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Cell surface changes associated with *in vitro* capacitation and acrosome reaction of goat epididymal sperm by a marine bio-active compound from the snail *Telescopium telescopium*

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ABSTRACT

A protein factor, spermatheca fraction fifty, isolated from the spermatheca/ovotestis gland of a marine mollusc *Telescopium telescopium* by 50% ammonium sulphate precipitation was capable of inducing a capacitation and acrosome reaction of washed goat cauda epididymal spermatozoa *in vitro*. The effect of this isolated bio-active compound on goat sperm cells was comparable to heparin and concanavalinA. The number of acrosome reacted spermatozoa treated with heparin, concanavalinA and spermatheca fraction fifty increased steadily and significantly ($P<0.001$) over the extended period of incubation. Observation concluded that spermatheca fraction fifty could be used for the evaluation of sperm plasmamembrane integrity and/or acrosomal integrity *in vitro*.

Key words: *Telescopium telescopium,* sperm, capacitation, acrosome reaction

Introduction

Before fertilization, mammalian spermatozoa undergo a variety of morphological and biochemical changes which lead to the acquisition of progressive motility and fertilizing ability (BEDFORD, 1966; JOHNSON, 1975). These modifications occur during transit through the epididymis and continue even after ejaculation during the residence of spermatozoa in the female reproductive tract (BOSTWICK et al., 1980; OLSON and DANZO, 1981). The processes of capacitation and acrosome reaction (AR) of spermatozoa in the female reproductive tract are essential for successful fertilization and are characterized

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by removal of epididymal and seminal plasma proteins, alterations in the biochemical characteristics of sperm surface proteins, as well as further alterations in the morphology of the male gametes (REYES et al., 1975; JOHNSON and HUNTER, 1976). Modifications of the sperm surface glycoproteins have been described in some mammalian species during formation, maturation, capacitation and AR of spermatozoa (LASSALLE and TESTART, 1994; DENG et al., 1999).

Sperm glycocalyx modifications/alterations accompanying removal or appearance of specific proteins or glycoproteins in the female reproductive tract resulting in capacitation and AR that are important for gamete recognition and fertilization in mammals have been studied *in vitro* using various lectins (agglutinins) that binds specifically to carbohydrate residues (TÖPFER-PETERSEN et al., 1984; YANAGIMACHI, 1974; PUROHIT et al., 2008). The lectins *Pisum sativum* agglutinin, *Concanavalia ensiformis* agglutinin and *Arachis hypogea* agglutinin have been used to assess acrosome integrity in several mammalian species (AHLUWALIA et al., 1990; FARLIN et al., 1992). *In vitro* lectin-binding assay is very important to predict the functional activity of the spermatozoan surface characters since glycoconjugates are believed to be functionally important in sperm-egg recognition and interaction. As lectins have high specificity to the accessible carbohydrate side chain, they are widely used in studying the architectures of the cell surfaces during cell development and in other biological events (SHARON and LIS, 1974). Lectins labelled with different fluorochromes have been used to analyze the expression, distribution and alteration of the membrane glycoconjugates and exhibited that the distribution of lectin binding sites are restricted to certain sperm domains (CROSS and OVERSTREET, 1987; AHLUWALIA et al., 1990). Due to their ability to bind tightly and reversibly to the cell surface without entering into cells, lectins have gained prominence in probing membrane structures and functions of glycoconjugates.

Lectins have been isolated from a variety of plants, animals and microbial sources (GILBOA-GRABER et al., 1985; LIS and SHARON, 1986). Invertebrates are also rich sources of different types of lectins (YEATON, 1981) and are also found in their haemolymph, albumin gland and in sex organs.

A new sialic acid specific lectin, spermatheca fraction fifty (SF_{50}) obtained from a marine gastropod, *Telescopium telescopium,* from the delta region of the Indian Ocean in West Bengal, India, was found responsible for dose dependant agglutination and immobilization of murine, ovine and human spermatozoa *in vitro* (PAKRASHI and DATTA, 2001). Our present investigation is primarily concerned with the identification of changes in the sperm plasmamembrane (SPM), especially to the outer acrosomal membrane (OAM) if any, after incorporating SF_{50} and comparing its effects with Con A and Heparin by two staining methods to study the changes in the SPM integrity and the occurrence of AR in washed goat cauda epididymal spermatozoa.

Materials and methods

Collection of the snail and preparation of SF_{50}. The cone snails, *Telescopium telescopium* under phylum-mollusca, class-gastropoda, were collected from the estuaries of inter-tidal zone during low tide in the Bay of Bengal near Sagar Island (22^0 19′ N; 80^0 03′ E) in West Bengal, India. 50 adult snails were collected randomly before the rainy season.

Crude cytosol fraction from the spermatheca/ovotestis glands of the snails were isolated (DATTA and PAKRASHI, 2000). Briefly, each gland was dissected out from the body of the snail after breaking the outer shell. Dissected glands were minced carefully in a Petri dish, homogenized at 4 °C with 4 volume of 0.15 M phosphate buffer saline (PBS; 8.76 g/L NaCl, 0.3 g/L NaH_2PO_4, 1.07 g/L Na_2HPO4), pH 7.2. The grinded tissue fragmented further by sonicator at 0 °C set at 100% output for four 15 s brusts at ≥30 s interval for a total period of 1 hour. The sonicated material was spun at 15,000 g for 30 min at 4 °C to discard the cellular debris. Cytosol fraction was aspirated carefully from the centrifuge tubes, passed through a membrane filter (150 µm pore size) and transferred in a beaker with a magnetic stirrer. Ammonium sulphate solution, 50%, was added drop by drop and allowed to stir overnight at 4 °C . The solution centrifuged again at 10,000 g for 30 min at 4 °C. The precipitated protein pellet was collected carefully and dissolved in 2 mL of PBS. Dissolved protein solution was dialyzed against 6 changes of PBS overnight at 4 °C, transferred into a sterile glass vial, lyophilized and kept at -20 °C until experimentation. The lyophilized fraction was named SF_{50} .

Estimation of total protein and pH of SF_{50}. Total protein concentration of SF_{50} (1 mg/mL in distilled water) was estimated (LOWRY et al., 1951) against standard solutions of BSA (globulin free). The readings were recorded by a spectrophotometer at 280 nm. The pH value of SF_{50} solution was estimated by pH meter.

Preparation of goat cauda epididymal spermatozoa. Testes from two proven, healthy and adult Black Bengal bucks were collected from the local abattoir immediately after slaughter and were brought to the laboratory in a thermos flask containing normal saline solution (NSS; 0.9%) at 37 °C. Tunica albuginea were removed from the testes and washed thoroughly with NSS. The fat-pad, blood vessels, adipose and connective tissues were removed carefully. Whole epididymides (4 in number) were dissected out from each testis. As per anatomical position, each portion of the cauda epididymis was cut and placed separately into four different polystyrene Petri dishes containing 5 mL of PBS. Each cauda epididymis was minced separately and carefully and allowed to suspend in the medium for 10 min. To obtain sperm, gentle pressure on the excised tissues was given by clean glass rod. The resultant suspensions in the medium were filtered through separate nitex membrane to free the cellular debris. The filtrates were collected into different clean glass test tubes, centrifuged twice at 500 g for 10 min and the supernatants were discarded

each time. Finally, 2 mL of capacitation medium (DE JONGE et al., 1988) was added to each sperm pellet separately, vortexed gently for 3 s and kept at 37 °C in an incubator in humid condition containing 5% CO_2 in air for 30 min allowing sperm cells to swim-up into the medium. After incubation, 50 µL of the sperm suspension from each tube was aspirated carefully from upper layer of the medium and resuspended again with 500 µL of capacitation medium in other sterile test tubes separately to adjust the final concentration to 2.5×10^7 cell/ mL using Neubauer counting chamber and incubated again as above for subsequent experimentations.

Sperm cells were observed under Leitz phase contrast microscope (×200, ×400) and the percentage of progressively motile spermatozoa from each sample was determined subjectively by scoring 200 individual sperm cells randomly from 10 different microscopic fields. Only sperm samples exhibiting >60% progressively motile were considered to avoid non-specific agglutination. These procedures were performed at room temperature (25 ± 2 °C) and completed within 2 h after slaughter.

Sperm treatment. Fifty microlitres of sperm suspension from each tube was transferred into different Eppendorf tubes. Heparin, Con A and SF_{50} prepared in capacitation medium were mixed separately to the individual sperm suspension at a final concentration of 25 µg respectively. All the tubes were vortexed gently for 3 s and incubated at 37 °C as above. Observations were made under phase contrast microscope (×200; ×400; ×1000) after 30 min and 60 min respectively including the control (capacitation medium only). A total of 6 replicates were considered for each treatment including the control and their mean results were considered.

Assessment of spermatozoan motility and arosomal status. Spermatozoan motility and visualization of the AR were evaluated under a Leitz phase contrast microscope (×100, ×200 and ×400, ×1000) to assess sperm capacitation. The whiplash-like motion of the sperm tails was evaluated after the stipulated incubation periods by placing 5 µL of each sperm-protein suspension, including the control, on separate clean microscopic glass slides and observing them under the Leitz phase contrast microscope (×100, ×200). An arbitrary score of 0 to 100 was used to assess the spermatozoan motility. Sperm suspension, 10 µL, from each Eppendorf tube was placed on separate clean dry microscopic glass slides and smeared. Smears were air-dried and the slides were incubated at 37 °C for 10 minutes in a coupling jar containing 0.8% Bismark brown stain, (pH 1.8). After incubation, the slides were washed twice in distilled water, subsequently incubated again in 0.8% Rose Bengal stain (pH 5.3) for 25 min at room temperature (25 ± 2 °C). After incubation, the slides were washed in distilled water and finally passed through an alcohol dehydration series (50%, 70%, 95%, 100% ethanol) (DE JONGE et al., 1989). After drying, the smears were examined under oil immersion (×1000) and the acrosomal status evaluated. Three sequential stages of AR viz. acrosome swelling, acrosome vesiculation and acrosome

shedding were considered. Scoring was done randomly counting 100 spermatozoa from each slide and the percentage of spermatozoa that underwent AR were calculated. Giemsa stain (HANCOCK and CREVAN, 1957) was also performed simultaneously to observe the status of acrosome reacted spermatozoa.

All the data were analyzed statistically (SNEDECOR and COCHRAN, 1980; HERBERT, 1980).

Results

Total protein concentration and pH value of SF_{50} were 109.5 µg/mL and 7.4 respectively.

Although *in vitro* capacitation has been extensively studied in various species, the condition for optimal concentration varies with the medium and the time of incubation. Observations revealed that spermatozoan progressive motility improved after the addition of BSA and the whiplash-like motion of the spermatozoa was found immediately and was maximum in the samples treated with heparin, Con A and SF_{50} respectively, as compared with the control samples. The findings (Table 1) also revealed that SF_{50} treated sperm samples at different periods of incubation exhibited a reduced trend of motility (72% and 68% at 30 and 60 min, respectively), like the other treatment groups including the control, however, they were non-significant when compared with their corresponding values. However, spermatozoan motility in all the groups were to decrease but capacitation was achieved better after an extended time of incubation. Moreover, the percentage of acrosome reacted spermatozoa varied significantly ($P<0.001$) between the two incubation periods and increased steadily over the time in all the treated groups.

Table 1. Spermatozoan motility (%) and acrosome reacted spermatozoa (%) at different time intervals treated with heparin, Con A and SF_{50}

	Spermatozoan motility (%)		Acrosome reacted spermatozoa (%)	
	Capacitation period		Capacitation period	
Inducer	30 min	60 min	30 min	60 min
Capacitation medium (Control)	82.45 ± 0.27	80.15 ± 0.75	15.43 ± 0.4	25.35 ± 0.4**
Heparin (25 µg)	80.48 ± 0.26	78.22 ± 0.5	27.36 ± 0.23	51.46 ± 0.23**
ConA (25 µg)	77.46 ± 0.26	75.09 ± 0.73	23.16 ± 0.5	44.32 ± 0.38**
SF_{50} (25 µg)	72.43 ± 0.4	68.30 ± 0.37	21.57 ± 0.24	42.42 ± 0.26**

Values are expressed as Means ± SE; n = 6 (for each treatment). **P<0.001, Significant differences with corresponding values; according to independent *t*-test.

The data presented in Table 1 also exhibited that the number of spermatozoa that underwent spontaneous AR by heparin and Con A was also comparable with SF_{50}.

Different types of acrosomal architectures and/or vesiculations were observed by the double staining method (DE JONGE et al., 1988), where spermatozoan heads that had an intact acrosomal cap took red stain and the absence of it, i.e. acrosome reacted, had a tinged white colour over the head area. However, the Giemsa stained acrosome reacted spermatozoa had a faint violet in colour over the head area in contrast to non-reacted spermatozoa where the acrosome had a deep violet stain.

Discussion

Acrosomal exocytosis is a prerequisite for mammalian fertilization. During residence in the female genital tract spermatozoa undergo biochemical and functional changes collectively referred to as capacitation (YANAGIMACHI, 1974). Ejaculated or epididymal spermatozoa can also be capacitated *in vitro* by incubation in a chemically defined medium. *In vitro/in vivo* capacitation is a result of multiple molecular changes in sperm plasma membrane proteins/glycoproteins and lipid components allowing transflux of ions that are important in initiating hyperactivation, capacitation and the AR.

Lectins are useful tools as a probe for examining various modifications in the distribution of cellular glycoconjugates/cell surface receptors and the changes that occur in glycoconjugates during cell differentiation and maturation (SPICER, 1993). The most widely used lectin is Con A, which binds preferentially to oligosaccharides with glycosidically linked mannose residues. Con A binds especially to certain sugar residues, possibly by the formation of carbohydrate-protein-cross-linked complexes (MONDAL and BREWER, 1992) and Con A binding is found to be prominent at the anterior part of boars' sperm head (TÖPFER-PETERSEN et al., 1984), which is also similar to the present experiment with goat caudal spermatozoa treated with SF_{50}.

In the present experiment, we also included the known capacitator agent heparin, and glycosaminoglycans with highly anionic charge density. The pentasaccharide of heparin that possesses high affinity for antithrombin III has a specific sulfation pattern that is essential for binding (PARRISH et al., 1988). Heparin works on sperm cells by increasing Ca^{++} uptake, intracellular alkalization and elevation of cAMP levels as observed in bovine and hamster spermatozoa (FIRST and PARRISH, 1987; SUSKO-PARRISH and PARRISH, 1988). Moreover, the reason for inclusion of BSA in the capacitation medium was that this protein facilitates capacitation by altering fatty acids and/or cholesterol from the sperm plasma membrane.

The present *in vitro* study clearly indicates that SF_{50} was able to induce capacitation and AR of washed goat cauda epididymal spermatozoa, like Con A and heparin. How SF_{50} acts on spermatozoa is not known clearly, plausibly it could be that SF_{50} acts by the formation of glycoconjugates within the sialic acid moiety content in the membrane component of spermatozoa (PELEG and IANCONSCU, 1966), moreover it could be that acceleration of

spermatozoan motility, capacitation and the AR occurred through cAMP induced by Ca^{++} influx after the addition of SF_{50}. Other explanations could be that cross-linked complexes may form between SF_{50} and its specific multivalent carbohydrates and glycoconjugates on the sperm surface and are able to modify membrane permeability and thus to induce AR. Furthermore, another reason might be presumed that the biochemical characteristics of lectins (including sugar specificity, amino acid compositions, molecular size and valency) as well as the saccharide structures recognized by lectin or its biological effects on the cell (as mitogenicity or cytotoxicity) or its ability to induce signal transduction (GUPTA et al., 1994; RINI, 1995) may be responsible for inducing sperm AR. Plausibly, SF_{50} may act on the sperm cells through any of these pathways.

Importantly, sialic acid is known to exert important functions in spermatozoa conferring a net negative charge (MOORE, 1979), masking sperm maturation antigen (TOSHIMORI et al., 1988), preventing phagocytosis of spermatozoa by macrophages of the female genital tract (TOSHIMORI et al., 1991) allowing spermatozoa to bind to epithelial cells in the reservoir zone of the oviduct (DE MOTT et al., 1995) thus increasing the ability of spermatozoa to bind to zona pellucida (PETERSON et al., 1986) and egg plasmamembrane (DACHEUX et al., 1983).

The AR test is a stable parameter of sperm function test and is useful to predict fertilization success, where SF_{50} could be utilized for this test. Sperm glycocalyx modifications in this study observed after incorporating SF_{50} may provide insight into the molecular modifications accompanying capacitation and AR, and could improve the diagnosis of reproductive problems of subfertile goats and males of other species. Hence, the present study concluded that SF_{50}, a new and novel bio-active compound from a marine source, glycoprotein in nature and specific for sialic acid (PAKRASHI and DATTA, 2001) could be a useful tool for examining the ability of sperm cells to undergo capacitation and the AR, and this approach might also be useful for diagnosis of reproductive problems in the distribution of lectin receptors in spermatozoa of subfertile males. However, this is the first report that agglutinin obtained from *Telescopium telescopium* was capable of inducing capacitation and acrosome reaction in mammalian spermatozoa. Furthermore, SF_{50} needs more evaluation for a deeper understanding for future use.

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SAŽETAK

Bjelančevina, 50 frakcija spermateke, izdvojena iz spermateke/ovotestisa morskoga mekušca *Telescopium telescopium* u postupku precipitacije s 50%-tnim amonijevim sulfatom potaknula je *in vitro* kapacitaciju i akrosomsku reakciju ispranih spermija iz nuzjajeta jarca. Učinak ovoga bioaktivnog sastojka na spermije jarca bio je usporediv s učinkom heparina i konkanavalina A. Broj spermija s akrosomskom reakcijom obrađenih heparinom, konkanavalinom A i frakcijom 50 spermateke pouzdano je i značajno ($P<0,001$) povećao razdoblje inkubacije. Zaključuje se da bi se frakcija 50 spermateke mogla rabiti za procjenu integriteta plazmalne membrane i/ili akrosomskog integriteta *in vitro*.

Ključne riječi: *Telescopium telescopium*, sperma, kapacitacija, akrosomska reakcija