STUDIES ON THE MECHANISM OF NIDATION

XXVII. SPERM-DERIVED INCLUSIONS IN THE RAT BLASTOCYST

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Summary. One-fifth of rat blastocysts contain morphologically identifiable sperm heads or tails. In the electron microscope, the sperm remnants are seen to interpenetrate the cells as well as to lie free in the perivitelline space. On the background of these morphological findings, the possible role of sperm derivatives in nidation is discussed.

INTRODUCTION

The perivitelline space of the mammalian ovum—the fluid-filled hiatus between the inner surface of the zona pellucida and the outermost surface of the cells of the egg—contains certain inclusions. Immediately after fertilization, the two polar bodies are found in the perivitelline space. Later, during the period of tubal transport, small masses of yolk-like material are expelled from the blastomeres into this space by a process which has been termed deutoplasmolysis. Both of these inclusions are composed of material of maternal origin.

Odor & Blandau (1949) and Izquierdo & Vial (1962) have noted remnants of supernumerary spermatozoa in the perivitelline space and their presence raises questions concerning their persistence as well as their possible role. In the truest sense, the perivitelline cavity is the environment of the developing egg. The materials which it contains are liberated at the moment of lysis of the zona pellucida.

This study is a morphological investigation of the presence of sperm-derived elements in the perivitelline space of rat ova during L₄, the 5th day of pregnancy.

METHODS

Ova were obtained by rinsing the contents of the uterine horns into polyethylene tubes placed in the vagina (full details of the method are found in Kraicer, 1967). Donor rats were young adult females of the Biodynamics Institute colony. Their uteri were rinsed between 10.00 hours and 13.00 hours of L₄ (L₀ = day of spermatozoa in vaginal smear).

Three different fluids were used to rinse out the ova: (1) unbuffered Ringer solution, pH 7.2, (2) autologous blood plasma, and (3) 3% glutaraldehyde buffered with cacodylate (Sabatini, Bensch & Barrnett, 1963). Observation of the inclusions in the perivitelline space is facilitated by suspension of the ova in

blood plasma since the plasma and the zona pellucida have similar refractive indices; hence, the zona is not discernible and perivitelline inclusions stand out.

Once isolated, the ova were examined by interferential contrast microscopy using a Nachet model 300 microscope. Some specimens were photographed using Polaroid film type 47, a very high speed film which permits short exposures at moderate light intensities.

For study in the electron microscope, blastocysts were flushed out of the uterus with 3% glutaraldehyde in sodium cacodylate buffer, pH 7.2 (Sabatini *et al.*, 1963). Collected blastocysts were kept in the glutaraldehyde fixative at room temperature for 20 min and post-fixed with 1% osmium tetroxide in phosphate buffer, pH 7.3 (Millonig, 1961) for 1 hr. After dehydration in an acetone series, they were embedded in Durcupan ACM resin (Fluka AG., Switzerland). Thin sections, cut on a Danon Ultramicrotome, were mounted on formvar-carbon coated grids and were stained in lead hydroxide solution (Karnovsky, 1961). The sections were examined in a RCA EMU2A microscope.

RESULTS

Approximately one-fifth of ova isolated on L₄ are seen to contain sperm tails, heads, or both in the perivitelline cavity (Kraicer, 1967). This figure may be somewhat low, since the sperm heads are seen clearly only when they lie on the upper surface of the ova or in the perivitelline space surrounding the ovum as viewed from above. Sperm heads lying below the inner cell mass would probably be obscured. By using interferential contrast microscopy, the observation and resolution of the sperm heads and tails were greatly facilitated.

Ova were found which contained one or many spermatozoa (Plate 1). In the vast majority of ova in which both sperm heads and tails were found, the heads and tails were not joined.

Examination of an ovum with multiple spermatozoal inclusions by electron microscopy gave further proof of the identity of these inclusions and also added information on their fate. One particularly fortunate section passed through the head, midpiece and tail of a spermatozoon in the perivitelline space (Plate 1e). This section provides striking evidence that head and tail can remain attached in the perivitelline space. No mitochondrial midpiece is seen, and the head appears empty. When fixed ova are stained with methylene blue or toluidine blue, the sperm head inclusions do not stain. It would, therefore, appear that these supernumerary sperm inclusions have lost their chromatin.

In the same ovum, other sperm-derived inclusions were seen. One of these, found inside one of the trophoblast cells, was a middle-piece in which both mitochondria and the large electron opaque filaments were seen. The more central finer fibres of the tail filament were not seen (compare Plates 2 and 3b). The mitochondria appeared well preserved, and a few cristae were seen. This sperm inclusion was not surrounded by a membrane. Although loss of the membrane is typical of the fertilizing spermatozoon (Szollosi & Ris, 1961; Szollosi, 1965), it is doubtful whether this fragment is a remnant of the fertilizing spermatozoon, since the mitochondria of the fertilizing spermatozoon are not believed to survive beyond the third cleavage (Szollosi, 1965).

Other fragments of sperm tails were found in the segmentation cavity of the blastocyst (Plate 3a). In this location they were surrounded by a thin membrane. This membrane may be the original sperm membrane, or may be a film of material deposited on the outer surface of the tail by the ovum.

Some of the sections through this same blastocyst included a pair of sperm filaments lying $1\ \mu$ apart in the perivitelline space (Plate 4). Because of their spatial arrangement it was possible to identify these sperm tails with certainty in consecutive sections. When the course of these filaments was followed one of them was observed to penetrate into, or be engulfed by, a trophoblast cell. Whereas the portion of the tail in the perivitelline space was encased in a membrane, no membrane was seen around the engulfed portion.

Thus, from examination of this blastocyst it was seen that sperm elements are present not only in the perivitelline space, but also within the cells of the L_4 blastocyst. Certain observations make it unlikely that these intracellular inclusions are derived from the fertilizing spermatozoon: the persistence of the midpiece mitochondria and the observation of penetration of a filament into a trophoblast cell. Although such inclusions have been noted previously (Blandau & Odor, 1952; Blandau, 1961) they are seen far less frequently than are sperm inclusions in the perivitelline space. This may be due to the failure of the light microscope to resolve these intracellular sperm inclusions. Until the frequency of occurrence of engulfed remnants is known, further comment is superfluous.

DISCUSSION

The persistence of sperm heads and tails, in a state of good morphological preservation, in the perivitelline space raises several questions. The whole of the fertilizing spermatozoon enters into the vitellus. All of its components are incorporated, after appropriate changes, into the now-fertilized egg. The sperm tail usually separates into multiple strands which are then lost into the ooplasm (Austin & Braden, 1953). Many of these changes are presumably due to enzymatic action. Since supernumerary spermatozoa, most of which do not pass the vitelline membrane, do not break down and are not incorporated, the enzymatic apparatus which absorbs the fertilizing spermatozoon must be held in the ovum by the vitelline membrane. This is in accord with the concept of the vitelline membrane being a barrier; this concept is based on the reaction of the vitelline membrane which blocks entry of more than one spermatozoon into the vitelloplasm. Even in this instance of penetration of the remnants of supernumerary spermatozoa into the zygote, their remarkable state of preservation suggests late entry.

Although the persistence of structures derived from spermatozoa within the egg is demonstrably possible, its significance is obscure. On the other hand the presence of supernumerary spermatozoa in the perivitelline space raises provocative questions.

The persistence of these male-derived elements until the time of loss of the zona pellucida means that they will be liberated at or near the site of nidus formation. This suggests a possible role for this material in nidation. At the

time of mating, the uterine epithelium is exposed to a large number of spermatozoa. About 5 days later, when the fertilized eggs, now blastocysts, shed their zonae pellucidae, there is a second exposure of the epithelium to the same foreign materials. The analogy to the conditions for establishing a sensitization reaction is apparent (Shelesnyak, 1964).

In order, however, to postulate that the sperm fragments which we have observed are participants in an immunological mechanism of nidation, one would expect to find these fragments in all zygotes. Instead, they have been identified in only one-fifth of ova examined. Nor does it seem possible to argue that this low proportion of eggs containing identifiable sperm remnants is due to breakdown of supernumerary spermatozoa which were originally present in the perivitelline space of the other four-fifths of eggs. The proportion of ova with identifiable supernumerary spermatozoa immediately after fertilization is the same as in the L₄ blastocyst (Odor & Blandau, 1949; Kraicer, unpublished).

If nidation is induced by an immune mechanism, sources of male-derived factors other than the supernumerary spermatozoa should be considered. The supernumerary spermatozoa in the perivitelline space would nevertheless play an auxiliary role. When present, such sperm-derived inclusions would add to the stimulus supplied by other materials of male origin in the zygote. In this connection, it is significant that the sperm tails lying in the perivitelline space appear able to penetrate the trophoblast cells. In penetrating, the membrane of the tail vanishes. In this, the penetrated supernumerary spermatozoon resembles the fertilizing spermatozoon whose membrane is known to coalesce into the vitelline membrane at the time of sperm penetration. Thus, the part of the supernumerary sperm tail which penetrates into the trophoblast cell is incorporated in the same sense that the fertilizing spermatozoon is incorporated into the vitellus. The portion of such a sperm tail, which remains outside the trophoblast, in the perivitelline space, would be firmly adherent to the surface of the blastocyst. As a result, when the zona pellucida disappears, the epithelium of the uterus adjacent to the trophoblast would be placed in intimate contact with the sperm tail. At least in such a case, therefore, the outer surface of the blastocyst is covered in part with male-derived material of possible immunological significance.

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PLATE I

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PLATE 3

PLATE 4

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EXPLANATION OF PLATES

PLATE 1

Sperm inclusions in the perivitelline space. (a) to (d) The inclusions as seen in interferential microscopy, (a) and (b) ($\times 480$) showing sperm tails, (c) and (d) ($\times 720$) sperm heads, indicated by arrows. The ovum (b) was suspended in autologous plasma, in which the zona pellucida is not seen. (e) An electron microscopic photograph of a longitudinal section through a sperm inclusion in the perivitelline space. Zp = zona pellucida.

PLATE 2

Sperm tail inclusion in a newly fertilized ovum. (a) Photograph of a thin section of an ovum isolated approximately 12 hr after fertilization shows the middle piece in cross-section ($\times 6620$). The sperm middle piece (b) ($\times 33,110$) has a minimum outer diameter of 0.81μ . It is included for comparison with Plate 3(b).

PLATE 3

Sperm tail inclusions in the blastocyst.

(a) Three sperm tail inclusions (S = cross-section of tail), two in the segmentation cavity and one between the cells of the blastocyst. The tails in the segmentation cavity are surrounded by a membrane ($\times 25,300$).

(b) A sperm middle piece (cf. Plate 2) found in a thin section of a trophoblast cell. A few cristae were seen in the sheath mitochondria ($\times 40,200$). The sperm middle piece has a minimum outer diameter of 0.87μ . G = Golgi apparatus; M = mitochondrion; N = nucleus; S = sperm tails; Zp = zona pellucida.

PLATE 4

Penetration of a trophoblast cell by a sperm tail inclusion. Two sections of sperm tail (arrows) are seen lying in the perivitelline space, about 1μ apart (a). Note how the spermatozoon at the right approaches a projection of a trophoblast cell (b), and is ultimately engulfed, losing its membrane (c).