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THE SURVIVAL OF SPERMATOZOA IN BOVINE CERVICAL MUCUS AND MUCUS FRACTIONS

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Summary. Bull spermatozoa survived at extremely low cell concentrations (10 to 470 cells/μl) at 37° C in either cervical mucus or gel obtained by centrifugation of the mucus, but became immotile almost immediately after being suspended at 500 cells/μl in isotonic saline. Spermatozoa were adversely affected by suspension at low cell concentration in supernatant obtained by centrifuging mucus, or in mucus that had been liquefied by maceration. Although spermatozoa were slightly more resistant to the lethal effects of dilution in saline after passage through mucus, the resistance was quickly lost. The results suggest that the structural and physical properties of the mucus are responsible for the absence of the dilution effect on spermatozoa in cervical mucus.

INTRODUCTION

Many investigators (Emmens & Swyer, 1948; Chang, Casida & Barrett, 1949; Blackshaw, 1953; Bishop, 1954; White, 1954) have shown that mammalian spermatozoa are adversely affected by suspension in liquid media at low cell concentration. Even when seminal plasma is used as a diluent, there is a progressive decrease in the viability of the spermatozoa with increased dilution, 'the dilution effect', and progression by the spermatozoa ceases almost immediately at cell concentrations of 400,000 to 500,000 spermatozoa/ml (White & Wales, 1961).

Results reported by Tampion & Gibbons (1963) indicate that the dilution effect is absent when spermatozoa are in bovine cervical mucus *in vitro* and that spermatozoa are more resistant to the effects of dilution in saline after a short period within mucus. However, the techniques employed did not ensure that the spermatozoa observed at the end of the test period had, in fact, been present in the mucus from the beginning of the experiment. The phenomenon was therefore re-examined, using techniques that overcame these difficulties, and an attempt was made to assess the relative importance of the physical and chemical properties of mucus in relation to the phenomenon.

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MATERIALS AND METHODS

Collection and treatment of mucus

Mucus was drained from the anterior vagina of oestrous cows through a stainless steel speculum. Some samples were liquefied by maceration in a mechanical blender. Other samples were separated into supernatant and gel fractions by centrifugation (Tampion & Gibbons, 1962a). The mucus gel was expanded before use by allowing it to absorb saline; it then exhibited physical properties similar to those of whole mucus.

Semen

Bovine semen was collected with an artificial vagina and was held for 1 to 3 hr at 18 to 20° C before being used. Ejaculates were used only when more than 70% of the spermatozoa exhibited motility.

Estimation of concentration and motility of spermatozoa in suspensions

The concentrations of spermatozoa in liquid media were determined using haemocytometers. In such suspensions, the proportion of motile spermatozoa was determined from assessments of the concentration of non-motile spermatozoa and the known total cell concentration (Tampion & Gibbons, 1963). The proportion of motile spermatozoa in preparations of spermatozoa in the mucus or the mucus gel was assessed by spreading samples of the preparations on slides and examining 200 spermatozoa. The concentration of spermatozoa in such preparations was determined at the end of the test period. A few drops of sodium hypochlorite solution were added to liquefy each preparation so that the concentration of spermatozoa could be determined by the use of haemocytometers.

All observations on spermatozoa were made by phase-contrast microscopy with the microscope enclosed in a heated chamber and maintained at 37° C.

Preparation of suspensions

The saline used contained 0·9% sodium chloride. The saline, the mucus supernatant and the liquefied mucus were buffered to pH 7·2 with 0·014 M-sodium phosphate buffer solution.

Suspensions of spermatozoa at specific cell concentration in liquid were prepared by adding the appropriate amount of semen to the pre-warmed media (37° C) with calibrated constriction micropipettes.

Suspensions of spermatozoa in the mucus or in the mucus gel were prepared by incubating 5-ml samples of the test material with 1 ml of semen at 37° C for 15 to 25 min. Before being used, the material was washed with warm saline to remove semen adhering to it.

Mucus-passaged spermatozoa were obtained as follows. Suspensions of spermatozoa in mucus were prepared and then incubated in 5-ml aliquots for 30 min at 37° C. Each suspension was then placed in an upright glass cylinder (15-mm bore), the lower end of which was covered with nylon sifting mesh of average aperture size 30 μ (Henry Simon Ltd, Cheadle Heath, England) that retained the mucus. The lower end of the cylinder was fitted within a straight-

sided collecting flask containing 1 ml of saline so that the mucus and the saline were in contact. After each system had been incubated for 30 min at 37° C, the saline containing the mucus-passaged spermatozoa was removed. A similar procedure was used to collect spermatozoa from cervical mucus obtained from previously mated cows.

The suspensions of spermatozoa in the various media were incubated in stoppered test tubes under aerobic conditions at 37° C.

Analysis of data

The data were analysed by the analysis of variance method.

RESULTS

Survival of spermatozoa in whole mucus and in mucus gel

In a series of experiments, suspensions of spermatozoa in the whole mucus or the mucus gel and serial dilutions of semen in saline (all prepared from the same ejaculate) were incubated for 1 hr. The proportions of the spermatozoa that were motile at 0 to 5 min and at 60 to 65 min were determined. The mean values are shown and compared in Table 1.

TABLE 1

THE MOTILITY OF BULL SPERMATOZOA AT VARIOUS CONCENTRATIONS IN BOVINE CERVICAL MUCUS AND IN SALINE

Diluent	*Spermatozoa per µl*	*No. of experiments*	*Mean percentage (±S.E.) of spermatozoa motile at:*	
			0 *to* 5 *min*	60 *to* 65 *min*
Mucus	10 to 450	8	96·9±0·7	58·6±4·0
	500 to 1950	14	97·8±0·5	65·8±3·8
	2000 to 4950	12	97·6±0·5	61·9±3·7
	5000 to 9950	9	97·2±0·6	59·6±4·0
Saline	500	8	2·3±0·9	0
	2000	14	48·9±3·4	20·1±4·1
	5000	12	54·8±4·0	29·2±5·0
	10000	9	63·1±2·8	45·7±3·3

There was an immediate, deleterious effect on spermatozoa when they were suspended at low cell concentration in saline, but there was no association between the concentration of spermatozoa in the mucus and the proportion of the spermatozoa that were motile. At 60 to 65 min, the correlation coefficient was $r_{41} = +0{\cdot}257$, $P>0{\cdot}1$.

The concentration of spermatozoa in twelve suspensions of spermatozoa in mucus gel ranged from 3 to 470 cells/µl. The proportion of motile spermatozoa was 96·1 (S.E. 0·5)% at 0 to 5 min and was 49·3 (S.E. 3·5)% at 60 to 65 min and there was no obvious association between the concentration of spermatozoa and the proportion of motile spermatozoa in the gel. By contrast, all of the spermatozoa in the control suspensions containing 500 spermatozoa/µl of saline were immotile at 60 to 65 min.

Survival, in saline, of mucus-passaged spermatozoa

Fifteen suspensions of mucus-passaged spermatozoa containing 150 to 530 spermatozoa/μl of saline were obtained from laboratory-prepared suspensions of spermatozoa in mucus. These preparations, and control suspensions containing 500 spermatozoa/μl of saline that had been prepared by diluting semen directly with saline, were incubated for 1 hr.

Initially, 34·8±5·1 (S.E.)% of the mucus-passaged spermatozoa were motile. At 15 to 20, 30 to 35 and 45 to 50 min, the proportion of motile cells was 16·7±2·6%, 5·9±1·9% and 0%, respectively. In the control suspensions, 6·1±2·2% of the spermatozoa were motile at 0 to 5 min but none was motile at 15 to 20 min.

Five suspensions containing 195 to 415 mucus-passaged spermatozoa/μl of saline were prepared using five samples of mucus taken from oestrous cows mated 4 to 5 hr previously. At 0 to 5, 15 to 20, 30 to 35 and 45 to 50 min, only 25·6±4·3%, 15·8±3·6%, 1·8±1·3% and 0%, respectively, of the spermatozoa were motile.

TABLE 2

COMPARISON OF THE MOTILITY OF BULL SPERMATOZOA SUSPENDED IN BOVINE CERVICAL MUCUS SUPERNATANT AND IN SALINE

Spermatozoa per μl	*Mean percentage of spermatozoa motile at:*					
	0 *to* 5 *min*			60 *to* 65 *min*		
	In mucus supernatant	*In saline*	*S.E. of difference*†	*In mucus supernatant*	*In saline*	*S.E. of difference*†
20000	70·3	72·0	1·5	62·1	55·7	3·0
10000	70·6	69·3	2·3	59·9*	50·4*	3·3
5000	66·1**	56·9**	3·1	44·0**	30·1**	3·9
2500	57·3**	43·6**	4·5	25·5	22·3	3·5
1250	45·8***	28·7***	3·9	4·8	9·3	2·8
625	32·1***	7·7***	4·7	0·1	0	0·1

All means based on fifteen replications. † d.f. = 14. Significance of difference between means denoted thus: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

Survival of spermatozoa in mucus supernatant and in liquefied mucus

Fifteen semen samples obtained at random from six bulls were each diluted serially, and in duplicate, with saline and with mucus supernatant or liquefied mucus so that the cell concentration for each set of dilutions ranged from 20,000 to 625 spermatozoa/μl. The suspensions were incubated for 1 hr.

During the first 5 min after preparation of the suspensions, the mucus supernatant provided more protection to the spermatozoa against the effect of dilution than did saline (see Table 2). Nevertheless, there was an immediate dilution effect associated with the use of the supernatant and this effect became more severe with time. By 60 to 65 min, there was little difference between the supernatant-based and the saline-based suspensions of similar cell concentration in the relative numbers of motile spermatozoa.

A comparison of the relative proportions of motile spermatozoa in suspensions

based on liquefied mucus and on saline is given in Table 3. In the suspensions containing less than 5000 spermatozoa/μl, a significantly greater proportion of the spermatozoa remained motile in the liquefied mucus-based than in the saline-based suspensions of similar cell concentration at 0 to 5 min. However, the protection afforded spermatozoa by the liquefied mucus was only partial and temporary. Although a significantly greater proportion of spermatozoa were motile at 60 to 65 min in the mucus-based than in the saline-based preparations containing 2500 or 1250 spermatozoa/μl, most of the motile spermatozoa suspended at a concentration of 1250 or 625 cells/μl in liquefied mucus had evidently suffered damage for they displayed extremely sluggish or erratic progression.

TABLE 3

COMPARISON OF THE MOTILITY OF BULL SPERMATOZOA SUSPENDED AT VARIOUS CONCENTRATIONS IN MACERATED BOVINE CERVICAL MUCUS AND IN SALINE

Spermatozoa per μl	*Mean percentage of spermatozoa motile at:*					
	0 to 5 min			*60 to 65 min*		
	In macerated mucus	*In saline*	*S.E. of difference*†	*In macerated mucus*	*In saline*	*S.E. of difference*†
20000	68·8	66·5	2·3	53·3	60·5	4·3
10000	65·9	65·2	1·4	51·1	56·5	3·3
5000	59·1	60·7	2·4	41·8	37·3	4·3
2500	55·3*	45·3*	3·4	33·9**	18·8**	4·8
1250	47·1***	23.7***	4·8	16·2**	3·6**	3·3
625	40·0***	5·6***	3·7	1·9	0	0·9

All means based on fifteen replications. † d.f. = 14. Significance of difference between means denoted thus: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

DISCUSSION

Results obtained in the present study support the findings of Tampion & Gibbons (1963) indicating that a dilution effect does not occur with spermatozoa in bovine cervical mucus, but show that the resistance gained by spermatozoa in mucus to the deleterious effects of dilution in a liquid medium is only slight and temporary. While the former phenomenon should be of particular importance in preventing damage to the reserve of spermatozoa established in the cervix after mating in cows and certain other species (Quinlan, Maré & Roux, 1932; Mattner, 1963, 1968), the physiological importance of the latter phenomenon should be slight.

It is unlikely that a chemical action is involved in the protection afforded spermatozoa by mucus or that mucus contains a specific protecting substance. Rather, it appears that the protective action of the mucus occurs as a result of its structural and physical properties. Spermatozoa appeared to be immune from the harmful effects of dilution immediately upon entry into either whole mucus or mucus gel in which most of the fluid that occurs in mucus had been replaced by saline. An obvious dilution effect, however, occurred immediately when spermatozoa were suspended in either mucus supernatant containing the

soluble substances that occur in the native secretion (Gibbons, 1959; Gibbons & Roberts, 1963) or in mucus that had been liquefied by maceration.

The dilution effect may occur through excessive loss of vital material when spermatozoa are in low cell concentration in liquid media (Emmens & Swyer, 1948). Cervical mucus is not, however, a true liquid but is a visco-elastic gel or semi-solid in which the insoluble mucopolysaccharide molecules are cross-linked to form a three-dimensional network (Gibbons, 1964). This arrangement prevents random movement and distribution of spermatozoa in mucus (Tampion & Gibbons, 1962b; Mattner, 1966) and may ensure that spermatozoa are associated with only a minute amount of the liquid phase as they traverse microzones in mucus (Tampion & Gibbons, 1963). Further, diffusion of the 'sperm extract' away from spermatozoa in mucus may not occur readily as the diffusion of simple ions such as Na^{22} is slow in human cervical mucus (Odeblad & Westin, 1958) which is chemically and structurally similar to bovine cervical mucus.

Alternatively, the absence of a dilution effect in cervical mucus might be explained by a low concentration of oxygen in mucus. Shannon (1965) has shown that the harmful effects of dilution on spermatozoa are greatly reduced when the oxygen content of the diluent is minimal and observations by Olds & VanDemark (1957) indicate that oxygen does not readily diffuse through bovine cervico-vaginal mucus.

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