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ORIGINAL ARTICLE

Identification of lipid raft glycoproteins obtained from boar spermatozoa

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Abstract

The surface of the spermatozoa is coated with glycoproteins the redistribution of which during in vitro capacitation plays a key role in the subsequent fertilization process. Lipid rafts are membrane microdomains involved in signal transduction through receptors and include or recruit specific types of proteins and glycoproteins. Few studies have focused on identifying glycoproteins resident in the lipid rafts of spermatozoa. Proteins associated with lipid rafts modify their localization during capacitation. The objective of the study was to identify the glycoproteins associated with lipid rafts of capacitated boar spermatozoa through a lectin-binding assay coupled to mass spectrometry approach. From the proteomic profiles generated by the raft proteins extractions, we observed that after capacitation the intensity of some bands increased while that of others decreased. To determine whether the proteins obtained from lipid rafts are glycosylated, lectin blot assays were performed. Protein bands with a good resolution and showing significant glycosylation modifications after capacitation were analyzed by mass spectrometry. The bands of interest had an apparent molecular weight of 64, 45, 36, 34, 24, 18 and 15 kDa. We sequenced the 7 bands and 20 known or potential glycoproteins were identified. According to us, for ten of them this is the first time that their association with sperm lipid rafts is described (ADAM5, SPMI, SPACA1, Seminal plasma protein pB1, PSP-I, MFGE8, tACE, PGK2, SUCLA2, MDH1). Moreover, LYDP4, SPAM-1, HSP60, ZPBP1, AK1 were previously reported in lipid rafts of mouse and human spermatozoa but not in boar spermatozoa. We also found and confirmed the presence of ACR, ACRBP, AWN, AQN3 and PRDX5 in lipid rafts of boar spermatozoa. This paper provides an overview of the glycosylation pattern in lipid rafts of boar spermatozoa before and after capacitation. Further glycomic analysis is needed to determine the type and the variation of glycan chains of the lipid rafts glycoproteins on the surface of spermatozoa during capacitation and acrosome reaction.

Keywords Boar spermatozoa · Capacitation · Glycoprotein · Lipid rafts · Lectin

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Background

Spermatozoa acquire the ability to fertilize the oocyte after residing in the female reproductive tract for a period. Biochemical and physiological changes that occur during this period and that make the spermatozoa competent to bind, penetrate and fertilize the oocyte are called capacitation [1]. A bicarbonate-mediated signaling pathway controls the scrambling of phospholipids; bicarbonate directly activates a sperm-specific adenylate cyclase, thereby switching on protein kinase A. It has been demonstrated that cholesterol is depleted from the plasma membrane of sperm upon capacitation [2]. The spermatozoa surface is covered with glycoproteins that form a protective layer and stabilize the cell during its transit through both the male and female genital tracts [3].

During capacitation and acrosome reaction (AR) a redistribution of glycoproteins has been observed in vitro in several species [4, 5]. Glycoproteins play a key role in the fertilization process, especially in the recognition of proteins in the oviduct [6] and in the recognition between gametes [7]. In previous studies performed on human spermatozoa, we found that during AR, glycoconjugates containing N-acetylglucosamine (GlcNAc) residues and/or sialic acid (NeuAc), mannose (Man), galactose (Gal) and fucose residues changed their distribution on the membrane surface in human spermatozoa [4]. In boars, we showed that a diminution of Man and GlcNAc/NeuAc residues was correlated with infertility [8]. We demonstrated as well that glycoproteins modified with GlcNAc/NeuAc residues were located principally in the acrosome and in the flagella and were reduced following capacitation; the Man residues were concentrated in the head and the middle of spermatozoa, but were predominantly located in the acrosomal region [5]. It is suggested that the Arylsulfatase-A, expressed at the cell surface of sea urchin, mouse, boar, and human sperm [9–11], is probably glycosylated with Man and GlcNAc/NeuAc residues in the membrane of capacitated boar sperm [12].

Lipid rafts are defined as lipid-ordered micro-domains in the membrane that concentrate specific molecules while excluding others. These domains resist to the treatment with 1% (*v*/*v*) Triton X-100 at 4 °C and are thus considered as the detergent-resistant membrane (DRM) fraction [13–18]. Lipid rafts are highly dynamic, heterogeneous in length since ranging from 10 to 200 nm and enriched in cholesterol and sphingolipids which contributes to their insolubility in Triton X-100 at low temperature and to their low flotation density [19]. Lipid rafts may contribute to the localization, organization, and regulation of specific signaling pathways [20–22] in spermatozoa of various species ranging from sea urchin to human [9, 23–26]; thus they must be an enriched source of proteins with possible functions in fertilization. Proteins associated with these lipid rafts modify their localization during capacitation and the AR [27]. Kasekarn et al. [28] identified a highly glycosylated protein, called WGA-gp, with affinity for lectin WGA; this glycoprotein is localized mainly in the flagella and is enriched in membrane microdomains or lipid rafts of boar spermatozoa. Two principal membrane lipid rafts types are reported: those that form caveolae, small invaginations in the membrane containing caveolin, and those that do not (called "flat rafts") [29]. Boar spermatozoa exhibit lipid rafts containing caveolin-1 and CD55, two proteins considered as specific markers of lipid rafts [30]. Other spermatozoa lipid rafts markers are gangliosides, such as ganglioside M1 (GM1), which are found in human and boar spermatozoa [31]. The aim of this study was to identify some of the glycosylated proteins associated with lipid rafts in capacitated boar spermatozoa via a lectin binding assay and mass spectrometry.

Methods

All chemicals were purchased from the Sigma Chemical Company (St Louis, Mo) unless otherwise indicated.

Semen samples

Semen samples were obtained from the sperm-rich ejaculate fraction of healthy fertile boars. Only ejaculates classified as normozoospermic were used. The viability and morphology of at least two hundred spermatozoa were evaluated by eosin-nigrosin staining. Concentration and motility parameters were assessed under phase-contrast microscopy (ZEISS, Germany) at 400X magnification [8].

Capacitation

Semen samples were washed twice to remove the seminal plasma by the addition of 1 mL of phosphate-buffered saline to an equal volume of semen followed by centrifugation at 600 X g for 10 min. Washed spermatozoa were incubated at a concentration of 5×10^6 cells/mL and placed in a culture flask with 500 mL of the capacitation medium TALP-HEPES supplemented with bovine serum albumin for 4 h at 39 °C in a humid 5% (*v*/*v*) CO_2 atmosphere; capacitation was assessed by chlortetracycline staining under a 495-nm UV epifluorescence microscope (ZEISS, Germany) at 400X magnification; only the samples containing more than 80% of capacitated spermatozoa were used [12].

Isolation of membrane lipid rafts

A low-density Triton X-100 insoluble membrane fraction was prepared according to Shadan et al. [24]. Capacitated (C) and non-capacitated (NC) boar spermatozoa were concentrated to 2×10^9 cells in 1 mL TALP, then mixed with 1 mL of ice-cold MBS (50 mM MES, pH 6.5, 150 mM NaCl, 1% (*v*/*v*) Triton

X-100, 2 mM Na_3VO_4 and 4 mM AEBSF) and incubated for 20 min at 4 °C. The samples were centrifuged for 10 min at 900 X g (4 °C). The supernatant (1.5 mL) was mixed with an equal volume of 85% (*w*/*v*) sucrose in modified MBS (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Na_3VO_4 and 2 mM AEBSF). The mixture was overlaid with 6 mL of 35% (*w*/*v*) sucrose followed by 3 mL of 5% (*w*/*v*) sucrose in modified MBS and centrifuged at 200×10^3 X g for 18 h (4 °C) in a SW41Ti rotor (Beckman, Palo Alto CA, USA). Fractions of 1 mL were collected from the top of the gradient. The protein content of each fraction was assessed by the micro Lowry method with Peterson's modification [32].

Lipid raft marker assays

Dot blot immunoassays were performed to examine the lipid rafts marker GM1. Two microliters of each fraction were laid down to a nitrocellulose membranes and air-dried; the membranes were blocked with 5% (*w*/*v*) non-fat dry milk powder in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% (*w*/*v*) Tween 20) for 1 h and incubated with Cholera enterotoxin subunit B (CTxB) conjugated to horseradish peroxidase (HRP) for 1 h [31]. To visualize GM1, we used a chemiluminescence assay (Immobilon™ Western, Millipore Corporation, Billerica, MA 01821, U.S.A.) and detected the signal with an imaging system (KODAK Gel Logic 1500 Imaging System, USA).

Equal amounts of protein were subjected to Western blotting. Samples were analyzed by SDS-PAGE under reducing conditions, and proteins were electroblotted on a nitrocellulose membrane (Amersham Protran Supported 0.45 μm NC, GE Healthcare). Membranes were first saturated for 45 min with 5% (*w*/*v*) non-fatty acid milk in Tris-buffered saline (TBS)-Tween buffer [15 mM Tris/HCl, 140 mM NaCl, and 0.05% (*v*/*v*) Tween 20, pH 8.0]. The following antibodies were used: rabbit polyclonal anti-flotillin-1 (sc-25,506, Santa Cruz Biotechnology, 1:2000) and mouse monoclonal anti-caveolin-1 (sc-70,516, Santa Cruz Biotechnology, 1:2000). Membranes were incubated with the different antibodies overnight at 4°, washed 3 times with TBS-Tween for 10 min and incubated with the appropriate secondary antibody (mouse IgG-HRP linked antibody or rabbit IgG-HRP linked antibody, GE Healthcare, 1:10,000), for 1 h. Finally, 3 washes of 10 min each were performed, and detection was performed with enhanced chemiluminescence (ECL Prime, GE Healthcare).

Polyacrylamide gel electrophoresis

Electrophoretic protein analysis was performed according to Laemmli [33]. Twenty micrograms of sample for each condition was used: the total protein of non-capacitated spermatozoa (TPNC), total protein of capacitated spermatozoa (TPC), raft proteins (RP) of NC (RPNC) and RP of C (RPC). Two gels were run in parallel; one was visualized by silver nitrate staining, and the other was transferred to a nitrocellulose membrane (Immobilon, Millipore, Bedford, MA) for western blot.

Lectin blot analysis

For the detection of carbohydrate residues associated with raft proteins, a lectin blot was run according to Vercoutter-Edouart et al. [34], with some modifications. Membranes were incubated with Digoxigenin (DIG)-conjugated lectin (Glycan Differentiation Kit, Roche™, Mannheim, Germany).

The lectins used were: peanut agglutinin (PNA, 100 μg/mL), which recognizes the disaccharide Gal (β1–3) N-acetylgalactosamine of the core of *O*-glycans, and *Sambucus nigra* agglutinin (SNA, 0.25 μg/mL) that recognizes NeuAc linked via α2–6 to gal and NeuAc linked via α2–6 to N-acetylgalactosamine, which are mostly found on *O*-glycoconjugates. *Galanthus nivalis* agglutinin (GNA, 0.25 μg/mL) recognizes terminal Man linked via α1–3 or α1–6 to Man on *N*-glycans, and *Datura stramonium* agglutinin (DSA, 0.25 μg/mL) recognizes Galβ1-4GlcNAc in complex and hybrid *N*-glycans. Positive and negative controls were used for lectin blots (5 μg of fetuin (Ft), asialofetuin (Aft), transferrin (Tf) and carboxypeptidase Y (CPY), provided with the kit; Roche™, Mannheim, Germany). Lectin blots were revealed by chemiluminescence after incubation with Fab fragments of polyclonal anti-DIG antibodies conjugated to HRP (30 mU/mL) (Roche Life Sciences).

Densitometry measurements were performed using ImageJ software (NCBI, USA) [35]. The optical density (OD) was determined and normalized against the OD of the whole lane of proteins from RPC or RPNC stained with Ponceau red [36]. These results are representative of five independent replicates.

Protein sequencing by nano-HPLC-tandem mass spectrometry

RPNC and RPC spermatozoa were separated by SDS-PAGE on 12.5% gels; 150 μg of each sample condition was used. The protein bands of interest were cut, destained, reduced and alkylated before *in-gel* trypsin digestion. Peptide mass spectrometric analyses were performed on an ion trap mass spectrometer (LCQ Deca XP +, Thermo electron, San Jose, CA) equipped with a nano-electrospray ion source coupled to a nanoflow high-pressure liquid chromatographic system (LC Packings Dionex, Amsterdam, The Netherlands) [37]. Database searches and proteins identification were performed for the tandem mass spectrometry (MS/MS) spectra using the Mascot Software (http://www.matrixscience.com) and the following parameters: "other mammalian" as taxonomy, 60 ppm tolerance for the parent ion mass and 60 amu for the fragment ions, one missed cleavage allowed, and

carbamidomethylation of cysteine and methionine oxidation as possible modifications. Only candidate proteins with a significant Mascot score were taken into consideration (significance threshold for candidate <0.05 using the MudPIT scoring method).

Statistical analysis

Lectin blot data were analyzed with the Student t-test using NCSS 2007 software, and differences were considered significant when $P < 0.05$.

Results

Spermatozoa protein extraction and isolation of lipid rafts

Spermatozoa from ten clinically healthy boars were analyzed. The total protein amount obtained after extraction was 1.72 ± 0.23 μg/10^6 from non-capacitated spermatozoa (TPNC) and 1.13 ± 0.11 μg/10^6 from capacitated spermatozoa (TPC). After ultracentrifugation, twelve fractions from the sucrose gradient were collected (1 mL each). When we performed a dot blot to visualize GM1 (an established specific marker of rafts), a signal was observed in the fractions 3 and 4 (Fig. 1A) for both conditions TPC and TPNC, corresponding to the DRM fraction, appeared as an opalescent band in the low-density fraction of the gradient; a signal was observed in the TPNC and TPC and in the rat brain cell homogenate (positive control), while no signal was found for the bovine serum albumin (negative control). These results are representative of three independent replicates of each sample condition with distinct ejaculates.

We obtained 0.27 ± 0.1 μg/10^6 spermatozoa of protein from RPNC and 0.32 ± 0.02 μg/10^6 spermatozoa from RPC. These fractions were analyzed for the immunodetection of two well-established markers of DRM, calveolin-1 and flotillin-1. Both proteins were detected in the lipid raft-enriched (RPNC and RPC) and total protein (TPNC and TPC) samples from non-capacitated and capacitated spermatozoa (Fig. 1B). Proteins from fractions 5 to 12 were not associated with GM1, and no protein was detected in fractions 1 and 2.

Lipid raft proteomic profiles

Proteins of spermatozoa were analyzed by SDS-PAGE and silver-stained. Bands with *Mr* ranging from 250 to 15 kDa were found in all conditions. Each SDS-PAGE was replicated at least three times with distinct ejaculates. In TP extractions, we observed minimal changes after capacitation, indicating that this process does not affect the global profile of proteins (Fig. 2, left panel). In contrast, in RP extractions, we observed that after capacitation, the intensity of several bands increased (arrows with dotted line at ~80, 70, 65, 48, 40, 36–34 and

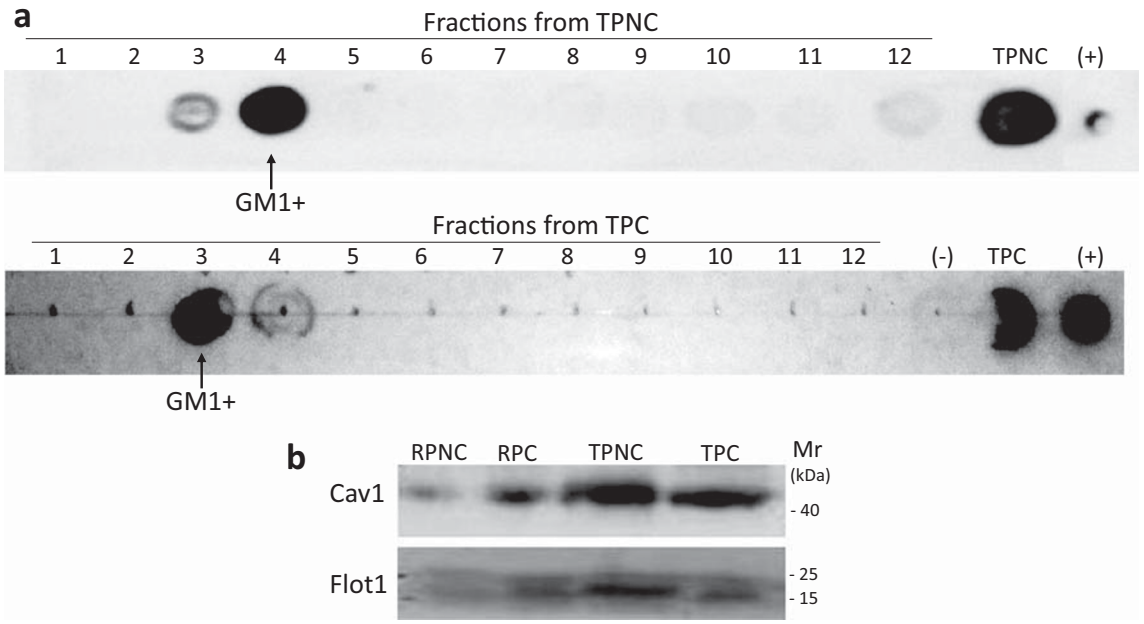

Fig. 1 **A.** Dot-blot for GM1. It was found in the total extract of capacitated spermatozoa (TC), total extract of non-capacitated (TNC), and in fraction 3 or 4. (−) Bovine serum albumin (negative control); (+) rat brain cells homogenate (positive control). A representative image of three independent experiments is shown; each was performed in capacitated (C) and non-capacitated (NC) spermatozoa. **B**. Western-blot detection of Caveolin-1 (Cav1) and Flotillin-1 (Flot1), found in raft proteins of NC (RPNC) and C spermatozoa (RPC), total protein of NC (TPNC) and C spermatozoa (TPC). Molecular mass markers are indicated in kDa

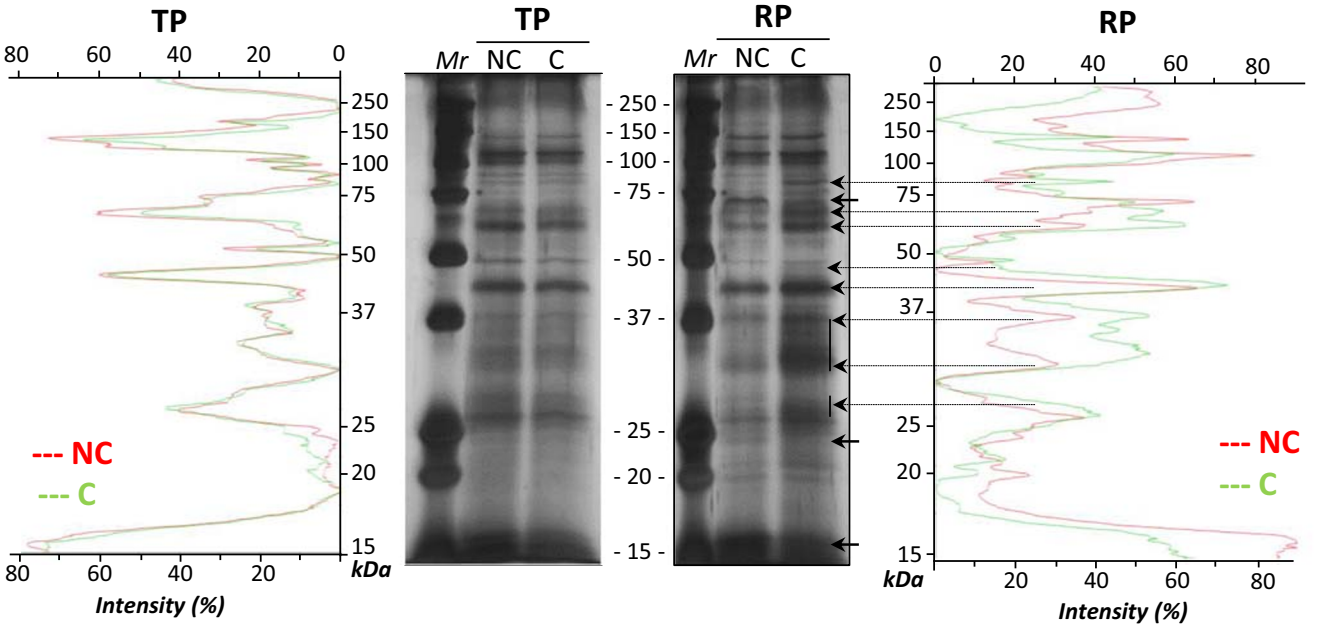

Fig. 2 SDS-PAGE and densitometry analysis of silver-stained boar spermatozoa proteins from total proteins (TP) of non-capacitated (NC) and capacitated (C) spermatozoa (left panel) and raft proteins (RP) of C and NC spermatozoa (right panel). Each SDS-PAGE was replicated at least three times with distinct ejaculate samples. In RP extractions, capacitation induced an increase of intensity for the ~80, 70, 65, 48, 40, 36–34 kDa bands (arrows with dotted line), and a decrease of intensity for the ~74, 23 and 15 kDa bands (arrows with full line)

28 kDa), whereas the intensity of a few bands decreased (arrows with full line at ~74, 23 and 15 kDa bands) (Fig. 2, right panel).

Lectin blot analysis

To determine whether the proteins obtained from lipid rafts are glycosylated or not, lectin blot assays were performed. Ponceau red staining of nitrocellulose membranes before performing lectin blots confirmed equal loading (see Additional file 1). We used PNA and SNA lectins for *O*-glycan labeling, and GNA and DSA lectins for *N*-Glycan labeling (Fig. 3). Each lectin blot was replicated at least five times with distinct ejaculates and representative data are shown. Positive and negative control glycoproteins were used for each lectin to confirm the specificity of the signal. For each lectin blot, we measured the OD of bands and calculated the fold change between both conditions (RPNC and RPC in at least five independent experiments; $P < 0.05$). Table 1 shows the quantification of the changes of glycosylation that were detected in boar spermatozoa lipid raft glycoproteins after capacitation.

We observed major changes in glycosylation for a protein with a low molecular weight (15 kDa) that showed a very strong signal with all lectins in the RPNC sample but that disappeared or strongly decreased (from 0 to −31-fold) after capacitation (RPC) (Fig. 3, Table 1). The detection of the *O*-glycan's core with PNA showed no significant changes except for a band of 18 kDa that significantly increased nearly 3.8-fold after capacitation. This band was only detected with PNA, suggesting that the *O*-glycan chain(s) of these glycoproteins may not be sialylated with α2,6 linkage since there was no recognition with SNA (Fig. 3A-B, Table 1). In contrast, there was a strong signal with SNA specifically on the bands with apparent molecular weight of 100, 79, 64, 26–24 kDa in RPC, compared with RPNC (from 2.6 to 20-fold increase) while the band at 36 kDa decreased significantly (−2.3-fold) (Fig. 3B, Table 1).

The signal of other lipid raft glycoproteins containing oligomannose residues, detected with GNA, decreased significantly after capacitation for the 45, 36 and 34 kDa bands (from −1.3 to −6.7-fold), whereas it increased significantly (1.3-fold) for the 64 kDa band (Fig. 3C, Table 1). Finally, with the DSA lectin that recognizes Galβ1-4GlcNAc in complex and hybrid *N*-glycans, we observed that most of the bands decreased after capacitation, showing a significant difference for the bands at 30 kDa (−4.6-fold) (Fig. 3D, Table 1). These results indicate that the *N*-glycosylation and/or *O*-glycosylation patterns are strongly modified during the capacitation process.

Mass spectrometry analysis

Protein bands from lipid rafts isolated from NC or C boar spermatozoa with a good resolution and showing significant modifications in their glycosylation content after capacitation were analyzed by mass spectrometry. The bands of interest had an apparent molecular weight of 64, 45, 36, 34, 24, 18

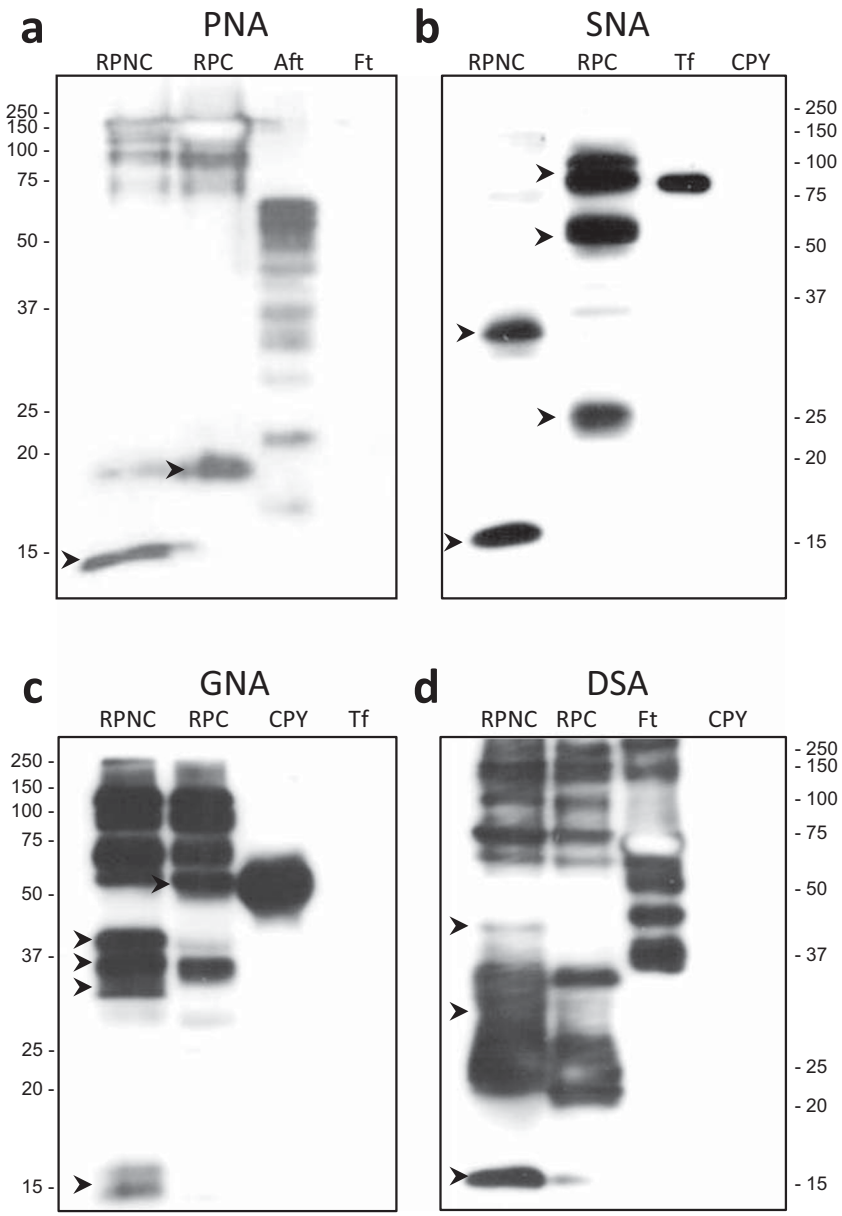

Fig. 3 Detection of glycoproteins from boar spermatozoa lipid rafts by lectin blotting. PNA **(A)**, SNA **(B)**, GNA **(C)** or DSA **(D)**. All significant changes ($p < 0.05$) observed after capacitation are indicated by arrows. Asialofetuin (Aft), fetuin (Ft), transferrin (Tf) and carboxypeptidase Y (CPY) were used as positive or negative controls depending on lectin and according to the manufacturer's indications (5 μg). RPNC = raft proteins of non-capacitated spermatozoa, RPC = raft proteins of capacitated spermatozoa. Molecular weights are indicated in kDa. Photographs representative of five independent experiments are shown. The optical density (OD) was determined and normalized against the OD of the migration of the total proteins from RPC or RCNP stained with the Ponceau red staining according to Romero-Calvo et al. [36]

and 15 kDa. These results are representative of five independent replicates of each sample condition.

We sequenced 7 bands and 20 known or potential glycoproteins were identified (see Additional file 2). Among them, 9 are potential novel *O*- and/or *N*-glycoproteins in boar sperm: Sperm adhesion molecule-1 (SPAM-1), testicular angiotensin-converting enzyme (tACE), disintegrin and metalloprotease domain-containing protein-5 (ADAM5), acrosin-binding protein (ACRBP), seminal plasma sperm motility inhibitor precursor (SPMI), sperm acrosome membrane-associated protein-1 (SPACA1), seminal plasma protein pB1, spermadhesin AWN (AWN) and Ly6/PLAUR domain-containing protein-4 (LYPD4). We identified 5 proteins whose glycosylation sites were already reported in boar: Lactadherin (MFGE8), acrosin (ACR), zona pellucida-binding protein-1 (ZPBP1), carbohydrate-binding protein AQN-3 (AQN3) and major seminal plasma glycoprotein PSP-I (PSP-I). All of them participate in the fertilization process except LYPD4 that has an unknown function.

We have also found six non plasma membrane proteins: Heat shock protein-60 (HSP60), peroxiredoxin-5 (PRDX5), testis-specific phosphoglycerate kinase-2 (PGK2), succinyl-CoA ligase subunit beta (SUCLA2), malate dehydrogenase-1 (MDH1) and adenylate kinase isoenzyme-1 (AK1).

Among the known or potential glycoproteins, for ten of them, this is the first time that their association with lipid rafts of spermatozoa is described (ADAM5, SPMI, SPACA1, Seminal plasma protein pB1, PSP-I, MFGE8, tACE, PGK2, SUCLA2, MDH1). Moreover, LYDP4, SPAM-1, HSP60, ZPBP1, AK1 were reported in lipid rafts of mouse and human

Table 1 Changes in protein glycosylation in boar spermatozoa lipid rafts based on lectin blot analysis

	OD											
Mr (kDa)	PNA (Gal)			SNA (NeuAc)			GNA (Man)			DSA (GlcNAc)		
	RPNC	RPC	FC	RPNC	RPC	FC	RPNC	RPC	FC	RPNC	RPC	FC
145							0.049	0.047	**-1**			
120	0.036	0.057	**+1.6**				0.061	0.050	**−1.2**	0.075	0.052	**−1.4**
110										0.062	0.048	**−1.3**
79	0.034	0.061	**+1.8**	0.021	0.054*	**+2.6**	0.078	0.067	**−1.2**			
64				0.012	0.089*	**+7.4**	0.025	0.032*	**+1.3**	0.061	0.050	**−1.2**
59	0.023	0.042	**+1.8**									
45							0.205	0.094*	**−2.2**	0.076	0.054*	**−1.4**
36				0.043	0.019*	**−2.3**	0.058	0.043*	**−1.3**	0.120	0.108	**−1.1**
34							0.034	0.008*	**−4.2**			
30										0.055	0.012*	**−4.6**
26				0.011	0.039*	**+3.5**				0.088	0.110	**+1.2**
24				0.001	0.020*	**+20**				0.088	0.177	**+2**
18	0.013	0.050*	**+ 3.8**									
15	0.149	0*	**0**	0.031	0.001*	**−31**	0.054	0.008*	**−6.7**	0.064	0.016*	**−4**

The Mr. was determined using Quantity One 4.6.8 software with molecular mass markers and optical density (OD) values determined using ImageJ (average values are shown). Significant differences (*) between RPNC and RPC were obtained using a t-test ($P < 0.05$). The fold change (FC) was calculated relative to RPNC values. Mr. = Relative molecular mass. RPNC = Raft proteins of non-capacitated spermatozoa. RPC = Raft proteins of capacitated spermatozoa. These results are representative of five independent replicates of each sample condition

spermatozoa but not in boar spermatozoa. We found and confirmed the presence of ACR, AWN, ACRBP, AQN3 and PRDX5 in lipid rafts of boar spermatozoa.

Discussion

Capacitation is a process that involves biochemical and physiological changes by which spermatozoa acquire the ability to fertilize the oocyte. During this process, lipid rafts migrate to the apical ridge of the spermatozoa head [38] and the periacrosomal region [31]. Glycosylated proteins are redistributed in the spermatozoa, suggesting that changes in the exhibition of these receptors could be used as indicators of the capacitation process and AR. We achieved the isolation of lipid rafts in non-capacitated and capacitated boar spermatozoa and identified the DRM material, often linked with lipid rafts, in the low-density fraction, as reported previously [24, 39].

In this paper, we found proteins associated with lipid rafts in non-capacitated and capacitated spermatozoa, in agreement with previous findings in boar [39] and human spermatozoa [31]. However, this finding is controversial, in light of what happens in mice: after capacitation, there is a total loss of proteins associated with lipid rafts, it is therefore thought that lipid rafts dissociate during mouse spermatozoa capacitation [25] and in other species [40–43].

Some of the identified proteins are non-plasma membrane proteins, such as SUCLA2 and HSP60, but it has been reported that lipid rafts are not confined to the plasma membrane; indeed, lipid microdomains are formed on subcellular organelles, including endoplasmic *reticulum*, Golgi and mitochondria (referred to as raft-like microdomains) [44, 45]; Mitochondrial PRDX5 identified in p15 has already been reported in the lipid rafts of boar spermatozoa [39]; we identify these three DRM-associated mitochondrial proteins and consider it unsurprising if we remember that the boar spermatozoa have many mitochondria.

Moreover, HSP60 has been found at the surface of mouse spermatozoa and is involved in zona pellucida (ZP) recognition [46]; PGK2 and AK1 were identified in vesicles of anterior head plasma membrane (AHPM) of capacitated and non-capacitated boar sperm [47]. MDH1 is a cytoplasmic enzyme, which to date has not been associated with lipid rafts. The extraction of the lipid rafts is a complex procedure, and it seems that this protein may co-purify with rafts, either by binding to *N*-glycosylated proteins or by association with cholesterol-sphingolipid-enriched microdomains [48]. The protein interaction with lipid rafts is not only of transmembrane or intrinsic proteins, but also for extrinsic proteins that are cytoplasmic and depend on signals so that they can interact with lipid rafts. This could be because the subcellular compartments are highly dynamic, with diverse and intricate

architectures that are not always preserved during membrane isolation procedures [49].

In our work, we found that some glycoproteins remain in the lipid rafts fraction after capacitation. The *O*-glycosylation of some proteins strongly increased, while the *N*-glycosylation of several proteins was dramatically affected. These results allow us to assume that the proteins remaining in the DRM or lipid rafts fraction should be involved in subsequent events in the fertilization process.

Carbohydrate residues play an important role in regulating cell-cell recognition and in activating surface receptors on the plasma membrane that trigger a variety of signal transduction pathways depending on the cell type. In particular, in male gametes from several species, the glycosidic chains of membrane proteins are involved in the adhesion of spermatozoa to the oviduct, the recognition and the fusion of the spermatozoa with the oocyte, and the embryo implantation [50]. The lectins we used bind to carbohydrates that participate in an important way in the processes by which the sperm acquires its fertilizing capacity and in the recognition between gametes. We then focused on protein bands that presented glycosylation changes during capacitation. In some cases, we found the same protein in different apparent molecular weight bands, like lactadherin (in p45, p36, p34 and p24) and ZPBP1 (in p36, p24 and p18). This could result from differences in glycosylation site occupancy and/or variation in glycan chain structures that influence the rate of migration in the gel as previously described in other cells [51]. Moreover, as several proteins were identified in each band, it is not possible to determine the contribution of each glycoprotein to the differences in lectin detection that we observed between capacitated and non-capacitated spermatozoa.

We demonstrated that several proteins glycosylated with Gal and Man residues remain after capacitation and that others increase like SPAM-1, and MFGE8 found in p64 and AQN3 found in the p18 band; this band contains proteins that participate in the adhesion and binding with the egg's ZP. These glycoproteins are related to the sperm binding to the egg and they were found in AHPM vesicles of boar sperm [47, 52]. Interestingly, several studies have highlighted the importance of carbohydrates and glycan-modifying enzymes in these processes. Indeed, Gal residues participate in spermatozoa-oviduct interactions in boar [53] and Man residues are essential for bovine egg-sperm binding [54]. In mice, galactosyltransferase is linked to the AR and is relocated to the plasma membrane of the spermatozoa head by acrosomal exocytosis [55]. Furthermore, in human, PNA labeling is used to determine the acrosomal status of sperm [56]. The expression of tACE at the cell surface depends on the functional and morphological characteristics of spermatozoa [57] and is important for the transport across the oviduct and in the ZP union [58]. We identified tACE in p64 band which gave signals for both Man, GlcNAc and NeuAc residues, in agreement with its *N*-glycosylation described previously in human [59] and with its characterization in AHPM vesicles of boar sperm [47].

In humans, WGA-recognized GlcNAc/NeuAc residues cover the surface of spermatozoa plasma membrane and decrease after AR [4]. In boars, these residues decreased considerably after capacitation and AR [5]. In the present study, we found that in lipid microdomains, some of the proteins glycosylated with GlcNAc are conserved after capacitation, suggesting that these proteins may be required for AR and during the process of fertilization. Other authors have found that the distribution and concentration of WGA receptors are directly associated with male fertility; they decrease in semen samples with morphological abnormalities [60].

We also found that in the lipid rafts of boar spermatozoa some sialylated glycoproteins increased after capacitation while others decreased, and we identified some glycoproteins whose sialylation is potentially regulated during boar sperm capacitation. The sialylated glycoproteins were previously detected on the surface of motile human spermatozoa [60]. Sialylation of glycoconjugates contributes to the negative charge that spermatozoa acquire during the epididymal maturation and plays a dual role in sperm maturation and fertilization. Indeed, spermatozoa surface sialoglycoconjugates can help in joining with oviductal crests by protecting or masking other membrane proteins that could play a role in spermatozoa-oocyte recognition during capacitation. For example, NeuAc residues of the rooster's spermatozoa correlate with the ability to pass through the vagina and to be sequestered in the reproductive tract of the hen [61]. Moreover, NeuAc can protect the surface of spermatozoa from immune recognition during capacitation [60].

In this work we identified SPMI, pB1, PSP-I, AQN3 and AWN, all belonging to the spermadhesin family, and ACR, ACRBP, ZPBP1, MFGE8 that participate in sperm-egg interactions. Most of these proteins were previously characterized in AHPM vesicles of capacitated and non-capacitated boar sperm [47]. Their presence in the DRM associated with capacitation in boar sperm lipid rafts supports the idea that they are effectively associated with rafts, to participate in initial sperm-ZP recognition. Spermadhesins are present at the spermatozoa surface and constitute the major component of seminal plasma; most of them disappear during capacitation. The spermadhesins that persist at the surface of capacitated spermatozoa participate in the binding to the ZP [62], whereas the AQN1 spermadhesin has a role in the formation of the oviductal spermatozoa pool [63]. The seminal plasma sperm motility inhibitor precursor AQN3 protein has already been reported in the lipid rafts of boar spermatozoa [39], but no previous report has mentioned its glycosylation. Our results suggest that AQN3 might be *N*- and *O*-glycosylated. The spermadhesin PSP-I triggers the recruitment of immune cells after mating, thereby initiating a cascade of immunological events [64]; we show for the first time that PSP-I and seminal

plasma protein pB1 are likely to be associated with lipid rafts of boar spermatozoa.

Kongmanas et al. [47] reported that a fraction of the acrosomal ZP-binding proteins were abundant in capacitated sperm; they showed that zonadhesin, proacrosin/acrosin and ACRBP were transported to the surface of the plasma membrane of live acrosome-intact sperm during capacitation; interacting with each other, they may traffic as a complex from the acrosome to the sperm surface. The acquisition of the ability to adhere to the ZP is a complex process done in post-testicular maturation events, it has been hypothesized that a cohort of sperm-based receptors may require active assembly and/or presentation in the form of a dynamic multimeric zona recognition complex [65, 66]. So, the study of carbohydrates and glycoproteins expressed in sperm, that participate in the processes of gamete recognition is a subject that still needs to be studied in depth.

Conclusions

This paper provides an overview of the glycosylation pattern in boar spermatozoa lipid rafts before and after capacitation. Our results indicate that some glycoproteins associated with lipid rafts are modified during capacitation, in agreement with data showing that glycosylation allows these molecules to function as receptors or as anti-recognition factors during AR and fertilization. However further glycomic analysis is needed to determine both the type and the variation of glycan chains of the lipid rafts glycoproteins on the spermatozoa surface during capacitation and AR. In addition, quantitative proteomic experiments could help in determining whether changes in glycosylation are related or not to variation of protein expression. This study could facilitate the identification of new markers of spermatozoa capacitation process.

List of abbreviations AR: acrosome reaction; C: capacitated; DIG: digoxigenin; DRM: detergent-resistant membrane; DSA: *Datura stramonium* agglutinin; Gal: galactose; GlcNAc: N-acetylglucosamine; GM1: ganglioside M1; GNA: *Galanthus nivalis* agglutinin; Man: mannose; NC: non-capacitated; NeuAc: sialic acid; OD: optical density; PNA: peanut agglutinin; RP: raft proteins; RPC: raft protein of C; RPNC: raft protein of NC; SNA: *Sambucus nigra* agglutinin; TPC: total protein of capacitated spermatozoa; TPNC: total protein of non-capacitated spermatozoa; WGA: wheat germ agglutinin; ZP: zona pellucida.

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Authors' contributions Direction of the study and established the experimental design: RF, IJM, HGM, ASVE, TL, JCM. Performed the experiments: JBLS, OMP, SFB. All authors analyzed the results, contributed to the finalized manuscript and approved the manuscript.

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Compliance with ethical standards

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate Not applicable.

Consent for publications Not applicable.

Competing of interest The authors declare that they have no competing interests.

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