Remodelling of Lipid Rafts during In vitro Capacitation and Acrosome Reaction of Ram Spermatozoa

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Abstract

**Background:** Lipid rafts are often known as Detergent-Resistant Microdomains (DRMs). We report for the first time the presence of two lipid raft markers, caveolin-1 and ganglioside GM1, on the ram sperm surface, and the effect of in vitro capacitation and acrosome reaction on these marker distributions, the protein content and lipid composition of DRM and non-DRM fractions.

**Methods:** Caveolin-1 and ganglioside GM1 were evidenced by immunocytochemical and fluorescence analysis, respectively. DRM and non-DRM fractions were separated by an OptiPrep™ density gradient. Cholesterol by fluorometry, GM1 by peroxidase reaction, protein content by spectrophotometry, and fatty acid profiling by gas chromatography were determined.

**Results:** Caveolin-1 was evidenced at the acrosome of 59.2 ± 4.3% fresh spermatozoa, and the proportion of stained cells increased (P<0.05) after capacitation. GM1 was detected at the post-acrosome and tail of all spermatozoa, and no change was found after capacitation. Cholesterol and GM1 were distributed all along the gradient, with a peak in DRM fractions. A higher proportion (P<0.001) of saturated fatty acids was found in DRM fractions, confirmed by the unsaturation index and a higher lipid/protein ratio. In vitro capacitation induced a decrease in the content of saturated fatty acids in both DRM (P<0.001) and non-DRM (P<0.01) fractions. Polysaturated fatty acids increased in DRMs after the acrosome reaction. All treatments resulted in lower content of cholesterol and proteins in DRM (P<0.01) and non-DRM fractions (P<0.01), and a higher GM1 content in DRMs (P<0.05).

**Conclusions:** Lipid raft-like microdomains were isolated in a discrete region of the gradient. Their high content of saturated fatty acids confers a highly ordered environment. Their composition is modified during in vitro capacitation and acrosome reaction.

**General significance:** These results represent the first characterization of ram sperm DRM, and may contribute to a better understanding of the sperm fertilizing potential acquisition mechanism.

Keywords: DRMs; cholesterol; fatty acids

Introduction

Spermatozoa are cells with a limited biosynthetic capability [1,2]. Hence, their functionality is mainly controlled by external factors acting through cell surface and plasma membrane components. Proteins from the epididymal and seminal plasma are adsorbed onto the sperm surface as they pass through the male and the female reproductive tracts. The final result of this remodelling is a functional mature sperm with an adequate plasma membrane for specific interactions with the egg during fertilization.

Moreover, sperm are highly polarized cells with two functional parts, and their membranes can be categorized into different compartments. In the sperm head, the plasma membrane surrounds the acrosome, the equatorial segment and the post-acrosomal region. These sperm surface areas are known to be involved in zona pellucida binding, acrosome reaction, and membrane fusion during fertilization. Several experimental approaches have shown that the sperm surface is regionalized in these areas [3,4] in spite of the absence of a bilayer spanning structure to prevent mixing of diffusible membrane proteins or lipids [5].

The fluid mosaic model [6] used since the 1970s to understand the cell plasma membrane structure and functions, has gained in complexity with the increasing knowledge of compartmentalized domains of biological membranes. For spermatozoa in particular, the Singer-Nicholson model does not adequately explain the restricted lateral diffusion of their membrane surface molecules. Instead, a relatively new concept known as lipid rafts is increasingly being investigated in sperm cells as well as in mammalian cells to gain a better understanding of these phenomena.

Membrane rafts are defined as small, heterogeneous, highly dynamic domains that serve to compartmentalize cellular processes [7]. These membrane microdomains are associated with a heterogeneous group of proteins which float in the bulk sphingolipid-cholesterol phase of...
the membranes. The unique, ordered properties of these domains are due to hydrophobic interactions between their saturated fatty acids and the rigid structure of intercalated cholesterol. These properties result in the resistance of lipid rafts to solubilisation by a number of non-ionic detergents, commonly Triton X-100. These microdomains float in a discontinuous density gradient, due to their low density. This is the reason why lipid rafts are generally identified with DRMs (detergent-resistant membranes). Because the definition of raft is based on the methodological method for their isolation and to avoid confusion, we should make the distinction between membranes that can be isolated from detergent lysates (DRMs), and domains that may exist in cells (lipid rafts) [8].

Mammalian sperm cells have a highly differentiated morphology, which is reflected in their membrane surface properties [9]. When mammalian spermatozoa are ejaculated, they are unable to fertilize the Oocyte. They acquire fertilizing potential by the process known as capacitation that takes place during the sperm transport throughout the female genital tract. During capacitation, the sperm membrane protein and lipid organization changes dramatically [10-12]. These changes result in the capacitated state, which allows sperm to bind to the zona pellucida and the acrosome to react.

The plasma membrane of ram spermatozoa displays some particularities. The cholesterol:phospholipid ratio (0.38) is considerably lower than that in other mammalian species [13], which could be the reason why albumin-mediated cholesterol efflux might not be necessary during ram sperm capacitation [14,15]. This special low content in cholesterol might also be related to the increased time required for ram sperm capacitation [16], and the high density of cryopreservation [17,18]. Likewise, the phospholipid-bound fatty acid composition and, particularly, the very high polyunsaturated:saturated fatty acid ratio in the ram sperm membrane may also influence membrane susceptibility [19,20].

Several sterols, sphingolipids, glycolipids and glycosylphosphatidylinositol-(GPI-)anchored proteins have been reported in lipid rafts of different types of cells, including spermatozoa [21-23]. GM1 ganglioside and proteins such as caveolin [24,25] or flotillins are commonly used as lipid raft markers. However, although these microdomains generally possess a distinctive composition, not all rafts are identical in terms of either the lipids or the proteins that they contain [21].

Lipid rafts are highly dynamic and lateral movements have been described in various cell types in response to physiological stimuli. A multiplicity of cellular functions has been associated with these lipid microdomains, such as membrane trafficking, cellular signal transduction or viral entry [26]. In gametes, lipid rafts have been related to sperm maturation, fertilization, and early embryogenesis [27]. Furthermore, certain proteins involved in gamete membrane fusion and zona binding are enhanced in DRMs [28], which suggests that lipid rafts could be involved in sperm–zona pellucida binding and the acrosome reaction [29]. Following in vitro capacitation, membrane rafts become highly enriched in zona binding proteins [28-30]. However, contradictory results have been reported, and although some authors have described a redistribution of DRMs after in vitro capacitation [29-31], others [32] found no change in the distribution of raft markers after the induction of this process.

Despite the numerous studies on lipid rafts, there is to our knowledge no published account of the composition of these microdomains in ram spermatozoa. In this study, we describe for the first time the localization of two lipid raft markers, caveolin-1 and GM1, on the ram sperm surface, and the influence of in vitro capacitation and the acrosome reaction on their distribution. Moreover, we analyze the protein content and lipid composition of DRM and non-DRM fractions isolated from ram spermatozoa in three physiological states, namely fresh, capacitated and acrosome-reacted.

**Materials and Methods**

**Sperm preparation**

All the experiments were performed with fresh semen taken from nine mature Rasa aragonesa rams using an artificial vagina. All the rams belonged to the National Association of Rasa aragonesa Sheep Breeders (ANGRA) and were 2-4 years old. They were housed under uniform nutritional conditions at the Experimental farm of the University of Zaragoza in compliance with the requirements of the European Union Directive for Scientific Procedures. All experimental procedures were performed under the supervision of the Ethics Committee of the University of Zaragoza. The sires were kept apart, and semen was collected every two days, in two successive matings each day. Under these conditions, and using second ejaculates, individual differences are very low, as we have already reported [33]. We used pooled ejaculates of 3 rams that provide a good quality uniform sperm sample, suitable for representative studies of ram semen avoiding individual differences. A seminal plasma-free sperm population was obtained by a dextran/swim-up procedure as already reported [34] with a medium devoid of CaCl₂ and NaHCO₃ (fresh sample).

**In vitro capacitation**

For the induction of in vitro capacitation, aliquots of 1.6 × 10⁸ cells/ml were incubated for 3 hours at 39°C in a humidified incubator with 5% CO₂ in air. Incubations were performed in complete TALP medium [35] containing 100 mM NaCl, 3.1 mM DRMs (detergent-resistant membranes) M KCl, 25 mM NaHCO₃, 0.3 mM NaH₂PO₄, 21.6 mM Na lactate, 3 mM CaCl₂, 0.4 mM MgCl₂, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose, and 5 mg/ml bovine serum albumin, pH 7.2. Sperm aliquots incubated in this medium were defined as control samples.

To induce capacitation, a previously tested cocktail for capacitating ram spermatozoa [14,15] was added to the sperm aliquots, and defined as capacitated samples. The cocktail consisted of caffeine and theophylline (both inhibitors of phosphodiesterases, Sigma Chemical Co., Madrid, Spain; 1 mM each), okadaic acid (OA, a broad spectrum phosphatase inhibitor, Sigma Chemical Co., Madrid, Spain; 0.2 µM), methyl-β-cyclodextrin (M-β-CD, Sigma Chemical Co., Madrid, Spain; 2.5 mM) and 1 mM of the cAMP-elevating agent dibutyryl-cAMP (db-cAMP, Sigma Chemical Co., Madrid, Spain).

**Acrosome reaction induction**

The acrosome reaction was induced by the addition of calcium ionophore A23187 (3.4 µM containing 0.3% DMSO; Sigma Chemical Co., Madrid, Spain) to the swim-up obtained sample (1.6 × 10⁸ sperm/ml) and further incubation at 39°C for 1 h [36]. Control tubes had DMSO added (0.3%) but no ionophore, which has been shown to have no effect [36].

**Evaluation of sperm samples**

The sperm concentration was calculated in duplicate using a Neubauer’s chamber (Marienfeld, Germany).

The capacitation status was assessed by the Chlorotetraycline-
fluorescence (CTC) assay that we had previously validated for the evaluation of capacitation and acrosome reaction-like changes in ram spermatozoa [15]. Three sperm types were studied [37]: non-capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (AR, showing no fluorescence on the head). We examined the samples, within 12 h, using a Nikon Eclipse E-400 microscope under epifluorescence illumination with a V-2A filter. All samples were processed in duplicate and we scored at least 150 spermatozoa/slide. No fluorescence was observed when CTC was omitted from the preparation.

Detection of lipid raft markers on ram spermatozoa

To analyse the presence of caveolin-1, flotillin-1 and flotillin-2, sperm samples were exposed to immunocytochemical staining by an avidin-biotin-peroxidase technique (Vector, CA, USA). Fresh, control and capacitated samples were cytospun onto slides. The cells were fixed in methanol for 15 min and permeabilized in 3.7% Triton X-100. After rehydration in PBS pH 7.2, endogenous peroxidase was inactivated with 1.7% hydrogen peroxide in 100% ethanol for 30 min. Subsequently, the samples were washed in PBS pH 7.2, and then incubated with a blocking reagent provided with the kit for 45 min, followed by incubation for 2 h with the primary antibodies: mouse anti-caveolin-1 (1/40), mouse anti-flotillin-1 (1/60), and mouse anti-flotillin-2 (1/60) (BD Biosciences, Madrid, Spain). After washing, the slides were incubated with biotinylated anti-mouse antisera for 40 min. The avidin-biotin-peroxidase complex was then applied for 45 min. The binding sites of the primary antibodies were visualized by Diaminobenzidine (DAB) and hydrogen peroxide solution (20 mg DAB in 100 ml of 0.05 M Tris-HCl buffer pH 7.6, containing 0.005% H_2O_2) for 5 min. After the each incubation, the slides were rinsed with PBS. The samples were visualized under a Nikon Eclipse E-400 microscope (Nikon, Tokyo, Japan). Positive staining, indicated by dark brown deposits in some cells, was evaluated according to the obtained sperm pattern and the percentage was estimated. As a positive control, we used an endothelial sample, and as negative controls the primary antibodies were omitted, with the remaining procedure being the same. These negative controls did not show any positive staining.

The distribution of the GM-1 ganglioside on the sperm plasma membrane of the fresh, control and capacitated samples was studied by staining with the Cholera Toxin Subunit B (CTXB)-Alexa488 conjugate (Molecular Probes Inc., Eugene, OR, USA). CTXB binds to GM1 with a high affinity and has been widely used as a reporter of the distribution of lipid rafts [38-40]. Sperm aliquots of 8 x 10^9 cells were allowed to settle on Poly-L-lysine-coated slides (Sigma Chemical Co., Madrid, Spain). The spermatozoa were firstly incubated for 30 min with 10 µg/ml CTXB-Alexa 488 conjugated at room temperature (RT) in the dark. Secondly, fixation was performed for 10 minutes with 3.7% formaldehyde at RT. After the each incubation, the slides were rinsed with PBS. Finally, the spermatozoa were mixed with 5 µl 0.22 M triethylenediamine (DABCO, Sigma Chemical Co., Madrid, Spain) in glycerol:PBS (9:1) in order to enhance and preserve the cell fluorescence. Finally, the preparations were covered with coverslips, sealed with colourless enamel, and visualized by means of a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under epifluorescence illumination using a B-2A filter. At least 200 cells per sample were evaluated and the percentage of cells displaying positive fluorescence was scored. Negative controls were performed by omitting CTXB-Alexa488 and did not reveal positive cells.

**Lipid raft isolation**

We used the method described by Hinzpeter et al. [41] with minor modifications. Sperm samples were lysed in cold extraction buffer (TEN buffer (25 mM Tris-HCl pH 7.3, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail [Sigma Chemical Co, Madrid, Spain], plus 1% Triton X-100 [Sigma Chemical Co., Madrid, Spain]) for 30 min and cavitated at 15,000 PSI for 15 min. The lysate was mixed with Optiprep^TM^ (Axis-Shield, Dundee, Scotland, UK) to make 1.2 ml of 40% Optiprep^TM^ solution and placed at the bottom of an ultracentrifuge tube. The mixture was overlaid with 2.4 ml of 30% OptiprepTM in TEN buffer followed by an additional 1.2 ml of TEN buffer without Optiprep^TM^.

The samples were spun at 4°C overnight (20 h) or 2 h at 200,000 xg in a TLA-110 rotor (Optima TLX Ultracentrifuge). A total of 13 fractions of 400 ml were collected and numbered starting at the top of the gradient.

**Analysis of the Gradient Fractions**

**Quantification of cholesterol**

Cholesterol was measured with the Amplex® Red Cholesterol Assay Kit (Molecular Probes Inc., Eugene, OR, USA), which provides a simple fluorometric method for the sensitive quantitation of cholesterol. Briefly, cholesterol esters are broken down by cholesterol esterase, and then all the cholesterol in the sample is oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone product. The H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent, AR), a highly sensitive and stable probe for H_2O_2 [42]. In the presence of horseradish peroxidase (HRP), Amplex Red reagent reacts with H_2O_2 to produce highly fluorescent resorufin [42,43], which has maxima absorption and fluorescence emission at approximately 571 nm and 585 nm, respectively. To measure cholesterol in the gradient fractions, 25 µl of each fraction were made up to 50 µl with PBS, and then mixed with 50 µl of a reaction mixture containing 2.087 U/ml of cholesterol oxidase, 2.087 U/ml of cholesterol esterase, 2.087 U/ml of HRP, and 0.312 mM ARr. The reaction was performed for 30 min at 37°C, protected from the light. The fluorescence was measured at 550 nm and 590 nm for absorption and emission, respectively. The concentration of cholesterol was calculated by extrapolating the fluorescence data in a cholesterol standard curve ranging from 0 to 10 µM.

**Ganglioside GM1 measurement**

Ganglioside GM1 was detected in gradientfractions by dot blot using the cholera toxin subunit B (CTXB) as already reported [44]. Briefly, 5 µl of each fraction were spotted onto previously activated Immobion-P membranes (Millipore, Bedford, MA, USA). After drying the samples, the membranes were blocked with 5% skimmed milk for 1 h, at RT. Cholera toxin subunit B-Horseradish Peroxidase conjugate (CTXB-HRP, Sigma Chemical Co., Madrid, Spain), commonly used as a marker for membrane fractions in sub cellular fractionation [45], was added to the membranes at 1/1000 dilution, and incubation was carried out for 1 hour at RT. Extensive washes were made after the incubations. GM1 that bound the CTXB was visualised using the Gel Doc System with Molecular Analyst software (Bio Rad, Hercules, CA) and processed for analysis with PD-QuestTM 2D analysis software (Bio Rad, Hercules, CA) to determine the relative GM1 content of the spots. Negative controls omitting CTXB-HRP showed no reactivity.

**Total protein measurement**

The total protein content in gradient fractions was measured using...
the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL), according to the manufacturer’s indications.

**Fatty acid analysis**

Lipids were extracted from gradient fractions by the addition of 6 volumes of chloroform-methanol (2:1, v/v), centrifugation at 800 g for 3 min, and aspiration of the resulting lower phase. Heptadecanoic acid was added to all samples as an internal standard (15 mg from chloroform-methanol (1:1, v/v) stock solutions) (Sigma Chemical Co., Madrid, Spain) prior to the extraction. Lower phases were evaporated to dryness under nitrogen. Fatty acids were transmethylated by alkaline methanolysis using the BF3 reagent kit (Supelco, Bellefonte, PA). Dry fractions were resuspended in 0.5 ml of methanolic-base and incubated at 100°C for 3 min, followed by the addition of boron trifluoride-methanol (0.5 ml), incubation at 100°C for 1 min, addition of hexane (0.5 ml), incubation at 100°C for 1 min, and addition of 6.5 ml of saturated NaCl. The samples were centrifuged at 800 x g for 4 min. The hexane upper layer was transferred to a new glass tube and an aliquot injected in a Hewlett Packard 5890A gas chromatograph. A Supelcowax column of 30 m length and 0.5 mm internal diameter was used. The initial temperature was 150°C and the final temperature 260°C. The FID temperature was 300°C and the total running time 27 min. Fatty acid methyl ester peaks were identified by comparison with retention times of standard mixtures (Sigma Chemical Co., Madrid, Spain), and quantified in comparison with the internal standard detector response.

**Statistical Analysis**

Results are shown as mean ± SEM of the number of samples indicated in each case. Statistical analyses were carried out using GraphPadInStat software (3.01; San Diego, CA, USA) and SPSS software (v.15.0, IBM, New York, NY, USA). A Kolmogorov-Smirnov test was performed to establish whether the values came from a Gaussian distribution and then-test was applied when two groups were compared. Because the lipid/protein ratio data were not normally distributed, and had a lognormal distribution, logarithm transformation of these data was carried out and the Bonferroni test was used. ANOVA tests were performed to determine whether there were significant differences among groups. Raft marker determination data, expressed in percentages, were analysed by means of the chi-square test.

**Results**

**Raft markers in ram spermatozoa**

In order to identify raft markers in ram spermatozoa, we investigated the presence of caveolin-1, GM1 ganglioside and flotillin-1 and 2.

Caveolin-1 was located by immunocytochemistry at the acrosomal region of fresh sperm samples, with two different distribution patterns: at the most apical part of the acrosome (type A, Figure 1A), and a more extensive staining on the whole acrosome [type B, Figure 1B]. The quantitative analysis of both patterns revealed 38.5 ± 3.6% of type A, 20.6 ± 6.3% of type B, and a high percentage of non-stained cells, 40.8 ± 4.3% (mean values ± SEM; n=4). Positive (Figure 1C) and negative (Figure 1D) controls confirmed the specificity of results. After capacitation, the proportion of type B stained sperm increased (37.7 ± 4.3%, P<0.05) with a concomitant decrease in the percentage of non-stained cells (27.2 ± 1.1%, P<0.01).

Ganglioside GM1 was evidenced by fluorescence staining in all spermatozoa of fresh samples. The reactivity signal of GM1 was located at the post acrosomal region and tail (Figure 2), and only very occasionally at the most apical ridge of the acrosome. Although the intensity of staining was not identical in all spermatozoa, we were not able to discern reliable different staining patterns. Furthermore, we did not find any significant change after capacitation.

Flotillin-1 and flotillin-2 were not detected in ram spermatozoa by either of immunocytochemistry, fluorescence or western-blots analysis.

**Isolation of DRMs**

To isolate DRMs from ram spermatozoa, we assayed different times of ultracentrifugation using sperm lysates obtained by incubating fresh samples with Triton X-100. A total of 13 fractions were isolated from the Optiprep™ gradient after ultra centrifuging for either 2 or 20 h. Analyses of cholesterol, ganglioside GM1 and the total protein content were carried out in all fractions. The results obtained (Figure 3) revealed that the lowest-density fractions (1-3) were not enriched in cholesterol and GM1, and that DRMs reached their isopycnic point at the interface between 0-30% Optiprep™ that correspond to the...
fractions 4-5 (DRM fractions). The 30-40% interface corresponded to fractions 9-10, and non-DRMs (Triton X-100 soluble fractions) were located at the bottom of the gradient.

The comparison of both methods (Figure 3) indicated that 2 h of ultracentrifugation enables a better discrimination between low- and high-density fractions, while after 20 h, cholesterol (Figure 3A) and GM1 (Figure 3B) were concentrated in the medium density fractions (mainly fractions 7, 8, and 9). Based on these results, and given that quantitative protein determination showed that proteins were concentrated in the high-density fractions (10-13, non-DRMs fractions, Figure 3C) with both centrifugation times, 2 h was established as the ultracentrifugation time for the following experiments.

Changes in DRMs composition after treatments

The analysis of cholesterol in the fractions separated after 2 h of ultracentrifugation from lysates of fresh, control, capacitated and acrosome-reacted samples revealed substantial differences (Figure 4A). The cholesterol content in fraction 5 (DRMs) of the fresh sperm samples (25.2 ± 8.5 µM) was decreased (P < 0.01) to 4.8 ± 1.1, 3.5 ± 1.5 and 3.9 ± 0.8 µM in the control, capacitated and acrosome-reacted samples (25.2 ± 8.5 µM) was decreased (P < 0.01) to 4.8 ± 1.1, 3.5 ± 1.5 and 3.9 ± 0.8 µM in the control, capacitated and acrosome-reacted samples, respectively. In the non-DRM fractions, a significant loss of cholesterol was also observed in the treated samples compared to the fresh ones (P < 0.001) (Figure 4A).

Despite the fact that CTC staining was able to significantly discriminate different capacitation patterns (Table 1), the cholesterol content in the gradient-separated fractions was not significantly different between the control, capacitated and acrosome-reacted samples (Figure 4A).

GM1 distribution was different from that of cholesterol (Figure 4B). It is noteworthy that, unexpectedly, it was more abundant in the non-DRM fractions, independently of the physiological state of the sperm. The induction of capacitation and the acrosome reaction resulted in a change in GM1 distribution, which decreased in the non-DRM fractions, especially in fractions 10 and 11, with a concomitant increase in fraction 4 (DRMs) from 3.2 ± 0.9% in fresh samples to 6.3 ± 0.6, 6.0 ± 0.5, and 7.7 ± 0.4% in the control, capacitated and acrosome-reacted samples, respectively (P < 0.01). Concomitantly, a significant increase in fraction 4 (DRMs) from 2.5 ± 0.2% in fresh samples to 6.3 ± 0.6, 6.0 ± 0.5, and 7.7 ± 0.4% in the control, capacitated and acrosome-reacted (P < 0.01) samples, respectively (Figure 4B).

Total protein concentration was measured in all fractions (Figure 4C). The results revealed a very low protein content in the DRM
fractions. Similarly to that found with cholesterol, the incubation in capacitating conditions (control sample), induced capacitation and acrosome reaction led to a loss of proteins from all the fractions, although significant differences were only found in the fractions 5 (DRMs) and 10 (non-DRMs) (P < 0.05).

As observed in the cholesterol analysis, no significant differences were found between the control, capacitated and acrosome-reacted samples in either GM1 or protein content in any fraction.

Fatty acid analysis and saturation index

The results of fatty acid analysis are summarized in (Table 2). As a general observation, DRM fractions contain a significantly higher level of saturated fatty acids than the heavier fractions (P < 0.05). In addition, the induction of capacitation accounted for a decrease in saturated fatty acid content in both DRM and non-DRM (P<0.01) fractions (Table 2).

Regarding the percentage of polyunsaturated fatty acids (PUFA), a significantly higher content (P< 0.05) was present in non-DRM fractions, in both fresh and capacitated samples, while no difference was found in the acrosome-reacted samples (Table 2).

The unsaturation index corroborated the results obtained by the fatty acid analyzing it was higher in the non-DRM than in the DRM fractions in the fresh (181.4 ± 17.9 vs 127.8 ± 21.2), capacitated (166.3 ± 8.0 vs 136.6 ± 23.2) and acrosome-reacted (209.7 ± 12.8 vs 178.2 ± 55.0) samples.

The total fatty acid/protein ratio was significantly higher (P<0.001) in DRM fractions in the fresh, capacitated and acrosome-reacted samples with respect to non-DRM fractions (Table 3). Furthermore, this ratio was significantly increased (P<0.001) in the capacitation-induced samples in both DRM and non-DRM fractions as compared to fresh and acrosome-reacted samples (Table 3).

Discussion

In this study, we report for the first time the presence of two lipid raft markers, Caveolin-1 and GM1, in ram spermatozoa. Caveolin-1 is a cholesterol-binding protein [46], and a key raft organizer [25-47] that has been identified in the acrosomal region of the mouse [48,49], guinea pig [49], stallion [50] and boar [30] spermatozoa. We have identified, by immunocytocchemical analysis, caveolin-1 in fresh ram spermatozoa freed from seminal plasma by swim-up. This result indicates the presence of raft-like microdomains in the ram sperm membrane. Caveolin-1 was located at the acrosomal region, although not all sperm were stained. These results corroborate our previous observations about the heterogeneity of the sperm sample obtained by swim-up [51,52], and it is also consistent with those reported for stallion semen in which a relationship between a lack of positive caveolin-1 staining and a reduction in fertility was found [50]. Our results also showed an increase in the proportion of stained sperm at the acrosome, occasionally including the equatorial region, after capacitation. This observation is consistent with previous results showing that the spatial distribution of lipid rafts within the sperm membrane is influenced by the capacitation status [31-33].

Although the presence of flotillin-1 [30] and flotillin -2 [53] has been reported in boar, flotillin-2 in human [54] and, without specifying the type, in ram [55] spermatozoa, our numerous attempts (immunocytochemical, indirect immunofluorescence and western-blot analysis) to identify both flotillins (-1 and -2) failed. However, we succeeded in identifying another raft marker of lipid nature, the ganglioside GM1. GM1 has been reported as a mouse sperm receptor for the seminal plasma protein SVS2 (semen-coagulating protein) that acts as a decapacitating factor [56], which makes GM1 an attractive target for studying sperm physiological changes. Nevertheless, the reported results about the location of GM1 in sperm are contradictory. GM1 has been found at the postacrosomal of epididymal mouse [57] and rat [58] spermatozoa, and at the acrosomal area of epididymal murine sperm [59]. Conversely, GM1 was reported as nonexistent or patchy and inconsistent throughout the whole sperm cell of human and boar [31-54]. One likely difference among these studies could be the fixation procedure [60], which might affect viability. In fact, it has been reported that GM1 moves rapidly from its position overlying the acrosome to the post-acrosomal area after cell death in unfixed or lightly fixed samples [59]. Our results show that GM1 is located at the postacrosomal region and tail of all ram spermatozoa. Furthermore, we were unable to observe changes in GM1 location associated with capacitation, although a trend toward the reorganization of GM1 has been suggested in methyl-beta-cyclodextrin-induced capacitated boar sperm [31].

To approach the isolation of DRMs, we considered two main features: their resistance to solubilization with detergents due to the presence of phospholipids containing saturated fatty acyl chains, which confers on them a highly ordered environment [22]; and their low density, a consequence of a relatively higher lipid/protein ratio than bulk membranes. The typical isolation method involves the extraction of cells with 1% Triton X-100 followed by centrifugation in a discontinuous 5-30% density gradient [22]. In our conditions, cholesterol and GM1 were distributed all along the gradient, and according to our own interest, a peak of both was found in fraction 5, just in the upper region of 30% Optiprep™ and very near to the 0-30% Optiprep™ interface. The proportion of saturated fatty acids

| Fraction | % saturated fatty acids | % PUFA | Unsaturation Index |
|----------|------------------------|--------|------------------|
|          | Fresh | CAP | AR | Fresh | CAP | AR | Fresh | CAP | AR |
| F4       | 55.9 ± 6.9a | 40.4 ± 2.7b | 55.8 ± 9.1b | 22.3 ± 3.5 | 20.3 ± 10.7 | 15.8 ± 5.4 | 133.6 ± 19.1 | 130.0 ± 52.8 | 123.6 ± 18.6 |
| F5       | 57.4 ± 8.4a | 41.1 ± 7.6a | 49.2 ± 18.4a | 18.0 ± 8.5a | 18.2 ± 2.3a | 39.8 ± 15.5a | 121.9 ± 43.0 | 143.1 ± 5.7 | 232.8 ± 108.9 |
| DRMs     | 56.6 ± 4.8a | 40.8 ± 3.6a | 52.5 ± 8.6a | 20.1 ± 4.2a | 19.2 ± 4.9a | 27.8 ± 9.6a | 127.8 ± 21.2 | 136.6 ± 23.9 | 178.2 ± 55.0 |
| F10      | 42.8 ± 6.8a | 35.6 ± 4.3a | 52.0 ± 2.0a | 33.4 ± 3.8 | 29.5 ± 6.2 | 30.3 ± 3.7 | 211.6 ± 23.8 | 185.6 ± 17.7 | 208.4 ± 40.7 |
| F11      | 47.5 ± 7.4a | 40.1 ± 3.9 | 46.9 ± 1.7 | 24.8 ± 10.3 | 28.0 ± 7.0 | 27.6 ± 8.2 | 165.8 ± 53.9 | 158.7 ± 21.9 | 226.1 ± 8.0 |
| F12      | 45.6 ± 5.8a | 42.6 ± 5.1 | 46.0 ± 11.5 | 24.6 ± 9.5 | 24.2 ± 7.6 | 26.2 ± 0.6 | 165.7 ± 47.7 | 150.8 ± 14.7 | 200.9 ± 36.2 |
| F13      | 45.3 ± 5.8a | 36.3 ± 2.6a | 40.0 ± 7.9a | 27.7 ± 3.4a | 35.8 ± 3.3a | 23.6 ± 3.9a | 182.2 ± 26.0 | 169.7 ± 8.9 | 203.4 ± 34.4 |
| Non-DRMs | 45.3 ± 2.8a | 38.7 ± 1.9 | 46.2 ± 3.1a | 27.8 ± 3.2a | 29.3 ± 2.9a | 26.9 ± 1.0 | 181.4 ± 17.9 | 166.3 ± 8.0 | 209.7 ± 12.8 |

Different superscript letters between columns indicate significant differences (P< 0.01). Different superscript numbers between rows indicate significant differences (P< 0.05).

Table 2: Analysis of fatty acids by gas chromatography inflections obtained from the Optiprep gradients (n=3).

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the results of this study confirmed a loss of cholesterol from DRMs to evidence its efflux linked to capacitation in ram spermatozoa [14]. An interesting feature about cholesterol is that, while we were not able to detect any significant differences in agreement with previous reports [63], the presence of m-β-CD, a cholesterol-depleting agent, in the capacitation inducing medium did not seem to have an influence in the general decrease in cholesterol content, as no significant differences in cholesterol were found between control, capacitated and acrosome-reacted samples.

The protein composition of lipid rafts has been analysed [22,67,68]. The identified and raft-associated proteins have been involved in numerous biological processes, including signal transduction events, membrane component sorting, viral budding, toxin entry into the cells, prion action, and amyloid formation [69]. Our results showed that the amount of proteins in DRMs of ram spermatozoa is lower than that reported in boar [30] and mouse [48]. Furthermore, proteins from DRMs and non-DRMs are lost after capacitation and the acrosome reaction. These results are consistent with previous reports that describe the release of sperm membrane proteins during capacitation of bull [70], boar [71,72], rabbit [73] or ram [74] spermatozoa.

We have already shown that RSV14 and RSV20 (ram seminal plasma proteins) are adsorbed onto the ejaculated ram sperm surface [75], and that they are redistributed and partially lost from the plasma membrane during in vitro capacitation and the acrosome reaction (only approximately 35% of them remained on the spermatozoon after both processes, [76]). Thus, we can speculate that there is both a loss and a redistribution of proteins in discrete plasma membrane domains. In fact, some proteins found in the epididymal fluid have been located in lipid rafts of spermatozoa: Prp in ram [55], and AQN-3 (also known as spermathesin) [77] and P47 (the SED1 porcine homologue) [78] in boar spermatozoa.

It has also been demonstrated that DRMs isolated from spermatozoa possess the ability to bind with high affinity and specificity to the zona pellucidae of homologous oocytes [53-79]. Although caution is required in equating DRM association of a protein with its residence in membrane raft domains in situ [80], such findings encourage the speculation that sperm membrane rafts serve as constitutive platforms for the spatial constraint of key recognition molecules and that the remodeling events associated with capacitation lead to their assembly and presentation on the outer leaflet of the sperm plasma membrane [81]. Likewise, it has been reported that DRMs of ejaculated spermatozoa are reorganized by seminal plasma proteins which are responsible for the dissociation of certain proteins from DRM domains [61]. In this context it is tempting to speculate that RSV14 and RSV20 might associate with discrete plasma membrane microdomains, and that seminal plasma might play an important role in the behaviour and composition of lipid rafts.

The study of sperm membrane surface remodelling based on the new concept of membrane lipid microdomains may contribute to a better understanding of the mechanism leading to the acquisition of sperm fertilizing potential. The association of specific proteins involved in gamete interaction with these lipid microdomains represents a promising matter for future research.

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