DISCUSSION
5. DISCUSSION

Infertility is an increasingly significant health problem in many areas of the world. The etiology of infertility is of major importance if any therapeutic or preventive measures are to be implemented. Substantial number of patients with unexplained infertility attend infertility clinics, with reported prevalence ranging from 6% to almost 60%. The possible causes of unexplained infertility could be anatomical (fixed retroversion, uterine abnormality), abnormalities of follicular development, ovulation and fertilization immunological factors, cervical mucus abnormalities, occult infectious mycoplasmas (cervical or seminal) ureaplasmas or chlamydial and occult endometriosis, psychogenic and emotional factors. Although various reasons for unexplained infertility are known, no definite aetiological factor can be found. Inability to identify a specific cause might be due to lack of knowledge, technology or both. Therefore, additional techniques, should be advocated to further the infertility investigation.

Among the many causes of infertility, those related to infection are the most difficult to treat, yet the most amenable to prevention. Bilateral tubal occlusion in female patients is the most common infertility etiology associated with infections. Tubal infections are due to C. trachomatis, N. gonorrhoeae or other sexually transmitted pathogens.
Bacterial vaginosis also puts the patient at increased risk of upper genital tract infection, with severe consequences to fertility and outcome of pregnancy (Spiegel, 1991). However, the importance of microbial colonization in infertility or subfertility remain controversial.

5.1 MICROFLORA OF THE FEMALE GENITAL TRACT

Genital tract of the females has its own microflora which is present normally without causing any apparent disease. The question arises whether these microorganisms have any role to play in fertility/infertility of females. It is now known that the organisms which comprise the normal flora also represent the major pathogens in nonvenereal infections of the female genital tract. This supports an endogenous route of infection. It is possible that the normal flora in the genital tract of females suffering from unexplained infertility could be producing certain metabolites interfering the normal process of fertilization in some way or the other.

Therefore, in the present study, the comparison of endocervical isolates from infertile females suffering from primary unexplained infertility was done with the endocervical isolates from fertiles. The results (Table 1) showed the presence of different types of microorganisms. Micrococcus luteus, S. aureus, S. epidermidis and Streptococcus faecalis could be isolated from fertile as well
as infertile females. *Micrococcus sedentarius* and *Planococcus* sp. were present only in the fertiles while *Bacillus subtilis*, *Pseudomonas maltophilia*, *Micrococcus agilis*, *E. coli* and *Kluyveromyces* sp. were absent in the fertiles and present only in the infertiles. However, from the results, it cannot be concluded that these are the specific microorganisms which are present in fertiles and infertiles, because in a number of cases these were absent. For example, although *B. subtilis* was the most common of the microorganisms that were present in infertiles and not in fertiles, it could be isolated only from 4 cases. In the rest 7 cases, it was absent.

In the earlier reports, *S. viridans*, *S.aureus*, Streptococci (entrococcal, hemolytic and gamma type), *P.mirabilis*, *P.aeruginosa*, Micrococci, *S.faecalis*, Lactobacilli, *S.epidermidis* and *Diphtheroides* could be isolated from cervices of fertiles as well as cervicitis patients (Kaye et al., 1954; Hok et al., 1967; de Louvois et al., 1975). *E.coli* has been reported either from only fertiles (de-Louvois et al., 1975) or only from infertiles (Kaye et al., 1954). The presence of *B.subtilis* only in infertile is in accordance with the work of Kaye et al. (1954). Lindner et al. (1978) postulated that enterobacteriaceae and yeasts are not part of the normal flora. At the same time, other workers showed its presence in the genital tract of healthy fertile females. Thus there is
no clear difference in the genital tract flora of fertile and infertile women apart from the cases where infertility was due to the known pathogens like *T. vaginalis*, *C. trachomatis*, *U. urealyticum* etc.

It is quite clear from the above results that there are some microorganisms which are present only in the infertiles and absent in the fertiles. These microorganisms did not cause any vaginal or cervical infection in the females. But these microorganisms do produce some extracellular substances that directly come in contact with the spermatozoa and cause lowering of fertility by inhibiting sperm motility. Lowering or complete loss of sperm motility has been shown by microorganisms by direct agglutination of spermatozoa (Teague et al., 1971; Gupta et al., 1978) or by certain microbial factors released by these organisms (Paulson and Polakoshi, 1977; Eschenbach and Sweet, 1985; Kaur et al., 1988a).

5.2 CULTURE SUPERNATANT, WASHED CELLS, CELL WASHINGS AND THEIR EFFECT ON HUMAN SPERMATOZOA

The results (Table 2 and 3) show that bacterial culture supernatants inhibit the motility of spermatozoa which suggests that some of the factors are present in the culture supernatants of microorganisms. It is also known that microorganisms produce a wide variety of pharmacologically active substances which can affect sperm motility (Matthews and Wade, 1977).
The effect of cell free supernatants of 24h and 48h cultures of endocervical isolates from infertiles females on human spermatozoal motility was compared with that of the isolates from fertiles (Table 4 and 5). The number of isolates showing significant decrease in motility were significantly higher (60% isolates) than those from fertiles (20%).

Cell washings of 40-43% and 10-20% of the endocervical isolates from infertiles and fertiles were effective in decreasing the sperm motility (Table 13 and 14). It seems that the extracellular metabolites of the bacteria that are loosely attached on the cell surface are released in the cell washings which are responsible for inhibiting spermatozoal motility.

The heat inactivated (100°C for 10 min) supernatants failed to inhibit sperm motility suggesting the presence of a heat labile protein that was responsible for causing the effect.

Many workers have shown the spermicidal activity of bacteria from cervices of infertiles. Matthews and Buxton (1951) reported that only the pathogenic bacteria were spermicidal while every organism tested by Kaye et al. (1954), including nonpathogens as Staphylococcus albus, diphtheroids, and Doderlein’s bacillus, immobilized or killed all spermatozoa within 1 hour. Buxton et al. (1954) also showed that all organisms belonging to the genera
*Escherichia, Aerobacter, Paracolobactrum and Alcaligenes* were highly spermicidal and clumping began immediately after mixing the spermatozoa and the organisms. It was suggested that further investigations on the effects of spermicidal organisms on fertility were required since adequate control studies also showed similarity in incidence of spermicidal organisms in good postcoital tests. Our results also show that in case of fertiles, there are organisms that decrease the sperm motility. However, the number of such isolates in fertiles is significantly low as compared to the infertiles.

When washed cells were used to study their effect on sperm motility (Table 6 and 7), it was observed that 17 isolates out of 30 isolates from infertiles inhibited motility of spermatozoa and 8 out of which decreased the motility by agglutination. Only 5 isolates from the total 20 isolates from fertiles inhibited motility and 2 out of these 5 agglutinated spermatozoa. It was observed that sperm agglutination of spermatozoa occurred only with washed cells and not with supernatants or cell washings. These findings suggest that there is some factor which is present on the cell surface which is responsible for sperm agglutination. The degree of agglutination was found to be maximum at 37°C (Table 10) and in citrate egg yolk solution. Agglutinating capacity was inhibited when cells were treated for 10 min at 100°C in case of *B.subtilis* and *E.coli* and at 70°C in case of *S.faecalis, S.aureus* and *M.sedentarius* (Table 11).
Rosenthal (1943) presented evidence to indicate that the sperm agglutinating factor of E. coli is within the cell itself. The reaction occurs with fresh or formalized bacterial cells in acidic or alkaline medium, and with cells repeatedly washed in saline. He found that the Berkefeld filtrate of E. coli cultures was inactive. If these cells were killed by boiling, its sperm agglutinating capacity was destroyed (Rosenthal, 1943; Buxton et al., 1954).

In our laboratory, similar results were observed with B. subtilis and E. coli. Other sperm agglutinating microorganisms (S. faecalis, S. aureus, M. sedentarius) could be inactivated at lower temperatures. It is believed that the active principle may be due to a special protein substance or an enzyme in the bacteria.

The foregoing results have shown that motility of spermatozoa is affected directly by agglutination or indirectly by the metabolic end products. The reasons given for these observations are scarce. In an earlier report, Brokaw (1980) has suggested that the enzyme elastase could be causing immobilization of spermatozoa due to the breakdown of elastin like molecules in the tubules. The effect of microbial elastase on spermatozoal motility has also been reported by Kaur et al. (1988a).

5.3 PROTEASES AND ELASTASES

Elastase is one of the proteases that has the
specific property of degrading elastin. Different types of elastases could be obtained from both eukaryotic and prokaryotic cells e.g. human alveolar macrophages (Reilly et al., 1989), neutrophil elastase (Takahashi et al., 1988), pancreatic elastase (Bieth et al., 1989) and elastase from different microorganisms. Now the question is whether it is the elastase alone which is causing immobilization of spermatozoa or it is the protease. Therefore, in the present study, the production of caseinolytic protease (P) and elastases degrading insoluble elastin (E) and soluble elastin (S.E.) by all the endocervical isolates were screened.

The results obtained in both fertile and infertile groups were compared (Table 18). The number of isolates producing (E and S.E.) significantly higher (14, 5 and 9 respectively) in infertiles than in fertiles (4, 1 and 4 respectively). B. subtilis, P. maltophilia and M. sedentarius produced both protease and elastases.

On the basis of the production of the above enzymes, various groups were made and their effect on sperm motility was compared. 100% isolates in the group P+E+S.E.+ showed 100% decrease in motility of human spermatozoa (Table 19). Only 50% of P+E−S.E.+ and 60% of P+E−S.E.− groups lowered the sperm motility significantly. The isolates producing only the elastase degrading soluble elastin (P−E−S.E.+  ) had no effect. This indicates that soluble elastin degrading elastase is not spermicidal whereas the other elastase and caseinolytic
proteases play some role in inhibiting the sperm motility. Elastase degrading insoluble elastin (E+) seems to be most important. The results further suggest that besides the proteases and elastases, there are certain other factors that have sperm immobilizing capacity as 33.3% of P^-E^-S.E.^- group also showed decrease in sperm motility.

On the basis of the screening of various isolates for protease and elastase production and keeping in view the importance of elastase (degrading insoluble elastin), two isolates B.subtilis isolate no.4 and P.malophilia that produced maximum elastase were selected for further study.

5.4 OPTIMIZATION OF CONDITIONS FOR PRODUCTION OF ELASTASE

Whenever, the enzyme has to be obtained from a microbial source, it becomes important to know various environmental conditions under which the enzyme is obtained in large quantities. Moreover, in vitro, environmental factors affecting production of elastase, by an organism provide information for possible parameters affecting elastase production by an organism, in vivo.

Different organisms are able to grow in different type of media but their growth and activity might be varying. Since the organisms face different environmental conditions, it is possible that under a given set of conditions, it might not be producing a particular metabolite, while basically it is being positive in nature.
When both the organisms were grown in different media (synthetic medium, BHI, TSB, nutrient broth and peptone water), the best medium which supported optimal production of elastase and protease was BHI for \textit{B. subtilis} whereas in case of \textit{P. maltophilia}, TSB was found to be the best medium (Table 21). Synthetic medium did not support the growth of \textit{P. maltophilia} and therefore enzyme production was zero. The production of elastase and protease was more under shake conditions as compared to stationary conditions (Table 22 and 23). It is also observed that the elastase produced by \textit{B. subtilis} was more in cultures grown at 37°C than at 30°C. \textit{P. maltophilia} produced 5.12 units ml$^{-1}$ at 30°C and only 2.75 units ml$^{-1}$ of elastase at 37°C under shake conditions in 72h. Protease activity was also maximum at the same temperatures at which elastase production was maximal. None of these isolates could grow at 45°C.

In an earlier observation, Morihara and Tsuzuki (1977) reported that production of protease and elastase by \textit{P. aeruginosa} isolated from patients is affected by the type of medium and conditions of the cultures. Various strains of \textit{P. aeruginosa} were divided into the following groups by the above workers: the first group producing only elastase in complex medium and both protease and elastase in semisynthetic medium; the second group could not produce any proteolytic enzyme in complex medium but did produce both the enzymes in semisynthetic medium, the third group could not
produce any proteolytic enzymes in either of these media. Wretlind and Wadstrom (1977) also reported production of protease by \textit{P. aeruginosa} strain PAKS-I. Its production was affected by shaking conditions. Kaur et al. (1988b) reported maximum yield of extracellular elastase by \textit{B. subtilis} (a human endocervical isolate) in shake-cultures grown in syncaze medium at 37°C.

The results (Fig. 2 and 3) also show that the time of incubation (i.e. age of culture) influences elastase production even if the organism is growing in a medium showing sufficient growth. The enzyme is released only in the late log or stationary phase of growth. Protease activity could be detected at 8h of growth in both organisms though the maximum protease activity was also observed in the stationary phase.

5.5 \textbf{PURIFICATION OF ELASTASE}

Purification of elastase from \textit{B. subtilis} and \textit{P. maltophilia} was done by ammonium sulphate precipitation of the culture supernatants followed by gel filtration (section 3.9). The specific activity of elastase in case of \textit{B. subtilis} was found to be maximum with 60% ammonium sulphate saturation (Table 28) as also reported by Kaur et al. (1988b). It was found to be maximum at 70% saturation when \textit{P. maltophilia} culture supernatant was taken. 19.6 folds increase in elastase activity was seen after the ammonium
sulphate precipitate was subjected to gel filtration using G-100 (Fig.4) in case of *B.subtilis* and it was 22.43 folds for *P.maltophilia* elastase (Fig.5, Table 29).

The purity of both the elastases was confirmed by the single band obtained after subjecting the enzyme to polyacrylamide gel electrophoresis. The molecular weight of *B.subtilis* elastase was approximately 25,000 and that of *P.maltophilia* elastase was 24,000 (Fig.6).

5.5.1 **Heat stability**

The heat stability of the purified enzyme was studied and the results (Table 30) showed that as the temperature was raised, the elastase activity decreased and the enzyme was totally inactivated at 70°C in 10 min in both the cases. Morihara et al. (1965) reported the stability of *P.aeruginosa* elastase upto 70°C. However, the enzymes from *B.subtilis* and *P.maltophilia* were stable only upto 60°C. The reason for the difference in results could be the difference in the isolates used.

5.5.2 **pH optima**

In literature, the pH optima of elastase have been reported to vary although obtained from a single source, depending on the basis of the type of buffer used in the assay system. For example, *Pseudomonas* elastase had optimum pH values of 8.0 in Tris buffer, 7.5 in phosphate buffer, 7.0 in carbonate buffer (Morihara et al., 1965) while Wretlind
and Wadstrom (1977) reported its optimum pH values of 6.5 in Tris-maleate and 8.0 in phosphate buffer. Similarly the optimum pH for Bacillus elastase has been reported to be 9.0 (Morihara et al., 1965; Kaur et al., 1988b).

The optimum pH for activity of *B. subtilis* elastase was 8.5 and that for *P. maltophilia* was 7.5 (Table 31).

5.5.3 **Effect of purified elastase on spermatozoa**

The earlier results showed that the supernatants from isolates producing elastase inhibited motility of spermatozoa. To confirm whether the effect was due to elastase only purified elastase from *B. subtilis* and *P. maltophilia* was used at different concentrations. The decrease in motility was more when higher concentrations of elastase was used (Fig. 7 and 8) and was found to be concentration dependent. 100ug *B. subtilis* elastase and 50ug of *P. maltophilia* elastase inhibited the motility of human spermatozoa completely in 4h. At 200ug or above concentrations, the motility was zero in 5 min.

It is generally agreed that ejaculated spermatozoa are less viable in vitro than those taken directly from the epididymis or vas deferens (Emmens, 1947). Therefore, rat spermatozoa were directly derived from the epididymis and effect of purified elastase on their motility was observed. The results (Fig. 8) showed that 200ug of elastase inhibited the rat spermatozoal motility to zero in 5 min. 100ug was able to do so in 15-30 min.
The observations on the effect of elastases on spermatozoal motility indicate that nexin is present in the flagellar axonemes. The nexin of sperm axonemes seems to be sensitive to all those enzymes which can hydrolyze elastin. The loss of sperm motility by proteases and elastases is perhaps because of hydrolysis of the interdoublet linkages. The active sliding of microtubules can occur over distances much greater than those normally encountered during flagellar bending, if sperms flagella are first digested with trypsin (Summers and Gibbons, 1971). The trypsin digestion causes damage to both the interdoublet linkages and the radial spokes (Summers and Gibbons, 1973). The elastic resistance given by interdoublet linkages responsible for sperm motility is perhaps nullified by hydrolysis with elastase and other proteases attacking nexin and with this more specific action on the interdoublet linkages, the microtubules are made free.

5.5.4 ATPase activity

The motility of spermatozoa requires energy in the form of ATP (Nelson, 1980; Gibbons, 1988) and this energy is provided by the breakdown of ATP through hydrolysis into adenosine diphosphate (ADP) and phosphoric acid. Gibbons and Rowe (1965) named adenosine triphosphatase as dynein. Dynein is known to be activated or inhibited by various chemicals (Baccetti and Afzelius, 1976). Since sperm motility is one of the most important parameter in evaluating the fertility
potential of a semen specimen (Amelar et al., 1980) and in the female genital tract the cervical microflora tend to inhibit the motility of spermatozoa, there is every possibility that the ATPases are inhibited by the microbial products. Therefore, the effect of elastase on ATPase of human and rat spermatozoa was studied (Table 32 and 33). There was decrease in Na\(^{+}\)-K\(^{+}\) and Mg\(^{2+}\) ATPases of both rat and human spermatozoa. The inhibition was also observed to be concentration dependent as the ATPase inhibition was higher when higher concentration of elastase was used in the reaction mixture.

5.6 DECAPITATION

In the earlier experiments on the effect of culture supernatants on human spermatozoal motility, culture supernatants from \textit{B. subtilis}, \textit{P. maltophilia} and \textit{M. sedentarius} not only decreased the motility of spermatozoa but also decapitated a few spermatozoa. All these isolates showing this effect were elastase producers. Experiments were performed to study whether it is elastase or any other decapitating factor that caused decapitation. Not only human spermatozoa were tested but other spermatozoa from rat, cow bull and buffalo bull were also used to identify this factor. It was found that rat spermatozoa were highly susceptible to cleavage where 100% cleavage could be observed. Only 10-17% of human spermatozoa were decapitated and there was no
prominent effect on cow bull and buffalo bull spermatozoa. However, for further studies rat and human spermatozoa were used because in case of rat spermatozoa the effect was most pronounced. Human spermatozoa was used because our main aim was to find out the factor responsible for causation of unexplained infertility in human females.

When the effect of incubation time on decapitation by \textit{B. subtilis} and \textit{P. maltophilia} supernatants (Table 34) was studied, it was observed 100\% cleavage of rat spermatozoa was achieved by \textit{P. maltophilia} supernatant in 1h of incubation while \textit{B. subtilis} supernatant required 4h of incubation at 37°C for causing decapitation of all spermatozoa. However, only 12-15\% of human spermatozoa could be decapitated even after 4h of incubation.

The reason for this could be the structural variations seen in the neck regions of mammalian species (Zamboni and Stefanini, 1971; Fawcett and Phillips, 1969). The membranous folds found in the neck are more pronounced in the spermatozoa of some species than in other (Fawcett and Phillips, 1969).

Although pH is known to affect the spermatozoa (Millette et al., 1973), there is no decapitation between pH 6.0 and 10.0. To confirm whether the decapitation with culture supernatants was due to pH or any other factor, the supernatants were autoclaved (121°C for 15 min) and their pH was noted. It was observed that the pH was 8.85 and 7.7 in
case of *B. subtilis* and *P. maltophilia*, respectively. Both the autoclaved and unautoclaved samples that had the same pH were tested for decapitation of rat and human spermatozoa. The results (Table 35) showed that the autoclaved supernatant failed to decapitate the human as well as rat spermatozoa. These results prove beyond doubt the decapitation not due to pH and suggest that the factor is a heat labile protein. When the exact temperature of its inactivation was studied, it was found to be inactivated at 70°C in 10 min and at 56°C in 60 min (Table 36 and 37). The elastase activity in the samples was also checked simultaneously. It was observed that with samples having no elastase activity, decapitation also ceased. Further experiments were done to prove the role of elastase in decapitation.

Supernatants from *B. subtilis* and *P. maltophilia* cultures of different ages, was used to study decapitation (Table 38 and 39). Since the enzymes under study were protease and elastase, their activity was also determined in the samples. The results have shown that only protease with non-elastolytic activity has no effect on spermatozoa. Protease is produced earlier than elastase but the elastase activity is increased, there is increase in the cleavage of spermatozoa. The results (Table 40) showed that maximum decapitation occurred when supernatants from 96h old cultures of *B. subtilis* and 72h old *P. maltophilia* cultures were used. This was corresponding to the maximum elastase activity.
units of elastase per ml could cause 100% cleavage of rat spermatozoa. Human spermatozoa seem to be less susceptible to the enzyme.

Proteolytic enzymes like trypsin have been used for chemical dissection of mammalian spermatozoa to yield free heads, tails with attached midpieces, and tails from which the mitochondrial components of the midpiece were removed (Millette et al., 1973). Mouse and rat spermatozoa were cleaved by brief treatment with trypsin to yield free heads and tails, while human, guinea pig, and rabbit spermatozoa were cleaved by trypsin only after incubation with 2-mercaptoethanol or dithiothreitol. This is due to the differences in the chemical composition of spermatozoa from different species. Earlier studies indicated that proteolytic cleavage occurred at a specific location in the neck of the spermatozoan leaving the basal plate attached to the head of the cell. Proteolytic treatment had no apparent effect on any other spermatozoan structures, whereas acid or base treatment results in damage to the plasma membrane, the acrosome, and other structures (Millette et al., 1973; Gall et al., 1973). Their results suggested that trypsin acts on a particular protein or group of proteins that may be responsible for maintaining the physical connection between the head and the tail. From our results also it seems that in the rat spermatozoa, this site is more accessible to elastase than in the human spermatozoa.
The decapitating factor was concentrated by ammonium sulphate precipitation of the culture supernatants from 96h old *B. subtilis* and 72h *P. maltophilia* culture supernatants. At 60% saturation with ammonium sulphate, the elastase activity was maximum (0.7 units/mg) and the decapitation was also maximum (Table 41). In case of *P. maltophilia* it was at 70% (3.5 units/mg, Table 42).

The ammonium sulphate precipitates were further purified by molecular sieving through G-100. The fractions were checked for their protease and elastase activity and also their ability to decapitate human and rat spermatozoa. Decapitation of rat spermatozoa was observed with fractions that showed protease and elastase activity (Fig.9 and 10). These results show that elastase is the enzyme responsible for decapitation.

To further confirm these findings, supernatants were taken from various groups of endocervical isolates (*P+E−S.E.−*, *P+E−S.E.+* and *P−E−S.E.+*) grouped on the basis of production of enzymes like proteases (caseinolytic), elastase degrading insoluble and soluble elastin. The effect of these supernatants on rat and human spermatozoal cleavage showed that proteases are not involved in decapitation (Table 43). As far as elastases are concerned, only the elastases degrading insoluble elastin (*E+*) seem important and not the others degrading soluble elastin. The group *P−E−S.E+* was unable to decapitate spermatozoa. This proved beyond doubt
that it is only the elastase degrading insoluble elastin that is responsible for decapitation. The elastases degrading soluble elastin and insoluble elastin seem to be different. Soluble elastin is a single chain structure while insoluble elastin has a double chain structure which is connected by a short chain indicate that the neck region of spermatozoa could be having structures similar to that of the connecting chain of insoluble elastin.

Antibodies against elastase and microbial elastase inhibitor, elastatinal when added to elastases could inhibit the decapitating ability of the elastase enzyme. These results (Table 43) indicate that it is the elastase which is involved in decapitation. Further investigation was done by Scanning Electron Microscopy.

Minimum amount of column purified elastase required to decapitate 2x10^6 rat spermatozoa was found to be 3.12ug of B.subtilis elastase and 1.56ug of P.maltophilia elastase (Fig.11 and 12). Proteolytic cleavage of rat and mouse spermatozoa (1x10^7 cells ml^-1) was achieved by 0.05 mg/ml trypsin (Millette et al., 1973). But trypsin did not cause any damage to the acrosomal membrane.

5.7 SCANNING ELECTRON MICROSCOPY

Scanning Electron Microscopy was done to study the extent of damage caused to human and rat spermatozoa. With B.subtilis and P.maltophilia elastase the human spermatozoa
as well as rat spermatozoa showed extensive damage to the head and neck membranes. Not all spermatozoa were cleaved in case of humans (Plate no.5a to 5c), though the disruption of the membrane around the neck and postacrosomal region was quite prominent after 4h of elastase treatment. In case of rat spermatozoa, though the cleavage occurred within 1h of treatment, the plasma membrane showed prominent disruption and loosening after 2h of treatment. With he increase in reaction time the damage also increased.

The heat inactivated elastase, non-elastolytic protease and non-proteolytic elastase degrading only soluble elastin were found to have no effect on the morphology of rat spermatozoa (Plate no.13, 18a, 18b, 19a, 19b and 19c).

5.8 INHIBITOR STUDY

In the reproductive tract of both males and females there is a close association of proteinases and various activators and inhibitors. The acrosomal protein acrosin has the capacity to penetrate the zona pellucida of ovum (Stambaugh and Bukley, 1969; Zaneveld et al., 1970; Stambaugh et al., 1969; Zaneveld et al., 1971). Trypsin and chymotryptic like enzymes are also involved in sperm penetration of the vitelline coat (Green and Summers, 1980). In the female genital tract, the implantation process is aided by proteolytic enzymes (Dabich and Andary, 1976; Denker, 1977; Hoversland and Weitlauf, 1982). These enzymes
are required in optimal concentration and any imbalance has an adverse effect of fertilization (Deman et al., 1974).

Elastase producing microorganisms have been demonstrated in the cervices of infertile females (Kaur et al., 1988a) and it has also been shown that purified elastase from these organisms (B. subtilis and P. aeruginosa) has detrimental effect on sperm motility of human, rat, cow bull and buffalo bull spermatozoa. The presence of elastase producing organisms in the female genital tract could lead to the imbalance in the proteolytic system, which is otherwise essential in the process of fertilization and implantation. Normally proteinase inhibitors in the male (Hirschhauser and Baudner, 1972; Polakoshi and Williams, 1974; Kramer et al., 1992) and female genital tract (Zaneveld et al., 1975; Haendle et al., 1970) tend to provide the required balance between proteinase and proteinase inhibitors.

In cases of unexplained female infertility, where the usual criteria for fertility is fulfilled and yet the female is infertile, more than one factor(s) could be responsible for its causation. One of the factor observed in the present study is the protolytic enzyme, elastase. But elastase producing organism could also be isolated from the fertiles (Table 18), though it was isolated only from 1 out of 9 fertile females while in case of infertiles it was isolated from 5 out of 11 cases. The question that arose was that how
could this female harbouring an elastase producer be fertile. One possibility in the present study was that the amount of elastase produced by that particular isolate (*M. sedentarius*) was lower than that produced by isolates from infertiles (Table 18).

The cervical washings of infertile females showed elastase activity when elastin-orcein was used as a substrate (Table 44). These washings together with the microbial elastase showed enhanced activity. From these results it is quite obvious that elastase has some role to play in the fertility status of a woman. The cervical mucus of infertiles has some type of an activator. The cervical washings from fertile females did not affect the elastase activity while in some cases it was inhibited, when higher amounts of cervical mucus was used in the reaction mixture.

The cervical mucus from cases of unexplained infertility was taken in different concentrations and its effect on elastase activity as well as the corresponding effect on sperm motility was studied (Table 45). It was observed that as the elastase activity decreased, there was increase in the motility of spermatozoa indicating the detrimental effect of elastase in the cervical area and that the effect was concentration dependent.

Earlier reports have shown the presence of inhibitors of trypsin, chymotrypsin and neutral proteinases from human leukocytes, in cervical mucus (Polakoshi and Williams, 1974).
In one of the studies (Casslen, 1986), the inhibitors of trypsin, chymotrypsin and elastase have been shown in luteal phase uterine fluid in humans. This was in connection with the proteinases concentration required during implantation. In the present study, the intra-uterine fluid from fertiles and infertiles was checked for its inhibitory activity against microbial elastase. In fertiles, the inhibitor was found (Table 46) but it could not be detected in the infertiles. Further experimentation was limited due to the difficulties associated with the availability of volunteers to give intra-uterine fluid.

Seminal plasma is known to contain proteinases as well as its inhibitors (Guraya, 1987; Kramer et al., 1992). The full seminal plasma is known to contain certain factors that affect the viability and motility of spermatozoa (Baas et al., 1983). It is important that seminal plasma should be studied for the presence of elastase activity and also for elastase inhibitors. There are couples where the male is fertile and the female too is fertile but together, they are infertile. This could be due to the presence of certain factors in seminal plasma. Seminal plasma from normal and abnormal semen samples was tested for its elastase activity. There was no elastase activity in the individual samples. It was also observed (Table 47) that 3 out of 13 samples increased the activity of microbial elastase while 9 decreased it and one had no effect. The presence of elastase
inhibitor in seminal plasma seems important in couples when the female harbours elastase producing microorganisms in the lower genital tract. The seminal plasma neutralizes the elastase in the cervix and hence the female is fertile even in the presence of elastase producers. Why three out of 13 samples increased the elastase activity cannot be explained because these samples were normal (according to WHO standards) with good progressive motility. It could be possible that there are some other factors that increase the activity of microbial elastase. All the samples tested did not have elastase activity of their own. Therefore, the only possibility could be the presence of some activator that enhances the elastase activity.

The seminal plasma of abnormal semen samples also decreased the activity of B. subtilis elastase in 6 out of 7 cases. This inhibitor was found to be heat labile (Table 48). One sample had no effect. This could explain why males with very few number of motile spermatozoa are found fertile.

5.9 IN VIVO EXPERIMENTS

Some microorganisms from cervices of infertile caused immobilization and agglutination of spermatozoa. The elastase purified from these organisms was also found to inhibit sperm motility of human and rat spermatozoa. It was thought that in some human volunteers with unexplained infertility complaints, the infertility might be solely due to such
microflora which could cause sperm immobilization. To test this hypothesis, experiments were planned with the idea that if these elastase positive opportunistic organisms are established in fertile female laboratory animals, they might cause a condition of infertility in them. And if so when they are eliminated by antibiotic treatment, the fertility should be restored.

The genital tract microflora was screened for microorganisms producing caseinolytic proteases and elastase (degrading insoluble elastin). 70% of the isolates were protease producers (Table 51). Only 4.6% were elastase producers. The rats harbouring elastase producers in their genital tract were not used for experiment.

*B. subtilis* and *P. maltophilia* (elastase producers), *M. luteus* (non-elastolytic) protease producer and *S. aureus* (non-protease were non-elastase producers) were selected from the organisms that were isolated from cervices of fertile and infertile females (Table 49). Rat was taken as the animal model. Therefore, the effect of all these organisms on rat spermatozoal motility and agglutination was studied (Table 50). Excluding *S. aureus*, rest of the isolates showed inhibition of sperm motility and the elastase producers agglutinated and decapitated the rat spermatozoa.

Elastase producers could be established in 40-66.6% of the rats. In others, the possibility why these organisms were unable to establish themselves in the genital tract of
female rats could be the competitive inhibition by the normal flora in those animals. *Micrococcus* sp. could not be established in the specified time during which it was instilled. The reason for this could be that this was a species specific microorganism (Table 53). After mating these groups with male rats with proven fertility, the animals were seperated and observed for their fertility. 80% and 100% of the rats became infertile with *B.subtilis* and *P.maltophilia*. The infertility caused by elastase producing organisms established in laboratory animals, might mainly be due to two reasons (i) immobilization and agglutination of the spermatozoa which are in the genital tract during the mating period, (ii) by action of their elastase on nexin and ATPases. These reasons seem possible because in the control groups: (a) without any isolate from human cervices (b) with *S.aureus* from fertile human females established in the rat genital tract only 1-2 rats were infertile out of the 15 rats in each group. This again suggests that *B.subtilis* and *P.maltophilia* have some role to play in infertility. The animals (0-20%) which could give birth to offsprings even after establishment of elastase positive microorganisms, might be due to the inhibition of elastase by various unknown biological factors present in the mucus (Schiessler et al., 1977a; Fritz, 1980) or due to less degree of contact between spermatozoa and such organisms. To prove, the conviction that bacterial elastase might be active in vivo against spermatozoa causing their
immobilization the genital tract of female rats was directly instilled with elastase from *B. subtilis* or *P. aeruginosa* during the mating period. In these experiments none of the animals selected did have elastase positive microorganisms naturally present in their genital tract. None of the animals instilled with elastase could conceive in comparison to the control group which remained normally fertile (86.7-93.4%).

The organisms which were established (*B. subtilis* and *P. maltophilia*) were found to be sensitive to a number of antibiotics (Table 52). Though the antibiotics used to study the antibiogram of these organisms are not totally related to their clinical applications it gives some idea about their use in curing the infertile cases. Tetracycline is observed to be the most effective antibiotic against all the organisms studied in concentrations ranging between 80-100ug ml\(^{-1}\). Local application of 200 ug of the antibiotic per day for 7 days eradicated the elastase producing organisms that were established in the genital tract. 62.5-66.6% of the treated animals resumed their fertility. The low rate of fertility could be due to the effect of the antibiotic on sperm motility as a gap of 3 days was given after the antibiotic treatment before keeping the rats for mating. It is known that antibiotics inhibit the motility of spermatozoa (Matthews and Wade, 1977). The inhibitory effect of 21 antibiotics on the motility of spermatozoa of various animals and man was studied by Fuska et al. (1973). Frequentin
(isolated from Penicillium frequentans) and cyclopaldic acid totally stopped the movement of spermatozoa within 5 min. The effect was different when spermatozoa from different animal species was used. An evaluation of the biochemical effect of frequentin on sperms indicated a decreased viability of the sperm by this substance.

In general the results have shown that specific organisms could be found in infertile females, which might be responsible for immobilization of spermatozoa, directly or indirectly due to release of compounds like elastase. It is also suggested that routine microbiological status of the infertile females may be carried out and the effect of the organisms on sperms may be studied. If the organisms are found to immobilize spermatozoa then antibiotic therapy might be tried. Many workers have reported that antibiotic treatment resulted in improved fertility (Quesada et al., 1968; Friberg, 1980; Cassell et al., 1983). It is possible that in their study the infertility was cured due to the eradication of elastase producing organisms in the genital tract of women.

The medical applications of microbial enzymes have been considered with reference to systems of the body or in some cases where it is impractical to deal with individual disease (Sizer, 1972). In most types of infection and trauma there is an accumulation of pus, microorganisms blood cells and other kind of debris. In view of this situation, it is
not surprising to learn that enzymes, especially proteases, have been widely used clinically to treat such conditions. With the results of the in vivo experiments in rats, it can be speculated that bacterial elastase could be of use as an antifertility agent. Since elastase hydrolyse the elastin, a commonly occurring protein in the cervical tissue, it might prove dangerous to assume its medical application, based on these preliminary results. On the basis of present results and the above facts, a search should be directed towards microbial elastases which could be safe in its application as an antifertility agent.