Amelioration of Sperm Agglutinating Factor (SAF) Induced Sperm Impairment by Anti-SAF Polyclonal Antibody

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Abstract

**Background:** We have previously isolated a Spermagglutinating Factor (SAF) from *Escherichia coli*, which was capable of causing sperm agglutination and impairment of sperm parameters viz. apoptosis, premature acrosome loss and inhibition of Mg\(^{2+}\) dependent ATPase activity in vitro. In addition, intravaginal administration of Balb/c mice with SAF resulted in infertility. To provide evidence that SAF plays an important role in impairment of sperm parameters and infertility, anti-SAF antiserum was raised and its application as a therapeutic intervention against SAF induced damage was evaluated.

**Methods:** Effect of anti-SAF antiserum was evaluated against SAF mediated adverse effects on sperm parameters. Spermagglutination was observed using light microscopy and Mg\(^{2+}\) dependent ATPase activity was estimated in terms of release of inorganic phosphate. Sperm apoptosis and acrosome status were evaluated by means of flow cytometry and fluorescent microscopy, respectively. Further, the impact of anti-SAF antiserum was also seen on fertility outcome in mice.

**Results:** Results showed that immunization of mice with SAF lead to the generation of high titer specific antibodies. Raised anti-SAF antiserum could neutralize all the biological effects of SAF in contrast to control antiserum. Furthermore, intravaginal application of anti-SAF antiserum along with SAF rendered mice fertile.

**Conclusion:** Here we provide evidence that SAF plays an imperative role as all the detrimental effects induced by SAF whether in vitro or in vivo were blocked on simultaneous incubation with anti-SAF antiserum. Present work also highlighted the efficacy of the anti-SAF antiserum as a curative measure against SAF.

Keywords: Apoptosis; *Escherichia coli*; Spermagglutinating factor; Acrosome reaction; Mg\(^{2+}\) dependent; ATPase activity

Abbreviation: SAF: Sperm Agglutinating Factor

Introduction

The significance of bacteriospermia has gained increasing importance in recent years. Acute and chronic infections in the male reproductive system may compromise the sperm cell function [1]. Spermagglutination can subsequently be affected by these infections at different points in their development and maturation. A variety of pathogenic bacterial species are isolated from the semen of fertile and infertile patients with the ability to interact directly with spermatozoa [2]. These interactions are typified by attachments between bacteria and spermatozoa, agglutination phenomena, and morphologic alterations of spermatozoa [3]. Some investigators have also reported evidence for soluble spermatoxic factors.

Among the bacterial species that interact with spermatozoa are well known causative pathogens of genitourinary infections such as *E. coli*, *U. urealyticum*, *M. hominis* and *C. trachomatis* [4,5]. The most discussed and tested organism concerning male infertility is *Escherichia coli*, as the most important pathogen causing prostatitis and epididymitis. Several authors have postulated a negative effect of *E. coli* on sperm motility. Further, Moretti et al. carried out electron microscopic studies that revealed the ultrastructural damage of the sperm membrane in all parts of the spermatozoon, especially in the mid-piece, causing both swelling of the mid-piece and tail invagination on incubation with *E. coli* [1]. Thus, it was suggested that *E. coli*/spermatozoon-interaction may be a two-step process: i.e. adhesion to and subsequent destruction of the sperm membrane [3].

In an earlier work done in our laboratory, we had also isolated a strain of *E. coli* from semen sample of male attending infertility clinic, impeding the motility of spermatozoa by agglutination in vitro. The ~71 kDa Sperm Agglutinating Factor (SAF), responsible for agglutination could also be isolated and purified [6]. The SAF was found to be spermicidal at higher concentrations and could lead to adverse effects on various sperm parameters. Intravaginal administration of SAF in Balb/c mouse led to infertility [7]. Because SAF is specifically responsible for the above mentioned phenonemonas, we hypothesized that generation of anti-SAF antibodies could be exploited as a preventive measure against SAF induced damage. To experimentally evaluate this hypothesis, anti-SAF antiserum was raised in Balb/c mouse and examined as an antidote against SAF mediated effects.

Material and Methods

**Animals**

Sexually mature 5-6 weeks old male (25 ± 2 g) and 4-5 weeks old female (22 ± 2 g) Balb/c mice were used in the present study. The animals were maintained under standard laboratory conditions (12 h
light: 12 h darkness cycle) and were housed in polypropylene cages. Food and water were available ad libitum. All the experiments were approved by Institutional Animal Ethical Committee, Panjab University vide no. IAEC/156 dated 25.08.11 and were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Semen sample**

Semen samples were obtained by masturbation, into a sterile wide mouth container, from men undergoing fertility evaluation at Post Graduate Institute of Medical Education and Research (PGIMER), Sector-12, Chandigarh, India. Ejaculates showing normal parameters according to World Health Organization criteria were used [8]. The protocols for the present study were approved by Panjab University Institutional Ethics Committee vide letter No. 02/PUIEC dated 25.03.09.

**Isolation and purification of Sperm Agglutinating Factor (SAF) from E. coli**

The SAF was extracted and purified from 48h old cell culture of E. coli by the method earlier standardized in the laboratory [6]. Briefly, the cell culture was washed with Phosphate buffered saline (PBS; pH 7.2, 50 mM) and pellet was given salt treatment with 3 M NaCl for 12 h which released the factor into the solution. After dialysis and concentration of salt treated sample, further purification of SAF was carried out using gel permeation chromatography and ion exchange chromatography.

**Generation of antibodies**

Antiserum was raised in Balb/c mice against SAF according to a method of Castle et al. [9]. A group of 5 mice received SAF at a concentration of 25 µg per mouse per dose. The immunizing protein preparation was emulsified in an equal volume of Freund's Complete Adjuvant (FCA; Sigma) to achieve required concentration and 0.1 ml of the emulsion was injected intraperitoneally. Four booster injections in Incomplete Freund's Adjuvant (IFA) were given at an interval of two weeks. Four days after the final booster, blood was drawn from tail vein and allowed to clot for 1 h at 37°C, followed by centrifugation at 5000 g for 10 min for the serum collection. Bleedings from mice prior to the first injection provided control serum. Serum from several bleedings from the same or different animals was pooled to provide sufficient immune and control material respectively, for several experiments. Serum aliquots were kept at 4°C for immediate use and at -20°C for long term storage.

**Estimation of titer using ELISA**

For titer estimation, non competitive (direct binding) ELISA was carried out [10]. For this, a 96-well polystyrene microtiter ELISA plate was coated with 5 µg/100 µl of the purified protein SAF dissolved in carbonate buffer (pH 8.0) and incubated overnight at 4°C. After the incubation, wells were washed three times with phosphate buffered skimmed milk (PBSM; PBS with 1% skimmed milk). Each protein coated well was blocked using 100 µl of 2% Bovine Serum Albumin (BSA) in PBSM in aliquots having 4x106/ml motile spermatozoa in each tube and incubated for 30min with either a) BWW medium (serving as control); b) Cal (physiological AR (10 µmol/l) as positive control); c) SAF (50 µg); d) SAF (50 µg) + anti-SAF antiserum (undiluted) and e) SAF+control serum (undiluted). Further after treatment, spermatozoa were washed with 10 ml 0.9% sodium chloride twice with centrifugation at 800 × g for 10 min and the sperm pellet was smeared on a glass slide and fixed using 95% ethanol for 30 min. Fixed sperm were stained with PSA-FITC and observed by means of a fluorescence microscope (Nikon, Japan) and at least 200 spermatozoa were differentiated blindly according to the fluorescence pattern of their acrosomes (bright fluorescence: acrosome intact; no fluorescence or only fluorescence of the equatorial segment: loss of acrosomal membranes).

**Annexin V-PI assay**

Staining of cells was carried out using a combination of Annexin V and Propidium Iodide (PI) as it is possible to simultaneously distinguish live, apoptotic and necrotic sperm populations. Staining was performed using a commercial kit (Annexin V–FITC Apoptosis detection kit, Sigma Chemical). In brief, aliquots of semen samples containing 4x10^6 sperm/ml were suspended in 500 µl of binding buffer and labeled with 10 µl of annexin V–FITC plus 5 µl of PI. After incubation for 10 min in the dark, samples were immediately analysed by flow cytometry.
Flow cytometry: Flow cytometry analysis was performed using the flow cytometer BD Canto I™. Ten thousand events were measured for each sample at a flow rate of 50-100 event/s and analysed using the BD DIVA™ Software. The debris was gated out, by drawing a region on the forward versus side-scatter dot-plot enclosing the population of cells of interest. The compensations and the settings were adapted according to the assay utilized.

Samples were evaluated by FL-1 (Annexin V–FITC) and FL-3 (PI) detectors. Labeling patterns identified the different cell populations where FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as apoptotic cells; and FITC positive and PI positive as late apoptotic or necrotic cells.

**Mg**²⁺ dependent Adenosine Triphosphatase (ATPase) activity: Motile spermatozoa selected by swim up procedure were washed twice (500g, 5min) in PBS and the final working concentration of sperm obtained was adjusted to 1×10⁹ spermatozoa/ml in Tris-HCl buffer (0.2 M, pH 7.6). Sonication of washed spermatozoa (1×10⁹/ml) was carried out at 50 Hz for 10 min. SAF at a concentration of 80 µg resulted in blockage of ATPase activity.

To determine effects of anti-SAF antiserum on Mg²⁺ dependent ATPase activity, 100 µl of different concentrations of anti-SAF antiserum (undiluted or 1:2) were added in combination with SAF (80 µg) to 0.2 ml of sonicated sperm suspension along with the reaction mixture (0.2 ml Tris-HCl buffer, 0.2 ml of MgCl₂ (5 mM) and 0.2 ml of ATP (6 mg/ml)) [11,12]. After incubation at 37°C for 1 h, the reaction was stopped by adding 1ml of cold 10% Trichloroacteic Acid (TCA) and then incubated at 4°C overnight for protein precipitation. Also, the effect of control serum as well as PBS was checked in same manner as described above. In addition, another control tube was set up containing 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. [12]. One unit of ATPase was expressed as the µmoles of Pi released after 1h of incubation at 37°C.

**Fertility outcome:** Sexually mature female Balb/c mice were given intravaginal administration during proestrus-estrous transition phase for fertility outcome studies. SAF at a concentration of 5 µg when administered intravaginally before mating led to infertility. To evaluate the anti-SAF antiserum mediated blockage of SAF induced infertility, female Balb/c mice (n=15) were divided into different groups. Group I (n=3) received single administration of 5 µg of SAF alone while Group II was divided into three subgroups, each receiving 5 µg of SAF in combination with undiluted or 1:2 dilution of anti-SAF antisera or control serum (n=3/group). The control group III received 20 µl of PBS (n=3/group). All the animals were allowed to mate overnight with proven fertile males in a ratio of 2:1. After confirmation of mating (presence of vaginal plug), female mice were separated and were observed for consistent weight gain as an indicator of pregnancy. Each experiment was repeated twice using fresh animal’s everytime amounting to total of 30 animals.

**Results**

**Purification of SAF**

Purification of sperm ligand was carried out using salt extraction method and column chromatographic techniques. Molecular weight estimation of purified SAF showed the molecular weight to be ~71 kDa.

**Generation of antibodies**

When the titer of anti-SAF antibodies was estimated using ELISA, the data showed decrease in absorbance values of the oxidized TMB with increasing dilutions of up to 1: 16,000. While, the absorbance values in case of control serum viz. pre-immune was significantly lower in comparison to immunized serum (Figure 1).

**Sperm agglutination**

Addition of anti-SAF antiserum till dilution of 1:2 could lead to 50% blockage of SAF induced agglutination within 20sec in contrast to undiluted antiserum wherein, complete blockage was obtained.

**Acrosome status**

Assessment of acrosome reaction was carried out using PSA-FITC staining. SAF at 50 µg resulted in a premature Acrosome Reaction (AR) to the extent of 82.8 ± 1.38% (p < 0.01) in contrast to control (DMSO) samples wherein the percentages of spermatozoa with disrupted acrosomes were significantly lower (15 ± 0.79%) as seen by fluorescence on complete acrosomal region. Percentage of acrosome reacted spermatozoa in presence of SAF was comparable to positive control (90.01 ± 0.99%) i.e. Cal treated sample. However, co-incubation of anti-SAF serum (undiluted) along with SAF (50 µg) and spermatozoa resulted in blockage of SAF induced acrosomal loss. Presence of anti-SAF antiserum resulted in increased percentage of intact spermatozoa to 70.6 ± 0.9% (p < 0.01) (Figure 2). While, no significant effect on percentage AR spermatozoa was observed in case of control serum.

**Apoptotic index**

Incubation of anti-SAF antiserum along with SAF and spermatozoa decreased significantly the percentage of apoptotic spermatozoa in contrast to spermatozoa incubated with SAF alone. SAF at concentration of 150 µg significantly decreased percentage of live cells and led to a significant increase in percentage of late apoptotic as well as necrotic spermatozoa to 70.6 ± 0.9% within 30min of incubation. While...
in presence of anti-SAF antiserum, SAF could not lead to significant shift of live cells towards apoptosis as the percentage of early apoptotic spermatozoa was found to be 50.5 ± 2.1% in contrast to 70.6 ± 0.9% (p<0.05) in presence of SAF alone after incubation of 30 min. Though, no significant differences were found between percentage apoptotic cells in case of control serum (Figure 3).

**Discussion**

Bacterial infections play an important role in male infertility. These infections may lead to deterioration of spermatogenesis, impairment of sperm functions (motility/morphology), obstruction of genital tract and production of potentially destructive Reactive Oxygen Species (ROS) [13-15]. Amongst various microorganisms, *E. coli* represents to be the frequent microorganism isolated from prostatitis and epididymitis [16]. Though, studies have highlighted the negative impact of *E. coli* on sperm parameters, very few could establish the factors involved in this interaction [17]. Furthermore raising antiserum against these factors might result in generation of a therapeutic intervention against sperm damage since use of passive immunization has been quite successful for various infectious diseases [18]. On similar lines, earlier studies carried out in our laboratory led to the isolation and purification of a Spermagglutinating Factor (SAF) from *E. coli*. SAF compromised various sperm parameters such as motility, viability, and Mg²⁺ dependent ATPase activity in vitro and lead to infertility in vivo.

In order to investigate the potential function of SAF leading to impairment on human spermatozoa, the ability of the anti-SAF

| S. No. | Sample | Mg²⁺ ATPase activity (units) |
|-------|--------|-----------------------------|
| 1.    | Control| 1580 ± 2.0                  |
| 2.    | SAF (80µg) | 812.67 ± 2.08*             |
| 3.    | SAF+ anti-SAF antiserum | 1189 ± 0.05#              |
| 4.    | Control serum | 824 ± 4.0                  |

The results are expressed as mean ± S.D (*p<0.001; # p<0.05)

**Table 1:** Effect of anti-SAF antiserum against SAF induced inhibition of Mg²⁺ dependent ATPase activity.

| Dose instilled | No. of treated mice | Fertility outcome |
|----------------|---------------------|------------------|
| PBS/Control serum | 3 | 3/3 (100%) |
| SAF (5µg) | 3 | 0/3 (0%) |
| SAF+anti-SAF antiserum (undiluted) | 3 | 3/3 (100%) |
| SAF+anti-SAF antiserum (diluted) | 3 | 0/3 (0%) |

Experiment was repeated twice and identical results were obtained.

**Table 2:** Effect of anti-SAF antiserum on fertility outcome in mice
anti-serum to inhibit SAF induced damage was tested. Anti-serum raised against SAF in Balb/c mice specifically resulted in amelioration of SAF induced detrimental effects. Co-incubation of anti-SAF anti-serum with SAF resulted in blockage of spermagglutination in vitro. While no effect on spermagglutination was seen on incubation of human spermatozoa with control serum. These results pointed that agglutination of human spermatozoa might be induced by SAF only, while introduction of anti-SAF anti-serum efficiently inhibited agglutination of human spermatozoa. These results also highlighted the generation of SAF specific anti-serum which was further exploited as a curative measure against SAF.

A crucial aspect of sperm function is the Acrosome Reaction (AR) as premature AR and/or AR failure are important causes of male infertility. The AR is induced by binding to the zona pellucida, probably after ‘priming’ by progesterone released from the oocyte and surrounding cells. Spermatozoa that lose their acrosome prematurely (spontaneous AR) during reside in vagina; lose their fertilizing capacity as fertilization is restricted primarily to sperm that reach the egg intact [19]. The incubation of SAF with spermatozoa at a concentration of 50μg resulted in higher percentage of acrosome reacted spermatozoa comparable to positive control (Ca2+) as observed by fluorescent microscopy. But co-incubation of anti-SAF anti-serum along with SAF significantly inhibited the percent acrosome loss and increased the percentage of acrosome intact spermatozoa. These results again highlighted the potential of anti-SAF anti-serum to be exploited as a preventive measure.

Apoptosis, a programmed cell death, has been considered as potentially useful indices of male infertility. One of the commonly used methods for detecting spontaneous and induced apoptosis in the sperm is annexin V/PI staining. Apoptosis is associated with loss of membrane asymmetry which further results in externalization of Phosphatidyl Serine (PS) on the outside of plasma membrane. Annexin V labelled with FITC has high affinity for PS and identifies cells with deteriorated membranes [20]. In addition, PI is a vital dye that is used to measure viability and to distinguish apoptotic from necrotic cells. In the present study, SAF immediately shifted a majority of live sperm population to late apoptotic stage as significantly higher number of sperm had membrane PS externalization. Though, simultaneous incubation of spermatozoa in the presence of SAF and anti-SAF anti-serum showed a noteworthy decrease in the percentage of apoptosis along with a concomitant increase in percentage of live spermatozoa (p<0.05). While no significant differences were found between percentage apoptotic cells in case of control serum.

Motility is an expression of the viability and structural integrity of the cell. If spermatozoa prematurely lose motility, they also lose their natural fertilization potential since they cannot travel to meet oocyte. The significance of ATP hydrolysis in flagellar beating is well-known [21]. This hydrolysis is being carried out by dyenin ATPase which is an Mg2+ dependent enzyme localized on the axoneme [22]. The importance of these dyenin arms in motility was clearly established by several experiments showing a direct correlation between the quantity of dyenin arms present on the axoneme and the sliding velocity [23]. SAF described in this paper has a potential in inhibiting Mg2+ dependent ATPase. Co-incubation of sonicated spermatozoa with anti-SAF anti-serum along with SAF showed a remarkable increase in reduction of ATP, as the ATPase units rose from 812.67 ± 2.52 to 1189 ± 3.05 (P<0.05). These results are in concordance with earlier studies carried out in our laboratory wherein incubation of SAF and receptor collectively with human spermatozoa led to blockage of SAF induced immotility [7].

The results obtained so far highlighted that SAF has detrimental effects on the above-mentioned sperm parameters which could be blocked by the application of anti-SAF anti-serum. The mechanism of action of the antibody is consistent with the inhibition of the binding of SAF on spermatozoa in presence of anti-SAF anti-serum. As these factors play a very important role in successful fertilization, these might be responsible for infertility induced in Balb/c mice after intravaginal administration with SAF. From a therapeutic point of view, the inhibition of SAF activity would be one of the favoured methods for rescuing infertility. So, in vivo studies were carried out to study whether SAF induced infertility could be inhibited using anti-SAF anti-serum. It was observed that simultaneous administration of anti-SAF anti-serum with SAF (5 μg) led to conception in female mice. Similarly, the group of mice serving as control, receiving PBS or control serum along with SAF remained fertile and delivered pups after the completion of gestation period. While groups of mice instilled with SAF remained infertile.

Conclusion

The present work highlighted that SAF might be responsible for the sperm impairment and infertility. The study also enlightened the potential of anti-SAF anti-serum to be exploited as a therapeutic intervention against SAF induced adverse effects on human spermatozoa both in vitro and in vivo.

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References

1. Moretti E, Capitani S, Figura N, Pammmoli A, Federico MG, et al. (2009) The presence of bacteria species in semen and sperm quality. J Assist Reprod Genet 26: 47-56.
2. Fleisch FM, Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469: 197-235.
3. Diemer T, Ludwig M, Huwe P, Hales DB, Weidner W (2000) Influence of urogenital infection on sperm function. Curr Opin Urol 10: 39-44.
4. Sanocka D, Frazczek M, Jedrzejczak P, Szumała-AKakol A, Kurpisz M (2004) Male genital tract infection: an influence of leukocytes and bacteria on semen. J Reprod Immunol 62: 111-124.
5. Cunningham KA, Beaglely KW (2008) Male genital tract chlamydial infection: implications for pathology and infertility. Biol Reprod 79: 180-189.
6. Prabha V, Thakur N, Kaur S, Kaur N, Singh A et al. (2009) Agglutination of human spermatozoa due to human semen culture bacterial isolates bearing sperm ligand. Am J Biomed Sci 1: 126-132.
7. Kaur K, Prabha V (2013) Impairment by sperm agglutinating factor isolated from Escherichia coli: receptor specific interactions. Biomed Res Int 2013: 548497.
8. Department of Reproductive Health and Research World Health Organization (WHO) (2010) Laboratory manual for the examination and processing of human semen, (5th edn.) WHO Press, Geneva, Switzerland.
9. Castle PE, Whaley KJ, Moencher TR, Hildreth JE, Saltzman WM, et al. (1991) Monoclonal IgM antibodies against rabbit sperm for vaginal contraception. J Androl 12-S27.
10. Wattersson JJ, Shull JM, Kifles AW (1993) Quantitation of α-, β- and γ-kafirins in vitreous and opaque endosperm of Sorghum bicolor. Cereal Chem 70: 452-548497.497.
11. Kohn FM, Mack SR, Schill WB, Zaneveld LJD (1997) Detection of human sperm acrosome reaction: comparison between methods using double staining, Pium salivum agglutinin, concanavalin A and transmission electron microscopy. Hum Reprod 12:714-721.
12. Boyce A, Casey A, Walsh G (2004) A phytase enzyme-based biochemistry practical particularly suited to students undertaking courses in biotechnology and environmental science. Biochem Mol Biol Educ 32: 336-340.

13. Everaert K, Mahmoud A, Depuydt C, Maeyaert M, Comhaire F (2003) Chronic prostatitis and male accessory gland infection—is there an impact on male infertility (diagnosis and therapy)? Andrologia 35: 325-330.

14. Tremellen K (2008) Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update 14: 243-258.

15. Isaiah IN, Nche BT, Nwagu IG, Nnanna II (2011) Current studies on bacterospermia the leading cause of male infertility: a protégé and potential threat towards mans extinction. N Am J Med Sci 35: 562-564.

16. Golshani M, Taheri S, Eslami G, Suleimani AA, Fallah F, et al. (2006) Genital tract infection in asymptomatic infertile men and its effect on semen quality. Iranian J Publ Health 35: 81-84.

17. Paulson JD, Polakoski KL (1977) Isolation of a spermatozoal immobilization factor from Escherichia coli filtrates. Fertil Steril 28: 182-185.

18. Zeitlin L, Cone RA, Moench TR, Whaley KJ (2000) Preventing infectious disease with passive immunization. Microbes Infect 2: 701-708.

19. Anderson RA, Feathergill KA, Waller DP, Zaneveld LJ (2006) SAMMA induces premature human acrosomal loss by Ca2+ signaling dysregulation. J Androl 27: 568-577.

20. Shen HM, Dai J, Chia SE, Lim A, Ong CN (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod 17: 1266-1273.

21. Harper CC, Cheong M, Rocca CH, Darney PD, Raine TR (2005) The effect of increased access to emergency contraception among young adolescents. Obstet Gynecol 106: 483-491.

22. Gibbons BH, Gibbons IR (1974) Properties of flagellar “rigor waves” formed by abrupt removal of adenosine triphosphate from actively swimming sea urchin sperm. J Cell Biol 63: 970-985.

23. Mencarelli C, Lupetti P, Rosetto M, Mercati D, Heuser JE, et al. (2001) Molecular structure of dynein and motility of a giant sperm axoneme provided with only the outer dynein arm. Cell Motil Cytoskeleton 50: 129-146.