) gonads of the sea urchin and of suspension of the sperm (Panel C, x100) and spawned eggs (Panel D, x100), all stained with toluidine blue. Note the strong metachromasy associated with the sperm (arrows in Panel C) and with the egg jelly coat (arrows in Panel B). The arrows in Panels B and C indicate the egg jelly coat and the head of the sea urchin sperm, respectively. E-G: Light micrographs of the sea urchin eggs stained with biotinylated lectins. The eggs were stained with the following biotinylated lectins: AAA (from *Aleuria aurantia*, Panel E), LCA (from *Lens culinaris*, Panel F) and PNA (from *Arachis hypogaea*, Panel G). Note the staining around the eggs (arrows), denoting their occurrence at the jelly coat.

DISCUSSION

The acidic polysaccharides from the sea urchin gametes have structures distinct from the vertebrate glycosaminoglycans, as indicated by their electrophoretic mobility on agarose gel and their resistance to chondroitin ABC lyase and to nitrous acid deamination. This conclusion was supported by the presence of polysaccharides composed by sulfated fucose units, as reported by previous studies (Vilela-Silva et al. 2002; Cinelli et al. 2007). Sulfated fucans and sialic acid-rich glycoconjugates are the only carbohydrate-rich molecules found around the egg jelly of sea urchins (Mikami-Takey et al. 1991;Vilela-Silva et al. 2008). The sea urchin sperm contains a single fraction of acidic polysaccharide sensitive to metachromasy. Therefore, the positive staining of the sperm in Figure 3, Panels A and C, was associated exclusively with the sialic acid-rich polysaccharide. In contrast, the egg jelly coat contained two fractions of acidic polysaccharides and the positive metachromasy could be associated with both or either one of them.

Two sialic acid-rich polysaccharides from sea urchin gametes differed significantly in their sugar composition. Hexoses and deoxy hexose (fucose) were found in the sialic acid-rich polysaccharide from the egg jelly coat but they were absent in the sperm molecule. These polysaccharides also differed in their type of sialic acid: *N*-acetylneuraminic acid (Neu5Ac) in the sperm and *N*-glycolylneuraminic acid (Neu5Gc) in the egg jelly coat polysaccharide. In order to define the exact location of the sialic acid-rich polysaccharide from the egg jelly coat, the additional sugars found in their structure was considered (Table 1). For identify the carbohydrate residues, specific lectins were used: lectins LCA (from *Lens culinaris*), PNA (from *Arachis hypogaea*) and AAA (from *Aleuria aurantia*) that recognized specifically α-mannose, α-galactose and α-fucose, respectively. The biotinylated lectins bound to the sea urchin egg jelly coat (Fig. 3, Panels E-G), denoting the localization of the sialic acid-rich polysaccharide, although this result would need a carefully interpretation because other glycoconjugates might also bind the lectins. However, sulfated fucan and the sialic acid-rich glycoconjugates seemed the only macromolecules rich in carbohydrate found around the egg jelly of sea urchins (Mikami-Takey et al. 1991; Vilela-Silva et al. 2008).

Carbohydrates containing sialic acid are present on the surface of vertebrate cells. They participate in the recognition and interaction events that are essential for the growth, development and immunological reaction of these organisms (Nasirikenari et al. 2006; Nacher et al. 2010). Occasionally, sialic acids may be linked to each other to form polymeric structure, as in the capsules of some pathogenic bacteria and in few animal structures (Miyata et al. 2006).

Sialic acid-rich glycoconjugates are found in the zona pellucid of mammalian eggs and also in the jelly coat and vitelline layer overlying sea urchins eggs (Vilela-Silva et al. 2008). The egg jelly coats of the sea urchins, *S. purpuratus* and *H. pulcherrimus* contain polysialic acid composed of sulfated Neu5Gc (Kitazume et al. 1994). The sea urchin *P. depressus* contains a similar polysialic acid structure but is composed of a mixture of Neu5Gc and 9-*O*-acetyl-*N*-NeuGc in an equimolar ratio (Kamerling et al. 1980). These sialic acid-rich molecules potentiate the acrosome reaction in the sea urchin sperm, which is induced in a species-specific way by the egg jelly sulfated polysaccharides (Hirohashi and Vacquier 2002). Interestingly, one particular species of sea urchin, *Glyptocidaris crenularis*, sialic acid-rich polysaccharide is absent in the egg jelly (Castro et al. 2009).

Several studies have been conducted on the characterization of sulfated polysaccharides from the egg jelly coat of sea urchins and characterized the unique polysaccharide structures of well-defined units, composed of sulfated fucose or sulfated galactose (Alves et al. 1998; Vilela-Silva et al. 1999 2002). These sulfated polysaccharides induce the sperm acrosome reaction in a species-specific way and constitute a barrier for interspecific fertilization in these invertebrates. The proposition of a model system for the involvement of sulfated polysaccharides from the sea urchins egg jelly as a mechanism for regulation the species-specificity in fertilization requires identification of target molecules in the sperm surface. The description of group of proteins named “receptors for the egg jelly” in the sperm of the sea urchin *S. purpuratus*, which bound the egg jelly sulfated fucan (Vacquier and Moy 1997), was a possible way to clarify this issue. However, it was not possible to find similar proteins in the sperm of sea urchin species studied using a similar methodology. Attempts were made to extend the study of sea urchin sperm molecules

looking for carbohydrate structures, which showed a sialic acid-rich polysaccharide in the sperm. It was highly sulfated but differed from a similar molecule found in the egg jelly due to the occurrence of Neu5Ac instead of Neu5Glc and also due to very low proportion of other constituent sugars (Table 1). The sialic acid-rich polysaccharide from the sperm head had similarity with another glycoconjugate, named flagellasialin, which also contained Neu5Ac (Miyata et al. 2006, 2011).

Studies on whether the sialic acid-rich molecule found in the sperm head interacted with the sulfated fucan from the egg jelly followed the analogy of the interaction of sulfated polysaccharides that regulated sponge cell-cell adhesion (Vilanova et al. 2009). Affinity chromatography was used to investigate possible interaction between the sulfated polysaccharides, as described in the previous publication. The sulfated fucan was linked to a Sepharose matrix and the sperm sialic acid-rich polysaccharide was applied to the affinity column, which was eluted with increasing salt concentration. No interaction was observed when the column was run in the presence or absence of calcium and other bivalent cations. Even in the view of these negative results, it seemed still necessary to investigate the possible role of the new sialic acid-rich polysaccharide from the sea urchin sperm in the biology of fertilization; in particular to pursue alternative methods to investigate carbohydrate-carbohydrate interaction using sea urchin sulfated polysaccharides.

In conclusion, a distinct sialic acid-rich polysaccharide in the sperm of the sea urchin *L. variegatus* was found, which differed significantly from a similar molecule found in the egg jelly. It was not possible to demonstrate the interaction of this sperm polysaccharide with the egg jelly sulfated fucan. However, alternative methods should be studied to investigate possible interactions among these molecules. It could help to characterize the mechanisms of fertilization in the sea urchin model system.

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