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EXPLOITATION OF MOLECULAR MIMICRY BETWEEN BACTERIA AND SPERMATOZOA FOR AMELIORATION OF SPERM IMMOBILIZATION FACTOR INDUCED INFERTILITY IN MALE MICE

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ABSTRACT
Earlier in our laboratory sperm immobilization factor (SIF) isolated from *Staphylococcus aureus* was found to induce the impairment of male mice's reproductive vigour. SIF has also shown immobilization of mouse spermatozoa and motile bacteria in vitro. Thus, indicating the presence of common SIF binding receptor on both spermatozoa and bacteria. After these encouraging findings, the present study was intended to evaluate SIF-binding bacterial receptor from *E. coli* (SBRE) as an ameliorating agent against SIF-induced impairment of male mice's reproductive vigour. For this, mice were divided into four groups consisting of three animals in each group. Each mouse was administered via intra vas deferens route on the right side with 10 µg of SIF preincubated with either of the following: PBS (group I); 10 µg SBRE (group II); 25 µg SBRE (group III); 50 µg SBRE (group IV). Mice were sacrificed after 24 h by cervical dislocation, and the results in terms of body weight, seminal parameters, tissue somatic indices (TSI) and histology were studied. No significant body weight changes were observed in all the experimental groups on all the days of sacrifice. In the case of seminal parameters, azoospermia was observed on the right side of group I, II, and III compared to the left side in all the experimental groups. However, restoration of sperm count and motility occurred only in the case of group IV. No significant changes TSI (%) of various reproductive and non-reproductive organs were observed in all the test Groups (I-IV) except in case of SBRE (Group III and IV) where a significant increase in TSI values of caudal epididymis and vas deferens of the right side was observed. Histological studies showed hypo spermatogenesis and inflammation in the right-side reproductive organs of group I, II and III while the left side of all the experimental groups and right side of group IV showed normal tissue histology. In conclusion, SBRE from *E. coli* was able to ameliorate the deleterious effect of SIF on the reproductive potential of male mice.

INTRODUCTION
Majority of regulatory networks found in cells are based on protein-protein interactions since proteins form the significant bulk of macromolecules involved in molecular recognition (Yugandhar et al. 2014). Such interactions rely majorly on high specificity and high affinity between correct interacting partners (Perozzo et al. 2004). A breach of this pathway is known to occur by the phenomenon of mimicry. Molecular mimicry between proteins is a well- documented event in which bacteria, viruses or parasites evade the immune response; agonists and antagonists bind to receptors; or when an autoimmune response is initiated because a pathogen shares sequence similarity with native proteins (Tsonis and Dwivedi, 2008). The mimicry concept has come a long way with its roots originating from ecological mimicry to the now-prevalent, autoimmune diseases. In today's scenario, mimicry has also been hypothesized to play a crucial role in infertility (Berwary, 2017). Molecular biology approaches have demonstrated the existence of sequence homologies between genes encoding bacterial proteins and mammalian sperm proteins (Kalaydjiev et al. 2007). Several reports have surfaced up in support of this. To cite a few, the studies conducted by Kurpisz and Alexander (1995), have suggested that *Escherichia coli* cells of serotypes 08, 09 and 086 contain heterogenetic antigens similar to the cellular antigens of human spermatozoa. Similarly, a linear homology was observed at the amino acid level between β-tubulin of human spermatozoa and three Helicobacter pylori proteins, flagellin, Vacoulating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) (Dimitrina et al. 2016). Further, in the case of Chlamydia trachomatis, Heat Shock Proteins (HSPs) which serve as essential antigens of infectious agents, are among the most conserved molecules in phylogeny. Since bacterial and human HSP share ~50% amino acid homology (Shinnick, 1991), prolonged exposure of the immune system to cHSP60 may lead to the formation of antibodies against spermatozoa (Witkin et al. 1997).

Finally, the ureaplasma urease subunit has been found to cross-react with human nuclear autoantigen sperm protein (hNASP), providing evidence for the mimicry at the molecular level. In this regard, in an earlier work done in our laboratory, sperm immobilization factor (SIF) isolated from *Staphylococcus aureus* has been found to cause in vitro immobilization of not only human spermatozoa and mouse spermatozoa but motile bacteria as well (Prabha et al. 2009; Thaper et al. 2018). The binding studies with FITC-labelled SIF confirm that SIF binds to spermatozoa and various gram-positive and gram-negative bacteria (Prabha and Vander, 2012) indicating the presence of common SIF binding motifs on spermatozoa and bacteria.

Moreover, SIF has been able to cause impairment of reproductive vigour of both male (Sharma et al. 2017) and female mice (Gupta et al. 2016). Further, the SIF-binding receptor (SBR) from human spermatozoa could block SIF-induced sperm impairment in-vitro. It could alleviate SIF induced infertility in female Balb/c mice following a single intravaginal application before mating. Hence, the amelioration of SIF-induced impairment by bacterial receptors, instead of receptor from spermatozoa, was thought of. In this regard, the SBR from *E. coli* has been purified and found to ameliorate SIF-induced sperm impairment in vitro further providing evidence for molecular similarities between bacteria and spermatozoa. This analogy is very suggestive and merits further investigation in exploiting the bacterial receptor as an ameliorating agent against SIF-induced impairment of reproductive potential in the male mouse model. With this aim, impact of intra vas deferens route of administration of SIF and SIF-binding receptor from *E. coli* on body weight profile, seminal parameters, tissue somatic indices (%) and histological evaluation of male mice was carried out.

MATERIALS AND METHODS

Microorganisms

Standard strain of *Escherichia coli* (MTCC1687) procured from Microbial Type Culture Collection, IMTECH, Sector-39, Chandigarh, India) used for isolation of SIF-binding receptors was available in the laboratory.

Animals

For the present study, 5-6 week old sexually mature (25±2g) male BALB/c mice were used. Mice were maintained under standard laboratory conditions. They were kept in propylene cages (430×270×150 mm3, six animals per cage) at 20-25°C. Cages were bedded with clean rice husk in well aerated animal room of Department of Microbiology, Panjab University, Chandigarh vide letter no PU/IAEC/S/16/141. Standard diet and water was given to all mice and water. Atleast for one week, animals were allowed to adapt to the new housing and experimental conditions. Institutional Animals Ethics Committee of the Panjab University, Chandigarh approved all the experimental protocols. All experiments were performed in accordance with the guidelines of Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation.

Isolation and purification of sperm immobilization factor(SIF) from *S. aureus*

The sperm immobilization factor (SIF) used in the present study was already available in the laboratory in the purified form.

Extraction of SIF-binding receptors (SBR) from bacteria

SBR from *E. coli* was also available in the laboratory in the purified form.

Evaluation of SIF-binding receptor (SBR) from *E. coli* as ameliorating agents against SIF-induced impairment of reproductive vigour

For this, male BALB/c mice were divided into 4 groups with 3 mice in each group. The administered volume for each mouse was 20µl. Briefly, mice were administered with 10µg of SIF pre-incubated for 30min at 37°C with Phosphate buffer saline PBS (Group I), 10µg SBRE (Group II), 25µg SBRE (Group III) or 50µg SBRE (Group IV) in the right vas deferens using a 27-gauze needle under anaesthesia of ketamine (75mg/kg) and xylazine (12mg/kg) under surgical conditions whereas the left vas deferens served as control. Following a scrotal incision, the right testis, epididymis and vas deferens were externalized. The dose was inoculated into the lumen of the right vas deferens towards the direction of caudal epididymis. Incisions were closed with 3-0 silk suture and after the surgery animals were kept individually in isolated propylene cages. After 24h, mice from each group were sacrificed and evaluated for following parameters: body weight profile, seminal parameters, Tissue Somatic Index (TSI %) and tissue histology.

Body weight profile

In order to observe treatment related changes in the body weight of mice, initial weights were 22 recorded on 0h and final weights after 24h.

Sperm count

Mice from each group were sacrificed by cervical dislocation and were dissected. The vas deferens was pulled out and placed in freshly prepared 500µl of PBS buffer (50mM, pH 7.2). Gentle teasing was done to enable the spermatozoa to swim out into the buffer in a glass plate. A fixed volume of 10 µl of the sample was placed on a glass slide and examined under the light microscope at 400X magnification. Around six to eight fields were scanned and the mean number of spermatozoa in all the fields was multiplied by 10^6. The slides were also assessed for the morphology of spermatozoa in each field to evaluate the respective abnormalities (WHO, 2010).

Sperm motility
Motility of the sperms extracted from the sacrificed mice was determined by the method of Emmens, 1947.

Tissue somatic indices (TSI %)
After, 24h mice from each group were sacrificed by cervical dislocation and the various reproductive organs (viz. testis, caudal epididymis and vas deferens) and non-reproductive organs (spleen, kidney and bladder) were removed aseptically. The organs were grossly examined and weighed. The TSI (percent tissue/organ weight in relation to body weight) was evaluated (Krishnaiah and Reddy, 2007).

Histological studies
Histological analyses of the reproductive organs (testis, caudal epididymis and vas deferens) of mice from each group were carried out. The various reproductive and nonreproductive organs were harvested, fixed in 10% formaldehyde for 24h and then embedded in paraffin according to standard histological methods. Serial paraffin sections of 4mm were stained with haematoxylin eosin and observed at 400X magnification for any significant changes in reproductive organs.

RESULTS
Evaluation of SIF-binding receptor from *E. coli* (SBRE) as ameliorating agent against SIF-induced impairment of reproductive vigour
The reproductive health of male mice upon administration of SIF pre-incubated with PBS/SBRE *via* intra vas deferens route was determined by examining the changes in body weight, seminal parameters, TSI (%) and tissue histology.

Weight profile
The bodyweight of all the animals administered with SIF pre-incubated with PBS or different concentrations of SBRE was recorded at 0h and 24h. Results showed no significant change in body weight at the start of the experiment (i.e., at 0h) and final body weight taken at 24h in all the mice of all the experimental groups (I-IV) **(Figure 1)**.

Evaluation of seminal parameters
To assess the treatment related changes in seminal parameters, three mice from each group were sacrificed after 24 h.

Sperm count
The sperm count in right vas deferens of mice administered with SIF pre-incubated with PBS (Group I) showed complete spermatogenesis inhibition compared to left vas deferens. Even pre-incubation of SIF with lower concentrations of SBRE, i.e. 10µg (Group II) and 25µg (Group III) could not significantly ameliorate SIF-induced impairment of sperm count, with the count of right side being comparable to that of Group I. However, test Group IV, in which SIF pre-incubated with 75µg SBRE was administered, restoration of 93% of the sperm count occurred in the right vas deferens compared to left vas deferens (Figure 2) (Table 1).

Motility
The effect of intra vas administration of SIF pre-incubated with PBS or SBRE was studied in all the experimental groups after 24. Since complete inhibition of spermatogenesis was observed, in the case of SIF treated mice (Group I); hence, this parameter could not be assessed. In case of mice (SBRE) in test Group II and III, i.e. those administered with lower concentrations of SBRE, all the sperms extracted from right vas deferens were rendered immotile as compared to left vas deferens of Group II (44% motility) and Group III (87% motility). The ameliorating effect of SBRE was observed in Group IV mice, wherein the percentage of motile spermatozoa was comparable in both the left and right vas deferens (approximately 60%) (Table 2).

Morphology
The effect of SIF pre-incubated with PBS or SBRE on the morphology of mouse spermatozoa was studied after 24h of intra vas deferens administration. The results showed that morphologically normal sperms were observed in both right and left vas deferens of all the mice in all the experimental groups (Group I-IV).

Tissue somatic indices (TSI %)
Tissue somatic indices (%) of various reproductive (testis, epididymis and vas deferens) and non-reproductive organs were determined after 24h. It was found that in the case of non- reproductive organs, no significant changes were observed in all the experimental groups (I- IV) (Figure 3). However, in case of reproductive organs, in comparison to the left side, there were no significant treatment-related changes in the absolute weights of all the organs of the right side, except for a significant increase in TSI (%) values of right cauda and right vas deferens of both Group III and IV (Figure 4).

Histopathological examination
To check any adverse effect of SIF pre-incubated with PBS or SBRE on tissue morphology of various reproductive organs viz. testis, cauda epididymis and vas deferens non- reproductive organs viz. spleen, kidney, bladder and liver, histological analysis was carried out. The left set of reproductive organs revealed normal tissue histology, i.e., testis showed regular seminiferous tubule and germinal cell morphology (Figure 5a), epididymis displayed the well-vascularized loose connective tissue present around the epididymal duct (Figure 5b). Further, the normal columnar epithelium was seen in the vas deferens (Figure 5c). However, the right set of organs of mice in Group I showed degeneration of germinal epithelium and reduced mature sperms in the centre of tubules and active inflammation in testis (Figure 5d). In vas deferens, there was leukocyte invasion in the lumen (Figure 5f). Cauda showed evidence of mild to moderate interstitial inflammation

with no mature sperms present (Figure 5e). Receptor dependant amelioration was not observed in the case of Group II, and III since the tissue sections displayed changes similar to those observed in Group I. However, the histopathological examination of the right set of reproductive organs of Group IV animals receiving the highest concentration of SBRE showed normal tissue histology (Figure 5g, 5h, 5i).

In the case of tissue histopathology for non-reproductive organs viz. spleen, kidney and bladder, no abnormalities were observed. All the organs were found to be functional and histologically normal in all the experimental groups (Group I-IV) (Figure 6).

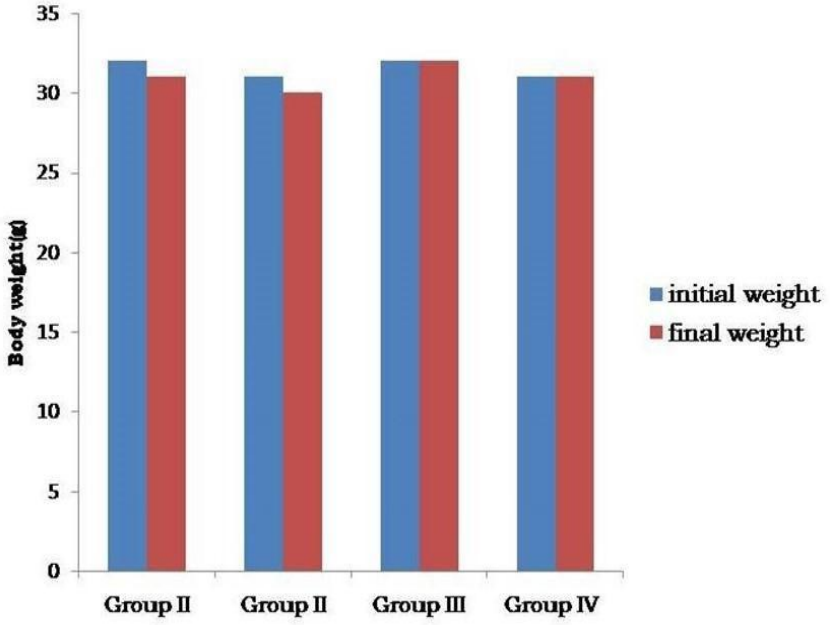

Figure 1: Weight profile of mice administered with SIF pre-incubated with PBS (Group I) or 10µg SBRE (Group II) or 25µg SBRE (Group III) or 50µg SBRE (Group IV) after 24h of administration.

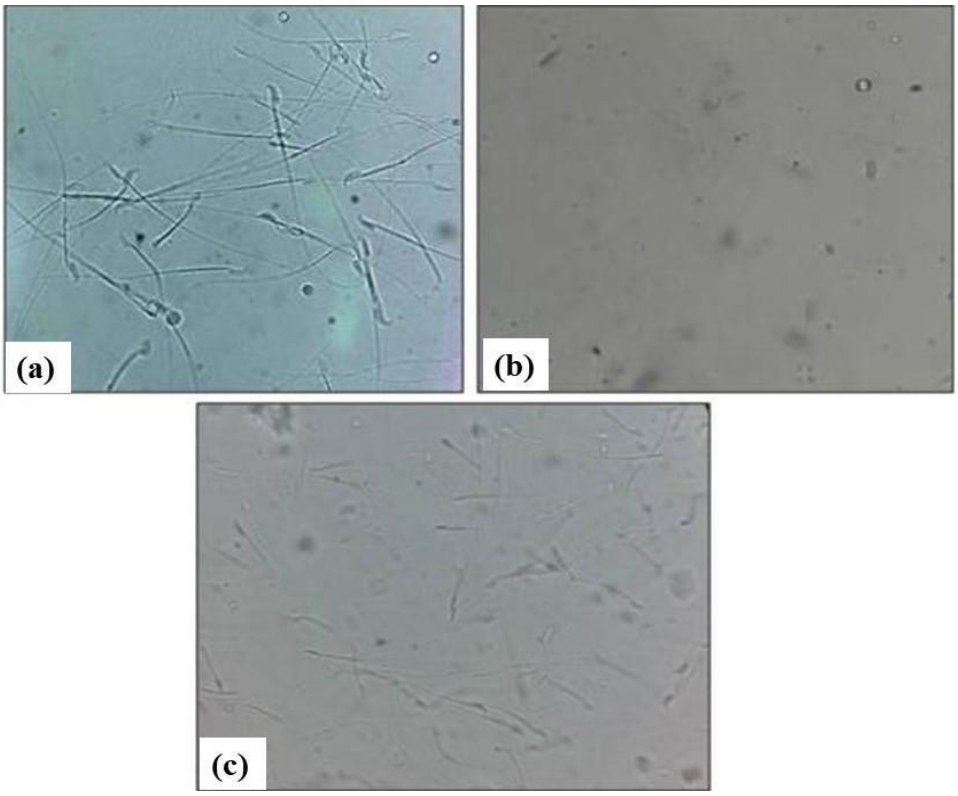

Figure 2: Photomicrographs of mouse spermatozoa showing A) normal sperm count in left vas deferens, B) hypo spermatogenesis in right vas deferens of Group I and C) normal sperm count in right vas deferens of mice in Group IV.

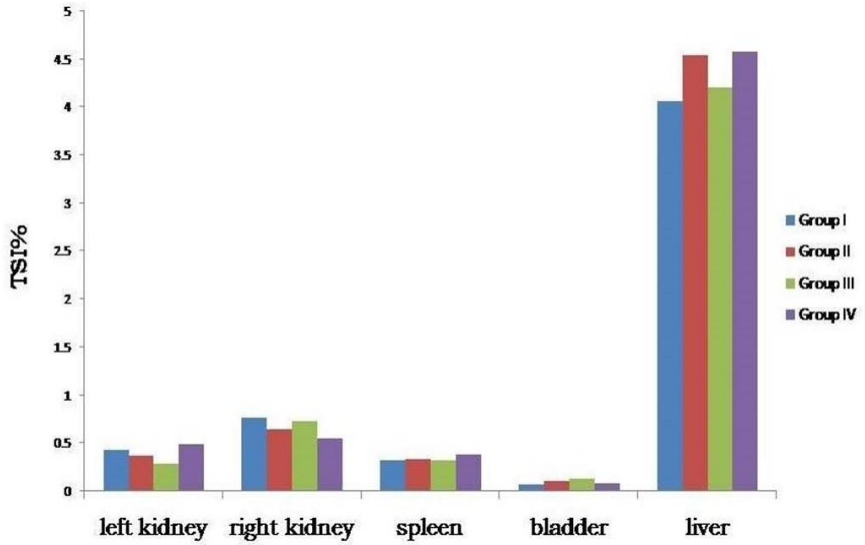

Figure 3: Tissue somatic indices (%) of various non- reproductive organs treated with SIF pre-incubated with PBS/SBRE.

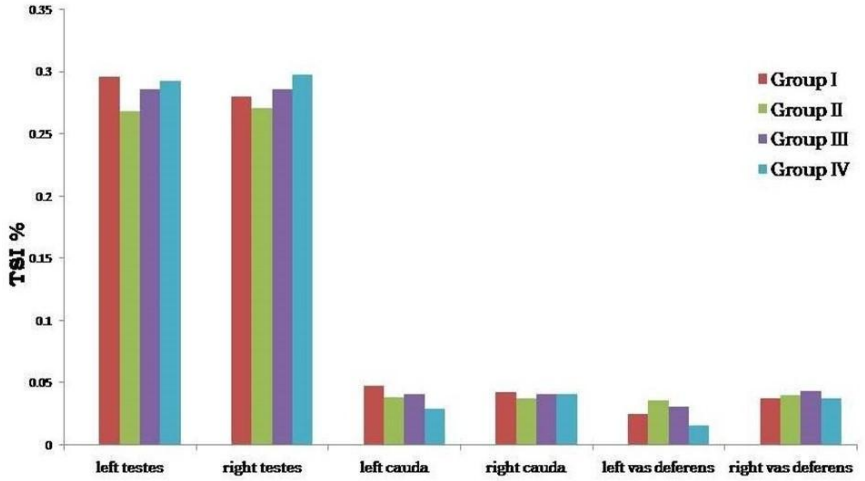

Figure 4: Tissue somatic indices (%) of various reproductive organs treated with SIF pre-incubated with PBS/SBRE.

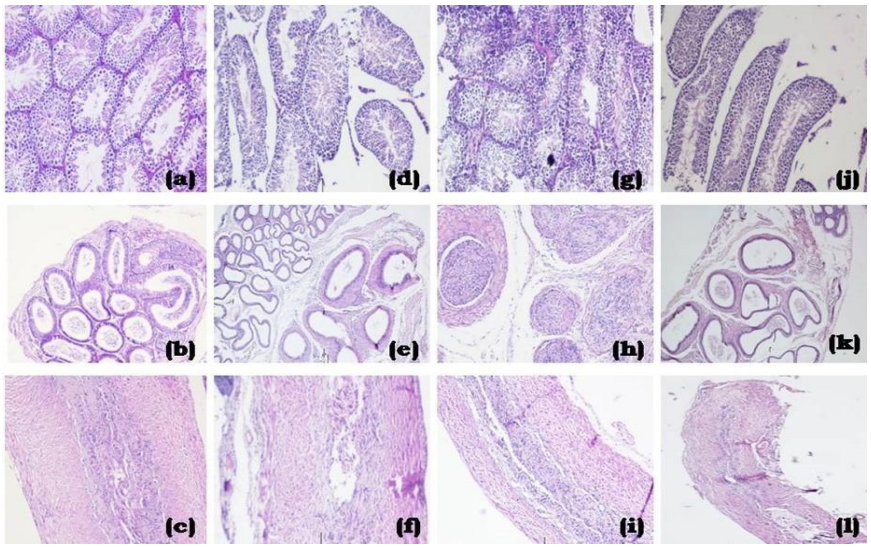

Figure 5: Representative photomicrographs of histological examination of right set of reproductive organs viz. testis, cauda epididymis and vas deferens of mice in Group I (d-f), Group IV (g-i), as compared to the left set of organs which served as control (a-c).

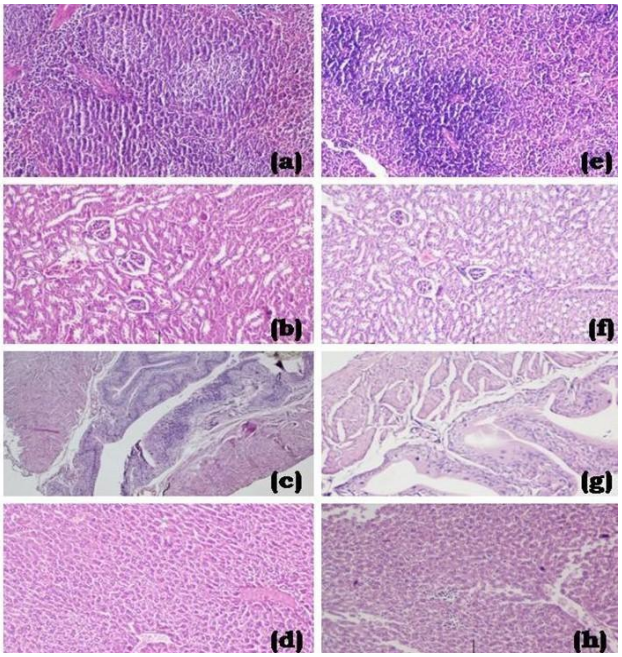

Figure 6: Representative photomicrographs of histological examination of non- reproductive organs *viz.*spleen, kidney, bladder and liver of mice in Group I administered with SIF pre-incubated with PBS (a-d) or SBRE (Group IV) (e- h).

Table 1: Sperm count in vas deferens of male mice after 24h of treatment with SIF and different concentrations of SBRE.

BACTERIAL RECEPTOR	SPERM COUNT(x 10^6 /ml)							
	Group I		Group II		Group III		Group IV	
	Left vas	*Right vas*	*Left vas*	*Right vas*	*Left vas*	*Right vas*	*Left vas*	*Right vas*
SBRE	57	0	23	1	24	0	28	19

Table 2: Percentage of motile spermatozoa in vas deferens of male mice after 24h of treatment with SIF and different concentrations of SBRE.

BACTERIAL RECEPTOR	SPERM MOTILITY (%)							
	Group I		Group II		Group III		Group IV	
	Left vas	*Right vas*	*Left vas*	*Right vas*	*Left vas*	*Right vas*	*Left vas*	*Right vas*
SBRE	70	0	44	0	87	0	66	60

DISCUSSION

Based on circumstantial evidence, it would not be inappropriate to say that molecular mimicry has remained an attractive explanation for autoimmune diseases for a considerable time. However, for researchers in molecular mimicry, challenges will always be there to prove that molecular mimicry can also be a mechanism in causing infertility (Acharya et al. 2010). Various studies have demonstrated that sharing antigens does occur between human spermatozoa and various prokaryotic microorganisms, leading to anti-sperm antibodies (ASA's) that damage sperm motion parameters. For instance, reports suggesting the presence of cross-reactive antigens between spermatozoa and bacteria viz. *E. coli* (Popivanov et al. 1981), *Salmonella typhi* (Kurpisz and Alexander 1995), *H. pylori* (Figura et al. 2002), *C. trachomatis* (Witkin et al. 1997) and *Ureaplasma urealyticum* (Shi et al. 2007) have surfaced up. However, from the clinical perspective, work in this area has not led to any treatments with proven efficacy.

In the light of this initiative, previously in our laboratory, a strain of *S. aureus* has been found to produce a sperm immobilization factor (SIF) that immobilizes human spermatozoa, mouse spermatozoa and various motile bacteria. Moreover, upon labelling with FITC, SIF was found to bind human and mouse spermatozoa along with various motile and non-motile bacteria indicating common SIF-binding receptors (SBRs) on both spermatozoa and bacteria (Thaper et al. 2018; Thaper et al. 2019). In vivo studies revealed the SIF-induced impairment of reproductive potential of male (Chauhan et al. 2020) and female mice (Gupta et al. 2016). Further, intravaginal application of SBR from human spermatozoa could alleviate SIF- induced infertility in Balb/c mice. On similar lines, SBRE from *E. coli* has been purified and found to ameliorate SIF-induced sperm impairment in-vitro further providing evidence for molecular similarities between bacteria and spermatozoa (Thaper et al. 2018). Hence, the present study aimed to evaluate SBRE as ameliorating agents against SIF-induced impairment of reproductive vigour in the male

mouse model. In the present study, doses of SIF pre-incubated with PBS or SBRE were given in the right vas deferens; however, the left vas deferens served as control. The effect of SIF with PBS or SBRE on male mice's reproductive potential was evaluated by monitoring their effects on changes in body weight, seminal parameters, TSI (%) and tissue histology.

The body weight profile of mice treated with SIF pre-incubated with PBS or SBRE showed no difference in body weight at the start of the experiment (i.e., at 0h) and final body weight taken at 24h indicating that neither SIF alone nor upon pre-incubation with the SBR had any tainting effect on the health of mice. These findings are in line with the study by Wahyuni et al. (2017). It was reported that no substantial weight loss was observed in any of the test groups compared to control groups when Tetraprenyltoluquinone, an anti-cancer agent, was administered intraperitoneally. The significance of body condition and TSI as indicators of the health status of the animals was investigated. Upon evaluation of weights of reproductive organs in comparison to the left side, a significant weight gain in right cauda and right vas deferens of Group III and IV was observed, a finding which was absent in the Group I and II. This indicates a good health status of the animals in the study group.

Similarly, in the case of non-reproductive organs, no significant changes in weight of all organs of all the Groups (I-IV) were observed. This is consistent with the study by Reddy et al. (2011). They stated that no statistically significant treatment-related differences were seen in the testis' total weights, epididymis, ventral prostate, and seminal vesicle in Nisin-dosed and control rats.

Analysis of seminal parameters provides a valuable diagnostic tool to assess the fertility status of an individual. In this regard, in the present study, when SIF pre-incubated with PBS or SBRE, the examination of sperm count 24h after cessation of intra vas treatment showed that in comparison to left vas deferens, sperm count in right vas deferens displayed the complete absence of spermatozoa in Group I. However, only Group IV showed recovery of spermatogenesis in the right vas deferens in comparison to left vas deferens. Further, when the other seminal parameter, i.e., motility was assessed, a pattern similar to sperm count was observed, i.e., motility was more severely affected, with not even a single motile sperm on the right side of test Groups I-III and restoration of the same in test Group IV. Lastly, the evaluation of sperm morphology revealed the absence of morphological defects in spermatozoa of mice in all the experimental groups (Group I-IV). From the above results, it can be concluded that SIF-induced alteration of sperm count and motility indicates that reproductive function was compromised. However, the ability of SBRE to ameliorate the SIF-induced impairment of reproductive vigour is purely concentration-dependent. Similar results have been reported by Reddy et al. (2010) wherein they have demonstrated testosterone-mediated amelioration of carboplatin-induced infertility when administered via the intraperitoneal route. They have reported that a significant decrease in sperm motility and viability in case of carboplatin treated rats. However, the above-mentioned sperm parameters' average percentages were significantly increased when the rats were treated with carboplatin and testosterone. Similar results have been obtained in another study conducted by Kaur and Prabha, (2013) in which they have highlighted the efficacy of the receptor from human spermatozoa as a corrective measure against the negative influence of sperm agglutination factor (SAF) on functional parameters of spermatozoa as well as fertility, thereby, presenting receptor as a potential therapeutic intervention against SAF induced infertility.

An in-depth analysis of treatment with SIF pre-incubated with PBS/SBRE was done by histological examination of reproductive and non-reproductive organs. Photomicrographs revealed that there was no gross histological difference between the right vas deferens of mice from Group IV and the left vas deferens of mice from all the experimental groups (I-IV) wherein normal tissue histology in all the reproductive organs was observed. However, the right set of organs in Group I, II and III showed vacant central part of seminiferous tubules with no mature spermatozoa in testes indicating hypo spermatogenesis. The epididymis and vas deferens showed focal inflammation. Hence, it can be concluded that lower concentrations of SBRE could not ameliorate the SIF-induced histological alterations in the reproductive organs of male mice. However, in striking contrast to this, tissue histology for non-reproductive organs viz. spleen, kidney and bladder, showed no structural abnormalities. All the organs were found to be functional and histologically normal, in all the groups. Similar histological findings have been reported by Najafi et al. (2014) wherein they have examined the protective role of Royal jelly, an antioxidant, in Oxymetholone-induced reproductive failure of male mice. They have reported the absence of histological alterations in testes of control and Royal jelly-only groups, whereas, drastic morphologic changes were observed in the testis of Oxymetholone-treated mice. Thus, from the present study, it can be concluded that the SIF binding receptor isolated from *E. coli* was able to ameliorate the deleterious effect of SIF on male reproductive vigour. Hence, SIF-binding receptors from bacteria, mimicking SIF-binding sperm receptors, might open up new opportunities for the treatment of infertility.

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