Factors from fluid of the ovarian pocket that stimulate sperm motility in domestic hens

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Summary. Fluid was collected from the region of the peritoneum surrounding the ovarian pocket of domestic hens at about the time of ovulation. This fluid, diluted to 10%, increased the motility of chicken spermatozoa by a maximum of sixfold at 40°C *in vitro*. Gel filtration revealed two peaks of motility-stimulating activity: one was identified as calcium and the other as a heat-labile substance of low M_r (200). It is suggested that this motility-stimulating activity may facilitate fertilization, as spermatozoa are normally stored in a quiescent state within the oviduct and can be passively transported by cilia to the site of fertilization in the ovarian pocket.

Keywords: spermatozoa; motility; domestic hen; fertilization

Introduction

Munro (1938) first suggested that avian spermatozoa may be immotile during most of their sojourn in the female reproductive tract. He noted that chicken spermatozoa remained immotile *in vitro* when suspended in a simple salt solution at the avian body temperature of 40–41°C. This view is supported by the observation that in the uterovaginal sperm storage tubules, where avian spermatozoa may remain for several weeks before fertilization (see Lake, 1975), spermatozoa appear to be mostly immotile (Bakst, 1987). In addition, sperm motility does not seem to be required in domestic hens for the movement of spermatozoa within the oviduct from the shell gland (uterus) to the magnum, as dead spermatozoa on inert particles, such as carbon powder, inserted in the shell gland are transported along the reproductive tract (Mimura, 1939, 1941). However, it has been shown that only motile spermatozoa can transverse from the posterior to the anterior vagina (Takeda, 1974) and it has been suggested that factors within seminal plasma, deposited with the spermatozoa, may enhance intravaginal sperm transport (Terada *et al.*, 1984). Two factors that stimulate sperm motility, calcium and an unidentified regulator of low M_r , have been identified in seminal plasma of chickens (Ashizawa & Wishart, 1987).

The penetration of an ovum by avian spermatozoa involves hydrolysis of the proteinaceous perivitelline layer by sperm acrosomal enzymes (Bakst & Howarth, 1977; Okamura & Nishiyama, 1978). In some mammalian species, development of hyperactivated sperm motility is associated with capacitation and is considered important for penetration of the zona pellucida (Yanagimachi, 1981). As penetration of the avian perivitelline layer appears to occur in an analogous manner to penetration of the zona pellucida, it is possible that chicken spermatozoa must also be motile to locate and penetrate the perivitelline layer. Furthermore, as these spermatozoa will have previously been immotile within the sperm-storage tubules (see Bakst, 1987), their motility will need to be stimulated by factors associated with the ovum or its surrounding fluids.

At the time of ovulation, fluid collects inside the ovarian pocket (P. E. Lake, pers. commun.) which is the portion of the body cavity immediately adjacent to the ovary and the infundibulum.

The ovarian pocket is enclosed by the body wall, the left abdominal air sac and the viscera (see Olsen & Nehrer, 1948). Fertilization occurs within this part of the peritoneum either before, or within 15–20 min of, entry of the ovulated ovum into the infundibulum (Warren & Scott, 1934; Olsen & Nehrer, 1948; Okamura & Nishiyama, 1978). After this, the outer perivitelline layer is laid down (Bain & Hall, 1969) and is impenetrable to spermatozoa (Bakst & Howarth, 1977). Since spermatozoa are found in ovarian pocket fluid taken at the time of ovulation (Bobr *et al.*, 1964) and since the infundibulum actively engulfs the ovulated ovum, or even surrounds the preovulatory follicle at this time (Warren & Scott, 1934; Phillips & Warren, 1937), it may be presumed that this fluid represents the milieu in which fertilization takes place. We examined the ability of this fluid to stimulate sperm motility and attempted to separate and identify the factors involved.

Materials and Methods

Animals

Male domestic chickens were a Rhode Island Red-type control strain (from Ross Poultry Ltd, Newbridge, Midlothian, UK) and hens were a commercial laying strain (Hi-Sex, Euribrid). All birds were caged individually, given 14:00 h light:10:00 h dark and fed a proprietary breeders' ration *ad libitum*.

Collection and treatment of ovarian pocket fluid

Hens were killed by cervical dislocation 15–20 min after oviposition and laparotomized. The viscera were carefully drawn aside to reveal the 5–10 ml of fluid lying within the region of the ovarian pocket, and this fluid was removed with a pipette. Only straw-coloured fluid was retained; blood-contaminated samples were discarded. The fluid was then centrifuged at 105 000 *g* for 1 h at 5°C. The supernatant was stored at –20°C directly, or after concentrating it tenfold by freeze drying. Calcium was removed from the fluid with Dowex 50 × 4-200 as described by Ashizawa & Wishart (1987). The resin was converted to the sodium form with 1 mol NaOH l⁻¹ and then washed and equilibrated with NaCl–TES buffer (0.15 mol NaCl l⁻¹ with 20 mmol TES (*N*-[hydroxymethyl]methyl-2-aminoethanesulfonic acid) l⁻¹ adjusted to pH 7.4 with 1 mol NaOH l⁻¹). This was then mixed well with the samples at 20% (w/v) to remove calcium. Characterization of the sperm motility-stimulating factors was achieved by further treating fluid from ovarian pockets by immersion in a bath of boiling water for 5 min and by organic extraction, by mixing with 5 volumes of chloroform.

Gel filtration

Sephadex G-15-120 (Pharmacia Fine Chemicals, Inc, UK) was hydrated and equilibrated with NaCl–TES buffer in a 1.8 × 100 cm column; 1 ml of concentrated (× 10) ovarian pocket fluid was passed through the column at 80 ml h⁻¹ at 5°C and 80 × 5 ml samples were collected and stored at 5°C before assay.

Preparation of spermatozoa

Samples of semen pooled from four to six males were diluted eightfold with NaCl–TES buffer at room temperature, mixed and centrifuged at 700 *g* for 12 min. The pellet of spermatozoa was reconstituted gently in the same buffer to give a concentration of about 1 × 10⁹ spermatozoa ml⁻¹. These preparations were incubated in 4 ml quantities in 25 ml Nalgene flasks in a shaking water bath at 40°C.

Assays

Sperm motility was assayed spectrophotometrically and was defined by the parameter %(Δ OD)_m, which represents the maximum proportional decrease in optical density at 550 nm occurring after the movement of a suspension of spermatozoa through a flow cell is arrested, and correlates with the percentage of motile spermatozoa. The constant OD_m, which represents the maximum optical density obtained as a suspension of spermatozoa is drawn through a flow cell, was used to estimate concentrations of spermatozoa (Wishart & Ross, 1985). All parameters were measured 15–20 s after exposure of spermatozoa to a given medium. Calcium was measured spectrophotometrically with the indicator Arsenazo III (Gratzel & Beaven, 1977) and protein concentration was measured with Folin's reagent (Lowry *et al.*, 1951). Concentrations of adenosine triphosphate (ATP) in spermatozoa were measured in boiled extracts using firefly extract luminescence as described by Wishart (1982). Osmotic pressure was measured by freezing-point depression with an Advanced Digimatic Osmometer (Advanced Instruments Inc., Needham Heights, MA, USA).

Statistical analysis

Data were subjected to analyses using Student's *t* test. All given estimations are shown as means $\pm$ SEM.

Results

Increasing concentrations of native or calcium-depleted fluid from the ovarian pocket stimulated motility of chicken spermatozoa (Fig. 1a). In the presence or absence of calcium, the relationship between the concentration of ovarian pocket fluid and spermatozoa motility was hyperbolic as shown by the linear nature of the reciprocal plot (Fig. 1b). This plot shows that calcium accounts for about 70% of the motility-stimulating activity of the fluid from the ovarian pocket. The concentration of free calcium in ovarian pocket fluid was $2.04 \pm 0.14 \text{ mmol l}^{-1}$ ($n = 5$) and was negligible in the Dowex-treated fluid. Maximum stimulation of motility was achieved with 8% fluid. Further increases in motility with higher fluid concentration were not limited by energy availability: samples

Fig. 1. Potency of motility-stimulating activity of hen ovarian-pocket fluid before (●) and after (○) Dowex treatment to remove calcium. (a) Each point represents the mean $\pm$ SEM of motility of three samples of spermatozoa after exposure for 20 s to differing concentrations of different fluid samples, before and after Dowex treatment; (b) reciprocal plot of mean estimations from the same data. The parameter % (ΔOD)_m represents the maximum proportional decrease in optical density at 550 nm occurring after the movement of a suspension of spermatozoa through a flow cell is arrested, and correlates with the percentage of motile spermatozoa.

Fig. 2. Separation of motility-stimulating activities of fowl hen ovarian-pocket fluid using Sephadex G-15-120. The elution profiles represent fractions from the same sample (a) before and (b) after Dowex treatment to remove calcium. Calcium concentrations ($\blacktriangle$) and absorbance at 280 nm ($\circ$) of samples are shown with motility-stimulating activity ($\bullet$) measured as the motility of a standard sample of spermatozoa suspended in a portion of each fraction. The M_r 'markers' ($\blacksquare$) were ethanediol (M_r 62) and polyethylene glycol (M_r 200 and 400). The parameter % $(\Delta OD)_m$ represents the maximum proportional decrease in optical density at 550 nm occurring after the movement of a suspension of spermatozoa through a flow cell is arrested, and correlates with the percentage of motile spermatozoa.

of spermatozoa in the presence and absence of 10% peritoneal fluid had ATP concentrations which, at 40.3 ± 2.4 and 38.4 ± 1.1 nmol per 10^9 spermatozoa, respectively ($n = 3$), were not significantly different ($P > 0.01$).

Gel filtration of the native fluid revealed two peaks of motility-stimulating activity, with peaks in fractions 18 and 27 (Fig. 2a). The effectiveness and specificity of calcium removal were evident (Fig. 2b); both calcium and the second peak of motility-stimulating activity disappeared, whereas the characteristic pattern of the other parameters remained unchanged.

In both native and Dowex-treated samples, osmolality was greatest in fractions 21, at 441 and 481 mosmol kg^{-1} , respectively, against a background buffer osmolality of 310–315 mosmol kg^{-1} . The peak of A_{280} in fractions 11–14 was confirmed to be protein, by the method of Lowry *et al.* (1951). Fractions 57–61, which also had a high A_{280} , did not contain protein. The motility-stimulating activity of 10% ovarian pocket fluid treated with Dowex was significantly ($P < 0.01$) reduced from 49.3 ± 5.8 to 15.7 ± 0.6 by boiling for 5 min, whereas boiling had no significant ($P > 0.01$) effect on the motility-stimulating activity of native fluid, although changing it from 68.8 ± 4.4 to 55.5 ± 6.7 % $(\Delta OD)_m$ units ($n = 3$). Extraction with chloroform also had no significant ($P > 0.01$) effect on the motility-stimulating activity of native (68.8 ± 4.4 versus 65.7 ± 3.6)

and of Dowex-treated (49.3 ± 5.8 versus 37.8 ± 2.9) fluid (parameters as above). Sperm motility measured after 5 min exposure to 10% Dowex-treated fluid at 40°C was significantly ($P < 0.01$) reduced with respect to motility measured after 15–20 s exposure (30.7 ± 4.4 versus 17.1 ± 2.7). With 10% native fluid, motility after 5 min exposure was not significantly different ($P > 0.01$) from that measured after 15–20 s exposure (40.5 ± 2.5 versus 40.9 ± 2.3 ; $n = 5$).

Discussion

The results show that the fluid that collects in the ovarian pocket at the time of ovulation contains two sperm motility-stimulating factors: one was identified as calcium and the other, which remains unidentified, is a substance of M_r about 200 which is heat labile and not extractable with organic solvents. The position of this factor within the fractions obtained from the Sephadex G-15-120 column is analogous to that of the motility-stimulating factor identified in chicken seminal plasma (Ashizawa & Wishart, 1987) and may be the same substance. Apart from calcium, two other low molecular weight factors have been shown to stimulate fowl sperm motility: caffeine (Wishart & Ashizawa, 1987) and bicarbonate (Ashizawa *et al.*, 1989). The stimulating effect of the unknown factor was transient, lasting less than 5 min. Thus its mode of action may be similar to that of caffeine, which also stimulates a transient response, rather than that of calcium or bicarbonate, which invoke a more sustained increase in sperm motility.

The source of this ovulation-associated, ovarian pocket fluid is not known. It is unlikely to have originated from the ovary since follicular fluid is not present in the follicles of laying hens and the follicular blood supply appears to be reduced at the time of ovulation (Phillips & Warren 1937). Although the fluid may be partly peritoneal in origin, another potential source is the oviduct. In many mammalian species, oviductal fluids flow into the peritoneal cavity, the greatest production occurring at the time of ovulation (see Hamner, 1973). In domestic hens, at the time of ovulation, the infundibulum becomes oedematous and extremely active, particularly in the ovarian pocket adjacent to the mature follicle (Warren & Scott, 1934; Phillips & Warren, 1937) and its fluid volume is maximum (Morzenti *et al.*, 1978). Furthermore, spermatozoa are most numerous within the infundibular (Morzenti *et al.*, 1978) and ovarian pocket (Bobr *et al.*, 1964) fluids at this time.

El Jack & Lake (1967) suggested that uterine fluids might contain sperm-motility-stimulating factors that could influence sperm movement within the reproductive tract in hens and Brillard *et al.* (1987) showed that uterine fluids stimulated motility of chicken spermatozoa *in vitro*. However the 'plumping' and 'oviposition' fluids from the uterus contained, respectively, seven and 12 times more calcium (El Jack & Lake, 1967) than the ovarian fluid, so direct transfer of the uterine fluid to the infundibulum and peritoneum seems unlikely.

Whatever the source of this fluid, evidence suggests that it is the fluid that surrounds the ovum at the time of fertilization and that the sperm motility-stimulating factors may be implicated in promoting fertilization in domestic hens.

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