Ultrastructural observations of preimplantation stages of the sheep

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SUMMARY

Sheep embryos were examined with the electron microscope in order to characterize their organelles and the changes that occur during the preimplantation period. The first sign of differentiation of trophoblast cells was the appearance of junctions between external cells at the 16-cell stage. Nucleoli developed a granular component suggesting the synthesis of ribosomal RNA at the 16-cell stage also. Centrioles were seen as early as the 8-cell stage. Intracytoplasmic vesicles were present in large numbers in all cleavage stages but disappeared at blastulation. Mitochondria progressed from a very electron-dense hook- or U-shaped form with a few cristae to a cylindrical or spherical form of light density with many transverse cristae. Microvilli were not seen until the blastocyst stage and then only on the exterior surface of the trophoblast cells. Crystalloid or virus inclusions were not observed. It was concluded that the fine structure and developmental changes in the early sheep embryo are very similar to those of other mammalian species.

INTRODUCTION

Cleavage stages of the sheep have been examined cytologically (Assheton, 1898) and in whole mount preparations (Clark, 1934) but, to our knowledge, only the unfertilized egg (Russe, 1975) and the blastocyst from days 8–18 (Winterberger-Torres & Flechon, 1974) have been described ultrastructurally.

On the basis of histological examination of the 8-cell stage, Assheton (1898) stated that one cell was lighter in appearance than the others and postulated that the future epiblast and hypoblast were differentiating at this stage. Clark (1934) noted differences in sizes of blastomeres during cleavage and felt that at the 16-cell stage these marked size differences represented the first evidence of differentiation of the trophoblast cells.

This paper presents the results of an ultrastructural study undertaken to determine when differentiative changes between the inner cell mass and trophoblast first occur. In addition, we present observations on the fine structure of some sheep cleavage stages and chronicle the changes that occur in subcellular organelles as development progresses.

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MATERIALS AND METHODS

Cleaving embryos were recovered from flushings of the reproductive tracts of pregnant Blackface ewes at known times after mating. An injection of 1000 i.u. pregnant mare's serum was given 3 days before mating. Heat-inactivated sheep serum was used as a flushing medium. The flushings were searched under a dissecting microscope, either immediately or after an interval of 1–1 1/2 h. The embryos were located and examined to determine the approximate stage of development. The cytoplasm was observed to be very granular and opaque. One-cell, 8-cell, approximately 16-cell stages, morulae and blastocysts were recovered respectively 12 h, 3 1/2, 4 1/2, 5 1/2 and 6 1/2 days after mating (i.e. on days 0, 3, 4, 5 and 6 of pregnancy). The embryos were transferred to 3 % glutaraldehyde in 0.1 M phosphate buffer.

The embryos in glutaraldehyde were sent by post from A. McL. in Edinburgh to P. C. in San Francisco, where they were fixed in 2 % osmium tetroxide in 0.1 phosphate buffer, dehydrated through a cold ethanol series and embedded in Epon 812 by a modification of the procedure described by Luft (1961). Serial thick (1 μm) and thin sections were cut so that areas showing potential differentiation with the light microscope could be located easily with the electron microscope. Cell counts were also made on some embryos which were thick-sectioned serially. Thick sections were stained with toluidine blue (Trump, Smuckler & Benditt, 1961). Contrast of thin sections was enhanced by treating the sections with saturated uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963). Grids were examined with a Hitachi HS-8 electron microscope at 50 kV.

OBSERVATIONS

A total of 14 out of 28 embryos collected on days 3–6 of pregnancy were examined by light and electron microscopy (Table 1). Because a cleavage stage was assigned to these embryos by observation of the whole embryo, an additional three embryos were serially sectioned and the cells counted with the light microscope to determine the actual number of cells present. There was reasonable

Figures 1–3

Fig. 1. Electron micrograph of a zygote, 12 h post-ovulation. The sperm midpiece can be seen in the lower part of the micrograph (S). Note the hooked mitochondrion \( (M) \). G, golgi; \( V \), vesicle. \( \times 17350 \).

Fig. 2. Cell surface of the same sheep zygote. Note the few short microvilli and the absence of cortical granules. \( V \), vesicle. \( \times 15770 \).

Fig. 3. Sheep zygote. The U-shaped mitochondrion (arrow) characteristic of sheep zygotes produces mitochondria which appear to have 'holes' when sectioned transversely. Note the endoplasmic reticulum \( (ER) \) closely associated with the mitochondria. \( \times 15770 \). Insert: light micrograph of the sheep zygote. Note the many light vesicles and the pronucleus. \( \times 340 \).
agreement between the expected number and observed number of cells (Table 1).

The embryos examined ranged from the 8-cell stage to the blastocyst, but also included one zygote obtained 12 h after mating. All embryos examined had the following general similarities: (1) They were surrounded by an amorphous zona pellucida and contained significant amounts of flocculent material and cellular debris in the perivitelline space and intercellular space. (2) They contained numerous vesicles (500 nm to 2 \( \mu \)m in diameter) which appeared to be membrane-bounded, although often the membrane was discontinuous, and which contained small amounts of a flocculent material and/or membranous profiles. Approximately 100 vesicles were present in an average thin section of all cleavage stages except the blastocyst. These were present in embryos whether fixed within minutes after flushing from the oviduct or after 2 h in sheep serum. (3) A small amount of lipid was seen in each stage in the form of round globules which were medium to darkly osmiophilic and not surrounded by membranes. (4) Caveolae were regularly seen in all stages along the plasma membrane.

The following description of cleavage stages will be in chronological order and will note the primary modifications in organelles observed in successive developmental stages.

**Zygote (12 h)**

One embryo was obtained 12 h after making. It had two polar bodies and electron microscope examination revealed no cortical granules so it was judged

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**Table 1**

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Estimated cleavage stage</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>½</td>
<td>Zygote</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>8 cell</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>16 cell*</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>16 cell†</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Morula</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Morula</td>
<td>2</td>
</tr>
<tr>
<td>6½</td>
<td>Blastocyst</td>
<td>1</td>
</tr>
</tbody>
</table>

* A cell count based on serial sections of one embryo revealed 20 cells.
† A cell count based on serial sections of two embryos revealed 18 and 15 cells respectively.

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**Figures 4 and 5**

Fig. 4. Electron micrograph of a portion of an 8-cell sheep embryo. The arrows indicate focal densities between two blastomeres. Mitochondria (M) are usually cylindrical in shape. V, vesicles. \( \times \) 19920.

Fig. 5. Eight-cell sheep embryo. ER cisternae (ER) remain associated with the mitochondria. Similar cisternae were occasionally seen near the plasma membrane (arrows). \( \times \) 18920.
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to be fertilized. In addition a portion of the sperm midpiece was seen near
the pronucleus (Fig. 1). A few short microvilli were present on the cell surface
(Fig. 2). Mitochondria were very electron-dense and were predominantly
hooked or U-shaped (Figs. 1, 3). Agranular membranous cisternae interpreted
to be endoplasmic reticulum (ER) were closely associated with the mitochondria
(Fig. 3). A few polysomes were observed and several small regions of Golgi
vesicles and cisternae were seen (Fig. 1).

The one pronucleus observed had no pores and was of light electron density
with occasional dense patches present in the nucleoplasm. No nucleoli nor
intranuclear annulate lamellae were observed.

**Eight-cell stage (day 3)**

Very few microvilli were present on the cell surface although some were
present in regions of blastomere contact. No tight junctions were seen between
blastomeres although focal densities on the cytoplasmic side of the plasma
membrane were occasionally seen where blastomeres were in close contact
(Fig. 4). These resembled the 'primitive' junctions described by Enders (1971) in
cleavage stages of the rat. Mitochondria were very electron-dense, had 2–8 trans-
verse cristae and occasionally several polar cristae. The usual shape of mito-
chondria was cylindrical (Fig. 6) but there were also hooked and U-shaped forms.
Several mitochondria also had intracristal vacuoles. The endoplasmic reticulum
(ER) was closely associated with the mitochondria (Figs. 4, 5) although occasion-
ally cisternae resembling ER were also observed tightly applied to the in-
ternal surface of the plasma membrane (Fig. 5). Very few polysomes were present
in the cytoplasm. Centrioles with associated microtubules were observed near
the nuclei in blastomeres of two different embryos (Fig. 7). Cytoplasmic annu-
late lamellae (AL) were present in stacks (Fig. 7) and as single sheets closely
applied to the nuclear envelope. There were many aggregations of small mem-
branous vesicles in the perinuclear region (Fig. 8) and a small amount of Golgi
material was occasionally seen.

Nuclei exhibited pores, dense small chromatin aggregates spaced along the
inner nuclear membrane (Fig. 8) and several intranuclear annulate lamellae. No
ribosomes were present on the outer leaflet of the nuclear envelope. Nucleoli

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**Figures 6–8**

- **Fig. 6.** Portions of four blastomeres of an 8-cell embryo. Note the occasional
  C shaped mitochondrion (*), the numerous vesicles (V) and the flocculent material
  in the intercellular space (ICS). × 5400.

- **Fig. 7.** Eight-cell embryo. Small chromatin aggregates are spaced along the inner
  nuclear membrane of the nucleus (N). Several annulate lamellae (AL) and a centriole
  with microtubules (C) are present near the nucleus. × 12050.

- **Fig. 8.** Electron micrograph of a portion of an 8-cell embryo, illustrating the
  spherical nucleoli (n), and the small chromatin aggregates along the nuclear mem-
  brane. N, nucleus; A, aggregations of small vesicles. × 2920.
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were spherical and agranular with no visible nucleolonema (Fig. 9). A second type of dense intranuclear body resembling the nucleolus but composed entirely of strands approximately 15 μm wide was also observed in one nucleus of this stage only (Fig. 10).

**Sixteen-cell stage (day 3)**

The most notable difference between this stage and the 8-cell stage was the appearance of a granular component in the nucleolus (Fig. 11). Endoplasmic reticulum was still largely agranular and polysomes remained rare. Annulate lamellae were not observed.

**Sixteen-cell stage (day 4)**

By this time some of the external blastomeres of the 16-cell embryo had developed peripheral junctional complexes. These complexes consisted of an amorphous dense substance located in the cytoplasm adjacent to the membranes and extending across the intercellular space (Fig. 12). Mitochondria were of medium density, usually spherical with a few transverse cristae and hooked forms were rare (Fig. 13). ER associated with these mitochondria bore a few ribosomes but polysomes were still rare. Occasional agranular cisternae were seen near the plasma membrane of adjacent cells. No annulate lamellae were present although large aggregations of small vesicles were seen in the perinuclear area (Fig. 13). The clumps of chromatin observed along the inner aspect of the nuclear envelope in earlier stages were usually absent (Fig. 13).

**Morula (day 5)**

Occasionally long microvilli projected from the external surface of the embryo near intercellular junctions (Fig. 15) but in general the external surface of the embryo was non-microvillous. Terminal bars were present between most peripheral cells observed just below the external surface, but they differed in size (Figs. 14, 15). However, adjacent cells were so closely adherent that with the light microscope it was sometimes difficult to discern cell boundaries (Fig. 16). Polysomes were more numerous and nucleoli remained granular and spherical.

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**Figures 9-13**

Fig. 9. Nucleolus of an 8-cell sheep embryo, no nucleolonema or granular components are present. ×25670.

Fig. 10. An intranuclear body from the same nucleus as Fig. 9. It is composed of strands 15 μm wide. ×25670.

Fig. 11. Nucleoli from a 16-cell embryo, day 3. A granular component is now present (g). ×19710.

Fig. 12. Junctional complex (J) between the peripheral portions of two blastomeres at the 16-cell stage, day 4. PVŚ, perivitelline space. ×19710.

Fig. 13. Sixteen-cell embryo, day 4. Mitochondria appear less dense (M). V, vesicles; N, nucleus; n, nucleolus; A, aggregation of small vesicles. ×5400.
Morula (day 6)

This embryo had very few cell junctions and was judged to be less advanced than the day 5 morulae. However, the Golgi apparatus was extensive in the perinuclear region (Fig. 17).

Blastocyst (day 6½)

Only one blastocyst was available for examination. The most obvious difference in this stage as compared to earlier stages was the almost complete absence of vacuoles from cells of the inner cell mass (ICM) and trophoblast (Figs. 18, 19). Numerous microvilli covered the exterior surface of the embryo (Fig. 18) and terminal bars were present between the peripheral portions of all exterior cells. Occasionally desmosomes were observed interior to the terminal bars. No terminal bars or desmosomes were present between ICM cells. Cells of the ICM were loosely bound to one another but also formed a few points of close contact with each other and with cells of the trophoblast (Fig. 19). Mitochondria were of medium to light density and of variable shape (cylindrical, spherical). The cylindrical mitochondria displayed many transverse cristae (Fig. 18). Membrane-bounded structures interpreted as degradation bodies were also observed. Nuclei and nucleoli were similar to those of the morulae. No organelle differences between ICM and trophoblast were observed except for the presence of junctional complexes and microvilli on trophoblast cells.

No differences in electron density were observed between cells of any embryo at any stage examined, nor were cells of variable size seen except those that could be explained by differences in plane of section and asynchronous cleavages.

DISCUSSION

Vesicles similar to those present in cleavage stages of the sheep have also been observed in unfertilized sheep eggs where they were termed ‘yolk globules’ (Russe, 1975). In the present study, there was no apparent decrease in vesicle number as cleavage progressed which might support a process of yolk utilization. Their nearly complete disappearance between the morula and blastocyst stages suggests they may be involved in the process of blastulation. A similar loss of

Figures 14–17

Fig. 14. Terminal bar (arrow) present between two cells of a morula. PVS, perivitelline space. × 19710.
Fig. 15. Morula. Microvilli are occasionally present near cell–cell junctions. PVS, perivitelline space. × 19850.
Fig. 16. Light micrograph of a morula stage. Vesicles are present throughout the embryo. The blastomeres are very closely adherent. × 340.
Fig. 17. Morula exhibiting extensive Golgi material in the region near the nucleus (N). × 14980.
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cytoplasmic vesicles has been reported in the mouse embryo at blastulation (Calarco & Brown, 1969). Fibrous inclusions characteristic of rodent cleavage stages (Enders & Schlafke, 1965) and suggested to be yolk (Szollosi, 1965) were not observed in the sheep.

We were not able to substantiate earlier reports of differentiation based on cell staining at the 8-cell (Assheton, 1898) and cell size at the 16-cell stages (Clark, 1934). The lighter staining interior cells observed by Assheton (1898) could be due to the manner in which his material was prepared. After fixation, the embryo was stained with carmalum or borax carmin in toto and then sectioned. Interior cells might not stain as readily in this manner. Differences in cell size observed in the present study could be attributed to different planes of section and to slightly asynchronous cell divisions. The first sign of differentiation apparent to us was the appearance of tight junctions between the apices of external cells at the 16-cell stage (day 4). This then creates a population of outer, trophoblast, cells (which will contain the blastocoel fluid) and inner cell mass cells. This is very similar to the process described in other mammals (Enders & Schlafke, 1965). Prior to this time cell adhesion appeared to be maintained by small adhesions between cells similar to those described in the rat (Enders, 1971). Microvilli did not appear until the blastocyst stage.

The presence of hook-shaped mitochondria has been previously noted in unfertilized sheep eggs (Russe, 1975) as well as cattle oocytes (Senger & Saacke, 1970). Stern, Biggers & Anderson (1971) showed that the structural changes in mitochondria during mouse preimplantation development were accompanied by a functional change. Since sheep mitochondria changed structurally during cleavage, perhaps a functional change is also occurring.

Granules first appear in sheep nucleoli at the 16-cell stage. The appearance of a granular component in the nucleolus has been correlated with the onset of nucleolar function, i.e. synthesis of ribosomal RNA, in Ascaris (Kaulenas, Foor & Fairbairn, 1969), Triturus (Karasaki, 1965), rat (Szollosi, 1971) and mouse (Hillman & Tasca, 1969) embryos. By analogy then, nucleolar function probably begins in sheep at the 16-cell stage. Subsequently, ribosomes were seen on the endoplasmic reticulum and polysomes appeared in increasing numbers in the cytoplasm. Nuclear heterochromatin decreased at the 16-cell stage and may be a morphological manifestation of a change in nuclear function.

Figures 18 and 19

Fig. 18. Blastocyst, day 6½. The outer surface of the trophoblast cells is microvillous (MV), mitochondria are cylindrical with many cristae (M), and the nucleus has no chromatin aggregates along its inner aspect (N). In addition, polysomes are numerous, granular endoplasmic reticulum is present and the outer nuclear leaflet is studded with ribosomes. Z, zona pellucida. × 14850.

Fig. 19. Blastocyst. Cells of the inner cell mass (ICM) and trophoblast cells (TC) are similar in organelle morphology, but different in cell adhesion. A portion of a polar body with vesicles (P) is seen exterior to the microvillous border of the TC. × 4100. Insert: light micrograph of the sheep blastocyst. Note the absence of vesicles. × 170.
The sheep embryo may differ from other mammals in the possession of centrioles by the 8-cell stage. To our knowledge centrioles have not been reported in early cleavage stages of other mammalian species.

In most respects then the fine structure and developmental changes in the pre-implantation embryo of the sheep are similar to other mammalian species.

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REFERENCES


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