

In vitro sperm agglutination and spermicidal activity of protein isolated from a marine mollusc *Telescopium telescopium* (Gastropoda)

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The crude extract of spermatheca gland and its 50% ammonium sulphate fractionated protein (SF-50) were found to have sperm agglutinating and immobilizing effect on murine, ovine and human spermatozoa *in vitro*. The effects were dose-dependent. Human unwashed and washed sperm cells were found dead within 20 sec after application of 6 mg/ml and 5 mg/ml of SF-50 respectively. Hemolymph of the mollusc, however, showed much less agglutinating effect than that of SF-50. SF-50 caused coiling of spermatozoan tail, swelling of acrosome and neck region and extrusion of the sperm tail membrane. The protein is highly soluble in water and contains sialic acid binding lectin.

The carbohydrate binding and cell agglutinating proteins have been isolated from a variety of animal and plant sources¹⁻⁴. Invertebrates are also rich sources of various agglutinins found mainly in the hemolymph and sex organs⁴. These agglutinins are known as natural antibodies as they can agglutinate cells or precipitate glucoconjugates. The purpose of the present study was to intercept fertility through agglutination or immobilization of spermatozoa by spermatheca extract of the snail *Telescopium telescopium* so as to hinder their forward progression and thus causing impediment to the final stage of approach to the ovum.

Materials and Methods

The snails, *Telescopium telescopium* (Gastropoda) were collected from the Sagar Island in West Bengal. They were allowed to empty their gut over a period of three days and then narcotized in isotonic MgCl₂ solution. After carefully removing the whole body, the hemolymph was collected by a tuberculin syringe and kept in a sterile container. Spermatheca and intestine were dissected from the body and thoroughly washed with PBS. All the organs were processed separately⁵.

For isolation of the partially purified protein, lyophilized 5 mg powder of spermatheca gland was dissolved in 20 ml of PBS and the protein (SF-50) was precipitated by 50% ammonium sulphate⁶. SF-50 then dissolved in 5 ml of PBS and dialyzed overnight with six changes of double distilled water at 4°C. The dialyzed protein solution was lyophilized and kept at

-20°C. To evaluate *in vitro* effect of the crude extract of spermatheca gland, hemolymph, intestine and also SF-50 on mammalian sperm cells, collected from rat, goat and human semen, test solutions were prepared in BWW medium⁷ (stock concentration 5 mg/ml; 0.5%).

For preparation of the sperm suspension, testes were dissected out from adult male, colony-bred Sprague-Dawley rats after anaesthetizing the animals. Goat testes were collected from local abattoir. Spermatozoa from rat and goat were collected from cauda epididymal region by mincing and kept separately at 37°C for 30 min in 1 ml of BWW medium preheated to 37°C in 15 cm polystyrene tubes.

Human semen samples collected from six fertile donors were allowed to liquefy at 37°C for 45 min and 0.5 ml of each semen sample was washed in 0.5 M PBS at pH 7.2. Spermatozoa from rat and goat were passed separately through double glass wool column to free the samples from debris prior to centrifugation.

For carbohydrate moiety test all the tubes containing sperm samples were washed twice by centrifugation at 1100 rpm for 10 min, discarding supernatant each time. Finally, 1 ml of BWW medium was added carefully to each sperm pellet. Washed human sperm pellets were also incubated with 1ml of BWW medium with human serum albumin (HSA). All the loosely capped tubes were incubated for 45 min at 37°C in an atmosphere of 5% CO₂ in air allowing the sperm to swim-up into the medium. Before experiments, sperm cells were carefully collected from the

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upper layer of the medium using Pasteur pipettes. Percentage of motile spermatozoa and their concentrations were assessed by Makler counting chamber⁸. Highly progressive motile sperm cells were collected to avoid non-specific agglutination. For observation, 60×10^6 /ml of sperm concentration was considered and for the agglutination and immobilization tests semen samples were diluted with BWB medium to a concentration of 60×10^6 sperm/ml. Only those samples having an overall sperm motility of more than 55% after dilution were used for *in vitro* tests.

Only preliminary agglutination and immobilization tests were done with rat and goat sperm but detailed works were performed on human sperm cells.

Sperm agglutination test—The slide agglutination test^{9,10} was performed by placing 50 μ l of washed spermatozoa from rat, goat and six human individuals including their semen separately on clean microscopic glass slides and 5 μ l of the different test solutions, prepared from lyophilized extracts of spermatheca gland, hemolymph, intestine and SF-50 (stock concentration 5 mg/ml; 0.5%) in BWB medium, adjusting pH at 7.4, were mixed separately and observed immediately under Leitz inverted microscope ($\times 100$). All the observations were recorded within 60 sec from 10 different microscopic fields.

Sperm immobilization test—Immobilization effect of different test solutions on sperm cells was ascertained¹¹ by placing 20 μ l of each washed sperm suspension and human semen samples individually into separate Eppendorf tubes. Test solutions, 100 μ l each, were mixed into different Eppendorf tubes and vortexed for 2 sec. Observations ($\times 200$) were made within 20 sec as above placing 20 μ l of each sperm-protein mixture on clean glass slides.

Evaluation of sperm agglutinating and immobilizing titer—To evaluate agglutination and immobilization titer of different concentration of SF-50, 20 μ l aliquots of washed human spermatozoa and human semen from six different donors were taken into different Eppendorf tubes and test solutions, 100 μ l of each were added (1:5) individually. The test solutions were prepared with SF-50 in BWB medium with double dilution at different concentrations (6, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 mg). The sperm protein mixture were mildly vortexed for 2 sec. Observations ($\times 200$) were made as above within 20 sec and 60 sec respectively for the (i) number of aggluti-

nating clusters and (ii) percentage of immobilized spermatozoa from 10 different microscopic fields.

Spermatozoa motility recovery test—For spermatozoan motility recovery test¹², 20 μ l of human whole semen from six individuals were mixed with 100 μ l of 5 mg/ml of SF-50 solution into six different test tubes and vortexed for 2 sec. Observations for spermatozoan motility were made within 20 sec. Any evidence for spermatozoan motility was interpreted as negative. When motility was absent, 0.25 ml of Baker's buffer (Na_2HPO_4 1.416 g/l, KH_2PO_4 0.12 g/l, NaCl 0.8 g/l, glucose 12 g/l, distilled water up to 1000 ml) was added to each test tube and incubated at 37°C on water-bath. Observations ($\times 400$) were made after 30 and 60 min.

Scanning electron microscopy—The nature of agglutination, i.e., the binding pattern of spermatozoa and their type of coiling and also the changes of sperm surface plasma membrane were studied by scanning electron microscopy. The washed human sperm cell (20 μ l) was mixed with 100 μ l of 0.312 mg/ml of SF-50 in an Eppendorf tube and vortexed for 2 sec. The sperm protein mixture was then allowed to incubate for 5 min at 37°C; the reacted sperm was then fixed overnight with 2.5% glutaraldehyde in PBS at 4°C and the mixture was centrifuged twice in PBS at 1100 r.p.m. for 5 min each. The sperm pellet then dehydrated and coated with gold before being examined by scanning electron microscope. The control study was run side by side with BSA (bovine serum albumin).

Lectin specificity (carbohydrate moiety test)—Lectin specificity test¹³ was performed to ascertain the lectin-like properties of the SF-50, if any. The specificity of sperm agglutination by SF-50 was tested using different inhibitors (competitive sugars) dissolved in BWB medium (1 mg/ml) containing human serum albumin (35 mg/ml), SF-50 solution (0.312 mg/ml) that showed the agglutination of washed human spermatozoa averaging 3 to 4 clusters in each field was considered. The protein solution (20 μ l) was taken into different wells of a round bottomed microtiter plate and 20 μ l of each sugar solution was added to each well, mixed thoroughly and incubated at 37°C in humid condition for 30 min. Thereafter, 20 μ l of the washed human sperm cells were added to each well and incubated again for 1 hr. Each sperm mixture was then examined as above for presence or absence of any agglutinating cluster. A control study without SF-50 was evaluated simultaneously.

Total protein¹⁴, osmomolality and pH of the crude spermatheca extract and "SF-50" were estimated after dissolving 1 mg lyophilized powder of each in 1 ml double distilled water separately. The pH of the solution was measured by pH meter (ELICO, India) and osmomolality was recorded by osmometer (Knauer Berlin).

Results

Sperm cells were collected from rat, goat and human by swim-up procedure and 60% progressively motile and $60 \times 10^6/\text{ml}$ spermatozoan concentration was considered. Total protein content of the crude extract of spermatheca gland and SF-50 were 285 $\mu\text{g}/\text{ml}$ and 115.5 $\mu\text{g}/\text{ml}$, and the pH values were 7.86 and 7.62 respectively. The osmomolality of SF-50 solution was 253 mOsm.

The sperm agglutinating property was observed in the crude extract of spermatheca and its purified fraction SF-50 as well as in the hemolymph. Significant variation ($P < 0.01$) in the number of sperm agglutinating clusters were observed in between hemolymph and spermatheca extract. The pattern of agglutination by sperm cells in all the species was tail to tail type.

Washed spermatozoa from the three species were immobilized within 20 sec by 5 mg/ml of crude extract of spermatheca and SF-50. The percentage of immobilized sperm cells treated with crude extract of spermatheca or SF-50 were found more significant ($P < 0.01$ and $P < 0.01$ respectively) than sperm cells treated with hemolymph. Other glandular extract had no effect on sperm cells.

Evaluation of sperm agglutination and immobiliza-

Table 1—Evaluation of agglutination and immobilization titer of "SF-50" on human semen *in vitro*

SF-50 (mg/ml)	Human Semen (Spermatozoa $60 \times 10^6/\text{ml}$)			
	No. of Clusters		Immobilized Spermatozoa (%)	
	20 sec	60 sec	20 sec	60 sec
6	3.5 $\pm$ 0.19	3.65 $\pm$ 0.19	100	100
5	3.6 $\pm$ 0.89	5.83 $\pm$ 0.79	62 $\pm$ 0.69	100
2.5	4.11 $\pm$ 0.14	66.23 $\pm$ 0.28	44 $\pm$ 0.96	83 $\pm$ 0.53
1.25	4.85 $\pm$ 0.17	5.88 $\pm$ 0.9	38 $\pm$ 0.65	62 $\pm$ 1.76
0.625	x	5.06 $\pm$ 0.22	24 $\pm$ 0.66	41 $\pm$ 0.94
0.312	x	4.05 $\pm$ 0.17	x	24 $\pm$ 0.584
0.156	x	3.05 $\pm$ 0.15	x	10 $\pm$ 0.53
0.078	x	x	x	x

Value expressed as mean $\pm$ SEM; n = 6

20 μl of the test sample and 100 μl of the "SF-50" solution
Observation ($\times 200$) from 10 different microscopic fields were made at 20 sec and 60 sec.

x = absent

tion titer with different amount of SF-50 revealed that the number of clusters and percentage of immobilized spermatozoa varied with the concentration of SF-50 used as well as with the incubation period. Moreover, with the increase in the amount SF-50, immobilization became more pronounced than agglutination. Unwashed and washed human sperm cells immobilized within 20 sec by SF-50 at a concentration of 6 mg/ml and 5 mg/ml respectively (Tables 1, 2).

The striking phenomenon of sperm agglutination observed was that when a treated sperm cell came in contact with another cell during movement, a sudden attachment of both tails occurred; the heads did not stick to each other. The size of the clusters became larger with more incubation time because free motile cells tended to adhere to clusters. It was also observed that the pattern of intense, vigorous forward motility of sperm cells changed after addition of "SF-50". Thus, just after addition, spermatozoan motility increased with whiplash like movement. The motility, however, decreased gradually and the free cells lost

Table 2—Evaluation of agglutination and immobilization titer of "SF-50" on washed human sperm cells

SF-50 (mg/ml)	Washed human sperm cells (Spermatozoa $60 \times 10^6/\text{ml}$)			
	No. of clusters		Immobilized spermatozoa (%)	
	20 sec	60 sec	20 sec	60 sec
6	x	x	100	100
5	2.5 $\pm$ 0.12	7.83 $\pm$ 0.6	100	100
2.5	2.95 $\pm$ 0.09	5.68 $\pm$ 0.18	42 $\pm$ 1.44	82 $\pm$ 0.37
1.25	4.75 $\pm$ 0.3	6.43 $\pm$ 0.27	40 $\pm$ 0.74	68 $\pm$ 0.71
0.625	4.9 $\pm$ 0.14	66.73 $\pm$ 0.22	20 $\pm$ 0.45	47 $\pm$ 0.91
0.312	3.9 $\pm$ 0.2	6.01 $\pm$ 0.18	11 $\pm$ 0.93	42 $\pm$ 0.52
0.156	3.5 $\pm$ 0.16	3.9 $\pm$ 0.08	x	9 $\pm$ 0.53
0.078	x	2.56 $\pm$ 0.13	x	x

Values expressed as mean $\pm$ SEM; n = 6

20 μl of the test sample and 100 μl (1:5) of the "SF-50" solution
Observations ($\times 200$) from 10 different microscopic fields were made at 20 sec and 60 sec.
x = absent.

Table 3—Inhibition activity of various sugars on human sperm agglutination

Inhibitor	Concentration	Sperm agglutination
D-galactose	1 mg/ml	0
D-galactosamine	-do-	0
D-glucose	-do-	0
Transferrin	-do-	0
Fetuin	-do-	0
C-fucose	-do-	0
Mannose	-do-	0
NeuAc	-do-	+
NeuGc	-do-	+

0" indicates non-inhibition of agglutination

"+" indicates inhibition of agglutination

forward progression to oscillatory movement of the tail or whirling or throbbing movement of the head or tail and ultimately became immotile and dead. The immobilized sperm cells did not revive motility after treatment with Baker's buffer.

Most of the sperm tails had more coiling than the control. The number of coiled tail spermatozoa varied with the doses of protein used, being higher at lower doses. Various types of loops were formed in the sperm tail after treatment with "SF-50". The pattern of binding or attachment with one sperm cell to another was due to formation of screw type notch between the tails. The "SF-50" treated sperm showed swelling in the acrosome and neck region as well as extrusion of the tail membrane. In the normal spermatozoa no such anomaly was observed. The carbohydrate moiety test revealed that sperm agglutination by "SF-50" was inhibited by NeuAc and NeuGc (Table 3).

Discussion

Both the agglutinating and immobilizing effects exerted by the extract obtained from the gland spermatheca of *Telescopium telescopium*, as well as by "SF-50" on murine, ovine and human spermatozoa *in vitro*, indicate the plausible presence of complex protein mixture. The nature of agglutination of sperm cells suggested the presence of membrane antigens on the sperm surface^{15,16}. The tail to tail agglutination pattern of spermatozoa in the present experiment suggests recognition of specific membrane antigens by "SF-50" on different parts of the sperm tail.

It is not clear as to how the surface antigen could be involved in agglutination. However, the plausible explanation could well be the formation of glycoconjugates with the sialic acid moieties contained in the membrane component of spermatozoa¹⁷ with "SF-50". Employing sialic acid analogs it was revealed that lectin from *Telescopium telescopium* is indeed specific for sialic acid. All other sugars did not inhibit this lectin. It has also been revealed from the experiment that number of sperm agglutinating clusters increased when incubation period extend in lower concentration. Lectin-induced agglutination is complex and the molecular basis for the observed increase in agglutinability is unknown¹⁸, it seems likely that the surface alterations are selective and not simply change in non-specific properties e.g. cell-surface charge, surface deformability or effects secondary to altered sperm motility¹³.

Baker's buffer failed to recover spermatozoan motility in treated sperm, indicating presence of spermicidal property in spermatheca or in its purified protein fraction. The evidence from SEM indicated presence of coil-tailed spermatozoa, swelling of the acrosomal cap and neck region and structural damage to the plasmamembrane of spermatozoan tail are the plausible factors for immobilization and/or death of the spermatozoa. It is known that irreversible bending or coiling of the sperm tail indicate the sign of structural damage, also swelling beyond the critical volume of spermatozoa cause rupture of the plasmamembrane, extrusion of the sperm tail leads to death of the sperm cells¹²⁻²⁰, which favour the above findings.

Because of its human sperm agglutinating and spermicidal effects within a few seconds of application without any apparent local irritability, observed antimicrobial properties (in press), sialic acid specificity and high solubility in water, "SF-50" deserves in-depth *in vivo* studies as a probable vaginal contraceptive.

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