Ultrastructural Changes in Preimplantation Rabbit Embryos

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Introduction

Follicular oocytes and penetrated eggs of several mammalian species have been the subject of numerous electron microscope studies (rat: Sotelo 1959, Sotelo and Porter 1959, Franchi 1960, Odor and Renninger 1960, Szollosi and Ris 1961, Guinea pig: Anderson and Beams 1960, Adams and Hertwig 1964, Austin 1965, Barron and Franklin 1968, cat: Liss 1964, man: Stegner and Waternberg 1961, Zamboni *et al.*, 1966, Baker and Franchi 1967, Baca and Zamboni 1967 and rabbit: Blanchette 1961, Merker 1961, Hadek 1963, Zamboni and Mastroianni 1966). However the segmentation process has been studied in only two laboratory animals: the rat (Izquierdo and Vial 1962, Schlafke and Enders 1963, and Mazanek 1965) and the mouse (Hillmann 1967). There is also a brief report by Enders and Schlafke (1965) in which several preimplanting mammalian embryos were examined and in which a number of species differences were found.

While segmentation in the rabbit has been the subject of numerous metabolic and physiological investigations (Adams 1958, Fridhandler 1961, Chang 1966, Daniel 1967), our knowledge of its ultrastructure during this process has received only limited attention (Austin 1965).

It is the purpose of this article to describe the cytoplasmic and nuclear changes which occur in the rabbit embryo from the two blastomere stage to the early blastocyst.

Materials and methods

3½ to 5 months old female rabbits were sacrificed at 24, 30, 36, 47, 64 and 72 hours after mating. Usually three does were used for each stage, and eggs of 3, 4, 8 and 16 blastomeres, early morula and near implantation blastocyte were obtained (Lewis and Gregory 1929). The embryos were recovered by the method of Pincus and Enzmann (1932) using either a saline solution (Brinster 1965) or phosphate buffered 4% glutaraldehyde. In the first case the cleaved eggs were recovered in a hollow slide with the aid of a stereoscopic microscope, then they were carried to another dish which contained

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Figs. 1-2. 1, abundant membraneous and vesicular structures inside the interblastomeric space. There are also numerous pinocytotic vesicles (arrows). Four blastomeres stage. $\times 18,900$. 2, prominent Golgi complex (G) in a blastomere at the eight blastomeres stage. $\times 18,900$.

1% buffered osmium tetroxide fixed for one or two hours at 4°C, and after a brief buffer wash they were quickly dehydrated in 50, 60, 70, 80 and 90% ethanol; five minutes in each; three changes of 30 minutes in absolute ethanol and two changes of 20 minutes in propylene oxide, and finally embedded in Epon 812. In the second method, the eggs were drained of glutaraldehyde and were carried to another dish containing fresh fixative at 4°C, to fix for one hour, after this they were left in phosphate buffer overnight. The next day they were postfixed in phosphate buffered 1% osmium tetroxide and were dehydrated and embedded in the same way as the others. Sections on copper grids were stained with uranyl acetate and lead citrate and were studied with either a Zeiss EM-9 or an Akashi TRS/80 electron microscopes.

Results

Egg envelopes

According to Boving (1963) there are three envelopes which can be distinguished by their origin and chemical composition; they are as follows: gloiolemma, mucolemma and oolemma. In our observation the first two were characterized by regularly distributed microfibrillae; while the oolemma or zona pellucida had a major concentration of irregular microfibrillae; in addition, during the early segmentation stages outstanding irregularities were seen both on the external and internal surfaces. Spermatozoa, single or in groups, were also commonly found widely distributed in the thickness of the zona pellucida.

The perivitelline space was clearly seen as a low density zone which, during early segmentation, included a number of spermatozoa and a considerable number of membranous and vesicular elements. These elements were particularly abundant in two zones; the area where the second polar body had separated and in the interblastomeric regions (Fig. 1).

Several microvillae were found at the two and four cell stage; during early divisions a number of evaginations were observed projecting both into the perivitelline space and into the interblastomeric space (Fig. 1). Around the interblastomeric space, the membrane showed a number of irregular evaginations between which were observed several structures and cytoplasmic organelles. In addition, zones were seen between some blastomeres in which the membranes are thicker (Fig. 3).

Occasional invaginations on the surface of the blastomere appeared to occur at all stages of segmentation. During the two blastomere stage, zones were found which appeared to show evidence of pinocytotic activity (Fig. 7). During the first divisions cytoplasmic extrusions were observed, the contents of which appeared to pass into the perivitelline space (Figs. 6 and 8).

Cytoplasmatic components

I. Mitochondria. These showed a very characteristic structure which did not change; in general their form was lobular although some were

Figs. 3-5. 3, sixteen blastomeres stage. Some thickening of cell membranes (arrows) between neighbouring blastomeres are seen. $\times 14,500$. 4, eight blastomeres stage. Portions

elongated. They measured approximately 1 micron and showed a very electron dense matrix. Cristae were found to run parallel to the outer membrane, although some penetrated into the matrix (Fig. 6). Intermitochondrial bridges and topographic relationships with other cytoplasmic structures were also commonly found (Figs. 9 and 11).

II. Cytoplasmic inclusions. Four main types of formations were found: a) Vesicles of approximately 1.5 microns in diameter, although they may attain much larger dimensions, with a well distinguished membrane. The contents were amorphous, more dense than the matrix and present throughout segmentation (Figs. 4, 9). b) Multivesicular bodies were particularly abundant during the first divisions; they were rarely seen during the later stages (Figs. 9 and 10). c) Dense lysosome-like bodies, which showed great polymorphism, were found during the early divisions in well localized zones, but their presence, although isolated, was evident during the whole segmentation process (Fig. 12). d) Inclusions of lipid nature were present throughout segmentation, but especially in the late stages in some of the blastomeres (Fig. 5).

III. Membranous structures. They were of three types: a) Saccules and tubules with a homogenous content, more dense than the matrix; these were irregularly distributed in the cytoplasm, and were frequently found very close to mitochondria although they could form real accumulations. b) Golgi complex; this structure was well developed. It showed its characteristic componentes, with the vacuolar portion which was the most developed (Figs. 2 and 9). Due to its size and its relationships with other cytoplasmic structures, it was difficult to distinguish the precise outline. However, its size decreased in the later stages although it was always evident. c) Annulate lamellae were present during the first two divisions both in the nucleus and in the cytoplasm (Figs. 11, 14 and 15), but were less common at the eight-blastomere stage, and were not found in the next stages.

With double fixation a greater wealth of structures were shown; the contents were finely granular and contained polysomal aggregates, which increased in number during the later stages. On the other hand, a remarkable fact in the late morula stage was a clear difference in matrix density; one third of the cytoplasm showed a very low density while the majority preserved the same density as in the early stages. Nevertheless, these cells with low density contained the same organelles as the others (Fig. 12). The presence

of cytoplasm of two blastomeres showing several bodies with an amorphous content (vb); one of which is greater than the others. Numerous microvesiculae (mu) can be seen (mu). $\times 5,000$. 5, sixteen blastomeres stage. Cytoplasm of three blastomeres showing white spaces where lipids were extracted (L). Numerous mitochondria (m) and vitellus like bodies (vb) can be seen. $\times 14,000$.

7, two blastomeres stage. Portion of the cortical zone showing abundant microvesiculae (mu) and invaginations of the cell membrane (arrows). $\times 19,500$. 8, cytoplasmic extrusion depositing its multivesicular content into the perivitelline space. Two cell stage. $\times 32,000$.

Figs. 6-8. 6, this micrograph shows a cytoplasmic extrusion with a multivesicular content. Several polysomes (arrows) and mitochondria (m) can be seen. Two cell stage. $\times 33,000$.

Fig. 9. Two blastomeres stage. Vesicular clusters of Golgi complex (G) occupying extensive areas of the cytoplasm. Some of the vesicles show association with multivesicular bodies (mvb), vitellus-like inclusions (vb) and mitochondria (m). Free membranes (arrows) and lipid inclusions (L) can be seen. $\times 29,000$.

Figs. 10-11. 10, four blastomeres stage. Two multivesicular bodies (mvb) and numerous dense microvesiculae in the ground substance (arrows) are seen. Note the two types of mitochondria (m). $\times 24,000$. 11, part of the cytoplasm of a blastomere showing a group of annulate lamellae (al). A lysosome-like body (li) associated with a mitochondrion can be seen. Numerous vitellus-like bodies are also present (vb). Two cell stage. $\times 28,500$.

Figs. 12-13. 12, portions of cytoplasm of two blastomeres showing difference in the density between them. Abundant ribosome-like clusters (arrows) and lysosomes (li), mitochondria (m) and lipid inclusions (L) can also be seen. 72 hours after mating. $\times 24,000$. 13, part of a nucleus containing an heterogeneous and reticular nucleolus (nu). Note a lysosome-like body in the cytoplasm (li). 72 hours after mating. $\times 24,000$.

Figs. 14-15. 14, portion of a nucleus showing a dense homogeneous nucleoli (nu) and several intranuclear annulate lamellae (arrows) two cell stage. $\times 18,000$. 15, four cell stage. Part of a nucleus showing two homogeneous spheroidal nucleoli (nu) and some intranuclear annulate lamellae (arrows). $\times 25,000$.

of microvesiculae was frequent (Fig. 9) but microfibrillae and microtubular structures were not found in these cells at any of the periods studied.

Nucleus

During the first stages the nucleus showed a very irregular shape, becoming globular by the blastocyst stage. The nuclear envelope showed the characteristic two-unit-membrane structure, with a perinuclear space of constant thickness. The nucleolus showed a dramatic transformation in structure, and during the two and four blastomere stage it was seen as dense spheroidal body (Fig. 15). However, from the eight blastomere stage on, spaces of low density began to form inside, and in the next divisions the nucleolus increased its volume and showed a reticular shape (Fig. 13).

Discussion

In the rabbit egg, Boving (1963), using the light microscope noted the presence of three distinct external capsules, colemma, mucocolemma and gloiolemma. However once the egg began to increase in size, the resolution of the light microscope was insufficient to detect the presence of the colemma. In our study with the electron microscope we have confirmed the presence of three layers. During all phases of segmentation we found abundant spermatozoa both in the external envelopes and the perivitelline space. However, we did not detect the presence of head filaments as described by Dickmann (1964) with the light microscope.

The plasma membrane did not show any interruptions through which the cytoplasmic material could flow to the perivitelline space as described by Sotelo and Porter (1959) during fertilization in the rat, and by Izquierdo and Vial (1962) during the first division. Our data agree with those of Mazanek (1965) who interpreted such observations as artefacts. However, the presence of abundant vesicular structures and membrane systems both in the perivitelline and interblastomeric spaces, after the first division in which the two polar bodies can still be observed intact, makes us think that the so called "pseudoplasmolitic process" really exists (Van der Stricht 1923). The mechanism of this process is different from the simple breakdown of the cell membrane, it could occur by the formation of multivesicular evaginations that are detached and later desintegrate in the perivitelline space as suggested by our micrographs (Figs. 6 and 8).

With regard to the interblastomeric relations, we found the presence of abundant filiform structures even in the most advanced stages of segmentation (Fig. 5) whereas Mazanek (1965) did not find them in the rat after the four blastomere stage. In addition to these structures, which greatly increase the free surface of the blastomeres, we saw a thickening of the membrane in certain zones (Fig. 3). The presence of vesicular structures in some regions of the cortex of the blastomeres during the two blastomere phase suggest

the existence of certain specialized areas of the membrane at which active pinocytosis takes place (Fig. 7). Zemboni and Mastroianni (1966) have found similar structures in preovulatory rabbit ova and interpreted them as ergastoplasmic structures which were destined to become organized into the endoplasmic reticulum.

Independent of the zones of intense activity, we found, throughout segmentation "caveolae" (Enders and Schlafke 1965) in the membrane, both on the side of the perivitelline space and in the interblastomeric space. This finding suggests the persistence of micropinocytosis during all phases of segmentation, although at a lower rate than in the two blastomere phase in which the incorporation of "heterosynthetic molecules" (Schechtman 1955) is greater, as shown by Glass (1963) in her work using immunofluorescent techniques.

Izquierdo and Vial (1962) and Mazanek (1965) have described the presence of two cytoplasmic zones in the blastomere of the rat. One zone was rich in organelles and inclusions while in the other these structures were scarce. In addition, the structurally rich zones were found radiating from the nucleus towards the cortex of the blastomere. In the rabbit a similar distribution of organelles and inclusions occurred only during the early divisions (Fig. 4).

The composition of the two cytoplasmic zones also differs from that found in the rat. In the agranular zone groups of five or more highly dense granules were found. These were very similar to the polysomes described by Mazanek (1965) and increased in number after the 16 blastomeres stage (Figs. 6 and 12). The granular endoplasmic reticulum was found only at the blastocyst stage. We did not see it at the 8 cell stage as reported by Hillemann (1967) in the mouse.

In the rich cytoplasmic zone multivesicular bodies were found consistently but were fewer in number than reported for the rat. It is difficult to ascertain whether these vesicles are left free in the matrix or whether a certain number are grouped in a sac and then transported to other areas of the cytoplasm (Fig. 10). Inclusions with amorphous contents were also found in this zone. Zamboni and Mastroianni (1965a) interpreted them as "ergastoplasmic cysternae." However we do not think there are good physiological or morphological grounds for such an interpretation, since ergastoplasmic cysternae have been well characterized with the electron microscope (Haguenau 1958, Porter 1961). The structures which we have described show similarities to those seen by Beams (1964) in the oocyte of the small milkweed bug *Lygaeus kalmii* Stal. Beams interpreted them as deposits of yolk which had been incorporated into the cytoplasm by micropinocytosis. Such an interpretation might apply to our observations in the rabbit. As mentioned previously, pinocytosis was observed throughout segmentation. These inclusions were often seen in areas near the membrane at different stages of organization. In addition there are a number of reports which describe the incorporation of

several substances of tubal origin into the egg during segmentation (Monesi and Salfi 1967, Fritz 1959, Mintz 1965, Glass 1963). However, whether these substances diffuse from the membrane or leave via pinocytosis remains to be determined. If pinocytosis is involved, do the vesicles move towards the bodies with amorphous contents or are they simply incorporated into the vesicular component to the Golgi complex? The persistence of these small bodies during the late stages of segmentation plus the large number of lipid inclusions present, appears to contradict the suggestion of Adams (1958) that the nutritive reserves of the egg become exhausted during the late morula stage.

In this material as well in the rat embryo, the Golgi complex is greatly developed, especially during the first divisions; this undoubtedly indicates an important role of this organelle in the embryo at this stage of development.

The modifications in the nucleus are somewhat different from those shown in the rat and mouse. In our material we did not find grooves in the external membrane of the nuclear envelope or in the zones near the nucleolus during the two cell stage, and there was no evidence of the passage of nucleolar material toward the cytoplasm. In spite of this, the nucleolus showed a structural transformation similar to that of the rat embryo, and the appearance of an "open" structure coincided, not only with a marked increase in the cytoplasmic polysomes, but with a change in density of the matrix of some of the blastomeres (Fig. 15). However, the transformation of the nucleolus in the rabbit begins later (8 cell stage) than in the mouse.

Finally, the presence of annulate lamellae within the nucleus in ascidian eggs (Kessel 1965, Everingham 1968a, b), within the pronucleus of penetrated human ova (Zamboni *et al.* 1966), mouse segmented ova (Hillemann 1967) and now in the rabbit embryos raises the problem of the role of these structures during the early stages of development.

Our data on the ultrastructural changes which occur during segmentation in the rabbit embryo agree with several of the observations made by Mazanek (1965) in the rat and by Hilleman (1967) in the mouse. However the development of the rat, mouse and rabbit differs in number of aspects and these differences warrant at more detailed study in other mammals before species characteristics can be firmly established.

Summary

An electron microscope study of the process of segmentation in the rabbit ova is described. During the first division cytoplasmic extrusions were observed, the contents of which appeared to pass into the perivitelline space. Vitelline inclusions were noted, as well as mitochondria with cristae which were positioned in an unusual manner. A well developed Golgi complex and several multivesicular bodies were also seen. During early cleavage annulate lamellae were observed inside the nucleus. The nucleolus changed from a

dense homogeneous globular structure to a heterogeneous reticular structure. This change coincided with the appearance of large numbers of cytoplasmic polysomes. At the morula stage differences were noted in the density of the fundamental matrix between the various blastomeres. The significance of these developmental changes is discussed.

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