



Decapitation of spermatozoa by endocervical isolates from cases of unexplained infertility

Grewal RK¹, Prabha V¹, Malhotra SK², Gupta KG¹

¹ Department of Microbiology, Panjab University, Chandigarh - 160014 and ² Department of Gynaecology and Obstetrics, PGIMER, Chandigarh.

OBJECTIVE(S): To explore the effect of extracellular products of cervical isolates from the cases of unexplained infertility on spermatozoa.

METHODS(S): Thirty endocervical isolates obtained from 11 cases of unexplained primary infertility were studied for the effect of culture supernatants on motility and decapitation of human spermatozoa. Effect on rat, cow bull and buffalo bull spermatozoa was similarly studied.

RESULTS: Sixteen isolates showed significant decrease in motility of spermatozoa. Out of 16 isolates, 5 isolates not only showed a decreased the motility of spermatozoa but also decapitated a few spermatozoa. This effect was more pronounced on rat spermatozoa where 100% decapitation could be observed whereas in humans only 14-17% spermatozoa were decapitated. There was no prominent effect on cow bull and buffalo bull spermatozoa.

CONCLUSION(S): Decapitation of spermatozoa occurred with supernatants of *Bacillus* sp. and *Pseudomonas* sp.

Key words : endocervical isolates, spermatozoa, decapitation

Introduction

Abnormalities of the cervix and its secretions are responsible for infertility in 15-30% of women. Therefore, the assessment of the interaction of sperm and cervical mucus is an important component of infertility evaluation and is tested early in the work up of infertile couple¹. Barros et al² reported that human spermatozoa can undergo certain modifications during their interaction with human cervical mucus and this interaction can have an important effect upon the process of fertilization. Several workers have given extensive consideration to the bacteriology of mucus^{3,4}.

Microorganisms present in the mucus do come in contact with spermatozoa and a obvious question arises, whether the micro-flora affect the functioning of spermatozoa in some way. In this regard since 1930's there have been sporadic references in literature with respect to agglutination and immobilization of spermatozoa by microorganism and their metabolic end products^{5,6}. However, up to now our knowledge in this field is very limited. Moreover, from the data available so far, it is not possible to say what are the different types of microorganisms present in the genital tract of infertile women having clean cervixes without disease and which compounds from microorganisms present in the cervical area specifically influence the functioning of spermatozoa.

Methods

Microorganisms

The bacterial isolates used in the present study were taken from cervixes of women with primary unexplained infertility. Women of unexplained primary infertility were those who

Paper received on 01/03/2005; accepted on 08/08/2007

Correspondence :

Vijay Prabha

Department of Microbiology

Panjab University,

Chandigarh 160014 India.

Tel. 2534147 Email : satishvijay11@yahoo.com

were unable to conceive after 2 years of unprotected coitus and all other factors (tubal factor, anovulation, luteal phase defect, cervical factor, antibodies against sperms, and other endocrine or medical disorders) were ruled out. Before taking the sample, the patient's recent medical history was taken into consideration. The cervical swab samples were taken only from those who had not taken any antibiotic for at least a week, and had no clinical evidence of vaginal or cervical infection.

Collection of cervical samples

Endocervical swab samples were taken by the method of Kehl et al⁷ from 11 cases of unexplained infertility. Samples were directly streaked on sheep blood agar and MacConkey's agar plates. The plates were incubated at 37°C for 48 hours and observed for bacterial growth. The isolates were picked up on the basis of their colony size and morphology and subjected to various tests for identification of their genera according to characteristics laid down in Bergey's Manual of Determinative Bacteriology. Various isolates so obtained were maintained on nutrient agar slant.

Collection of semen

Human semen ejaculates that satisfied the WHO criteria of normal standards were selected and the sperm count adjusted to 40×10^6 mL with sterile physiological saline.

Five samples of rat spermatozoa were collected from the vas deferens and cauda epididymis in minimum quantity of physiological saline by the method of Dye⁷. The number of spermatozoa was checked by counting in a hemocytometer and was adjusted to 40×10^6 sperms /mL. Sample were collected from five cow bulls and five buffalo bulls and similarly studied.

Effect of culture supernatants on motility and decapitation of human spermatozoa

All the endocervical isolates were grown in brain heart infusion (BHI) broth under shake conditions (90 rpm) at 37°C. The cultures were centrifuged at 10000 x g for 20 min at 4°C and clear supernatants were separated. The supernatants obtained were made cell free by passing through 2.2 mm sterile millipore filters.

The clear supernatant thus obtained was mixed with equal volumes of human or rat spermatozoa and incubated at 37°C. After 1,2,3 and 4 hours respectively, a drop of the sample from each tube was placed on a slide, covered with a coverslip and observed by light microscope under 400 x for motility and decapitation. Hundred spermatozoa were counted and the number of decapitated (heads without tail) ones and

nondecapitated ones was noted. The percentage cleavage of spermatozoa was calculated as follows –

$$\text{Percentage cleavage} = \frac{100 \times \text{No. of decapitated spermatozoa}}{\text{No. of decapitated spermatozoa} + \text{normal spermatozoa}}$$

Ammonium sulfate precipitation of decapitating factor

Bacillus sp. isolate no. 4 was grown in BHI for 96 hours at 37°C under shake conditions. The culture was centrifuged at 10000 g for 10 min at 4°C and to the clear supernatant varying amounts of $(\text{NH}_4)_2 \text{SO}_4$ were added so as to get 20 to 100% saturation. The flasks were kept at 4°C overnight and precipitates were collected by centrifugation at 10000 rpm for 20 min at 4°C. Precipitates were dissolved in minimum amount of Tris - HCl (0.05M, pH 8.0) buffer and dialysed against d.w. Decapitation was checked in all the samples as described earlier.

Purification of decapitating factor

Sephadex G-100 was packed with a bed volume of 94cm³ and equilibrated with Tris-HCl (0.05M, pH 8.0) buffer with a flow rate 24 mL/h. 4mg protein of 60% $(\text{NH}_4)_2 \text{SO}_4$ precipitate was loaded on the column and eluted with Tris-HCl (0.05 M, pH 8.0) buffer. Each fraction was screened for decapitation.

Scanning electron microscopy

Processing of sample for scanning electron microscopy was done according to the method described by Lohiya et al⁸.

Two hundred microliters of sperm suspension was incubated with 200 mg of purified decapitating factor for different time intervals till 4 hours. The spermatozoa were then settled by centrifugation at 500 rpm for 5 min. To each tube 4 mL of 2.5% phosphate buffered glutaraldehyde was added and the sperms mixed gently with the help of pasteur pipette. After 30 min the samples were centrifuged for 5 min at 500 rpm and washed twice in phosphate buffered saline. Control was prepared every time that contained only the sperm suspension and incubated for different time periods along with the other tubes before fixation was done.

One drop of fixed and washed spermatozoa was placed on a silver painted adhesive tape mounted on brass slugs and air dried; 100 Å thick gold coating was done in Jeol Fine Coat 10n sputter (JFC-1100). The specimen were observed in a Jeol Scanning microscope (JSM-6100, Japan) and operated at 20 kV.

Results

A total of 30 isolates were obtained from 11 cases of unexplained infertility (Table 1). All the isolates so obtained were grown in BHI broth for 24 hours and 48 hours at 37°C and the effect of cell free supernatants on human spermatozoa motility was studied. The results showed that out of 30 isolates, 16 produced significant decrease in motility, whereas, rest of the isolates showed no effect with respect to control. The results further showed that out of 16 isolates 5 not only decreased the motility but also decapitated 14-17% of the human spermatozoa.

Table 1. Endocervical isolates from cases of unexplained infertility

Microorganism	No. of cases
Bacillus sp.	4
Pseudomonas sp.	1
Micrococcus sp.	8
Staphylococcus sp.	8
Streptococcus sp.	2
Escherichia coli	2
Not identified	5

The effect was seen with the culture supernatants of 4 isolates belonging to genus Bacillus and one isolate belonging to genus Pseudomonas. However, maximum decapitation was seen with culture supernatant of Bacillus sp isolate No.4. Therefore, further studies on decapitation were done by selecting this isolate.

Decapitation of spermatozoa from other species as rat, cow bull, and buffalo bull was also studied and the results showed that rat spermatozoa were highly susceptible to cleavage, where 100% cleavage could be observed, whereas cow bull and buffalo bull spermatozoa showed no prominent effect.

Further, decapitating factor was concentrated and purified by subjecting to ammonium sulfate precipitation (60% saturation) of the 96 hour old culture of Bacillus sp. (isolate No.4) and molecular sieving through G-100. Fractions (20-27) showing decapitation of human and rat spermatozoa were pooled and lyophilized.

Scanning electron microscopy was done to study the extent of damages caused to human and rat spermatozoa by purified decapitating factor.

Normal human spermatozoa were characterized by flattened ovoid heads covered anteriorly by a rough, rigid surface and

posteriorly by a smooth surface. The heads appeared pointed at the posterior end and become somewhat round and flat anteriorly. Elevations appeared in the region between the anterior and posterior portions and a shallow furrow was observed as the dark area (Figure 1). There were considerable variations in the morphology and thickness of the neck. In some spermatozoa the neck appeared as a thickening or as a slight constriction connecting the midpiece. The midpiece comprised a small portion of the tail. The constriction between the mid piece and the tail was apparently due to the gap between the mitochondrial and fibrous sheaths. The tail was surrounded by a fibrous tail sheath.



Figure 1. Human ejaculated spermatozoa (X 12,000)



Figure 2. Human spermatozoa treated with decapitating factor showing decapitation of some spermatozoa (X 5,000)

When human spermatozoa were treated with decapitating factor for 4 hours it caused loosening and disruption of the membrane around the neck and postacrosomal region. There was breakage at the head and tail junction (Figure 2), Figure 3 shows the normal morphology of rat spermatozoa with intact head, neck, middle piece and tail. The acrosomal membrane was smooth and covered the entire acrosome. The membrane that covered the midpiece and continued on the tail was also smooth and continuous.

When rat spermatozoa were incubated with decapitating factor for 4 hours at 37°C, all the spermatozoal heads were cleaved from the tails. The plasma membrane was slightly damaged and there was loosening of the membrane over the mid piece. No effect on the membrane over the tail was observed (Figure 4).



Figure 3. Rat epididymal spermatozoa (X 6,000)

Heat inactivated decapitating factor was found to have no effect on the morphology of rat and human spermatozoa. There was no prominent effect on cow bull and buffalo bull spermatozoa.

Discussion

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 infertility of females. It is now known that organisms which comprise the normal flora also represent the major pathogens in non-venereal infection of the female genital tract. This supports the 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.



Figure 4. Rat spermatozoa treated with decapitating factor showing head tail separation of all spermatozoa.

A number of investigations have shown that microorganisms produce a wide variety of pharmacologically active substances which affect the sperm motility^{9,10}. In the present study also, it has been observed that culture supernatants of 16 out of 30 isolates obtained from cases of unexplained infertility decreased the motility for spermatozoa. The results further showed that culture supernatants of *Bacillus* sp. and *Pseudomonas* sp. not only decreased the motility of spermatozoa but also decapitated a few spermatozoa, which was unique in this study. Further, this factor was concentrated and purified, and scanning electron microscopic studies were done with the purified factor. These studies showed that rat spermatozoa were highly susceptible to decapitation than human spermatozoa, where 100% cleavage was observed whereas only 14-17% human spermatozoa showed decapitation. The reason for this could be structural variations seen in neck regions of mammalian species. The membrane folds found in the neck are more pronounced in the spermatozoa of some species than in those of others. Kozumplik¹¹ reports that morphological changes and sperm decapitation cause fertility disorder in breeding stock. In the present study also decapitation of spermatozoa has been observed by microflora present in cervix of infertile woman. As reported by Kamal et al¹² easily decapitated spermatozoa defect is a possible cause of unexplained infertility in women.

References

1. Zargar AH, Wani AI, Masoodi SR. Epidemiologic and etiologic aspects of primary infertility in the Kashmir region of India. *Fertil Steril* 1997;68:637-43.
2. Barros C, Jedlicki A, Fuenzalida I et al. Human sperm - cervical mucus interaction and the ability of spermatozoa to fuse with zona-free hamster oocytes. *J Reprod Fertil* 1988; 82: 477-84.

3. Eggert-Kruse W, Pohl S, Naher H et al. Microbial colonization and sperm-mucus interaction: results in 1000 infertile couples. *Hum Reprod* 1992;7:612-20.
4. Monga M, Roberts JA. Sperm agglutination by bacteria: receptor-specific interactions. *J Androl* 15:151-6.
5. Urata K, Narahara H, Tanaka Y et al. Effect of endotoxin-induced reactive oxygen species on sperm motility. *Fertil Steril* 2001;76:163-6.
6. Kehl SC, Georgakas K, Swain GR et al. Evaluation of the Abott LC assay for detection of *Neisseria gonorrhoea* in endocervical swab specimen from females. *J Clin Microbiol* 1998;36:3549-51.
7. Dye FJ. Preparation of mammalian meiotic chromosomes and spermatozoa/obtaining early mammalian embryos and preovulation oocytes. In: Glase JC (ed). *Tested studies for laboratory teaching*, Vol 18. Proceeding of the 18th workshop of the Association for Biology Laboratory Education, 1993;18:159-66.
8. Lohiya NK, Kothari LK, Manivannan B et al. Human sperm, immobilization effect of *Carica papaya* seed extracts: an in vitro study. *Asian J Androl* 2000;2:103-9.
9. Diemer T, Weidner W, Michelmann HW et al. Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. *Int J Androl* 1996;19:271-7.
10. Liu JH, Li HY, Cao ZG et al. Influence of several uropathogenic microorganisms on human sperm motility parameters in vitro. *Asian J Androl* 2002;4:179-82.
11. Kozumplik J. Morphological changes and sperm decapitation as a cause as a fertility disorder in breeding stock. *Veterinarni Medicina (Praha)* 1990;35:331-6.
12. Kamal A, Mansour R, Fahmy I et al. Easily decapitated Spermatozoa defect: a possible cause of unexplained infertility. *Hum Reprod* 1999;14:2791-5.0.