GROWTH AND OBSERVATIONS OF CHINESE HAMSTER SEMINIFEROUS EPITHELIUM IN VITRO

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SUMMARY
Seminiferous tubules from 1- to 3.5-month-old Chinese hamsters were cultivated under dialysis membranes in Rose chambers. The growth and development of the germinal cells was followed daily with phase-contrast microscopy and time-lapse cinemicrography. Spermatogonia lived for 2 or 3 weeks and underwent frequent mitoses. Spermatocytes in metaphase at culture initiation completed their meiotic division. These cells remained healthy for 3-4 days. Such phenomena as germinal cell/Sertoli cell association, nuclear rotation, multinucleated cell formation and spermatid formation were studied and photographed.

INTRODUCTION
The success of studies of growth and maturation of male mammalian germinal cells in tissue culture has been variable (Wolff & Haffen, 1965). The best results have been obtained using either (1) organ culture or (2) the mechanical or enzymic disruption of the testicular tissue and the cultivation of the dispersed cells. The organ-culture method has been more productive in that spermatogenesis has been initiated in testes from 4-day-old mice and has progressed to the pachytene stage of meiosis (Steinberger & Steinberger, 1966a). This method has the disadvantage, however, that it requires sectioning and staining of the representative tissue before development can be assessed. The second method yields germinal cells that remain viable for some time but they do not attach to the glass and grow; however, the non-germinal cells attach readily and grow prolifically (Jordan, Katsh & de Stackelburg, 1961; Kodani & Kodani, 1966; Steinberger & Steinberger, 1966b).

In the present study seminiferous tubules removed from Chinese hamsters were cultivated under dialysis membranes in Rose chambers (Rose, Pomerat, Shindler & Trunnell, 1958; Rose & Pomerat, 1960). Although the germinal cells probably did not attach to the glass, the membrane flattened the tubules and cells, thus permitting direct and continuous phase-contrast microscopic observation of both the germinal cells and non-germinal elements; these observations were documented with photomicrographs and time-lapse cinemicrographic films. All stages of the spermatogenic and spermiogenic cycle and the growth and association of the Sertoli cells will be described.
MATERIALS AND METHODS

Testes from Chinese hamsters 1-3.5 months old provided the most suitable tissue. Those from older animals did not grow as well and the presence of large numbers of spermatozoa interfered with microscopic examination of the germinal cells. The method of cultivation was essentially the same as that described previously by Yao & Ellingson (1969).

The nutrient solution was composed of 80% Eagle's minimum essential medium, 20% calf serum, 0.1 mM of non-essential amino acids, 10 mM sodium pyruvate, and 100 units of penicillin and 100 μg equivalents of streptomycin per ml. Just before use, 2-4 mM of L-glutamine were added (Steinberger & Steinberger, 1966a).

The tubules were placed in a small drop of Hanks's balanced salt solution, chick embryo extract, or whole egg ultrafiltrate on the bottom coverslip of the Rose chamber and were then covered with a cellophane dialysis membrane. This membrane separated the tubules from all components of the nutrient medium except those which were dialysable. In some experiments, the pituitary gland was also removed from the animal and small explants were placed in the chamber with the testicular tissue, generally underneath the membrane but sometimes on the other side of it.

Cultures were maintained 2-3 weeks on the average. A few were kept as long as 6 months. Fluids were changed twice weekly. The incubation temperature was 31 °C; the pH was 6.8-7.2.

All cultures were observed with a Zeiss inverted phase-contrast microscope. The photomicrographic and time-lapse cinemicrographic equipment were the same as that described previously (Yao & Ellingson, 1969).

Some of the cultures were fixed and stained at various times using PAS-haematoxylin (Regaud, 1901; G. G. Rose, personal communication), Carr's modification of methyl green pyronin (S. A. Carr, personal communication), and Elftman's direct silver method for Golgi substance (Lillie, 1965). Testes were fixed in Zenker's or Bouin's solution, embedded in paraffin, cut at 4-6 μm and stained with PAS-haematoxylin or haematoxylin-eosin.

RESULTS

General

The seminiferous tubules were delicate and easily flattened by the dialysis membrane. This compression permitted many of the cells within the tubules to be seen clearly. The degree of flattening varied, depending upon the location of the tubules in the chamber and upon the presence of wrinkles in some of the cellophane membranes. Those tubules which happened to lie in a wrinkle were too thick to study microscopically. In some instances the tubules were flattened excessively and the integrity of the tissue was completely disrupted.

In the cultures most of the spermatogonia and Sertoli cells remained within the tubules, while the majority of more mature germinal cells were expressed into the surrounding medium. The expressed cells formed a gradient with the smallest germinal cells closest to the tubules and the largest cells, the primary spermatocytes, farthest away. The primary spermatocytes and the developing spermatids were flattened the most and were the most difficult to cultivate.

While many Sertoli cells emigrated from the tubules and underwent mitosis, their migration was limited to a specific distance from each tubule, as probably dictated by the pressure of the cellophane membrane.

Along the basement membrane of the tubules lamina cells (Kodani & Kodani, 1966) were seen, having oval-shaped nuclei and homogeneous nucleoplasm. These cells generally were inconspicuous when viewed with phase-contrast optics and did
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not appear to divide. They were clearly visible by phase-contrast microscopy only when most of the germinal cells had been pressed out from the tubules.

The growth and development of the germinal cells in some cultures appeared to be enhanced by the presence of the pituitary gland explants. This tissue grew well under the membrane and lived more than 6 months.

Germin al cells and Sertoli cells inside the tubules

The Sertoli cells, with their characteristic large round-to-oval nuclei and one or more prominent nucleoli, were readily distinguished from the densely packed interphase spermatogonia with which they were closely associated (Fig. 1), or from larger spermatogonia in prophase (Fig. 2). The tubule membrane was sometimes wrinkled (Fig. 2), probably due to the loss of cells from the tubule, and this sometimes gave the illusion that the cells lay in cords. Type A and type B spermatogonia (Clément & Leblond, 1958; Regaud, 1901) could be distinguished and their presence confirmed by comparison with cells stained with PAS-haematoxylin and/or methyl green pyronin. When stained, the type A or 'dusty' spermatogonia were characterized by a nuclear membrane, which appeared thin, an oval nucleus and dust-like particles of chromatin distributed through the nucleoplasm; the type B or 'crust-like' spermatogonia had round nuclei with chromatin apposed against the nuclear membrane and also distributed throughout the nucleoplasm. The intermediate type of spermatogonium was not positively identified. The spermatogonia frequently underwent mitosis. At times many of these dividing cells were noted in certain areas of the tubule; 24 mitotic figures were seen in 1 microscopic field. The synchronous mitoses in a 5-day culture were filmed with time-lapse cinemicrography. When this film was projected, the metaphase plates of 7 cells in 1 field were seen to oscillate and 1 metaphase plate made a complete rotation before mitotic division was completed (Yao & Ellingson, 1969). Many spermatogonia and some spermatocytes, in prophase, rocked and/or rotated rapidly. These nuclear movements were less frequent in older cultures, but they were observed in some which were 2-3 weeks old.

Spermatocytes within the tubules of 24- to 72-h-old cultures were seen undergoing meiosis. Figure 3 shows 2 primary spermatocytes in the early diplotene stage after 48 h in culture. While these 2 spermatocytes did not complete division during a 5·5-h period of observation, others were seen to do so. Numerous secondary spermatocytes in metaphase were present in localized areas of the cultures and many of these frequently completed division. While we have not been able to confirm meiotic division of germinal cells within the tubules after 72 h in culture, we often have seen large round dividing cells in older cultures which were of the same size and morphology as spermatocytes.

The Sertoli cells were the longest-lived cells in the tubules. They eventually replaced or obscured the germinal cells and still appeared healthy in cultures 6 months old.
Spermatogonia, spermatocytes, and Sertoli cells outside the tubules

The germinal cells outside the tubules were flatter and therefore could be studied more easily than those within. While the spermatocytes generally were uninucleate, some had 2 or 3 nuclei. Their cytoplasm was filled with granules of various sizes and a prominent Golgi body was frequently seen during prophase (Fig. 4). The presence of the Golgi substance in these cells was confirmed using Elftman's silver impregnation technique (Lillie, 1965). In cells having 2–3 nuclei there was more than one Golgi body. Generally, there was one Golgi body per nucleus.

The primary spermatocytes were especially sensitive to this method of cultivation. Some of those in the metaphase stage at culture initiation completed division, but those in the leptotene stage generally were flattened so much (Fig. 5) that they degenerated in a few days and their cytoplasmic granules underwent rapid Brownian movement. They also exhibited blebbing or zeiosis. Remnants of zeiotic blebs which have detached from these cells are shown in Fig. 5.

The secondary spermatocytes generally were located closer to the tubule, were not flattened as much, and were more amenable to cultivation. On four separate occasions a number of these cells in the metaphase stage at culture initiation were seen to complete meiotic division. Although it was not determined when metaphase began, the time required for 1 of these cells to progress from metaphase to telophase was approximately 20 min. In another instance, secondary spermatocytes in metaphase had completed their division within 1 h. Photomicrographs and time-lapse sequences have been made to document these observations (Figs. 6, 7). In one 3-day culture, spermatocytes in metaphase were observed to complete meiotic division. It is believed that these particular spermatocytes had developed to this stage while in culture, since their presence had not been noted prior to this time.

The spermatogonia were also flattened but did not appear to be unduly stressed. Many had rapidly rotating and rocking nuclei (Yao & Ellingson, 1969). These cells became intimately associated with the Sertoli cells.

The Sertoli cells eventually formed a monolayer around each tubule, the cell-to-cell contact being by means of intercellular bridges as shown in Fig. 8. The exchange of cytoplasmic granules between these cells was noted in one time-lapse sequence. The Sertoli cells contained numerous cytoplasmic inclusions, including thread-like or fibrillar mitochondria (Fig. 8). The phagocytic property of these cells was readily apparent; at times their cytoplasm contained large amounts of cellular debris and their shape varied greatly. When adjacent to germinal cells they partially encircled them. At times they were greatly elongated, as they are in vivo. At others their cytoplasm appeared to contain immature germinal cells (Fig. 8) but close examination revealed that the germinal cells were superimposed on them. One Sertoli cell in prophase was followed with time-lapse. It entered metaphase 35 min after the first observation, anaphase 3 min later and completed cytokinesis 7 min later. The time from anaphase to early telophase was about 3 min in most of the cells. Blebbing or zeiosis was pronounced during division and particularly during telophase. After
cytokinesis the daughter nuclei were barely visible for the first 20–30 min. Mitotic figures were noted in cultures 3–4 months old.

The association of the Sertoli cells with the nearly mature spermia was not studied.

The developing spermatids and spermatozoa

We did not make a detailed study of the developing spermatids, but did observe and photograph all identifiable stages of spermiogenesis. Young spermatids in the Golgi phase are shown in Figs. 7 and 9. Figure 10 shows the cap phase of development. The developing spermatids were often binucleate or multinucleate. Binucleated ones are shown in Figs. 7 and 10. The majority of spermatids degenerated after 3 or 4 days in culture and the mature spermatozoa, when present, were active for only 4 or 5 days.

Multinucleated cells

Multinucleated cells were present in all the cultures. They were seen both inside and outside the tubules, in newly established as well as older cultures (Figs. 11, 12). They were seen also in impression smears made directly from the animal and stained with haematoxylin or Giemsa. In some cultures they appeared to increase in numbers with increasing time of cultivation. The number of nuclei per cell varied from 2 to as many as 75. These nuclei appeared to be arranged around a central ‘core’ of cytoplasm which contained many granules (Figs. 11, 12). The cell membrane was well defined; at times it appeared to be thicker than at other times. Many cells with multipolar spindles were present in localized areas, suggesting synchrony of this type of division also. The Sertoli cells outside the tubules were closely associated with these giant cells (Figs. 11, 12). In addition to formation by multiple divisions they also were formed by cell fusion. Two different time-lapse sequences showed fusion of these cells. In one instance 1 cell fused with a binucleated cell; in the second instance 1 nucleus separated completely from a cell with 6 nuclei and later, after forming an intercellular bridge, it fused with it again. Other cells in this microscopic field were also joined by bridges but did not fuse during this time interval. These giant cells underwent very active movements. They extended and retracted pseudopodia, their nuclei sometimes rocked and rotated, their cytoplasmic granules showed rapid movements which were not Brownian in nature, and in one time-lapse sequence, the whole ‘giant’ cell pulsed vigorously.

DISCUSSION

The seminiferous tubule is a morphologically unique tissue to cultivate since it contains only germinal and Sertoli cells. In our cultures the germinal cells expressed from the tubules generally remained ‘rounded-up’ and unattached to the cover glass and they could be easily distinguished from the Sertoli cells. Due to the membrane pressure, however, they were forced to rest on the glass substrate and this sometimes gave the appearance of attachment. All the cells in this system lived in a dialysate environment, since the large serum protein molecules were excluded by the dialysis
membrane which had a porosity of 2.4 nm. This was thought to be a beneficial factor since it helped to simulate *in vivo* conditions in which the basal membrane of the tubule separates the germinal cells from the vascular supply outside the tubule. The compression of the cells by the membrane was thought to be a beneficial factor also, since cells *in vivo* are normally compressed.

While this method of cultivating germinal cells had many disadvantages it appeared significant that the spermatogonia remained healthy for 2–3 weeks and that some spermatocytes in 24- to 72-h-old cultures completed meiotic division. It is possible that such factors as improved nutrition, use of hormones, and control of pH and oxygen tension will favour further growth and differentiation of these cells.

To our knowledge, this report is the first to describe the behaviour and growth of living germinal cells in a system which permits visual observation over extended periods of time with minimum disturbance to the cells. This is in contrast to the detailed *in vivo* studies of both the spermatogenic and spermiogenic cycles of the rat (Leblond & Clermont, 1952a, b; Clermont & Leblond, 1958) and the mouse (Oakberg, 1956a, b). All these studies were largely histological in nature as was the excellent work of Steinberger, Steinberger & Perloff (1964) using organ culture techniques.

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REFERENCES


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Figures 1–12 show germinal and Sertoli cells of the Chinese hamster cultivated under dialysis membranes in Rose chambers.

Fig. 1. Spermatogonia and Sertoli cells inside the seminiferous tubule. New culture, ×1200.

Fig. 2. Spermatogonia in prophase and Sertoli cells; note wrinkles of the tubule membrane. Four-day-old culture, ×1200.

Fig. 3. Two primary spermatocytes inside the seminiferous tubule in the diplotene stage of meiosis. Two-day-old culture, ×1200.

Fig. 4. A primary spermatocyte in the prophase stage of meiosis surrounded by Sertoli cells. Outside the seminiferous tubule. Three-day-old culture, ×1200.

Fig. 5. Primary spermatocytes outside the seminiferous tubule and compressed by the dialysis membrane. Two-day-old culture, ×750.

Fig. 6. Secondary spermatocytes in meiosis, outside the seminiferous tubule. New culture, ×750.
Fig. 7. Secondary spermatocytes and developing spermatids outside the seminiferous tubule. Two-day-old culture, $\times 1200$.

Fig. 8. A spermatogonium and Sertoli cells outside the seminiferous tubule. Eight-day-old culture, $\times 1200$.

Fig. 9. Developing spermatids outside the seminiferous tubule. New culture, $\times 1200$.

Fig. 10. Spermatocytes and developing spermatids outside the seminiferous tubule. New culture, $\times 1200$.

Fig. 11. Multinucleated cells surrounded by Sertoli cells. Outside the tubule. One-day-old culture, $\times 750$.

Fig. 12. A multinucleated cell outside the seminiferous tubule Eight-day-old culture, $\times 1200$. 