

A Method for Improvement of Sperm Quality of an Ejaculated Human Specimen Using Bovine Cervical Mucus as a Biological Filter

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A method is described for improving the quality of semen from subfertile men. Semen samples were filtered through bovine cervical mucus (collected at estrus) into spermatozoa-free seminal plasma (from the same ejaculate following centrifugation, or from azoospermic donor ejaculates). Evaluations were performed after 2 to 4 hours of filtration at 34 C. The filtered spermatozoa found in the seminal plasma reservoir were characterized by a significant increase in the percentage of morphologically normal spermatozoa, higher sperm motility, and a higher percentage of living sperm. Sperm density was in the range deemed adequate for fertility. After filtration into human cervical mucus, sperm penetration tests were performed with good results, which could be improved by the addition of caffeine to the semen. This method was found to be reliable and to enable the use of the filtered spermatozoa for insemination (AIH).

Key words: teratozoospermia, bovine cervical mucus, penetration test.

Studies of the chemical and physical characteristics of cervical mucus revealed that it consists of macromolecules of glycoproteins (Odeblad, 1968). These macromolecules form a random ensemble of loosely coiled chains with entanglements between the chains and between the parts of each chain (Lee et al, 1977). This arrangement results in the formation of micelles through which spermatozoa can penetrate (Elstein and MacDonald, 1970). Comparative studies of the characteristics of human cervical mucus (HCM) and bovine cervical mucus (BCM) revealed similarities (Gaddum-Rosse and Lee, 1978; Gaddum-Rosse et al, 1980;

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Bergman et al, 1981a), which enabled the establishment of an *in vitro* penetration test using BCM and ejaculated human spermatozoa (Bergman et al, 1981a).

Human cervical mucus serves as a natural filter to prevent penetration by certain abnormal forms of spermatozoa (Bergman, 1956; Ahlgren et al, 1973; Fredricsson and Bjork, 1977; Perry et al, 1977). It has recently been shown that BCM also acts as a biological barrier against abnormal forms of spermatozoa and prevents some abnormal spermatozoa from penetrating the cervical mucus (Bergman et al, 1981b). Four forms of spermatozoa were proven to penetrate easily: normal, tapering, microcephalic, and pinhead cells (Bergman et al, 1981b). Since BCM is abundant during estrus and easy to collect, it was logical to consider the possibilities of designing a device for filtering ejaculated human spermatozoa through BCM in order to achieve an improved sperm specimen.

The purpose of the present study was to establish such a device and to test its reliability.

Materials and Methods

Cervical Mucus

Specimens of BCM were aspirated from cows in estrus, using a veterinary speculum after careful cleaning of the vagina (Bergman et al, 1981a) and were transferred in cooled containers to the laboratory. Mucus

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specimens were divided into 0.3 ml samples and were frozen at -20°C for approximately three months. We did not find any changes in the properties of the cervical mucus after this length of time. Testing for longer time periods was not undertaken. Samples were thawed shortly before use and were tested for Ferning and Spinbarkheit scores, and for penetration by ejaculated human spermatozoa of good quality (as defined by the criteria of Homonnai et al, 1980). Samples of BCM that were characterized by Ferning + 3, Spinbarkheit + 3 (by Insler's score; Insler et al, 1972), and good penetrability were used in this study (see penetration test).

Semen Samples

Human semen samples were obtained by masturbation from patients referred to the outpatient clinic of this hospital because of the couple's infertility. Thirteen semen samples were used in this study. Sperm motility was measured at room temperature, 1 hour after ejaculation, and was defined as percentage of sperm cells with forward movement. The percentage of living sperm was estimated at the same time by the eosin method (Eliasson and Treichl, 1972), by counting sperm cells that exclude the dye. Sperm concentration was determined by hemocytometry (Amelar and Dubin, 1977). A spermocytogram was performed on every specimen of semen. A smear of the ejaculate was prepared and stained with Papanicolaou stain and the cells were classified according to the definitions introduced by MacLeod (1970), based mainly on head morphology.

Semen samples used in this study had the following characteristics: sperm concentration, 44.7 ± 3.6 million/ml; percentage of live sperm, $62 \pm 2.1\%$; sperm motility, $57.3 \pm 1.4\%$; percent of morphologically normal spermatozoa, $58.1 \pm 2.7\%$ (mean \pm SE of 13 samples). The semen samples were considered to be of good quality, as defined recently by Homonnai et al (1980).

Filtration Device

A glass capillary tube (1.4 mm inner diameter, 1.5 cm length) was filled with BCM by gentle aspiration by mouth, using plastic tubing. One end of the capillary tube was immersed in a reservoir containing 0.5 ml of human ejaculate. The other end of the capillary was immersed in a cup containing 0.5 ml of seminal plasma obtained from azoospermic semen or from an aliquot of the ejaculate used in the experiment after centrifugation at 3000 RPM for ten minutes. No abnormalities in seminal plasma of azoospermic men were found, as judged by its effect on the motility of spermatozoa from samples of good quality. This is in agreement with our earlier findings (Paz et al, 1977).

Semen was filtered for 4 hours at 34°C . At time zero and at 2, 3 and 4 hours, aliquots were taken from each cup for determination of sperm concentration and motility. The morphology of the spermatozoa was determined in samples taken from all sources after 2 hours of filtration.

Penetration Test

Six samples of spermatozoa that had penetrated BCM and entered the seminal plasma were tested for their ability to penetrate HCM. The resulting penetration values were compared with those obtained before filtration. In addition, we tested the effects of adding caffeine (10 mM) on the penetration values. Caffeine has been shown to increase sperm motility and penetration into cervical mucus. The penetration test and penetration value calculations are described in detail in our previous study (Yedwab et al, 1978). In brief, 0.3 ml of liquefied semen was placed in a plastic reservoir. A glass capillary tube was filled with HCM to a length of 44 mm and its tip was immersed horizontally in the reservoir of semen. The migration test was performed at 34°C for 1 hour. The number of motile spermatozoa that had migrated to 1, 2 and 3 cm was quantitated at $100\times$ magnification. These counts were used to calculate the penetration value (PV), which is the product of the number of spermatozoa at each mark multiplied by the distance, ie, $PV = C_i \times D_i$ where C_i is the number of sperm at L ($L = 1, 2, 3$ cm) and D_i is the distance (1, 2, 3 cm). At this magnification, 100 spermatozoa/field can be counted, and therefore, the maximum penetration value is 600.

Control aliquots of the same semen used for filtration were kept at room temperature throughout the period of filtration.

The paired t test was used for determination of the significance of results.

Results

Thirteen semen samples were filtered through BCM of good quality. The characteristics of sperm concentration, motility, and morphology in the semen and in the seminal plasma reservoirs were assessed. Fig. 1 summarizes sperm density in the seminal plasma and in semen reservoirs as a function of time of incubation. The mean concentration of sperm in the seminal plasma rose up to 18.4 million/ml after 4 hours of filtration, whereas the concentration of sperm in the semen reservoir decreased from a mean of 44.7 million/ml to 27.9 million/ml. It should be stressed that 2 hours of filtration were sufficient to achieve a concentration of cells that was above the minimal fertile value suggested recently (Zuckerman et al, 1977). For this reason, 2 hours was selected as a standard assay time.

Sperm morphology was assessed in aliquots taken from the semen cup at the time the experiment was started and from the seminal plasma into which sperm penetrated after 2 hours. Table 1 and Fig. 2 summarize the frequency distribution of the sperm cell types (%). In the seminal plasma, 80% of the sperm cells were of normal morphol-

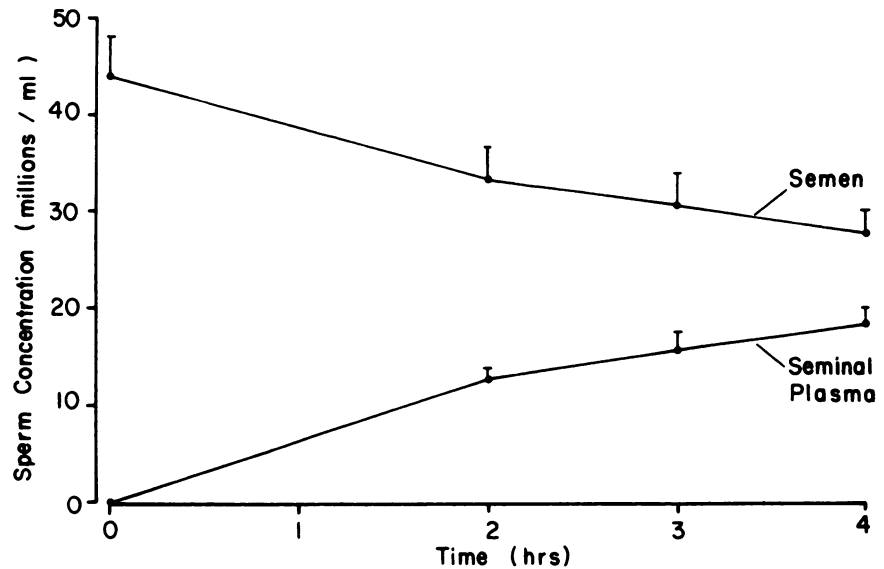


Fig. 1. Sperm concentration in semen and in seminal plasma reservoirs as a function of time of filtration. Values are the mean \pm SE of 13 runs.

ogy, compared with only 58.1% in the semen sample ($P < 0.0001$). Tapering, microcephalic, and pinhead sperm cells penetrated into the seminal plasma quite well, whereas macrocephals and cells with abnormal neck morphology penetrated much less easily ($P < 0.001$). Thus, the spermatozoa found in the seminal plasma were of a higher quality in terms of morphology. A spermocytogram of the contents of the semen reservoir after 2 hours of filtration revealed sperm of significantly decreased quality (Table 1). This led to the hypothesis that the percentage of living cells remaining in the semen reservoir is low. Indeed, a significant decrease was found in the percentage of live sperm cells in the semen after 2 hours of filtration (46 ± 3.8 ; mean \pm SE of six runs), compared with a

high percentage of live sperm cells in the seminal plasma (80 ± 2.1). Thus, most of the cells which did not penetrate the BCM were malformed or dead.

Table 2 summarizes sperm motility in the seminal plasma and semen samples. Motility decreased significantly in the semen samples, from a mean of 57.3% at the beginning of the experiment to 50% after 4 hours. Motility in the seminal plasma increased slightly from 59.6% to 65.4% after 4 hours.

A question arose concerning the ability of the filtered spermatozoa to penetrate a fresh HCM sample. Six post-filtration samples were tested in the *in vitro* penetration test with or without the addition of caffeine (10 mM). Table 3 shows that unfiltered spermatozoa exhibited a decreased

TABLE 1. Spermocytogram of Semen Specimens Before and After Filtration Through Bovine Cervical Mucus for 2 Hours, and in the Seminal Plasma Reservoir Collecting the Sperm*

	Sperm Cell Type (%)						
	Normal	Tapering	Macrocephal	Microcephal	Pinhead	Neck Pathology	Amorphous Cell
Semen							
Before filtration	58.1	17.3	12.7	6.2	0.3	5.2	0.1
	± 2.7	± 2.1	± 0.8	± 0.9	± 0.2	± 0.2	± 0.1
After filtration	44.7 ^b	19.8	16.4 ^c	8.7	1.3 ^a	8.0 ^c	1.1 ^a
	± 2.8	± 2.3	± 0.7	± 1.2	± 0.3	± 0.4	± 0.4
Seminal plasma reservoir							
	80.1 ^c	13.2	1.1 ^c	3.6 ^a	0.5	0.8 ^c	0.8
	± 1.8	± 1.5	± 0.3	± 0.5	± 0.2	± 0.2	± 0.4

* Values are the mean \pm SE of 13 runs.

Superscripts indicate values significantly different from the values found before filtration using the paired t-test: a = $P < 0.01$; b = $P < 0.001$; c = $P < 0.0001$.

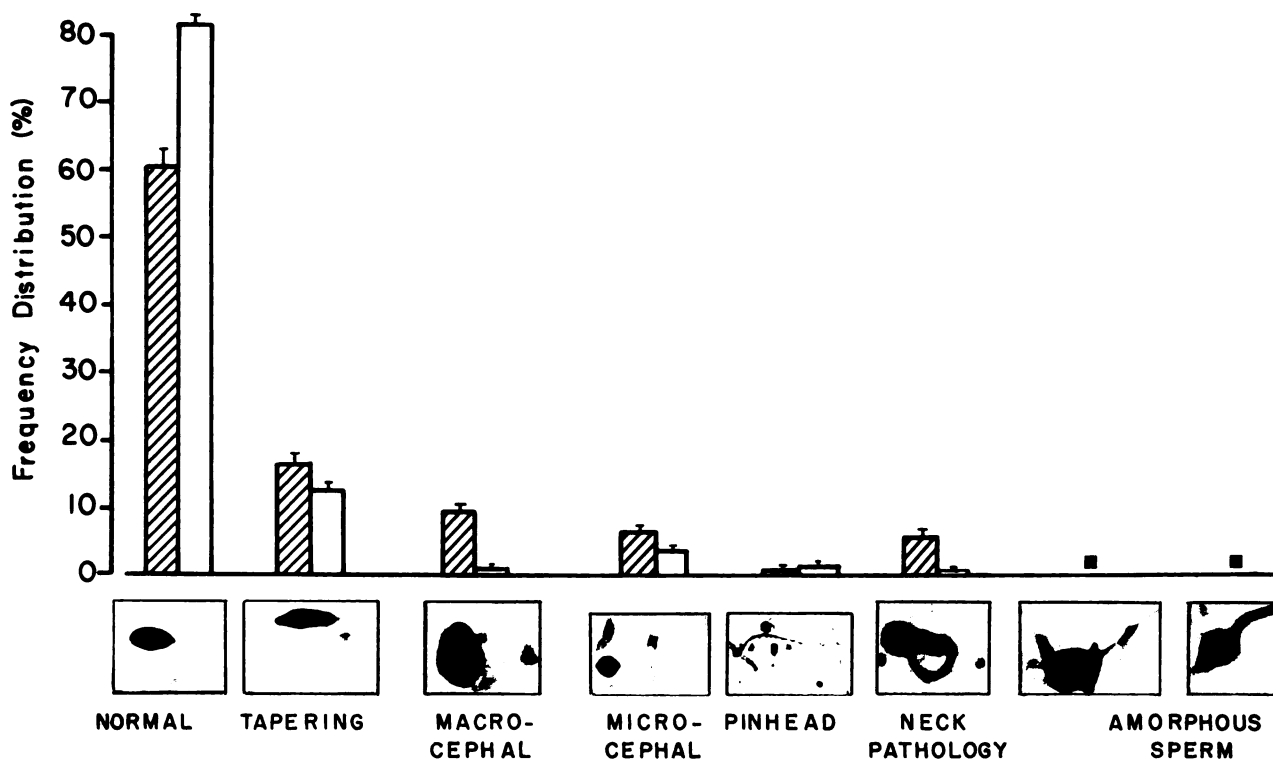


Fig. 2. Frequency distribution (%) of cell types from the spermocytogram of semen aliquots withdrawn from the semen before filtration through bovine cervical mucus (striped bars) compared with its pattern in seminal plasma (open bars) after two hours of filtration. The values are the mean \pm SE of 13 runs. Determination according to the t test of the significance of differences revealed the following: normal cells, $P < 0.0001$; tapering, $P < 0.05$; macrocephals, $P < 0.00001$; microcephals, $P < 0.001$; pinhead, nonsignificant; neck pathology, $P < 0.00001$; amorphous forms, $P < 0.005$.

penetration value when kept for 2 hours at room temperature. This could be improved by the addition of caffeine. Thus, filtration of spermatozoa through BCM did not affect their ability to penetrate HCM. Nevertheless, filtered sperm penetrated HCM much better following the addition of caffeine.

TABLE 2. Percentage of Motile Spermatozoa (%) in Semen Compared with the Values in Seminal Plasma after 2, 3, and 4 Hours of Filtration Through Bovine Cervical Mucus*

Time (hours)	Motility (%)	
	Semen	Seminal Plasma
0	57.3 ± 1.4	—
2	52.8 ^a ± 1.7	59.6 ± 2.3
3	50.8 ± 2.0	63.1 ^a ± 2.2
4	50 ^a ± 1.8	65.4 ^a ± 2.1

* The values are the mean \pm SE of 13 runs.

a = significantly different ($P < 0.05$) from the semen value recorded at zero time.

Discussion

Clinical studies in recent years have revealed the filtration capacity of the female reproductive system towards sperm cells with abnormal morphology. Sperm with good morphology were found in the uterine and abdominal cavities (Bergman, 1956; Ahlgren et al, 1973), as well as in specimens obtained from the internal cervix (Fredricsson and Bjork, 1977). *In vitro* studies of sperm penetration through a capillary tube containing cervical mucus confirmed these findings (Broer et al, 1975; Perry et al, 1977). Thus, it has been determined that cervical mucus acts as a natural filter, enabling normal sperm cells to penetrate through it and preventing the penetration of most abnormal forms. The exceptions are tapering forms (Bergman et al, 1981b) and microcephalic sperm, as well as pinheads, which constitute a very small percentage of the total number of sperm and are presumably without biological importance.

Bovine cervical mucus was found to be very similar to HCM in terms of physical and chemical

composition (Lee et al, 1977; Gaddum-Rosse and Lee, 1978; Gaddum-Rosse et al, 1980; Bergman et al, 1981a), as well as in the dynamics of human sperm penetration (Gaddum-Rosse et al, 1980; Bergman et al, 1981a), and, therefore, has been used as a convenient reference medium to study the penetrating ability of ejaculated human spermatozoa. In a recent study, we demonstrated the suitability of BCM as a reference cervical mucus for penetration tests (Bergman et al, 1981a), and showed its ability to exclude abnormal spermatozoa (Bergman et al, 1981b). These studies formed the basis for the present work, namely, development of a method for improving the quality of semen.

Joel (Joel, 1966; Joel and Chayen, 1971) proposed that abnormal sperm cells may be the major reason for male infertility. We have demonstrated that filtration for 2 to 4 hours through BCM into seminal plasma produced a semen sample with a significantly reduced percentage of abnormal forms and significantly improved motility. These are the two parameters that are most important in determining the ability of spermatozoa to penetrate human cervical mucus (David et al, 1979) and also appear to be related to their fertilizing capacity. Sperm density is of much less importance, since semen containing concentrations higher than 10 million/ml is considered fertile (Hommonai et al, 1980; Zuckerman et al, 1977). The addition of caffeine to the filtered spermatozoa significantly increased the ability of these cells to penetrate HCM. This was somewhat surprising to us because the motility of these cells was excellent. It would thus appear that during the long process of filtration and preparation (more than 4 hours after delivery of the semen specimen), the spermatozoa lost some of their capacity to penetrate the cervical mucus. This can be reversed, to some extent, by treatment with caffeine, which has been shown to increase sperm motility and penetrability into HCM (Yedwab et al, 1978).

Human cervical mucus is relatively difficult to collect in large quantities. The use of a spayed donor cow treated with estrogens (for regulation of cervical mucus production) is one possibility for collection of large quantities of BCM for the filtration device. Bovine cervical mucus can be preserved for long periods of time at -20°C and can be thawed and used on the day of the experiment.

It is concluded that BCM is an adequate medium for filtration of semen samples for use in artificial insemination by husbands' sperm (AIH). This fil-

TABLE 3. Penetration Values of Spermatozoa Filtered Through Bovine Cervical Mucus for Two Hours, Compared with Values in Aliquots of the Same Specimen Kept at Room Temperature for 2 Hours, in the Presence or Absence of Caffeine (10 mM) Added to the Semen Before the Test*

	Caffeine	Penetration Value
Semen	At the start of the experiment	— 323 ± 50
	After 2 hours at room temperature	— 168 ± 39 ¹
Seminal Plasma Reservoir	+	239 ± 32 ²
	—	200 ± 34
	+	276 ± 31 ³

* The values are the mean ± SE of six runs.

1 = significantly decreased ($P < 0.0001$) according to the paired t test from the value obtained before filtration; 2 = significantly increased ($P < 0.001$) according to the paired t test compared with the (—) caffeine test; 3 = significantly increased ($P < 0.0001$) according to the paired t test compared with the (—) caffeine test.

tration procedure can be performed on ejaculates obtained from subfertile men with semen characterized by a sperm concentration above 30 million/ml (it is advised to use ejaculates with counts much above this number in order to achieve high concentrations of filtered sperm), sperm motility greater than 50%, and sperm morphology with greater than 50% abnormal forms and a volume of more than 2.0 ml. It is recommended to employ this method only after all *in vivo* methods, including split ejaculates, have failed.

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