

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

Kiranjeet Kaur, Praveen Rishi and Vijay Prabha*

Department of Microbiology, Punjab University, Chandigarh, India

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: Spermagglutinating 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]. Spermatozoa 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 spermatotoxic 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*/spermatozoa-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 phenomenons, 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

*Corresponding author: Dr. (Mrs) Vijay Prabha, Professor, Department of Microbiology, Punjab University, Chandigarh, 160014, India, Tel: + 91-172-2534140; Fax: +91-172-2541770; E-mail: satishvijay11@yahoo.com

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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 semen 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 polyvinyl 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 buffer skimmed milk (PBBSM; PBS with 1% skimmed milk). Each protein coated well was blocked using 100 µl of 2% Bovine Serum Albumin (BSA) in PBBSM, incubated at 37°C for 2 h and washed three times with PBBSM. Serum samples were diluted 1 in 500 in PBS and two fold dilutions were made serially upto 1:128000. From these dilutions respective serum samples were added to different wells viz. 100 µl of anti-SAF serum was added in duplicates in wells coated with SAF. The plate was then incubated at 37°C for 1h, washed three times with PBST (PBS with 0.5 ml/L tween

20) and added 100 µl of 1:5000 dilution of goat anti-mouse IgG HRP conjugate in PBS. After 1 h, wells were washed again using PBST and 100 µl of Tetramethylbenzidine (TMB) plus hydrogen peroxide was used as substrate and the signal was amplified with 2N sulfuric acid to convert the product from a blue to a yellow colour. Absorbance of the oxidized TMB was read at 450 nm.

Control serum viz. serum collected prior to first immunization was proceeded in same way.

Anti-SAF antiserum mediated blockage of SAF induced sperm impairment

Sperm agglutination: Minimum concentration of anti-SAF antiserum required to block spermagglutination induced by SAF was determined. For this, semen sample at a concentration of 40x10⁶sperm/ml was added in different aliquots containing 120 µg of SAF. These aliquots were then incubated with undiluted or serially diluted anti-SAF antiserum in PBS (1:2, 1:4, 1:8, respectively). The mixture was immediately loaded onto slide and observed in a brightfield microscope (X400) for agglutination. As control, semen sample was also incubated with SAF alone or SAF+control serum or PBS alone.

Acrosome reaction: The effect of anti-receptor antiserum on Acrosome Reaction (AR) was verified using *Pisum sativum agglutinin-fluorescein isothiocyanate* (PSA-FITC) by the method of Kohn et al. with slight modifications [11]. Briefly, spermatozoa were kept for 3 h at 37°C in Ham's F-10 HSA medium containing HEPES (20 mmol/L) for capacitation. After incubation, only highly motile spermatozoa were collected and were divided in five aliquots having 1x10⁶ motile spermatozoa in each tube. Each aliquot containing capacitated sperm were incubated with either a) DMSO (spontaneous AR as negative control); b) CaI (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).

Apoptotic index: To study whether SAF induced death was associated with apoptosis, human semen samples were washed twice in Briggers Whitten Whittingam medium (BWW, pH 7.4) and divided in aliquots having 4x10⁶/ml motile spermatozoa in each tube and incubated for 30min with either a) BWW medium (serving as control); b) SAF (150 µg); c) SAF+anti-SAF antiserum (undiluted). Control serum was also incubated under same conditions along with SAF.

➤ 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 4x10⁶ 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 II™. 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.

Mg²⁺ 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 1×10^8 spermatozoa/ml in Tris-HCl buffer (0.2 M, pH 7.6). Sonication of washed spermatozoa (1×10^8 /ml) was carried out at 50 Hz for 10 min. SAF at a concentration of 80 μ g resulted in blockage of ATPase activity.

To determine effects of anti-SAF antiserum on Mg²⁺ dependent ATPase activity, 100 μ l of different concentrations of anti-SAF antiserum (undiluted or 1:2) were added in combination with SAF (80 μ g) 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% Trichloroacetic 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 μ moles of Pi released after 1h of incubation at 37°C.

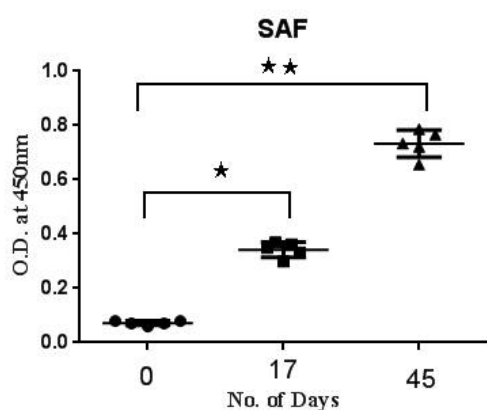


Figure 1: Specific antibody response to spermagglutinating factor (SAF) isolated from *E. coli* in mice. Group of BALB/c mice was immunized intraperitoneally. All serum samples were tested by ELISA at 1:500 dilution before and 45 days after a boost, using SAF as the coating antigen. The data are representative results of two independent experiments. * indicate significant differences ($p < 0.01$) between day 0 and day 17 and ** indicate significant differences ($p < 0.001$) between day 0 and day 45.

Fertility outcome: Sexually mature female Balb/c mice were given intravaginal administration during proestrous-estrous transition phase for fertility outcome studies. SAF at a concentration of 5 μ g 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 μ g of SAF alone while Group II was divided into three subgroups, each receiving 5 μ g of SAF in combination with undiluted or 1:2 dilution of anti-SAF antiserum or control serum ($n=3$ /group). The control group III received 20 μ l 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 upto 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

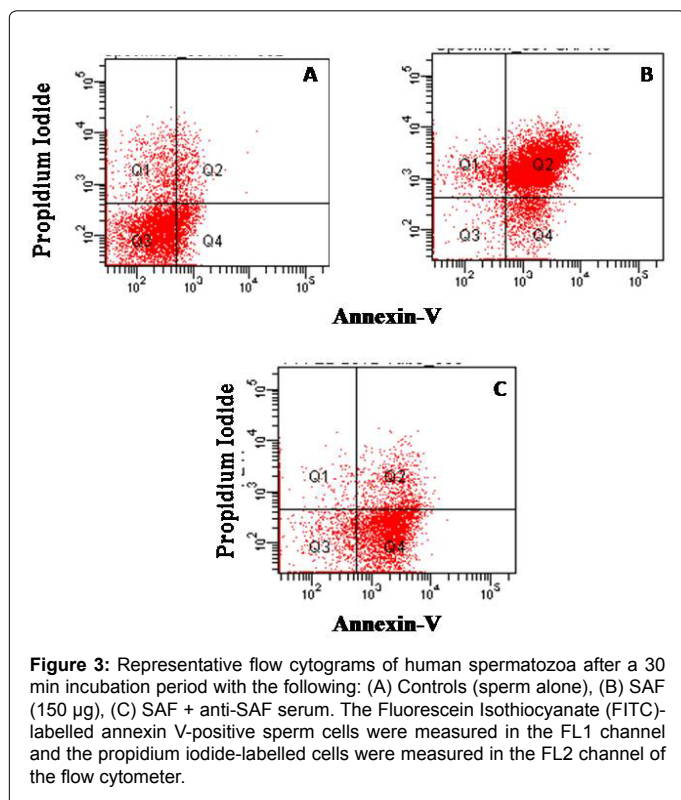
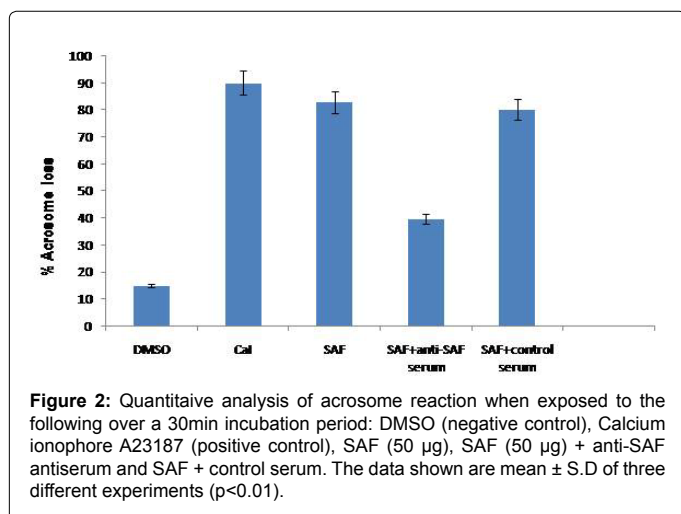
Addition of anti-SAF antiserum till dilution of 1:2 could lead to 50% blockage of SAF induced agglutination within 20sec in contrast to undiluted antiserum wherein, complete blockage was obtained.

Acrosome status

Assessment of acrosome reaction was carried out using PSA-FITC staining. SAF at 50 μ g resulted in a premature Acrosome Reaction (AR) to the extent of $82.8 \pm 1.38\%$ ($p < 0.01$) in contrast to control (DMSO) samples wherein the percentages of spermatozoa with disrupted acrosomes were significantly lower ($15 \pm 0.79\%$) as seen by fluorescence on complete acrosomal region. Percentage of acrosome reacted spermatozoa in presence of SAF was comparable to positive control ($90.01 \pm 0.99\%$) i.e. CaI treated sample. However, co-incubation of anti-SAF serum (undiluted) along with SAF (50 μ g) and spermatozoa resulted in blockage of SAF induced acrosomal loss. Presence of anti-SAF antiserum resulted in increased percentage of intact spermatozoa to $43.2 \pm 1.05\%$ leaving behind the percentage of AR spermatozoa to be $39.7 \pm 2.09\%$ ($p < 0.01$) (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 μ g significantly decreased percentage of live cells and led to a significant increase in percentage of late apoptotic as well as necrotic spermatozoa to $70.6 \pm 0.9\%$ 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 $50.5 \pm 2.1\%$ in contrast to $70.6 \pm 0.9\%$ ($p < 0.05$) 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).

Mg²⁺ dependent ATPase activity

Incubation of SAF with sonicated human spermatozoa could significantly inhibit Mg²⁺ dependent ATPase activity of spermatozoa in a dose dependent manner. At a concentration of 80 µg, there was decrease from 1580 ± 2.0 (control) ATPase units to 812.67 ± 2.52

($p < 0.01$) ATPase units within 1h of incubation with SAF (Table 1). Incubation of sonicated spermatozoa with anti-SAF antiserum led to blockage of SAF induced inhibition of Mg²⁺ dependent ATPase. It was observed that co-incubation of anti-SAF antiserum with SAF could sustain the ATPase units at 1189 ± 3.05 ($p < 0.05$), although; no such effect was seen in case of control serum.

In vivo studies

Evaluation of anti-SAF antiserum against SAF induced infertility was carried out. For this, female mice were divided in different groups. Results showed that Group I mice administered with SAF and Group II mice instilled with SAF+anti-SAF antiserum (diluted) or control serum were rendered infertile. While remaining Group II mice instilled with SAF+anti-SAF antiserum (undiluted) remained fertile. Mice belonging to group III receiving PBS remained fertile and delivered pups after completion of gestation period (Table 2). No differences in number of pups were found between Group II (undiluted) and Group III mice. The experiments were repeated twice and similar results were obtained.

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*. The present study was conducted to validate the involvement of SAF in sperm damage by using anti-SAF antiserum and raised antiserum was further evaluated as an antidote against SAF induced detrimental effects on human spermatozoa both *in vitro* and *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	Mg ²⁺ ATPase activity (units)
1.	Control	1580 ± 2.0
2.	SAF (80µg)	812.67 ± 2.08*
3.	SAF+ anti-SAF antiserum	1189 ± 0.5#
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

antiserum to inhibit SAF induced damage was tested. Antiserum raised against SAF in Balb/c mice specifically resulted in amelioration of SAF induced detrimental effects. Co-incubation of anti-SAF antiserum 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 antiserum efficiently inhibited agglutination of human spermatozoa. These results also highlighted the generation of SAF specific antiserum 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 (CaI) as observed by fluorescent microscopy. But co-incubation of anti-SAF antiserum 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 antiserum 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 antiserum 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 Mg^{2+} 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 Mg^{2+} dependent ATPase. Co-incubation of sonicated spermatozoa with anti-SAF antiserum 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 antiserum. The mechanism of action of the antibody is consistent with the inhibition of the binding of SAF on spermatozoa in presence of anti-SAF antiserum. 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 antiserum. It was observed that simultaneous administration of anti-SAF antiserum 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 antiserum 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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