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Microbial Sperm Immobilization Factor from *Pseudomonas aeruginosa* as a Contraceptive Agent: An Experimental Study

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Abstract

Various studies have shown *Pseudomonas aeruginosa* to be one of the most prevalent organisms involved in causing infertility in males and females. To explore the possible mechanism underlying this process, standard strain of *Pseudomonas aeruginosa* (MTCC 3542) was employed and it was found to cause complete immobilization of mouse spermatozoa *in vitro*. Further, only cell-free supernatant showed immobilization activity, indicating that the sperm immobilization factor might be released extracellular by the organism, as no activity was observed with the washed cells. This bioactive molecule from the supernatant was precipitated out with ammonium sulphate at the saturation of 60% to 80% and purified by gel permeation chromatography followed by ion exchange chromatography. The Sperm Immobilization Factor (SIF) was found to be a ~16 kDa protein. SIF at a concentration of 50 µg was able to cause 100% immobilization of mouse spermatozoa within 30 min of incubation at 37°C, whereas a concentration of 65 µg resulted in instant loss of viability of mouse spermatozoa and a total loss of Mg^{++} ATPase activity was observed at a concentration of 100 µg. Further, *in vivo* studies showed that a single intravaginal application of SIF (10 µg) before mating completely prevented conception in female mice. Also, no pregnancy related changes were observed from histological examination in the reproductive organs (i.e. ovary and uterus) of female mice receiving 10 µg of SIF in comparison to female mice receiving PBS. Thus, SIF with its spermicidal activity could be developed as a potent vaginal contraceptive for future use.

Keywords: Spermatozoa; *Pseudomonas aeruginosa*; Immobilization; Fertility outcome

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Introduction

Spermicides are the agents which immobilize or kill the spermatozoa in the female genital tract. They are deep inserted in the vagina near the cervix prior to intercourse to prevent pregnancy [1]. Currently available spermicides typically comprise detergent ingredients which can be either neutral surfactants *viz.* isononyl-phenyl-polyoxyethylene ether or nonoxynol-9 (N-9), p-menthanyl-phenylpolyoxyethylene (8,8) ether or menfegol, and isooctylphenyl polyoxyethylene (9) ether or octoxynol-9 (O-9) [2,3] or the cationic surfactant benzalkonium chloride or the anionic surfactant sodium docusate (dioctyl sodium sulfosuccinate) [4]. The spermicidal activities of these detergent-type contraceptives are associated with their structural affinity to the cell membrane lipids [5,6] thus have dire effect on the epithelial cells and normal flora of vagina [7-11]. Detergent-type spermicides have been used for many years in creams, gels, foams, and sponges, suppositories, and more recently in film and condom lubricants. The most commonly used spermicidal contraceptive worldwide is N-9 [12,13]. At cytotoxic level N-9 displays spermicidal and antibacterial/antiviral activity *in vitro* against pathogens responsible for sexually transmitted diseases (STDs) [14]. But frequent use of N-9 as a vaginal contraceptive/microbicide has been associated with an increased risk of vaginal or cervical infection, irritation, or ulceration [15-18]. Thus a new, effective, acceptable, and safe vaginal spermicide lacking detergent-type membrane toxicity may offer significant clinical advantage over the currently available detergent spermicides.

In a systemic search for developing a new spermicidal microbicide, an earlier study on a microbial origin antimicrobial peptide nisin has shown good contraceptive efficacy *in vitro* as well as *in vivo* [19]. Also, microorganisms such as Bacillus subtilis, *P. aeruginosa*, *E. coli*, and Candida albicans isolated from the cervices of infertile women have also been reported to inhibit sperm motility *in vitro* [20]. The underlying mechanism behind inhibiting the sperm motility was conjectured to be the sperm impairing factors of these organisms. Hence, the present study was intended with an aim

Table 1: Effect of SIF on Mg^{++} ATPase activity of mouse spermatozoa.

S. No.	Concentration of SIF (µg)	Units of ATPase (SIF)
1	25	540
2	50	180
3	100	0

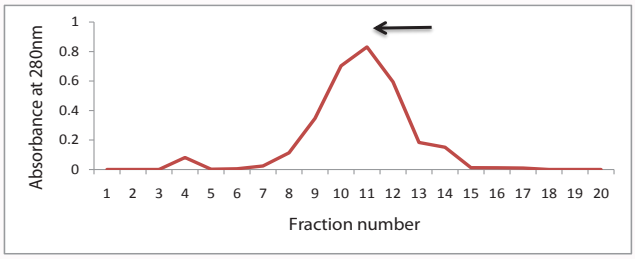

Figure 1: Elution pattern of SIF from *P. aeruginosa* after gel filtration through Sephadex G-200 column showing the presence of SIF in fractions 9-12 with a peak value in fraction 10.

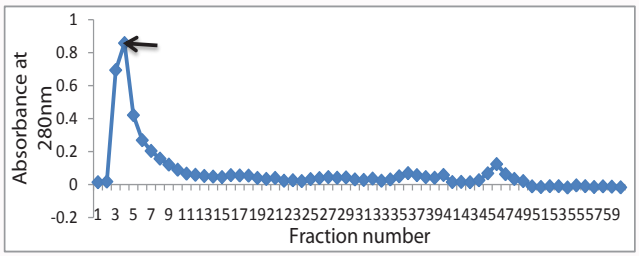

Figure 2: Elution pattern of SIF from *P. aeruginosa* after gel filtration through DEAE column showing the presence of SIF in fractions 3-5 with a peak value in fraction 4.

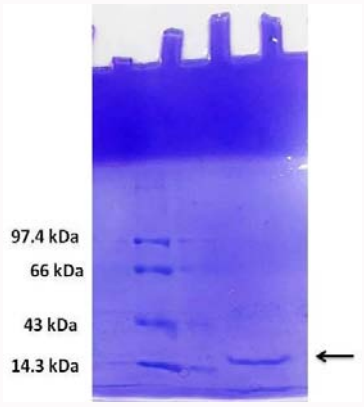

Figure 3: SDS-PAGE showing a purified band of SIF of ~ 16 kDa.
Lane 1: Standard protein marker
Lane 2: Purified SIF.

to isolate and purify the sperm impairing factor form *P. aeruginosa* and to evaluate its impact on mouse sperm parameters and on fertility outcome in female mice.

Materials and Methods

Microorganisms

The standard strain of *P. aeruginosa* (MTCC 3542) used in the present study was already available in the laboratory. *P. aeruginosa* was capable of significantly decreasing the sperm motility *in vitro*.

Experimental animals

Sexually mature, 4 to 5 week old female (22 g ± 2 g) BALB/c and 5 to 6 week old male (25 g ± 2 g) mice were used in the present study. The mice were maintained under standard laboratory conditions in propylene cages bedded with clean rice husk at 20°C to 25°C, in well aerated animal room of the department of microbiology, Punjab University, Chandigarh. All the animals were given standard pellet diet and water ad libitum. Animals were allowed to accustom to the new housing and experimental conditions for at least one week. All the experimental protocols were approved by the Institutional Animals Ethics Committee of the Punjab University, Chandigarh vide letter no PU/IAEC/S/16/142. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Extraction and Purification of Sperm Immobilization Factor (SIF)

72 h old culture of *P. aeruginosa* grown in BHI broth was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant so obtained was subjected to ammonium sulphate precipitation so as to get 20%, 40%, 60%, 80%, and 100% saturation. The precipitates so obtained were dissolved in a minimum amount of PBS (50 mM, pH 7.2). The precipitated protein was dialyzed against PBS under cold conditions and checked for sperm immobilization.

Molecular sieving

Further purification of SIF consisted of filtration through a Sephadex G-200 column (2 cm × 31 cm) equilibrated and eluted with PBS. The precipitated (60% to 80% saturation) and dialyzed protein dissolved in PBS was applied to column. Fractions of 3 ml each were collected and the absorbance of each fraction was read at 280 nm. Fractions (9-12) showing the immobilization activity were pooled and concentrated against polyethylene glycol at 4°C.

DEAE-cellulose column chromatography

The pooled and concentrated fractions after molecular sieving through G-200 were passed through DEAE cellulose, an anion exchange column. First of all, 80 mL of elution buffer (50 mM pH 7.2) were allowed to run down the column. Final elution was done with 0.05 M, 0.1 M, 0.2 M, 0.4 M, and 0.6 M NaCl dissolved in PBS (50 mM, pH 7.2). Fractions of 4 mL each were collected and read at 280 nm on U.V. spectrophotometer. The fractions causing immobilization of spermatozoa were pooled and concentrated.

Determination of molecular weight of SIF

The molecular weight of the protein was estimated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using the standard molecular weight marker [21]. A 10% gel was prepared and after the gel was run, coomassie blue staining was done and molecular weight was estimated.

Impact of SIF on various mouse sperm parameters: *in vitro* studies

Sperm motility: Motility of the sperms extracted from the sacrificed mice was determined by the method of Emmens [22].

Sperm viability: In order to estimate the percentage of viable sperms, an equal volume of mouse spermatozoa was mixed with 0.5% eosin and examined under the light microscope at 400X magnification.

Sperm Mg^{++} ATPase activity: Mg^{++} ATPase activity of

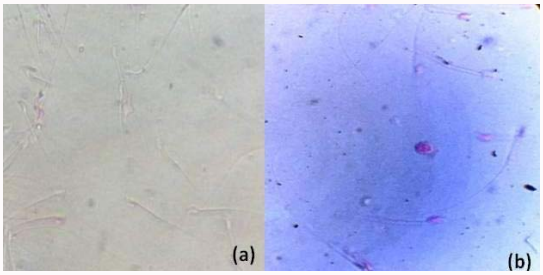

Figure 4: Photomicrograph showing eosin staining of mouse spermatozoa after incubation with (a) PBS; unstained live spermatozoa (b) SIF; pink stained dead spermatozoa.

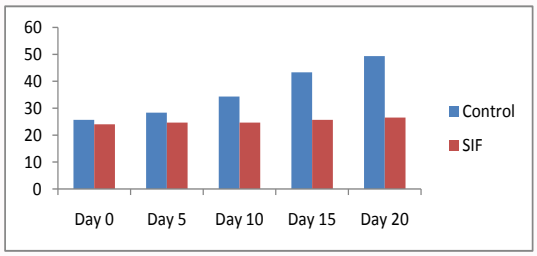

Figure 5: Representative weight profile of female Balb/c mice receiving PBS/ SIF during different days of gestation period.

Figure 6: Photomicrograph of pregnancy related changes in group of mice administered with PBS (a) abdominal distension on day 14 of gestation (b) delivery of pups at the end of gestation period.

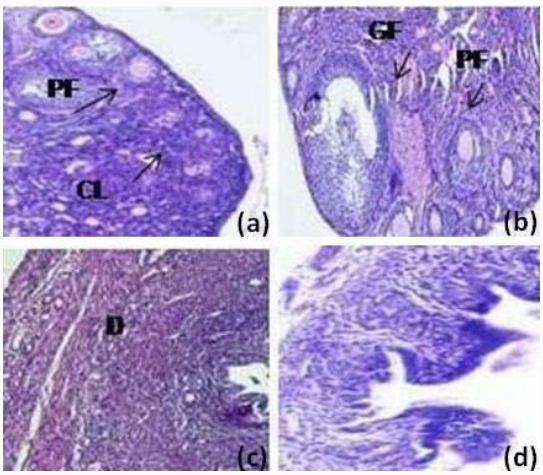

Figure 7: Histology of female reproductive organs (ovary and uterus) on day 14 in mice treated with PBS ((a), (c)), SIF ((B), (d)), ovary showing the presence of Corpus Luteum (CL) in sections (a) while section (b), that is, SIF treated ovary showing only the presence of Graafian Follicles (GF). Presence of Decidua (D) was also observed in control uterus in contrast to test group where no such changes were observed.

spermatozoa was studied according to Kielley [23] and Chappel [24] with slight modifications. Tris-HCl (0.2 M, pH 7.6) washed spermatozoa (1 × 108/mL) were sonicated at 50 Hz for 10min (10 cycles of 30s with 1min interval) at 4°C. The reaction mixture for ATPase consisted of 0.2 mL Tris-HClbuffer (0.2 M, pH 7.6), 0.2 mL of $MgCl_2$ (5 mM), 0.2 mL of ATP (6 mg mL-1), and 0.2 mL of sonicated sperm suspension. Different concentrations of SIF (12.5 µg, 25 µg, 50 µg, and 100 µg) were added to the reaction mixture. The mixture was incubated at 37°C for 1h. After this period of incubation, the reaction was stopped by adding 1 mL of cold 10% Trichloroacetic Acid (TCA) and then incubated at 4°C overnight for protein precipitation. The control tubes contained all the components of the reaction mixture but TCA was added in the beginning to stop the ATPase activity. Inorganic Phosphorus (Pi) released was determined according to the method of Boyce et al. [25]. One unit of ATPase is expressed as µg of the Pi released after 1h of incubation.

The experiment was repeated thrice.

Effect of intravaginal application of SIF on fertility outcome: *in vivo* study

For this study, a total of 36 female mice and 18 male mice were used in three fertility trials. Twelve healthy female mice were divided into two groups (control and treated), four for control and eight for treated. SIF (10 µg, 25 µg) was deposited with the help of auto pipette into the vagina of four female mice. The control animals received buffer only. The animals were mated overnight immediately after application of SIF without any delay with male mice of proven fertility (two female mice with one male mouse in each cage). The next morning, the mating was confirmed by the presence of a vaginal plug, and the mated animals were separated and kept until delivery.

Histological examination

Histological examination was carried out to further substantiate the results obtained from *in vivo* study. The female mice were synchronized in their oestrous cycle by the Whitten effect (Whitten, 1956). Female Balb/c mice were divided into two groups i.e. control group (receiving 20 µl PBS) and test group (receiving 10 µg SIF). The mice were allowed to mate with breeder male mice at a ratio of 2:1 to check the effect on fertility. One mouse from each group was sacrificed on day 14 and reproductive organs *viz.* ovaries and uterus were removed to study tissue histology. They were fixed in 10% formaldehyde for 24h and then embedded in paraffin according to standard histological methods. Serial paraffin sections were made, stained with haematoxylin/eosin and observed at 40X magnification.

Results

The standard strain of *P. aeruginosa* used in the present study was found to cause sperm immobilization *in vitro*.

Extraction and purification of Sperm Immobilization Factor (SIF) from *P. aeruginosa*

When the cell free supernatant of 72h old culture of *P. aeruginosa* was subjected to ammonium sulphate precipitation, the results showed that sperm immobilization factor could be efficiently precipitated at 60% to 80% saturation. When the precipitated protein, re-dissolved and dialysed against PBS (50 mM, pH 7.2) was subjected to purification through Sephadex G-200 column, the results showed that the immobilization activity was present in the fractions 9 to 12 with a peak value in fraction 10, where each fraction was of 3 ml quantity (Figure 1). Further, the fractions that showed immobilization

activity were pooled and concentrated using PEG 6000.

After gel filtration chromatography, the pooled and concentrated fractions so obtained were applied to DEAE-cellulose column. The results revealed that the bioactive component could be eluted with PBS. The fractions 3 to 5 showed sperm immobilizing activity with peak value in fraction 4 where each fraction was of 4 ml (Figure 2). These bioactive fractions were again pooled and concentrated by PEG-6000.

Molecular weight estimation of SIF using SDS-PAGE

The purification status and molecular weight of SIF obtained after ion-exchange chromatography was estimated by SDS-PAGE. 50 µg of SIF after denaturation was loaded on the gel along with the marker and electrophoresis was done using 10% separating gel. The coomassie blue stained gel showed a single band indicating the purified SIF with a molecular weight of approximately 16 kDa (Figure 3).

Impact of SIF on sperm parameters: *in vitro* studies

Motility: When the effect of SIF was checked on sperm parameters *in vitro*, the results showed that SIF at a concentration of 50 µg could lead to complete immobilization of spermatozoa within 30 min of incubation.

Viability: In order to assess the impact of SIF on sperm viability, dye-exclusion method using Eosin Y was employed. The results showed that upon incubation with SIF (65 µg), a significant decline in viability of spermatozoa as compared to control, could be observed (Figure 4).

Mg^{++} ATPase activity: The effect of SIF on Mg^{++} ATPase activity of mouse spermatozoa was also examined. It was observed that SIF could significantly inhibit Mg^{++} ATPase activity of spermatozoa. The activity reduced from 1,360 units (control) to 540 units, 180 units and 0 units in presence of SIF at a concentration of 25 µg, 50 µg and 100 µg, respectively (Table 1).

Effect of SIF on fertility outcome in female mice: *in vivo* study

SIF was also evaluated for its impact on fertility outcome of female mice. From the results, it could be observed that mice in Group I receiving 20 µl of PBS remained fertile as evident by consistent weight gain, abdominal distension and finally delivery of pups at the end of gestation period (Figure 5 and 6). While, in case of mice treated with 10 µg and 25 µg of SIF, the results showed that SIF at the concentration of ≥ 10 µg could efficiently block conception in female mice as mice did not show any pregnancy related changes and failed to deliver pups at the end of gestation period.

Histological examination

Histological examination of the reproductive organs of control mice on day 14 showed presence of corpus luteum in ovary (Figure 7a) and endometrial proliferation and deciduas formation in uterus (Figure 7c). These changes indicated conception. However, all these changes were absent in the group receiving 10 µg SIF as the ovary showed the presence of inactive follicles followed by the presence of fibrous tissue in the central cavity lined by a large number of primordial follicles (Figure 7b) whereas no changes in the uterus were observed (Figure 7d).

Discussion

The world population continues to grow at an alarming rate, with a projected 50% increase in current world population to approximately 9 billion by 2050 [26]. Spermicides are a biologically obvious way of interrupting fertility and have advantage that they do not depend on high skilled personnel for their prescription and use. Spermicidal agents have the ability to immobilize or kill the sperm upon contact. An ideal spermicide should immediately and irreversibly produce immobilization of the spermatozoa [27].

Earlier in our laboratory, blockage of conception in female mice as a result of sperm impairing uropathogens *viz. S. aureus*, *E. coli* and *S. marcescens* has been observed [28-30]. Further, the sperm impairing factors isolated and purified from *S. aureus* and *E. coli* have also been found to induce infertility in female mice upon vaginal administration. On similar grounds, the present work was carried out with an aim to isolate and purify the sperm impairing factor from *P. aeruginosa* and to study its impact on seminal parameters and on fertility outcome. An extensive literature suggests that immobilization of spermatozoa by microbial species can be a consequence of direct interactions or the release of secretory factors [31].

In our study, immobilization of spermatozoa in case of *P. aeruginosa* appeared to be linked with the factor released into the extracellular medium as no immobilization was observed with the washed cells. Thus, cell free supernatant of *P. aeruginosa* culture was used for extraction and purification of Sperm Immobilization Factor (SIF). It was observed that SIF could be efficiently extracted by ammonium sulphate precipitation and through chromatographic techniques it was purified to apparent homogeneity with a molecular of 16 kDa. Earlier, various research groups have isolated and purified sperm impairing factors from different microorganisms such as sperm immobilization factors from *E. coli* [32,33]; *S. aureus* [34] and Extracellular Polymeric Substance (EPS) from *T. vaginalis* [35].

Sperm motility and viability are thought to be the cornerstone of fertilization process. Premature loss of spermatozoa motility and viability results in loss of their natural fertilization potential as they are unable to travel to bind oocyte [36]. In the context of these reports, effect of SIF on sperm parameters was investigated *in vitro*. The results indicated that SIF has a considerable negative effect on the motility of mouse spermatozoa. SIF was found to immobilize spermatozoa directly. Further, SIF was found to induce sperm death. Recent observation by Barbonetti et al. [37] indicated release of an unidentified soluble factor by *E. coli* that inhibits mitochondrial membrane potential ($\Delta\Psi m$), motility and vitality of spermatozoa. Rennemeier et al. [38] revealed a new facet in the interaction of factors with male gamete. They demonstrated that quorum sensing molecules farnesol and 3-oxododecanoyl-L-homoserine lactone, released by yeast Candida albicans and the gram-negative bacterium *P. aeruginosa*, respectively, elicit multiple deteriorating effects on spermatozoa.

To accomplish the aim of fertilizing oocyte, as spermatozoa enters the female reproductive tract, male gamete senses the environment and adapts its motility, which is controlled partially by ATP. It possess cation dependant (Na^+, K^+, Mg^{++}) ATPases that account for the breakdown of ATP to release energy for flagellar contractile processes [39]. To address the possibility of involvement of cation dependant ATPases in SIF induced sperm impairment, the effect of SIF on Mg^{++} dependent ATPase was studied. From the results, a negative link between the SIF and Mg^{++} dependent ATPase activity was observed. It could be speculated that decrease in cation dependant ATPase activity might have a critical significance in the biochemical mechanisms underlying the decreased sperm motility as

a result of incubation with SIF. Similar results have been reported by Peralta-Arias et al. [40] wherein inhibition of the dynein-ATPase activity, an intracellular motor for sperm motility, led to sperm immobilization. In a recent study by Da Costa et al. [41], attributed the decreased sperm motility in individuals exposed to Cadmium (smokers) to inhibition exerted by Cd^{2+} on sperm ATPases associated with sperm motility.

In an attempt to explore the *in vivo* relevance of sperm impairing activities of SIF, fertility studies were carried out in female Balb/c mice. The use of female mice was determined by the fact that semen has a short exposure time to factors released by uropathogens in the male urethra after ejaculation while sperm can be exposed to these factors for several days through the female tract [35]. When SIF was intravaginally administered in female mice, they led to blockage of conception. Data emerging from fertility studies suggests that presence of sperm impairing factor in vagina may transform the female genital tract into a hostile milieu for spermatozoa, which may thwart natural conception and could play a critical role in induction of infertility. Nakano et al. [42] have outlined various parameters such as poor buffering capacity of acid vaginal pH, hormonal dysfunctions, and exogenous factors which can interfere with sperm movement through the vagina, therefore, be implicated in the pathophysiology of unexplained infertility. Histological examination also confirmed the presence of pregnancy related changes in control group in contrast to SIF treated group. These results further indicate the action of SIF both *in vitro* as well as *in vivo*. Thus, from the above preliminary observations, it can be concluded that SIF isolated and purified from *P. aeruginosa* was capable of compromising various sperm parameters *viz*. motility, viability and Mg^{++} dependent ATPase. Further, this factor also led to blockage of conception in female mice. Thus, this factor with enormous anti-fertility potential might be explored as excellent vaginal contraceptive.

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