The interaction of avian spermatozoa with the perivitelline layer

_in vitro and in vivo_

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The differences in the hydrolytic activity of chicken spermatozoa towards the inner perivitelline layer in situ and when isolated from the ovum were investigated. Points of hydrolysis produced by chicken spermatozoa on the inner perivitelline layer of laid and freshly ovulated eggs were viewed as dark holes after staining with fluorescein-conjugated wheat-germ agglutinin. In laid eggs that had been fertilized in vivo, these holes appeared more frequently on the perivitelline layer overlying the blastodisc at the animal pole than on that overlying the vegetal pole. However, in an assay in which fragments of the inner perivitelline layer from freshly ovulated eggs were incubated with spermatozoa in vitro, points of hydrolysis appeared with similar frequency on the perivitelline layer from over the germinal disc at the animal pole or from that over the vegetal pole. No difference could be demonstrated in the electrophoretic profile of peptides extracted from the inner perivitelline layer at the animal or vegetal pole. The factor(s) responsible for either increased attraction of spermatozoa to, or for increased activity at, the perivitelline layer of the animal pole does not appear to be associated with the perivitelline layer itself.

Introduction

The inner perivitelline layer of the avian egg may be considered to be analogous to the mammalian zona pellucida (Howarth, 1992); it is a proteinaceous investment surrounding the egg (Bellairs et al., 1963; Bakst and Howarth, 1977a; Kido and Doi, 1987) through which spermatozoa must penetrate to gain access to the oolemma and enter the ovum (Bakst and Howarth, 1977b; Okamura and Nishiyama, 1978a).

To characterize the interaction of spermatozoa with the inner perivitelline layer in the chicken, assays have been developed in which spermatozoa are incubated with fragments of the inner perivitelline layer in vitro and the time taken for the disintegration of the inner perivitelline layer (Koyanagi et al., 1988; Howarth, 1990), or the number of points of hydrolysis produced in a given time (Howarth, 1992; Bramwell and Howarth, 1992a; Steele and Wishart, 1992), are measured as the assay end-point. These assays have been used to show that the perivitelline layer contains factors that stimulate the acrosome reaction (Koyanagi et al., 1988) and that act as receptors for spermatozoa (Howarth, 1990, 1992). The location within the egg from which samples of the inner perivitelline layer for these experiments were taken has been either unspecified (Koyanagi et al., 1988; Howarth, 1990), or stated to be other than that over the germinal disc (Howarth, 1992). This ensures the maximum amount of material for the assay, since the inner perivitelline layer over the germinal disc, at approximately 8 mm², represents only 2% of its total area. However, the physiological relevance of such assays may be questioned since most evidence suggests that it is the inner perivitelline layer over the germinal disc that is likely to be the major site for attachment and penetration of spermatozoa in vitro: the germinal disc contains the female genetic material and organelles (Bakst and Howarth, 1977a; Perry et al., 1978) and penetration of the ovum by spermatozoa appears to occur only within this region (Okamura and Nishiyama, 1978). Howarth and Digby (1973) suggested that chicken spermatozoa might be chemotactically attracted to the animal pole, as they found that the inner perivitelline layer over the germinal disc of freshly ovulated eggs frequently ruptured during fertilization in vitro. Bramwell and Howarth (1992a) also briefly reported that the number of points of hydrolysis produced by spermatozoa in the inner perivitelline layer during fertilization in vivo were 14 times more concentrated in the perivitelline layer overlying the animal pole.

Bramwell and Howarth (1992b) compared the activity of spermatozoa in vitro towards the isolated inner perivitelline layer taken from different locations of the ovum. They reported that points of hydrolysis are concentrated in the areas of the inner perivitelline layer taken from over the germinal disc and conclude that this contains preferred sperm binding sites that are active in vivo and in the isolated inner perivitelline layer in vitro. However, their conclusions are based on a single result in which the concentration of points of hydrolysis in the inner

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perivitelline layer over the animal pole is only 1.33 times that found in the inner perivitelline layer taken from other areas of the ovum.

We investigated this differential hydrolysis of the inner perivitelline layer of chicken eggs more extensively. We found preferential hydrolysis of the inner perivitelline layer at the animal pole from laid eggs fertilized in vitro, but could not show increased sperm activity towards isolated fragments of the inner perivitelline layer taken from the animal pole during co-incubation in vitro. A preliminary account of part of this work has appeared in abstract form (Steele and Wishart, 1992).

Materials and Methods

Birds and semen collection

Male chickens (Gallus domesticus) from a Light Sussex strain and hens that were ISA-Brown commercial layers were caged individually, given free access to a breeder's ration and subjected to a photoperiod of 14 h light:10 h dark. Commercial lines of turkeys (Meleagris gallopavo) and guinea fowl (Numida meleagris) were kept under similar conditions at the Institute National de la Recherche Agronomique, Monnaie. Semen was collected and artificial insemination was performed as described by Lake and Stewart (1978).

Preparation of the inner perivitelline layer

Hens were killed 10–15 min after oviposition by injecting approximately 3 ml Euthatal (RMB Animal Health Ltd, Dagenham) into the wing vein. The animals were laparotomized and the newly ovulated ovum was carefully removed from the body cavity or from the first few centimetres of the infundibulum. Eggs that had traces of albumin associated with them were discarded. The ova were suspended in 0.15 mol NaCl I−1 with 20 mmol TES (N-Tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid) I−1 at pH 7.4 (NaCl–TES) and squares of inner perivitelline layer of approximately 1 cm² were cut from the animal and vegetal poles and washed free of adherent yolk by rinsing with NaCl–TES. A similar technique was used for preparing the inner and outer perivitelline layer from laid eggs. The inner perivitelline layer from follicular eggs was prepared by cutting similarly sized pieces from the appropriate area of the whole follicle wall and then isolating the inner perivitelline layer from the outer layers. The samples from the animal pole were trimmed to circles with a diameter of 0.5 cm.

Incubation of spermatozoa with the inner perivitelline layer in vitro

The pieces of inner perivitelline layer (or the perivitelline layer from laid eggs) were incubated with 1.0 ml modified Eagles’ minimal essential medium buffered with 10 mmol Hepes l−1 (MEM: Flow Laboratories, Irvine, CA) containing between 2×10⁶ and 2×10⁷ spermatozoa ml⁻¹ within a polycarbonate tube. The tubes were incubated at 40°C for 5 min, after which time the perivitelline layer fragments were removed and rinsed in NaCl–TES.

Staining of the inner perivitelline layer and assay of sperm hydrolytic activity

Samples of the perivitelline layer taken directly from laid eggs or the inner perivitelline layer from incubations in vitro were fixed for 2 h in NaCl–TES containing 9% formaldehyde. After a further rinse in NaCl–TES the fragments were stained overnight in 250 µl 10 µg wheat-germ agglutinin conjugated with fluorescein isothiocyanate (FITC) ml⁻¹ (Sigma Chemical Co., London) in NaCl–TES. Control samples were incubated without spermatozoa but otherwise were treated similarly.

The number of points of hydrolysis per 0.13 mm² (the area of the field viewed at ×400 magnification) was assessed by epifluorescence microscopy. Alternatively, samples were viewed unstained using dark background optics at a magnification of ×100. The inner perivitelline layer over the germinal disc or over the blastodisc was readily discernible by its apparently thinner structure, especially at the periblastic region. The concentration of points of hydrolysis at the animal pole was determined within this area.

SDS-PAGE of proteins of the inner perivitelline layer

Samples of whole and inner perivitelline layers were isolated and solubilized by incubating them at 100°C for 3 min in a solution containing 5.5% SDS and 0.14% β-mercaptoethanol. They were then centrifuged at 13,000 g for 5 min and samples of the supernatant (20 µl) were subjected to vertical SDS-PAGE on 5–15% gradient gels at a constant current of 35 mA for 4 h at 22°C, as described by Laemmli (1970). Molecular weight markers (SDS-7; Sigma Chemical Co.) in the range 14–66 kDa were also run on the gels.

After separation, gels were immediately fixed in water: methanol:acetic acid (4:5:1; v:v:v) and silver-stained using the method described by Blum et al. (1987).

Results

The FITC-conjugated wheat-germ agglutinin stained the inner perivitelline layer uniformly, with the holes hydrolysed by spermatozoa appearing as dark circles (see Fig. 1). The concentration of points of hydrolysis produced by spermatozoa per unit area of fragments of the inner perivitelline layer taken from over the animal pole (or, more precisely, from the inner perivitelline layer originally overlaying the periblastic region of the germinal disc) was similar to those in fragments taken from the vegetal pole of the ovum (Fig. 2). This occurred over a wide range of concentrations of holes. The different concentrations of points of hydrolysis were controlled partly by using the two concentrations of spermatozoa. However, variation in the ‘quality’ of the spermatozoa or the inner perivitelline layer also appeared to occur and was responsible for most of the variation seen.

The frequency of points of hydrolysis produced by 2×10⁷ spermatozoa ml⁻¹ in vitro on pieces of the inner perivitelline layer taken from the vegetal pole and from three equidistant locations at the equatorial region of a freshly ovulated ovum
were not significantly different (P > 0.1; Student’s t test) at 72 ± 9, 73 ± 9, 78 ± 7 and 76 ± 9 holes mm⁻², respectively.

In laid eggs the frequency of holes mm⁻² in the inner perivitelline layer from over the periblastic region at the animal pole was always considerably greater than in the layer from over the vegetal pole (Fig. 3). This was particularly exaggerated for eggs in which the frequency of holes in the inner perivitelline layer from the vegetal pole was low; for example, at a frequency of 2.0 holes per mm² the ratio of holes at the animal pole of the inner perivitelline layer to that at the vegetal pole was about 25. This ratio tended to decrease as the concentration of holes in the inner perivitelline layer from the vegetal pole increased, as the frequency of holes at the animal pole appeared to reach a maximum of about 75 holes mm⁻² in eggs fertilized in vivo (Fig. 3). Such a maximum was not apparent in fragments of inner perivitelline layer incubated with spermatozoa in vitro (Fig. 2).

For the interaction of spermatozoa with the inner perivitelline layer in vitro, the frequency of holes (mm⁻²) in the inner perivitelline layer from the vegetal pole and from three equidistant equatorial regions of a laid egg, fertilized in vivo, were not significantly different (P > 0.1; Student’s t test) at 8.6 ± 1.0, 9.5 ± 0.4, 9.9 ± 0.8 and 8.4 ± 0.5 (mean ± SD, n = 5).

When spermatozoa were incubated in vitro with the perivitelline layer from three laid eggs, the proportional frequency of points of hydrolysis (mm⁻²) produced was 0.06 ± 0.01 (mean ± SD) compared with that of the inner perivitelline layer from freshly ovulated eggs from the same hens. The proportion of holes similarly produced by spermatozoa in the inner perivitelline layer from the three largest follicles was 1.05 ± 0.03 (mean ± SD) compared with that of the inner perivitelline layer from freshly ovulated eggs. In a hierarchy of follicular ova from F1 to F6, the susceptibility of their inner perivitelline layers to hydrolysis by the same sample of spermatozoa was 102%, 93%, 110%, 96%, 121% and 95%, respectively, of that of the freshly ovulated ovum.

The ability to hydrolyse the inner perivitelline layer in vitro was also present in spermatozoa from early developmental stages. The mean (± SD, n = 6) number of points mm⁻² hydrolysed in the inner perivitelline layer by (2 × 10⁷ ml⁻¹) testicular, upper, middle, lower ductus deferens and ejaculated spermatozoa from the same bird were 7.9 ± 2.0, 11.0 ± 6.1, 12.0 ± 0.6, 21.4 ± 3.4 and 81.3 ± 5.1, respectively.

The activity of chicken, guinea fowl and turkey spermatozoa (all at 2 × 10⁷ ml⁻¹) towards their homologous inner perivitelline layer and that of the other two species was assessed in vitro using fragments from primary follicular ova from two different hens of each species. The mean number of points of hydrolysis mm⁻² made on chicken, guinea fowl and turkey inner perivitelline layers was, respectively, 365, 243 and 200 for chicken spermatozoa, 2.4, 13 and 2.4 for guinea fowl spermatozoa, and 4.0, 23 and 86 for turkey spermatozoa. The mean difference between each of the two estimations was 20%.
with a range of 3–39%. Thus, each species showed variation in sperm activity but, in each case, spermatozoa showed more activity towards the inner perivitelline layer of their homologous species.

The profiles of peptides of the inner perivitelline layer taken from the animal and vegetal pole of a freshly ovulated chicken egg and of the perivitelline layers from a laid egg are shown (Fig. 4). The profiles of the bands from the two samples of the inner perivitelline layer in vitro were identical, with major bands at 36 kDa and 76 kDa. In the perivitelline layers of the laid egg, major additional bands at 11 kDa and 14 kDa were observed; these were proteins of the outer perivitelline layer. In addition, the 36 kDa protein of the inner perivitelline layer protein appeared to have a greater mobility in this sample.

**Discussion**

The results reported here on the interaction of spermatozoa with the inner perivitelline layer in vivo support the hypothesis that chicken spermatozoa show more hydrolytic activity (Howarth and Digby, 1973; Bakst and Howarth, 1977b), in terms of numbers of points of hydrolysis (Bramwell and Howarth, 1992a), towards the inner perivitelline layer overlying the germinal disc in situ than that overlying other regions of the egg. However, our results do not support the conclusion of Bramwell and Howarth (1992b) that spermatozoa in vitro preferentially hydrolyse the region of the isolated inner perivitelline layer that overlies the animal pole and that this region is rich in receptors for spermatozoa. In support of our findings, we also demonstrated that the peptide profiles of inner perivitelline layer preparations from the animal pole and the vegetal pole are indistinguishable after SDS-PAGE. It is therefore possible that the factor responsible for more points of hydrolysis on the inner perivitelline layer over the germinal disc of eggs fertilized in vitro is not associated with the inner perivitelline layer and may originate from the ovum itself.

Clearly, the germinal disc underlying the inner perivitelline layer at the animal pole is quite distinct from other regions of the egg (e.g. Perry et al., 1978). Bakst (1978) found that microvillar projections of the oolemma were longer and more abundant at the animal pole and suggested that these might protrude through the inner perivitelline layer and, furthermore, that they might be the site of sperm receptors, although in a later report he considered that these projections might be remnants of granulosa cell microvilli (Bakst, 1979). Bakst (1978) also identified lacunae or ‘rounded concavities’, 60–100 µm in diameter and 20 µm deep, at the periblastic region of the germinal disc and suggested that these might contain some fluid responsible for activating spermatozoa at fertilization. It is particularly significant that these lacunae are situated under the region of the inner perivitelline layer where points of sperm hydrolysis are most concentrated. The identity of potential diffusible factors and whether they activate the acrosome reaction, encourage binding of spermatozoa to the inner perivitelline layer, or chemotactically attract the spermatozoa to the germinal disc remains unknown. Bakst and Howarth (1977b) suggested that potential cationic activators of sperm acrosomal activity, such as Ca$^{2+}$ or Mg$^{2+}$, might only be available at the animal pole. However, these ions are present in the medium used for fertilization of intact ova in vitro (Howarth and Digby, 1973) and Ca$^{2+}$ is present in a peritoneal exudate that has been suggested to be the fluid surrounding the ovum at fertilization in vivo (Ashizawa and Wishart, 1992). Thus, it seems unlikely that these ions would be limited only to the animal pole.

The attachment and acrosomal hydrolysis of the inner perivitelline layer from areas other than that over the germinal disc would seem to be futile, in terms of normal mechanisms of fertility. In several passerine species, points of hydrolysis are found only at the animal pole in eggs fertilized in vitro (T. Birkhead, personal communication). It may be that, in chickens, the points of hydrolysis in the inner perivitelline layer from regions other than the animal pole are formed by spermatozoa trapped in the outer perivitelline layer, but in close proximity to the inner layer, so that a normal acrosome reaction or disintegration of spermatozoa releases sufficient acrosomal enzymes to hydrolyse the inner perivitelline layer locally. In support of this hypothesis, we found a similar number of holes with associated remnants of spermatozoa per unit area of inner
perivitelline layer from both the animal and vegetal poles (G. Wishart, unpublished observations). However, although such interactions may prove to be ‘artefacts’, there does seem to be some specificity involved in the interaction of spermatozoa with regions of the inner perivitelline layer not associated with the animal pole in vitro, since spermatozoa have greater activity towards the inner perivitelline layer of their homologous species and since protein extracts of the perivitelline layer inhibit this activity (Bramwell and Howarth, 1992c).

The identity of the receptor for chicken spermatozoa in the inner perivitelline layer remains to be determined. Our studies show major peptide bands in this layer at 36 kDa and 76 kDa. Other studies using nonreducing SDS–PAGE have identified major proteins at 32 kDa, 183 kDa and 1000 kDa (Kido and Doi, 1987) and at 33 kDa, 54 kDa and 200 kDa (Howarth, 1992). The disparity between the size of these various fractions may be related to their reduction status and the fact that Kido and Doi used the inner perivitelline layer separated from laid eggs, while Howarth used material from freshly ovulated ova (as in the study reported here). In the preparation of the whole perivitelline layer from laid eggs, additional proteins of the outer perivitelline layer are also visible (such as lysozyme at 14 kDa); however, the major inner perivitelline layer peptide at 36 kDa bands at a higher molecular weight locus. Whether this represents a postovulatory deterioration in the structure of the inner perivitelline layer or a change in the inner perivitelline layer proteins induced by the outer perivitelline layer is not known. However, it is possible that such a change might result in a chemical block to the penetration of the perivitelline layer of laid eggs by spermatozoa, rather than in the simple mechanical barrier that the outer perivitelline layer is usually considered to provide (Howarth and Digby, 1973). The presence of sperm–receptor activity on the inner perivitelline layer of immature ova reflects the situation found in the development of the mammalian zona pellucida (see Wassarman, 1992). However, while the capacity to bind spermatozoa and eggs in mammals matures only within the excrurent ducts of the male reproductive tract (Jones, 1989), testicular chicken spermatozoa display some activity towards the inner perivitelline layer in vitro compared with ductus spermatozoa. This result supports the finding that chicken testicular spermatozoa can fertilize in vivo if they are inseminated intramagnically, since they are unable to traverse the vagina (Howarth, 1983), and that the surface changes to spermatozoa in the ductus deferens (Esponda and Bedford, 1985; Steele, 1992) are concerned with this ability to travel in the vagina rather than with the interaction of spermatozoa with the inner perivitelline layer.

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