



## Original Research Article

# Impact of chemical supplementation on the impairment of sperm parameters induced by sperm immobilizing factor

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## A B S T R A C T

### Keywords

Sperm immobilizing factor, spermatozoa, chemical supplements, *Staphylococcus aureus*, sperm parameters.

For successful fertilization, motility and viability are the most essential sperm parameters. Impairment of sperm motion parameters may be associated with sperm immobilization/ agglutination by mere presence of bacteria or their excreted toxic products. In an earlier work done in our laboratory, we have been able to isolate a sperm immobilizing strain of *Staphylococcus aureus* and the factor i.e. Sperm immobilizing factor (SIF) has also been purified. Further it is well known that supplementation with some agents viz. vitamins and antioxidants elicit beneficial effects on sperm motility *in vitro*. Therefore, when the present *in vitro* study was conducted to evaluate if chemical supplements could protect spermatozoa against SIF induced sperm immobilization. The results showed that all the vitamins tested (A, B1, B9, C, E) and N-Acetyl L-Cysteine were able to antagonise the effect of SIF thereby protecting sperm motility, viability and morphological defects. Conversely, Glutathione, Ferrous sulphate and Sodium selenite could not inhibit the sperm impairment induced by SIF. In addition, deterrence of SIF induced sperm immobilization was also observed on pre-incubation of Vitamin B1, E, Glutathione and Sodium selenite with SIF. These findings indicate the potential of chemical supplements in improving sperm parameters.

## Introduction

Infertility, a disease of reproductive system affecting both men and women is defined as the inability of a couple to achieve conception after one year or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al.*, 2009). In the past two decades, infertility has become a major clinical concern, especially in developed countries. The underlying causes may be ascribed to pathologic conditions affecting one or both members of a couple. Factors

associated with males that may preclude infertility include genital injury, genital tract obstructions, varicocele, genital malformations, endocrine and metabolic diseases, and allegedly psychiatric conditions. In females, infertility can be due to the factors that are usually divided into endocrine, vaginal, cervical, uterine, tubal and pelvic-peritoneal.

Besides these, genital infections are also one

of cause of infertility (Khalili and Sharifi-Yazdi, 2001). The negative impact of some microorganisms viz. *Staphylococcus aureus*, *Escherichia coli*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma genitalium*, *Candida albicans*, *Neisseria gonorrhoeae*, on sperm function has been claimed.

It has been reported that *S. aureus*, a frequently isolated microorganism in genitourinary infections (Sanocka-Macleiewska *et al.*, 2005), may exert adverse effect on human sperm motility. The negative influence of this species on sperm quality is partially due to its effect on sperm motility via sperm immobilization/agglutination (Diemer *et al.*, 1996). This may be due to direct adherence of bacteria with spermatozoa or by excretion of bacterial toxic products. In an earlier work done in our laboratory, strain of *S. aureus* causing sperm immobilization was isolated from the cervix of a woman with unexplained infertility and the factor responsible for sperm immobilization viz. Sperm immobilizing factor (SIF) was also purified (Prabha *et al.*, 2009). Also, it has been observed that SIF causes infertility in female mouse model (data not published).

However, there is a rationale to support the use of an array of chemical supplements, having the potential to improve sperm motion parameters, possibly fertilization rates and pregnancy outcomes. Thus the present *in vitro* study was conducted to evaluate whether chemical supplements could protect human spermatozoa against the sperm immobilization by SIF.

## Materials and Methods

**Semen Samples.** Semen samples were obtained from males attending infertility clinic at Government Multispeciality Hospital, Sector 16, Chandigarh, by

masturbation into sterile wide mouth container. Only ejaculates showing normal semen parameters according to WHO criteria (2010) were used. Samples underwent liquefaction at room temperature for 30 min. Experiments were performed within 1 h of obtaining samples. A preparation of washed sperm samples was done, in which the sperm cell pellet was retained after centrifugation at 500 rpm for 10 min and thereafter it was washed twice with sterile phosphate buffer saline (PBS) (50mM, pH 7.2). The protocols for the present study were approved by Panjab University Institutional Ethics Committee; vide letter no. 1485/1DS dated 08.06.2010.

**Microorganism.** The strain of *S. aureus* used in present study showing immobilization of human spermatozoa *in vitro* had already been isolated in our laboratory from the cervix of a woman suffering from unexplained infertility, attending the Department of Obstetrics and Gynecology, GMSH, Sector-16, Chandigarh, India (Prabha *et al.*, 2009).

**Isolation and Purification of SIF from *S. aureus*.** SIF was extracted and purified from 72 h old cell culture of *S. aureus* by the method previously standardized in the laboratory [5]. Briefly, the cell culture of *S. aureus* grown in brain heart infusion (BHI) broth, under shake conditions (220 rpm) at 37°C for 72 h, was centrifuged at 5,000 rpm for 15 min at 4°C. SIF was purified from the supernatant by ammonium sulphate precipitation, gel permeation chromatography, and ion exchange chromatography. To check the purification status, polyacrylamide gel electrophoresis (PAGE) was carried out.

**Minimum concentration of SIF showing 100% immobilization of spermatozoa.**

Minimum concentration of SIF showing 100% sperm immobilizing activity was determined by mixing different concentrations of SIF (5, 10, 20, 40, 60, 80 and 100 µg) with human spermatozoa. After 30 min of incubation, the highest dilution of SIF displaying 100% immobilization of motile spermatozoa was taken as the minimum concentration.

*Effect of chemical supplements on sperm impairment upon co-incubation with SIF.*

The effect of chemical supplements namely, retinyl palmitate (vitamin A), riboflavin (vitamin B<sub>2</sub>), folic acid (vitamin B<sub>9</sub>), L-ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, N-acetyl-L-cysteine, sodium selenite and ferrous sulphate (FeSO<sub>4</sub>.7H<sub>2</sub>O) was checked on sperm immobilizing activity of SIF. These solutions were freshly prepared at a concentration of 25mM. All these supplements were water soluble but, Vitamin A and E, due to their lipophilic nature, were prepared in olive oil. Each experiment was carried out thrice with semen samples from different donors.

*Motility.* To study the effect of these chemical supplements on sperm immobilizing activity of SIF, semen sample was incubated with 1) PBS (negative control) 2) SIF (positive control causing 100% sperm immobilization) 3) Chemical supplements 4) Chemical supplements and SIF. All these suspensions were incubated at 37°C for 30 min. On completion of incubation period, that is, after 30 min, one drop (10 µl) of each was placed on glass slide covered with a coverslip and checked for immobilization at X400 magnification under ordinary light microscope.

*Viability.* To assess the effect of chemical supplements on viability of spermatozoa upon SIF treatment, semen sample was incubated with same set of controls and tests

as mentioned above. All the suspensions were incubated at 37°C for 30 min. On completion of incubation period, that is, after 30 min, two drops of 1% eosin were added and mixed well. The mixture was covered with a coverslip on a clean glass slide and observed under light microscopy at X400 to differentiate unstained living cells from dead spermatozoa, which stained pink. At least 20 fields were screened per concentration.

*Morphology.* To evaluate spermatozoa morphological changes caused by co-incubation of SIF with chemical supplements, SEM was done by the standard method with slight modifications. Sperm suspension was centrifuged at 500X gravity for 10 min, mixed with either 1) PBS, 2) purified SIF, 3) chemical supplements, 4) chemical supplements and SIF, and incubated at 37°C for 2 h. To each tube, 1 ml of 2.5% phosphate buffered glutaraldehyde was added and mixed gently with a Pasteur pipette. After 30 min, samples were centrifuged for 10 min at 500X gravity and washed twice in PBS (50 mM, pH 7.2). One drop of fixed, washed spermatozoa was placed on a silver painted adhesive tape mounted on brass stubs and air dried. Gold coating was done at 100 Å on JFC-1100 fine coat ion sputter (Jeol, Tokyo, Japan). This gold coated stub was finally examined at different magnifications under the scanning electron microscope (model JSM-6100, SM-Jeol 20kV). SEM was carried out at sophisticated analytical instrumentation facility (SAIF), Panjab University, Chandigarh.

*Effect of chemical supplements on human spermatozoa when pre-incubated with SIF.*

In order to evaluate the effect of chemical supplements on SIF induced sperm immobilization, spermatozoa were exposed to SIF (10 µg) with and without 30 min pre-incubation with chemical supplements (25

mM). After 30 min of incubation, spermatozoa were checked for motility, viability and morphology.

## **Results and Discussion**

*Isolation and purification of SIF from S. aureus.* Sperm immobilizing activity was present maximally in the culture supernatant of 72 h old *S. aureus*. SIF could be precipitated at 60% to 80% saturation with ammonium sulfate. After filtration through a Sephadex G-100 column, immobilization activity was present in fractions 12 to 18 with a peak value in fraction 15. After further purification through diethylaminoethyl cellulose column, fractions 47 to 52 eluted with PBS containing 0.4 M NaCl were highly concentrated with SIF and the peak value was found in fraction 49. Concentrated fractions showing immobilization activity when subjected to molecular weight studies revealed that SIF was an approximately 20 kDa protein.

*Minimum concentration of SIF showing 100% immobilization of spermatozoa.* Minimum concentration of SIF showing 100% immobilization of spermatozoa after 30mins of incubation was found to be 10 µg.

### **Effect of chemical supplements on sperm impairment upon co-incubation with SIF**

*Motility.* Firstly, sperm sample (with initial 60% motility) when incubated with PBS at 37°C for 30 min showed reduced sperm motility (40%). On the other hand, SIF upon incubation with semen sample resulted in 100% immobilization of spermatozoa. Then a comparison of sperm motility in the presence of chemical supplements each with and without SIF was done. Supplementation of each one of these chemicals viz. Vitamin A, Vitamin B1, Vitamin C and N-acetyl-L-

cysteine with spermatozoa at 37°C for 30 min showed percentage of motile sperms same as that recorded for initial sample 60%. However, these supplements upon co-incubation with SIF protected sperm motility, thereby antagonizing the effect of SIF. Further, percentage motility on supplementation with Vitamin B9, Vitamin E and Sodium selenite was same as that with PBS 40%. Following SIF treatment, protective effect on sperm motility was seen in case of Vitamin B9 and Vitamin E, whereas no such effect was observed in case of Sodium selenite. On the contrary, Glutathione and Ferrous sulphate had negative effects themselves on the motility of spermatozoa, though no effect on efficiency of SIF induced sperm immobilization was found in the presence of these supplements (Fig 1).

*Viability.* When semen sample was incubated with PBS at 37°C for 30 min, a decrease in sperm viability was observed from initial 85% to 60%. Interestingly, upon co-incubation with SIF for 30 min, the resultant viability of spermatozoa was mere 10%. Supplementation of each of the chemicals viz. Vitamin A and N-acetyl-L-cysteine to sperm sample did not affect percentage viability compared to the initial sample at 30 min post incubation. However, upon co-incubation with SIF, viability was increased markedly by these supplements, thus counteracting the effect of SIF. Further supplementation of Vitamin B1, Vitamin B9, Vitamin C and Vitamin E to spermatozoa generated no differences compared to that with PBS with respect to sperm viability at 30 min post-incubation. Moreover, these supplements upon co-incubation with SIF protected sperm viability. Conversely, Glutathione, Ferrous sulphate and Sodium selenite themselves led to diminution of sperm viability. In addition, these supplements showed no effect on

sperm impairing activity of SIF (Fig 2, 3).  
*Morphology.* The effect of chemical supplements on the morphology of human spermatozoa co-incubated with SIF was studied using Scanning Electron Microscopy (SEM). The Scanning Electron micrographs of human spermatozoa treated with PBS showed normal morphology viz. intact head, neck, mid piece and tail. In contrast, treatment of semen sample with SIF resulted in prominent morphological defects i.e. loosening, disruption of membrane around the neck and mid-piece, and curling of tail. However, chemical supplement treated spermatozoa co-incubated with SIF showed morphology same as that of a normal sperm with intact head, neck, mid-piece and tail (Fig 4).

3.4. *Effect of chemical supplements on human spermatozoa when pre-incubated with SIF.* When the semen sample was added to the chemical supplements pre-incubated with SIF for 30 min at 37°C, it was observed that inhibitory effect of SIF on sperm motility was prevented in the presence of Vitamin B1 (55%), E (50%), Glutathione (30%), and Sodium selenite (35%) (Fig 5). These results were compared to positive control consisting of semen sample incubated with SIF showing 100% immobilization in 30 min. Similarly sperm viability was also increased by Vitamin B1 (61%), Vitamin E (62%), Glutathione (54%) and Sodium selenite (48%) (Fig 6). However, no significant difference in sperm motility and viability was observed with the rest of the chemical supplements.

Infertility is a common problem affecting one couple in six. Twenty-five percent of infertile couples have more than one factor that contributes to their infertility. In approximately 40 percent of infertile couples, the male partner is either the sole cause or a contributing cause of infertility. Another 40 percent is contributed by female

partner and the rest is attributed to unexplained causes. In females, infertility can be due to the factors that are usually divided into endocrine, vaginal, cervical, uterine, tubal and pelvic-peritoneal. Besides these, bacterial infections are relevant cause in the etiology of infertility (Khalili and Sharifi-Yazdi, 2001). Among bacterial species that are implicated in infertility are well known causative agents of genitourinary infections such as *Staphylococcus aureus*, *Escherichia coli*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma genitalium*, *Candida albicans*, and *Neisseria gonorrhoeae*.

Of the various bacteria, *S. aureus* probably represents the most frequently isolated microorganism in genitourinary infections (Sanocka-Macleiewska *et al.*, 2005). The direct inhibitory effect of *S. aureus* on progressive motility of spermatozoa has been reported. This microorganism in the cervix inhibits sperm motility either itself or by producing some extracellular metabolites. In our laboratory, strain of *S. aureus* causing sperm immobilization *in vitro*, has already been isolated from cervix of woman with unexplained infertility. The factor causing immobilization of spermatozoa has also been isolated and purified using the standardized method. The emerging reports of rapid spurt of various chemical supplements as a treatment for infertility have instigated us to find out the protective effect of a range of chemical supplements against SIF induced sperm immobilization.

Earlier reports by Agarwal and Sekhon (2010) have shown that vitamin C and E may protect loss of sperm motility as well as enhance the performance. Similar studies by Donnelly *et al* (1999) showed that the addition of Vitamin C and E to asthenozoospermic samples resulted in

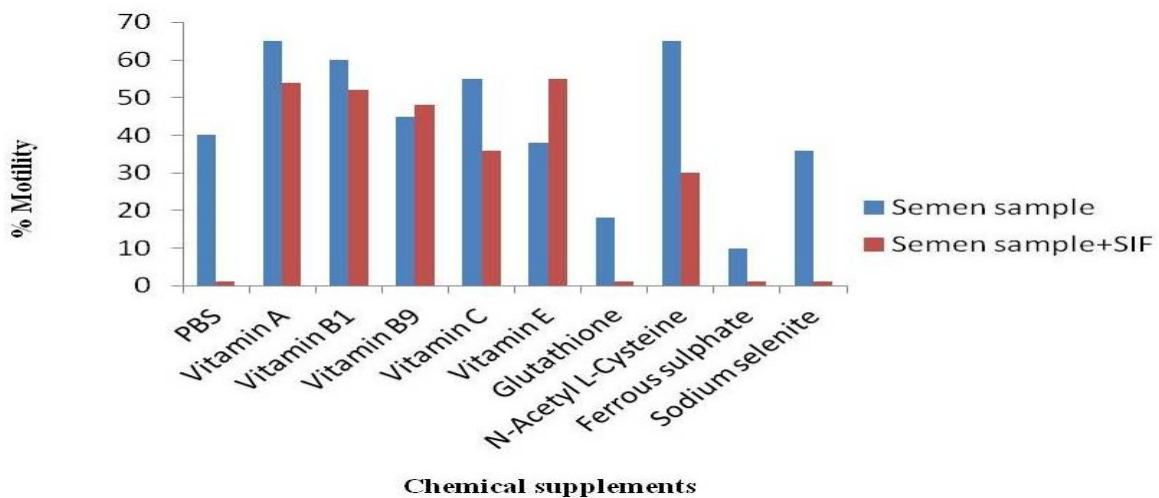
reduced H<sub>2</sub>O<sub>2</sub>- induced ROS production, leading to significant improvement in sperm motility and viability. Thus in present study when we examined the effect of different chemical supplements on sperm immobilization activity of SIF, all the vitamins and N-Acetyl L-Cysteine were able to antagonise the effect of SIF protecting sperm motility and viability. In this context, previous findings of Oeda *et al* (1997) also reported that incubating semen samples with N-acetyl-L-cysteine led to improved sperm motility and viability. Also, Bansal and Bilaspuri (2009) reported that supplementation with vitamin E improved the cattle sperm motility and viability. Similar observations have been made on humans (Verma and Kanvar, 1999), buffalo (Singh *et al.*, 1989) and boar (Slebozinska *et al.*, 1995).

Further, it is known that chemical supplements such as zinc and selenium have a large impact on the morphological integrity of sperm, especially the midpiece formation. Deficiency of these chemicals can lead to axonemal disruption, abnormal flagella and a poorly formed or absent mid-piece (Omu *et al.*, 2008). Therefore in the present study, the effect of chemical

supplements on the morphology of human spermatozoa when co-incubated with SIF was studied using SEM, prominent morphological defects were observed in case of spermatozoa treated with SIF while normal morphology was preserved on co-incubation with chemical supplements. Our results are in concordance with the studies by Cheah and Yang (2011) who reported that vitamin E has a remarkable effect to restore incomplete acrosomal membranes. Another major outcome of this study was the deterrence of SIF induced sperm immobilization on pre-incubation of chemical supplements with SIF. It was observed that Vitamin B1, E, Glutathione and Sodium selenite increased sperm motility and viability when pre-incubated with SIF. In line with our findings, Barbonetti *et al* (2013) reported prevention of sperm motility loss induced by soluble products of *E. coli* on pre-incubation with lactobacilli.

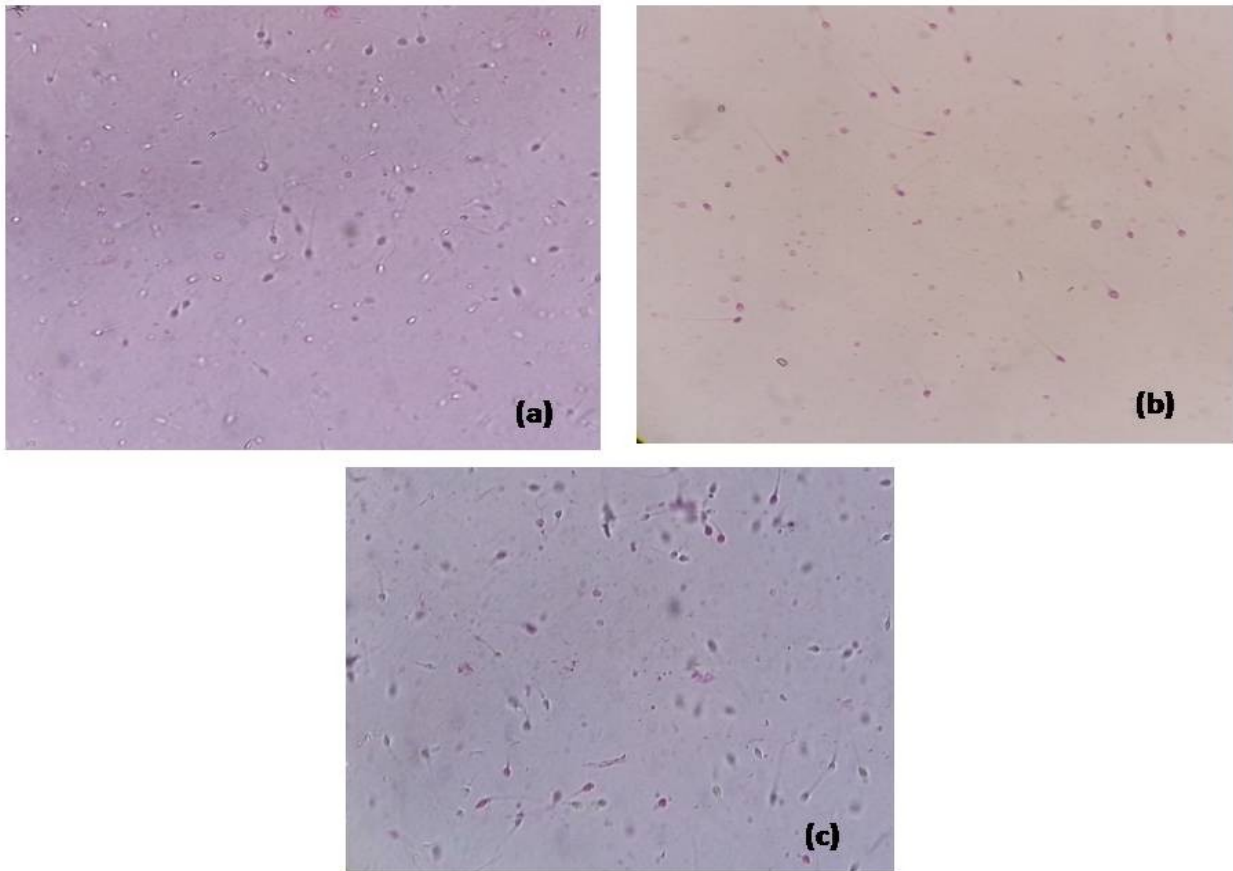
These findings shed light on potential of chemical supplements in improving sperm motion parameters, possibly fertilization rates and pregnancy outcomes in female host.

**Figure.1** Effect of different chemical supplements on sperm immobilization induced by SIF

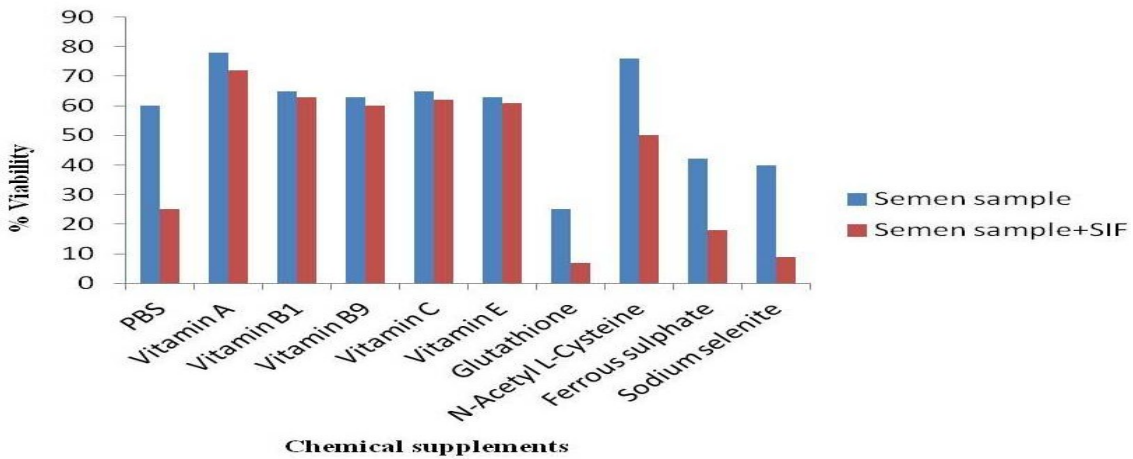




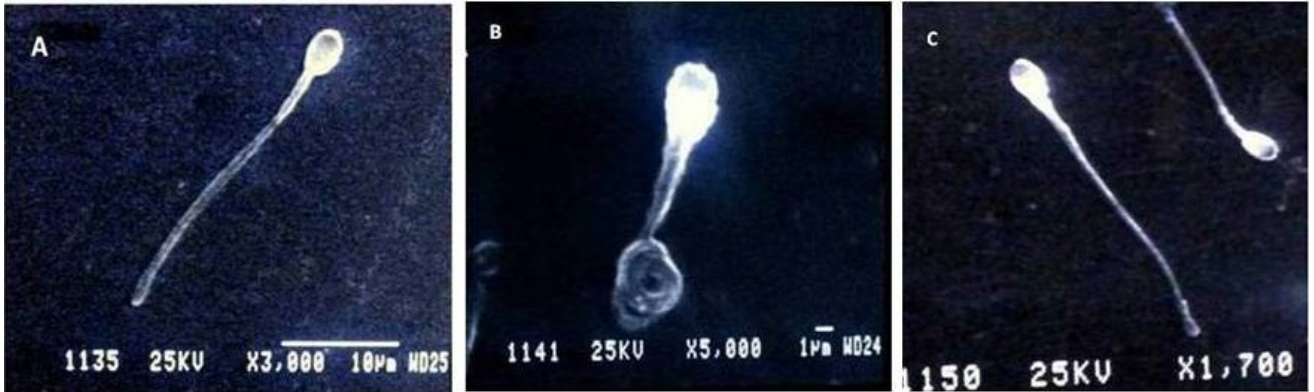
**Figure.2** Eosin staining of human spermatozoa with (a) PBS, unstained live spermatozoa (b) SIF (10µg), pink stained dead spermatozoa (c) SIF alongwith chemical supplements



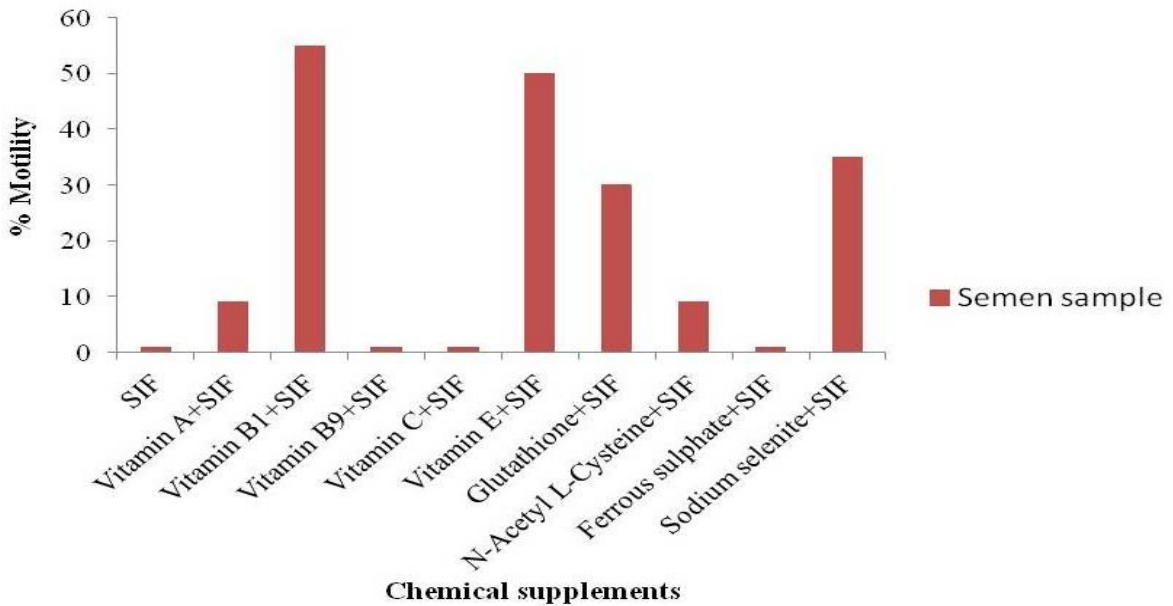
**Figure.3** Effect of different chemical supplements on the percentage of sperm viability when co-incubated with SIF



**Figure.4** SEM. (A) Normal washed human sperm sample. Reduced from X3,000. (B) Tail curling, and head and mid piece morphological changes in human sperm upon SIF treatment. Reduced from X5,000. (C) Vitamin E (representative) mediated prevention of tail curling, and morphological changes in sperm by SIF. Reduced from X1,700.

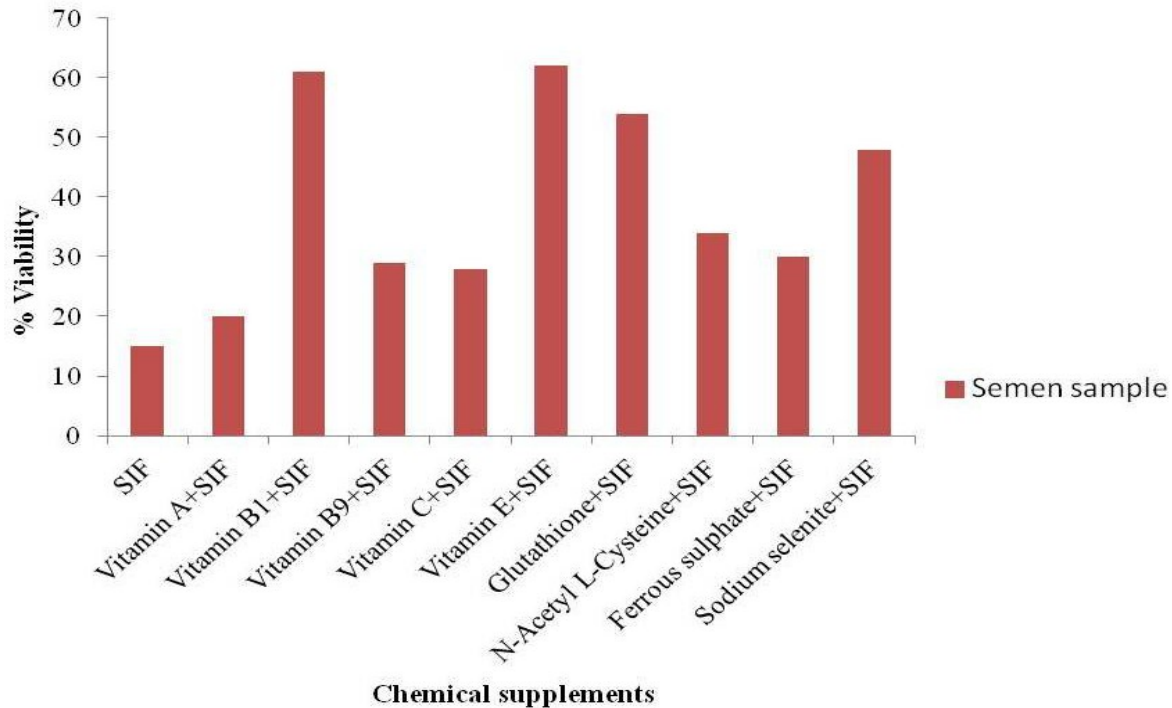


**Figure.5** Effect on percent motility of human spermatozoa when treated with chemical supplements pre-incubated with SIF.





**Figure.6** Effect of chemical supplements pre-incubated with SIF on the percent viability of human spermatozoa.



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