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Role of epididymal anti sticking factor in sperm capacitation

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ABSTRACT

Sperm capacitation depends on several features like hormones, ions, intracellular signaling, sperm associated molecules, etc. Anti sticking factor (ASF) is a novel sperm surface associated glycoprotein isolated from epididymal plasma. Function of ASF *in vivo* has not been revealed yet. The current study is an attempt to highlight the surface localization of ASF and corresponding biochemical changes that occurs in sperm cells during *in vitro* capacitation. In the presence of 1 nM ASF, percentage of bicarbonate and BSA induced capacitated cells in modified Tyrode medium (7.2) decreased from 72.45% to 16.25% as per Merocyanine 540 (M540)/DAPI stained flowcytometric analysis. Indirect immunocytostaining and western blot analysis shows that the amount of sperm surface bound residual ASF decline during *in vitro* capacitation. ASF at its effective concentrations notably reduced the bicarbonate and BSA induced cholesterol efflux. These data help in concluding ASF as a majorly responsible molecule that maintains caprine sperm membrane integrity by inhibiting cholesterol efflux. As the capacitation process, progress at *in vitro* condition, ASF is found to be released from the sperm surface and cell moved from non-capacitated to the capacitated state.

1. Introduction

Epididymal mature spermatozoa remain infertile despite of its healthy condition. Fertility potential is acquired by highly motile sperm within the female reproductive fluid [1]. The complex biochemical alteration of sperm cells occurring within this stage is collectively termed as capacitation [2], which transforms them from non-fertilizing to potentially fertilizing cells. Irrespective of the species, spermatozoa remain responsive to external factors-induced capacitation *in vitro* for 50–180 min only and then prepare itself for acrosome reaction [3]. Those external factors are mainly bicarbonate, BSA and Ca^{2+} [4]. This is essential to ensure that capacitation would not start before migration of the spermatozoa to the fertilization site. Thus, a tight control is necessary to inhibit premature capacitation. Epididymal plasma (EP) is a complex mixture of secreted proteins, glycoproteins, peptides [5] and is capable of maintaining spermatozoa in a nascent or decapacitated state [6,7].

Capacitation involves important changes in the sperm surface with loss, rearrangement and unmasking of various surface molecules [8]. These changes cause cholesterol efflux; membrane permeabilization followed by intra sperm biochemical signal activation and finally reaches the acrosome reaction [4]. Surface molecules released at the beginning of capacitation are often referred to as 'decapacitation factors' (DF). DF may be of either epididymal or seminal plasma origin and represent a mechanism for preventing the spermatozoa from becoming capacitated too quickly [9].

The earlier research reported several decapacitation factors and the biochemical property of these factors is distinct from each other [10,11]. Dostàlovà et al. [12], mentioned a specific acidic seminal fluid protein (aSFP) in bull, which belongs to spermadhesin group; aSFP gradually disappear from sperm surface during capacitation. Similar kind of surface depletion occurs for boar eoididymal anti-agglutinin during capacitation. The interaction of this particular anti agglutinin with sperm surface is carbohydrate (sialic acid) specific [13].

A glycoprotein that inhibits sperm sticking with glass was isolated from caprine epididymis by our group. Due to its activity it was designated as anti-sticking factor (ASF). Later it was found that ASF has high efficacy to inhibit distal corpus sperm *in vitro* agglutination [14]. This agglutination is in fact a sugar specific sperm–sperm interaction phenomenon [15]. However, little is known about its *in vivo* function and its importance in sperm physiology. From the primary data ASF believed to act as a surface associated factor. There are reports where sperm surface adhesive molecules play a role in sperm capacitation [12,13]. Thus, specific

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role of ASF in sperm cell capacitation is predicted. Till date, the potency of ASF on sperm capacitation system *in vitro*; has never been examined.

The main aim of this work is to elucidate the significance of this novel epididymal protein, ASF, in the final stage of maturation of sperm cell i.e. capacitation with special emphasis on sperm surface modulations using caprine sperm *in vitro* model.

2. Materials and methods

2.1. Chemicals

Merocyanine 540 [M540] (Cat. No. 323756), Ca^{2+} ionophore A23187 (C7522), 4′,6-diamidino-2-phenylindole (DAPI) (D9542), filipin III (F4767), BSA (Cat. No. 05470), $CaCl_2$ (C5670), Anti-Rabbit IgG–FITC antibody (F9887) were purchased from Sigma Chemical Co. (St. Louis, MO). Bicarbonate and other common chemicals were obtained from SRL, India.

Fresh epididymides of adult goats were obtained from the local slaughterhouse. Spermatozoa were extracted from these tissues within 2–4 h of slaughtering.

2.2. Isolation and incubation of spermatozoa

Unless otherwise specified mature sperm cells from caudal region of goat epididymis were extracted at 37 °C in Tyrode medium, washed twice immediately after extraction with the same medium and used for assay. The complete Tyrode medium composed of 96 mM NaCl, 3.1 mM KCl, 1.5 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1 mM potassium phosphate buffer, 50 mg penicillin/ml, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$, and 2 mg/ml BSA (pH 7.2). A modified Tyrode medium was prepared for incubating the spermatozoa in 'capacitating' conditions [Tyr + Bicarbonate + BSA] (solution A). For 'noncapacitating' conditions (as negative control), modified Tyrode medium without bicarbonate and BSA (solution B) was used. In both cases, stock solutions were prepared without $CaCl_2$ and pyruvate; filtered and stored at 4 °C. The remaining necessary components were added just before of the experiments.

2.3. Purification and polyclonal antibody generation of ASF

ASF was purified from epididymal plasma according to the protocol of Roy et al., [14]. Antiserum against the purified ASF was raised in rabbits by four successive injections. Pre-immune sera was collected from three month old female rabbits following immunization with 500 μg ASF with FCA followed by 200 μg, 200 μg, 500 μg ASF with FIA on days, 14, 21 & 28. Production bleed was done on the 35 day. Purified fraction was stored at −20 °C with 1 μM protease inhibitor cocktail [16].

2.4. Merocyanine 540/DAPI staining

We have developed Merocyanine 540 and DAPI combined staining for the first time to detect sperm capacitation. Live sperm cells (5×10^6) were incubated in Solution A (pH 7.2) in presence or absence of different concentrations of ASF. In vitro capacitation was performed in air-tight, 1.5 ml eppendroff tubes containing 400 μl of medium, in a shaking water bath at 37 °C for 180 min. After that, M540 solution (2.7 μM prepared in DMSO + 0.5 mg/ml polyvinylpyrolidone) was added and incubated for further 30 min in the dark at 37 °C. Then, the cells were washed and resuspended with PBS. Then 1 μg/ml DAPI was added and incubated for 5 min before analysis. Sperm cell analysis was performed using flowcytometer FACS Verse TM (Becton Dickinson). M540 fluoresces at about 570–590 nm. For M540 detection a 488 nm blue laser was used as the light source with filter 586/42. DAPI fluoresces is at about 460 nm. For DAPI detection a 405 nm violet laser was used as the light source with filter 448/45. The system was triggered by the forward light scatter signal (FSC). SSC and FSC were recorded so that only whole cell sperm specific scatter profile (devoid of debris), were selected for further analysis. Sperm cells were analyzed using PBS as sheath fluid. For each sample, 20,000 events were stored in the computer for further analysis with FACS Suite software (Becton Dickinson). DAPI negative cells with high M540 binding was designated as live cell with highly destabilized membrane or capacitated cells (UL), Cells with low M540 binding denoted as live uncapacitated cells (LL) and DAPI positive cells irrespective of M540 positive or negative denoted as dead (UR, LR).

2.5. Immunocytochemical observation by indirect immunofluorescence

Live spermatozoa were extracted to solution A or B (pH 7.2) and *in vitro* capacitation assay was executed. Roughly 100–120 spermatozoa from each sample were taken to prepare a smear on gelatin coated glass slide and fixed with 4% paraformaldehyde. After washing with PBS, cells were quenched with 0.1% sodium borohydride to minimize cellular autofluorescence. Cells were then treated with a blocking solution of 3% BSA in PBS. Next, cells were incubated with anti ASF antibody (at 1:50 dilution in 1% BSA). Following further washing with PBS, anti-rabbit IgG-FITC conjugate was added at a dilution of 1:20 in 1% BSA. Slide preparation was completed with the addition of 1–2 drops of anti-fader (DABCO, ChemSpider) and application of cover slip over it (avoiding air bubbles). Finally, cells were visualized under Ayrus epi-fluoroscent microscope at 600X magnification. Obtained images were processed with Adobe Photoshop 7.0 for better clarity.

2.6. ASF western blotting-densitometric analysis

Isolated washed sperm cells (5×10^6) were dispersed in solution A (pH 7.2) and *in vitro* capacitation assay was done for 3 h. An aliquot was taken from it at definite time intervals, washed with PBS twice and the pellet was resuspended in 50 μl PBS. Spermatozoa thus obtained was diluted with 50 μl 2X SDS-sample loading buffer and boiled for 3 min. After boiling undissolved material was removed by centrifugation at 10,000 × g for 15 min. Clear supernatants were concentrated and 10 μl loaded in 10% SDS-PAGE. Sample was ran at 90 V for 15 min and then at 150 V for 1 h. The SDS gel then transferred to PVDF membrane (Millipore) and western blotting was done using Anti ASF antibody. The detection was performed using ECL Western Blotting Substrate (Thermo Scientific). The Kodak X-ray film, developed through this blotting was further densitometrically analyzed by Thermo myImage analysis software.

2.7. Quantification of sperm membrane cholesterol

Spermatozoa (2×10^7) pretreated with ASF at a concentration range (0.5–1.5 nM). Treated cells were incubated in solution A or B 3 h at 37 °C. Incubated sperm suspension was washed thrice with PBS. Washed sample were treated with 6 volumes of chloroform: methanol (2:1 v/v). The mixture vortexed for 10 s and allowed to stand at room temperature for 1 h. After that the sample was vortexed again and centrifuged at 800 × g for 10 min to extract lipids. Chloroform extracts were pooled. The residual chloroform in the extract was removed in a rotary evaporator. Cholesterol content in the dried extract was measured by the method of cholesterol oxidase/peroxidase [17] using Autospan liquid gold cholesterol test kit

and analyzed in a spectrophotometer (Thermo Helios zeta) at 505 nm.

2.8. Statistical analysis

The results were expressed as means ± SEM. The data were statistically analyzed by Microcal Origin-pro version 9.0 (USA). Significance was tested using one-way ANOVA.

3. Results

3.1. Measurement of the percentage of capacitated cells in presence or absence of ASF

One of the major benefits of M540 staining is that it can be used to analyze the live spermatozoa rather than the fixed cells used in chlortetracycline (CTC) staining. Therefore, the alteration or

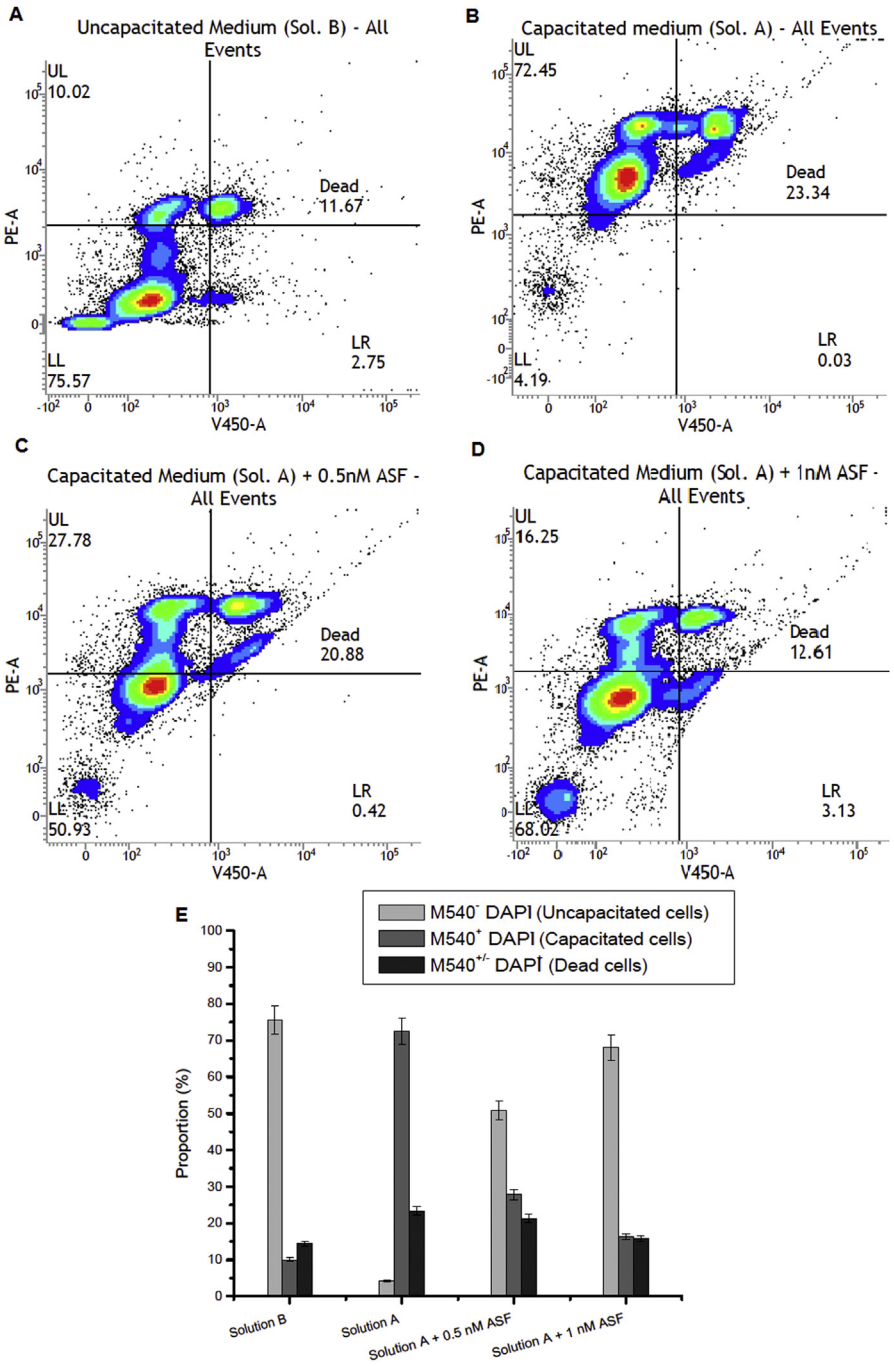

Fig. 1. The three patterns obtained for viable sperm stained with merocyanine 540 and DAPI. The highly fluorescent sperm heads demonstrate capacitated spermatozoa (UL), and the poorly fluorescent heads demonstrate noncapacitated spermatozoa (LL).UR and LR represents dead cell with high DAPI fluorescence. Flowcytometry analysis of sperm population incubated in non capacitating condition (A), capacitating condition (B), capacitating condition + 0.5 nM ASF (C), capacitating condition + 1 nM ASF (D) are provided here. A graphical representation of flowcytometry data where $M540^{-}$ $DAPI^{-}$ (Uncapacitated cells), $M540^{+}$ $DAPI^{-}$ (Capacitated cells), $M540^{+/-}$ $DAPI^{+}$ (Dead cells) was presented here (E). Data represent means ± SD of three independent experiments performed in duplicate.

damage of sperm cell membrane due to the use of fixative is nullified. From Fig. 1A it was found that a small population of live spermatozoa (10%) incubated in solution B responses to M540 staining (UL section/M540^{+}DAPI^{-}); whereas 72% cells in solution A showed high affinity for M540 staining in absence of ASF **(B)**. Proportion of M540^{+}DAPI^{-} population in presence of 0.5 nM and 1 nM ASF is 28% and 16% respectively **(C, D)**. Previously, Roy et al. [14], perceived that maximal activity of ASF on spermatozoa obtained at the concentration of 1 nM. We also found effect of ASF was saturated above 1 nM concentration (data not shown). A graphical illustration of M540/DAPI staining pattern is presented here Fig. 1E. The data represented the proportion of capacitated, uncapacitaed and dead (DAPI^{+}) spermatozoa in terms of M540/DAPI binding at different assay conditions. As M540 binds with membrane with greater lipid disorder, from this data it can be suggested that ASF reduce this disorder and is largely responsible to maintain caprine sperm membrane integrity.

3.2. Immunological determination of ASF localization on sperm surface

Antisera against ASF were used for the qualitative determination of ASF distribution on sperm surface at capacitating and uncapacitating condition. Microscopic photograph of fixed sperm samples were taken after 180 min of incubation in solution A or B using Lissview 7 software. Fixed sperm treated with pre immune sera was also investigated as control. In Fig. 2a homogeneous distribution of ASF was observed at the head region of sperm cells incubated in solution B (non capacitating medium). Strong signal coming from sperm proximal and acrosomal region actually imply its moderate localization in those portion at pH 7.2, though at this pH it was actually started to release from sperm surface. The data suggested ASF at physiological concentration has specific affinity for sperm membrane and no additional pretreatment with excess ASF is necessary. But in solution A (capacitating medium) minimal binding of ASF took place in sperm head region as well as signal intensity was significantly lowered. From the acquired data it can be stated that residual ASF was still present in capacitated sperm surface. Therefore complete dissociation of ASF is not essential for caprine sperm capacitation.

3.3. Western blot analysis of ASF release

We needed to quantify the relative amount of sperm-bound residual ASF after incubation in capacitating medium. Western blots of capacitated sperm whole cell extracts following densitometric evaluation with 'myImage Analysis' software (Thermo) was done. Image obtained from Western blots and relative density of sperm bound ASF as acquired from densitometric analysis are shown in Fig. 2b and Table 1. The density (intensity/area) was found to be rapidly decreased with the time of incubation. The relative density of sperm bound ASF decreased to 15% during the first 30-min and further gradual reduction thereafter with maximum decrease of 72% at the end of incubation periods i.e. 180 min.

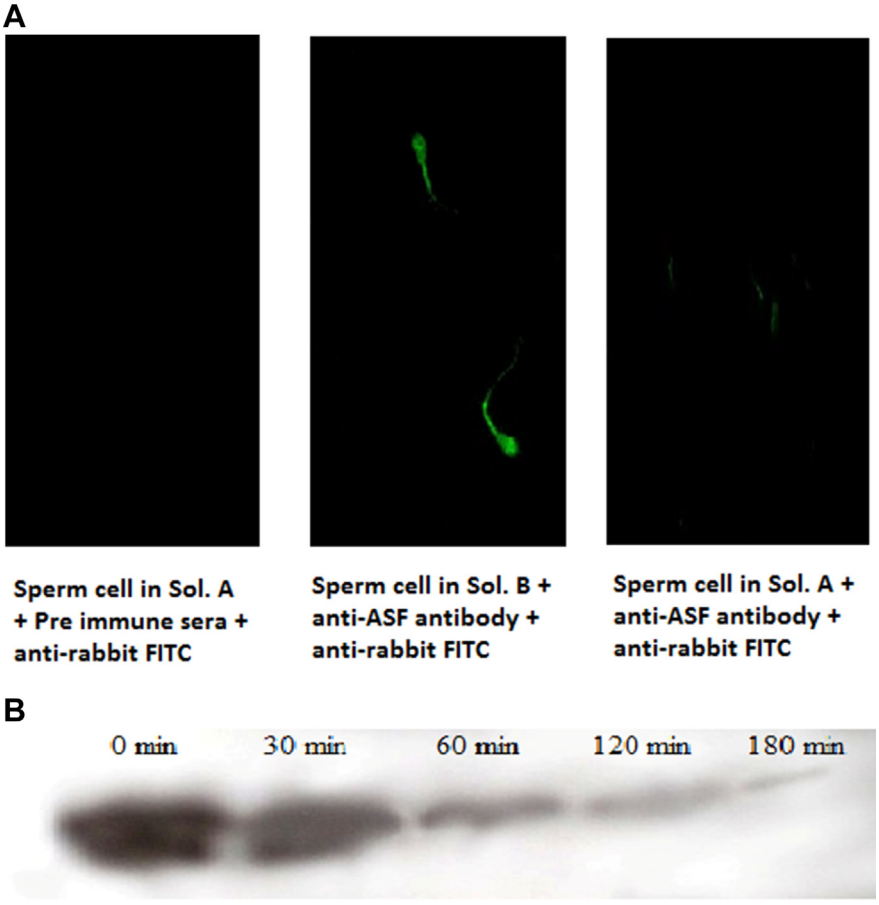

Fig. 2. 2a: Indirect immunofluorescence of mature caprine spermatozoa for ASF recognized by anti-ASF before or after capacitation incubation. Panels (a) capacitated spermatozoa treated with preimmune sera and (b): Non capacitated spermatozoa treated with anti-ASF; panel (c): capacitated spermatozoa treated with anti-ASF after the 180-min incubation. Data were representative of three replicates. **2b**: Incubation time-related decrease of the relative amount of the sperm-bound ASF. Extracts from spermatozoa (5×10^{6} cells/lane) were used for Western blotting techniques using anti-ASF antibody (1: 1000).

Table 1
Data represents amount of ASF present after incubation for a specific time in capacitation medium in terms of density (intensity/area). With the increment in time of incubation the density was continually decreased.

Region Id	Volume (intensity)	Area (pixels)	Density (intensity/area)	Median (intensity)	Local volume (intensity)	Local density (intensity/area)	%Purity
1 (0 min)	119,556,657	2625	45,545.39	48,059	43,484,842	16,565.65	56.28
2 (30 min)	85,116,858	2218	38,375.50	40,092	23,787,806	10,724.89	30.79
3 (60 min)	20,960,149	747	28,059.10	28,013	4,860,296	6506.42	629
4 (120 min)	16,354,195	860	19,015.51	19,018	4,264,453	4958.67	5.52
5 (180 min)	3,708,510	299	12,403.04	12,079	866,499	2897.99	112

3.4. Effect of ASF on sperm cholesterol content

Sperm cholesterol content was expressed as the amount of cholesterol in nM per 10^7 of spermatozoa. Sperm incubated in solution A or B in absence of ASF showed significant difference in cholesterol content. ASF at 0.5 nM concentration had little effect on sperm cholesterol content. However, at 0.75 nM or above concentrations of ASF, it significantly inhibited BSA/bicarbonate-induced cholesterol loss as compared with the control. The cholesterol content of spermatozoa in solution A that had been previously treated with 1 nM ASF or above remained significantly higher (18.2 ± 0.91 nmol/10^7 spermatozoa) than control i.e. without prior ASF treatment; (9.4 ± 0.47 nmol/10^7 spermatozoa).

4. Discussion

During the epididymal passage, the modulation of membrane associated factors seems to be one of the major events [18]. Epididymal plasma components play role during sperm protection and preservation during sperm storage. Most of these proteins are produced post-testicularly in the epididymis [5]. Such factors interact with the sperm membrane in the form of protein–protein interactions or lectin–sugar interactions. Some of these interactions might be specific and of high affinity, others may be of relatively low affinity, the proteins being later are associated with the membrane throughout the maturation and released from the sperm surface at the terminal point, for example, during capacitation [19]. These factors are commonly termed as decapacitation factor.

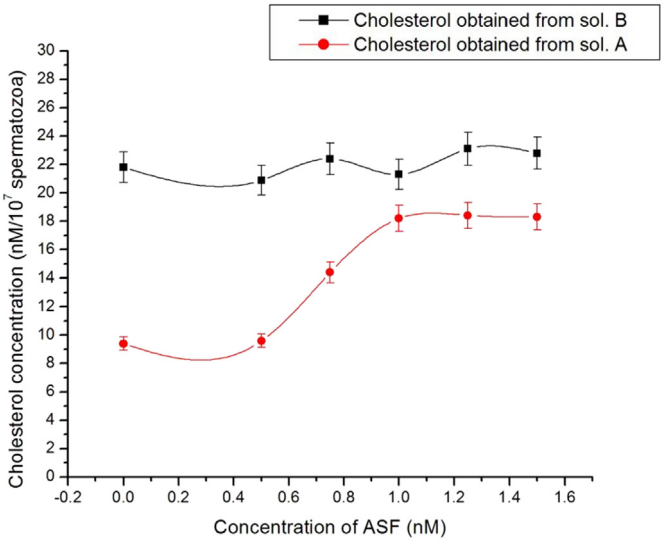

Fig. 3. Effects of different concentrations of ASF in BSA/bicarbonate-induced cholesterol removal of human spermatozoa. Data were represented as mean ± SD of three independent experiments.

Decapacitation factor binding with sperm membrane often found to be carbohydrate specific [20]. Runnebaum et al. [21] have described presence of a ConA-binding (Concanavalin A; *Canavalia ensiformis* lectin) molecules on the acrosomal region of the sperm surface across several species. Epididymal ASF identified and purified earlier is a novel ConA-binding glycoprotein [14]. Additionally we have found ASF has a D-galactose specific lectin like property at acidic pH 6.5 (Unpublished data). ASF could be a novel epididymal D-galactose binding molecule with a terminal mannose or mannopyranoside residue. Reports of pH dependent dissociation of surface molecules are available in other system [22,23]. Molecules characterized with pH dependent dissociation from sperm surface, are comparatively the lesser known entities [24]. By nature, pH of epididymal plasma is acidic [25,26]. So association of epididymal factor with the sperm surface *in vivo* has greater affinity at acidic pH [6,7,12]. All these reports inclined us to investigate the biological role of ASF in sperm cell capacitation.

Merocyanine 540 is a useful fluorescent hydrophobic probe for lipid packing as because it binds preferentially to membranes with highly disordered lipids [27]. It is also sensitive to heat-induced changes in the organization of membrane lipids. Thus M540 is an excellent probe to monitor the alterations in the lipid architecture of the cells. As per our knowledge we have used M540/DAPI combined staining for the first time to detect sperm capacitation. In BSA-bicrbonate induced *in vitro* capacitation study; we have found the proportion of highly destabilized membrane containing spermatozoa was decreased in presence of ASF (Fig. 1). The decline is dose dependent. Increased sperm membrane fluidity is a sign of sperm capacitation. It can be assumed here that ASF acts as a membrane stabilizing factor and increases the membrane rigidity by binding with it.

Epididymal components are majorly shed off from spermatozoa during their ascent to the oviduct [18]. Immunostaining as well as western blot data with anti-ASF antibody confirm ASF shading from sperm surface (Fig. 2a and b). In immunostaining experiments, we have done a qualitative analysis of the localization of ASF which provide pretty low signal in capacitating condition. In western blot the reverse data is actually obtained, i.e. the relative amount of ASF present on the sperm surface after incubation. The densitometric analysis of western blot image is emphasizing residual ASF amount reduction (Table 1). The disappearance of ASF in the capacitating conditions and the ability of ASF to suppress bicarbonate/BSA-induced capacitation are consistent with the view of a regulatory role of ASF on capacitation *in vitro*. It can be predicted that removal of ASF during capacitation allows BSA (albumin) to interact with sperm surface and exert its effect on sperm membrane and initiate capacitation.

As per the concerned reports, albumin specifically removes sterol components from sperm membrane and thereby increases membrane fluidity [28]. We had investigated the residual cholesterol content present in sperm membrane after BSA induced capacitation in presence of ASF. A significant increase in cholesterol concentration in presence of 0.75 nM ASF or above was found (Fig. 3).

Sperm surface lectins are factors present in epididymal or seminal plasma; these lectins interact with sperm membrane at unique microenvironment [29,30]. We have found that ASF possesses a D-galactose specific heamagglutinin activity at acidic pH and maintains D-galactose dependent interaction with sperm membrane (data not shown). The internal pH uprising due to the presence of bicarbonate changes the microenvironment of sperm membrane [31,32]. This might be a probable reason for ASF release. Because in Fig. 2a spermatozoa were incubated for equal time period in capacitating as well as non capacitating medium of same pH 7.2. So, it can be concluded that the removal of ASF allows BSA to access to the sperm membrane sterol. As cholesterol acceptor, BSA increase membrane fluidity and increases percentage of sperm containing highly destabilized membrane and sperm population propagates to capacitation state *in vitro*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.040.

Transparency document

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