Ultrastructure and Intercellular Vacuolization of Isolated Perfused and Control Rat Testes

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Isolated rat testes perfused in closed circuit with albuminated Krebs-Ringer bicarbonate buffer were examined by light and electron microscopic techniques in order to assess the structural integrity of the model. The extent of intercellular vacuolization in perfused testes was similar to that of control testes that had been prepared for electron microscopy by routine methods of perfusion fixation. The isolated perfused testes exhibit excellent preservation at the light and electron microscope level. The results indicate that albuminated Krebs-Ringer bicarbonate buffer is an excellent perfusate for *in vitro* studies of the isolated rat testis. This model of perfused testis can be used to study the early pathology of the effects of toxic compounds on the microscopic anatomy of the male gonad.

Key words: testis perfusion, rat, ultrastructure, hemoglobin-free perfusate, intercellular vacuolization.

Metabolic studies of the rat testis have been carried out *in vivo* in slices or in homogenates. In recent years, however, the isolated perfused rat testis has been used as a model for the study of drug and hormone metabolism in the male gonad (Baker et al, 1977a,b; Bardin et al, 1978; Ewing and Eik-Nes, 1966; Zirkin et al, 1980). Isolated organs are superior to slices and homogenates because they retain the intra- and intercellular structure of the organ *in vivo* while being perfused under carefully controlled conditions of oxygenation, temperature, substrate supply, and hormonal influence. To date, the metabolic integrity of the isolated perfused testis preparation has been characterized by determination of ATP concentration in the tissue at the end of the perfusion (Bardin et al, 1978) and by measurement of testosterone secretion (Ewing and Eik-Nes, 1966; Zirkin et al, 1980). Electron microscopic studies have not been performed to determine whether the fine structure of the germinal epithelium or the Leydig cells is damaged under the specific conditions of perfusion. The purpose of the present report is to describe the ultrastructural characteristics of the seminiferous epithelium and the Leydig cells in the isolated perfused rat testis following perfusion for one hour with artificial media of varying compositions.

Supported by grants from the American Heart Association (Established Investigatorship awarded to Dr. H. Brunengraber) and the Proctor Fund of Harvard Medical School.

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Submitted for publication May 4, 1983; accepted for publication June 13, 1983.

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic Farms), weighing 350 to 400 g, were kept in a controlled environment (24 C; 12 hours of light from 6:00 AM to 6:00 PM) for at least two weeks before being used as organ donors. Purina rat chow and tapwater were made available *ad libitum*.

Perfusions

The apparatus used for testis perfusion is based on one designed for perfusing mouse liver (Brunengraber et al, 1975). The association of two such apparatuses allows the parallel perfusion of four testes with the same recirculating perfusate. The apparatus is set up in a cabinet with a thermostat, which allows adjusting the perfusate temperature to 33 C.

Perfusion medium was Krebs-Ringer bicarbonate buffer, containing 4% bovine serum albumin (fraction V, fatty acid poor, Miles Biochemicals) and either 15 mM

glucose plus 100 mU crystalline insulin (Lilly) per ml, or 2 mM glucose and no insulin. Before use, the albumin was dialyzed as a 10% solution against 20 volumes of distilled water at 4 C with six changes in medium for 48 hours. After dialysis, the albumin was diluted to 4% with concentrated buffer.

Rats were anesthetized with 40 mg/kg of Nembutal (Abbott) injected intraperitoneally. After opening the scrotum, a 27½ gauge stainless steel needle connected to the end of a perfusion line was inserted into the spermatic artery and secured in place with two ligatures tied with 6-0 silk. Perfusion was started immediately at a flow rate of 1.0 ml/minute. The testis was separated from the epididymis, while about 5 ml of effluent perfusate was discarded in order to eliminate any pentobarbital present in the organ. The perfused testis was then transferred to a glass funnel, from which the venous effluent dripped back into the reservoir. Perfusion was conducted for 60 minutes following connection of the fourth testis to the apparatus. Duration of the surgical procedure was 30 minutes for four testes. Throughout the experiment, the adequacy of the perfusion was assessed by probing the softness of the organ. Testes that hardened through extravasation of perfusate were rejected.

Fixation and Processing of the Tissue

At the end of the perfusion, testes were prepared for morphological studies by first switching the perfusate from albuminated to non-albuminated medium for 3 minutes. This was followed by perfusion with 5% glutaraldehyde in 0.16 M collidine buffer (pH 7.2) for 10 to 15 minutes (Aoki and Hoffer, 1978). The testes were then removed from the perfusion apparatus, carefully diced into small cubes no larger than 2 mm^3, and transferred into 5% glutaraldehyde in 0.16 M collidine buffer at 4 C for an additional 1½ to 2 hours. Following a brief rinse in three changes of 0.2 M collidine buffer, the tissue was post-fixed in 1.3% OsO_4 buffered with 0.67 M collidine for 2 hours at 4 C, dehydrated in a series of increasing concentrations of cold acetone, and embedded in Araldite (Hoffer, 1976). For electron microscopy, sections showing pale gold interference colors were cut with a diamond knife on a Porter-Blum MT-2 microtome and stained with saturated aqueous uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggleshall, 1965). For light microscopic observations, sections 0.5 to 1.0 μm thick were cut from the same block and stained with toluidine blue.

For the control experiments, methods of testicular perfusion were the same as those used previously in comparable studies of the rat testis (Aoki and Hoffer, 1978). Sprague-Dawley rats weighing 350 to 400 grams were anesthetized with 40 mg/kg of Nembutal. An incision was made in the scrotum, and the testis and epididymis were exposed and gently dissected free. The spermatic cord was severed between two ligatures approximately 0.5 cm apart. The testis was transferred to a 10 ml beaker filled with saline and the testicular artery was cannulated with a 25 gauge needle attached to a 25 cm piece of polyethylene tubing (Clay-Adams, PE60). This tubing was connected to a three-way valve and then to a standard 140 cm high perfusion system. The testis was first briefly flushed with saline. As soon as saline entered the testicular artery, the spermatic cord was severed proximal to the ligature to provide a route for the effluent. After the testis blanched (about 10 seconds), saline was replaced with the same fixative as that used for the isolated testes, and perfusion was continued for 10 to 15 minutes. Further processing of the tissue was the same as that indicated above.

Evaluation of Intercellular Vacuolization

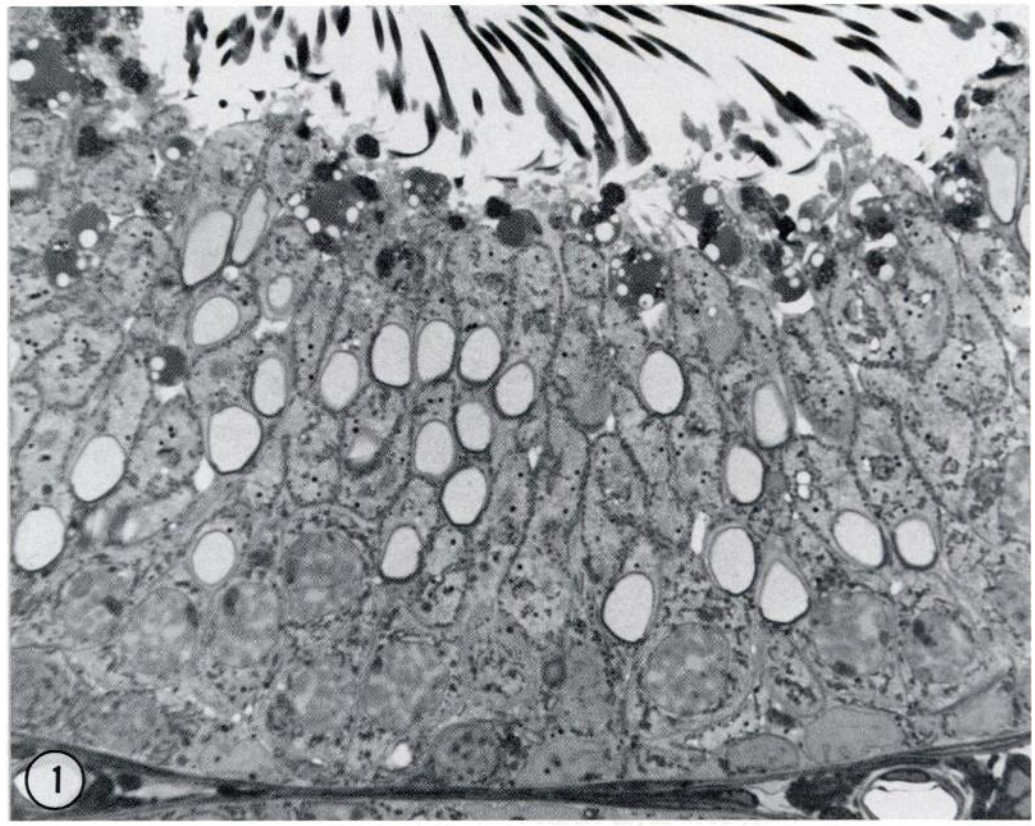
The degree of intercellular vacuolization was quantitated at the light microscope level on randomly selected tubular cross-sections. Scores of 1 (marked), 2 (moderate), and 3 (minimal) were assigned to each tubule, according to the frequency and size of vacuoles observed between the cells of the seminiferous epithelium. An average score was calculated for all the tubules examined in each organ. The intercellular vacuolization index of a group of control or perfused testes was then expressed as the mean ± standard deviation of the testes' scores in each group.

Results

Seminiferous Epithelium and Leydig Cells of Control Testes

The morphologic characteristics of the seminiferous epithelium in normal rats have been thoroughly documented; a detailed description need not be repeated here. In the present study, the seminiferous tubules and interstitial elements in the testes of control rats fixed by conventional perfusion techniques appear normal in all respects. Post-meiotic germ cells, spermatogonia, spermatocytes, and Sertoli cells at stage IX of the spermatogenic cycle are shown in Figure 1; no remarkable

Figs. 1–3. Light micrographs illustrating minimal, moderate, and marked degrees of intercellular vacuolization, which can be observed in control testes fixed under routine, optimal conditions by perfusion with collidine-buffered glutaraldehyde. Indices of 3, 2, and 1 correspond to minimal, moderate, and marked vacuolization, respectively, and are typed according to frequency and size of observed vacuoles. In all cases, the interstitial blood vessels were patent, and blood cells were not observed. **Fig. 1.** Light micrograph showing a cross-section through a normal Stage IX seminiferous tubule from the testis of a control rat. Spermatogonia, spermatocytes, and elongated spermatids are present and normal in appearance. Tubules such as these were assigned an intercellular vacuolization index of 3. **Fig. 2.** The seminiferous tubule shown in this light micrograph exhibits a moderate degree of intercellular vacuolization at all levels of the seminiferous epithelium. Tubules such as these were assigned an intercellular vacuolization index of 2. **Fig. 3.** Severe vacuolization between many germinal elements and Sertoli cells is readily observed in the seminiferous tubule shown in this light micrograph (asterisks). Note however that the cells themselves appear to be well-fixed. Tubules such as these were assigned an intercellular vacuolization index of 1.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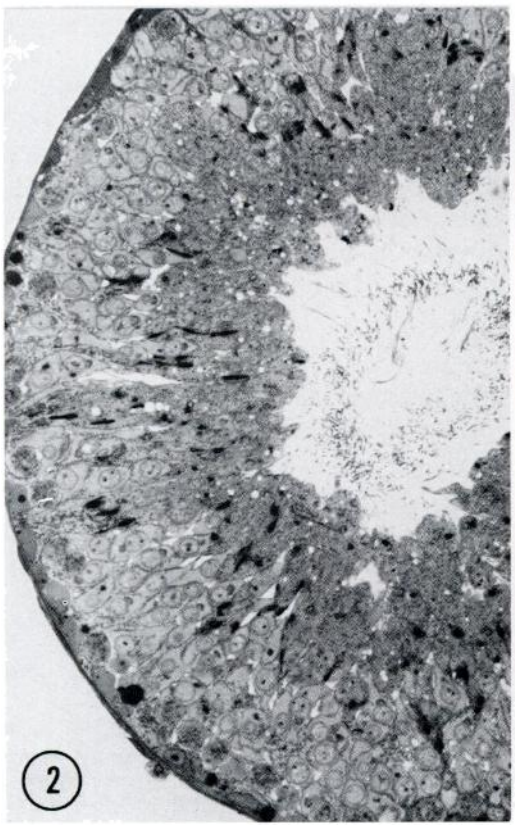
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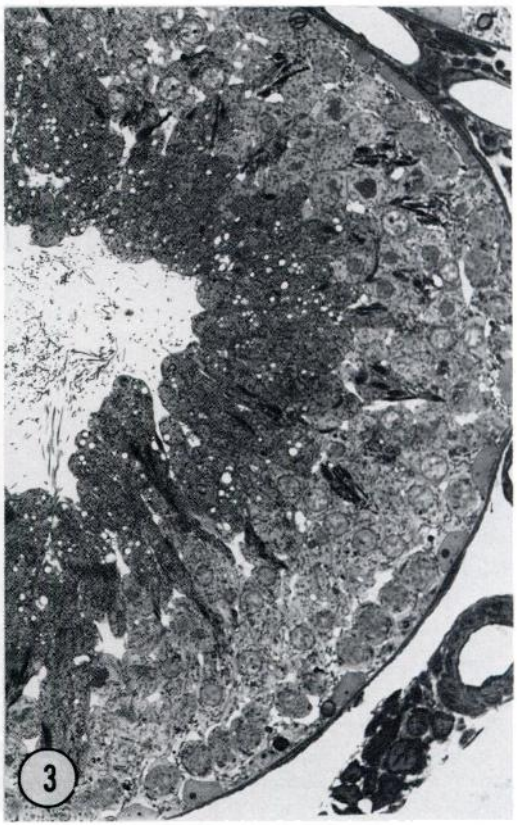
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TABLE 1. Quantitation of Intercellular Vacuolization in Tubules from Control and Perfused Testes

	Percentage of Tubules Showing Frequency of Intercellular Vacuolization			Intercellular Vacuolization Index*
	Minimal 3	Moderate 2	Marked 1	
Control (100 tubules in 4 testes)	57	29	14	2.44 ± 0.15
Perfusion with 2 mM glucose and no insulin (72 tubules in 4 testes)	51	38	11	2.30 ± 0.36
Perfusion with 15 mM glucose and 100 mU insulin/ml (142 tubules in 7 testes)	34	50	17	2.66 ± 0.29

* Indices of 3, 2, and 1 correspond to minimal, moderate and marked vacuolization, respectively, according to the observed frequency and size of the vacuoles. It is expressed as the mean ± SD of the testes' scores in each group.

features can be observed. Occasional intercellular spaces are found with the light microscope in the seminiferous epithelium of control rats. These are, of course, more evident at the ultrastructural level.

The morphologic criteria used as the basis for grading intercellular vacuolization in the tubules as minimal, moderate, or severe are illustrated in Figs. 1–3, respectively. Quantitative evaluation of intercellular vacuolization is shown in Table 1. It will be noted that, even under optimal conditions of fixation, only 57% of the tubular profiles in control testes are virtually free of intercellular vacuolization; the intercellular vacuolization index in these testes is 2.44 ± 0.15. In both groups of isolated perfused testes, the intercellular vacuolization index does not show a statistically significant change, although both groups exhibit a small decrease in the percentage of tubules having little or no intercellular vacuolization. Interestingly, the index for the testes perfused with both glucose and insulin actually increases somewhat over the control value.

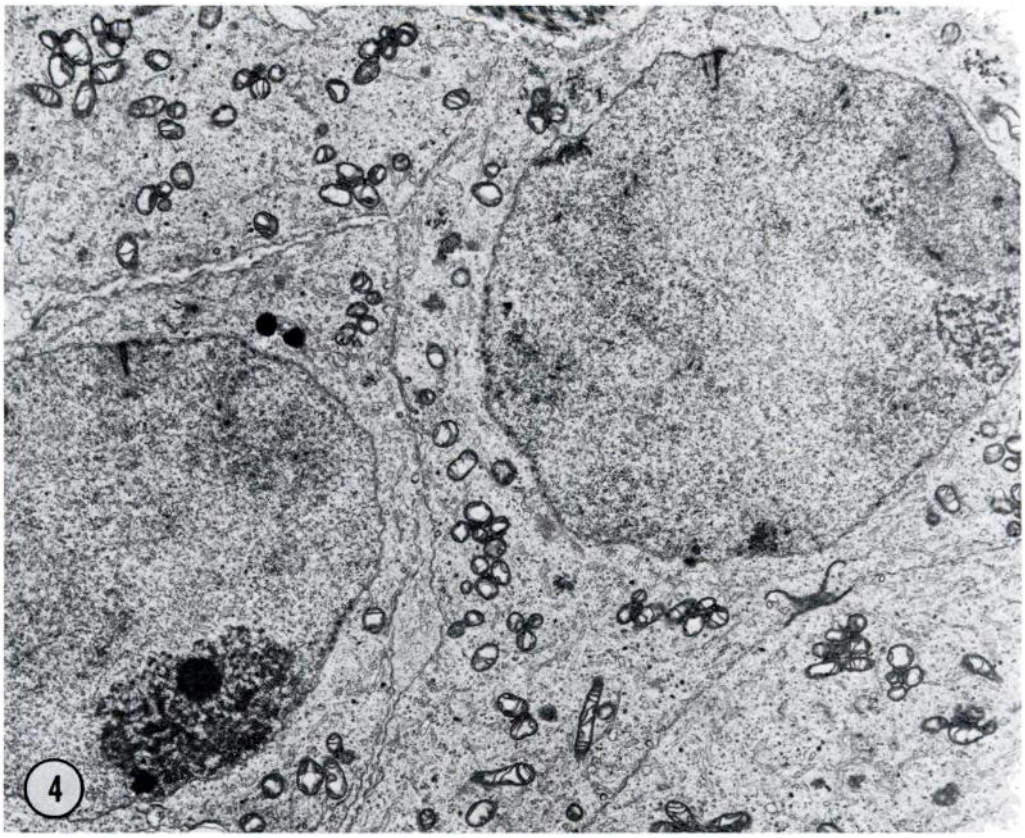
At the ultrastructural level, normal morphology is evident throughout the seminiferous epithelium of control rats. Briefly, spermatogonia and Sertoli cells are visible along the basement membrane. The Sertoli cells are characterized by large pleomorphic nuclei and prominent nucleoli, numerous mitochondria, lysosomes, and vesicular elements, and scattered profiles of endoplasmic reticulum and Golgi elements. Large lipid droplets are present according to the stage of the cycle. Primary spermatocytes (Fig. 4) can be recognized by their prominent nucleolar material and synaptonemal complexes. They have randomly dispersed mitochondria with extremely low-density matrices and few cristae. As spermatocytes mature into spermatids, the mitochondria migrate toward the cell border and tend to aggregate along the plasmalemma (Fig. 5). Vesicular elements of the endoplasmic reticulum increase, whereas cisternal profiles appear less numerous. With further cytodifferentiation (not shown here), the acrosomal granule characteristic of the cap phase of spermiogenesis increases in volume and elongates over the nucleus. In the interstitium, clusters of polyhedral Leydig cells are easily recognized by their abundant agranular reticulum and numerous mitochondria with lamellar cristae, dense matrix, and occasional mitochondrial granules; lipid droplets are rare (Fig. 6).

Seminiferous Epithelium and Leydig Cells of Isolated Perfused Testes

No significant differences are observed between testes perfused with 2 mM glucose and those perfused with 15 mM glucose and insulin. Therefore, the following morphologic description pertains equally to the testes of both groups.

Light microscope examination of each of the fourteen stages in the cycle of the seminiferous

Fig. 4. Electron micrograph of primary spermatocytes of a normal testis showing prominent nucleolar material and synaptonemal complexes. In the cytoplasm, mitochondria with extremely low density matrices and few cristae are dispersed at random. **Fig. 5.** Electron micrograph showing a round spermatid from a normal seminiferous tubule in stage VII of the spermatogenic cycle. Notice the aggregation of mitochondria along the cell border; this distribution of mitochondria is typical for round spermatids.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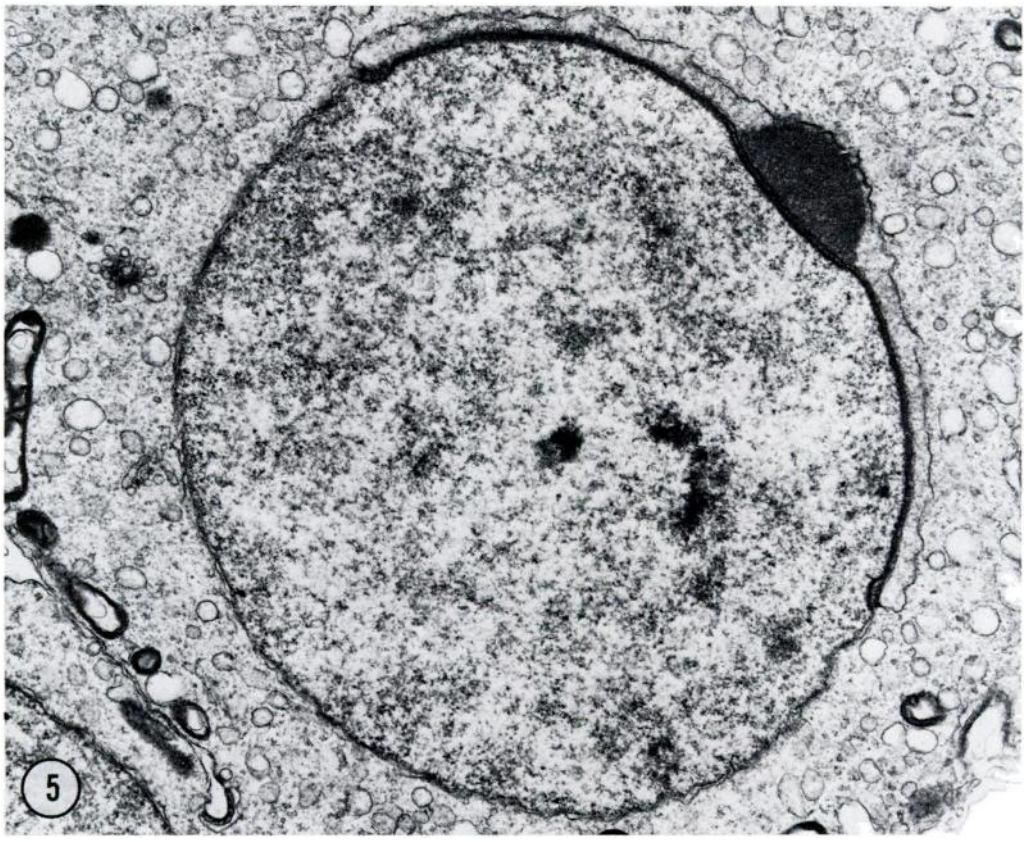
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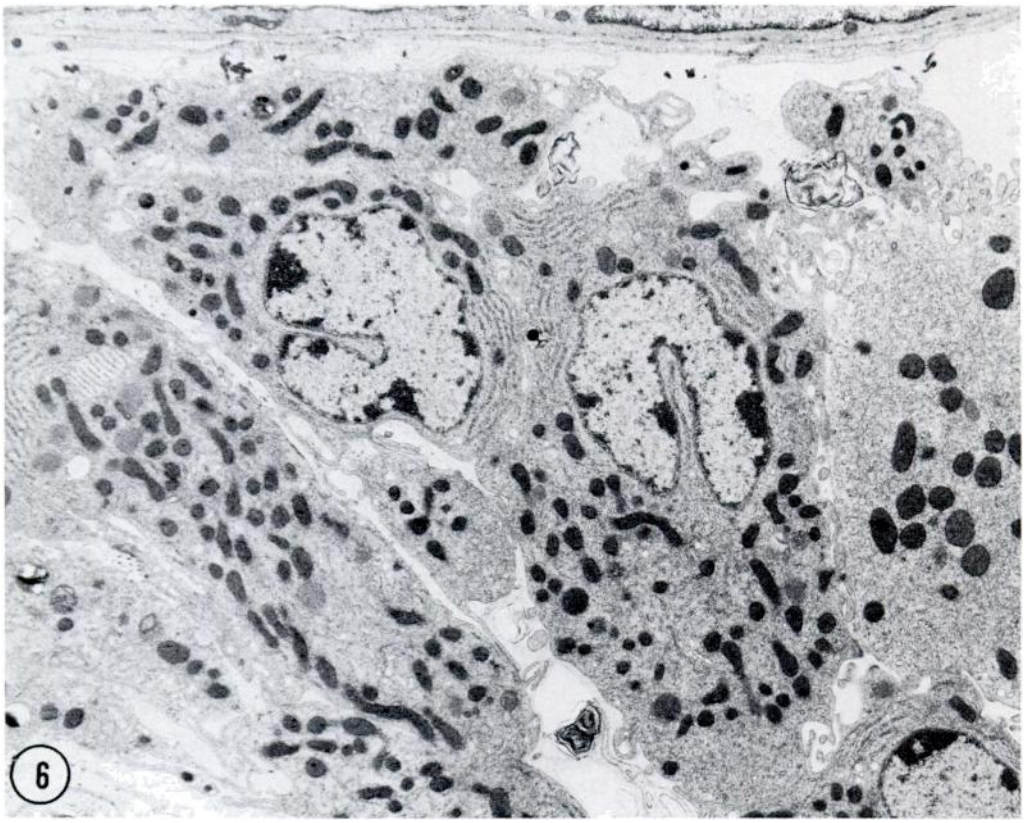

Fig. 6. Electron micrograph showing several Leydig cells in the interstitium of a control testis. The agranular reticulum is extremely well-developed and consists of an anastomosing network of smooth-surfaced tubules. Numerous mitochodria with lamellar cristae, dense matrix, and occasional mitochondrial granules are distributed randomly in the cytoplasm.

epithelium reveals that all elements of the germinal epithelium are present and normal in their appearance. Increases in intercellular spaces can be observed in occasional tubules, but the majority of tubules exhibiting intercellular spaces are within normal limits and are comparable in degree to those seen in control animals (Table 1). An example of normal-appearing seminiferous epithelium in the Golgi phase of spermiogenesis is shown in Fig. 7; similarly, Stage X elongating spermatids and pachytene spermatocytes exhibit normal morphologic characteristics (Fig. 8). The lipid droplets near the basement membrane are typical for this stage of the cycle.

With the electron microscope, ultrastructural features of germ, Sertoli, and Leydig cell morphology are normal and similar to controls in all respects. Intercellular spaces are minimal (Figs. 9 and 10). The nuclei of the Sertoli cells are large and pleomorphic, and they exhibit prominent nucleoli. The cytoplasmic ground substance of the Sertoli cells is uniform and homogeneous in appearance and contains numerous well-preserved mitochondria, elements of endoplasmic reticulum, and small dense lysosomes. Sertoli cell–Sertoli cell junctions are intact and well-preserved (Fig. 9). The ultrastructural features of the spermatogonia are unchanged (Fig. 9). Normal spermatocyte morphology is also evident; synaptonemal complexes, cisternal and tubular elements of the endoplasmic reticulum with a low density content and randomly dispersed mitochondria can be recognized. Round spermatids in the Golgi phase (Fig. 10) exhibit a well-developed Golgi apparatus, numerous vesicular elements, and mitochondria arranged along the cell periphery. As the spermatids continue to mature, elongation and condensation of the nuclear material takes place in the usual fashion (Fig. 10). In the interstitium, the Leydig cells exhibit no remarkable features; agranular reticulum, mitochondria, and nuclear morphology are all normal (Fig. 11).

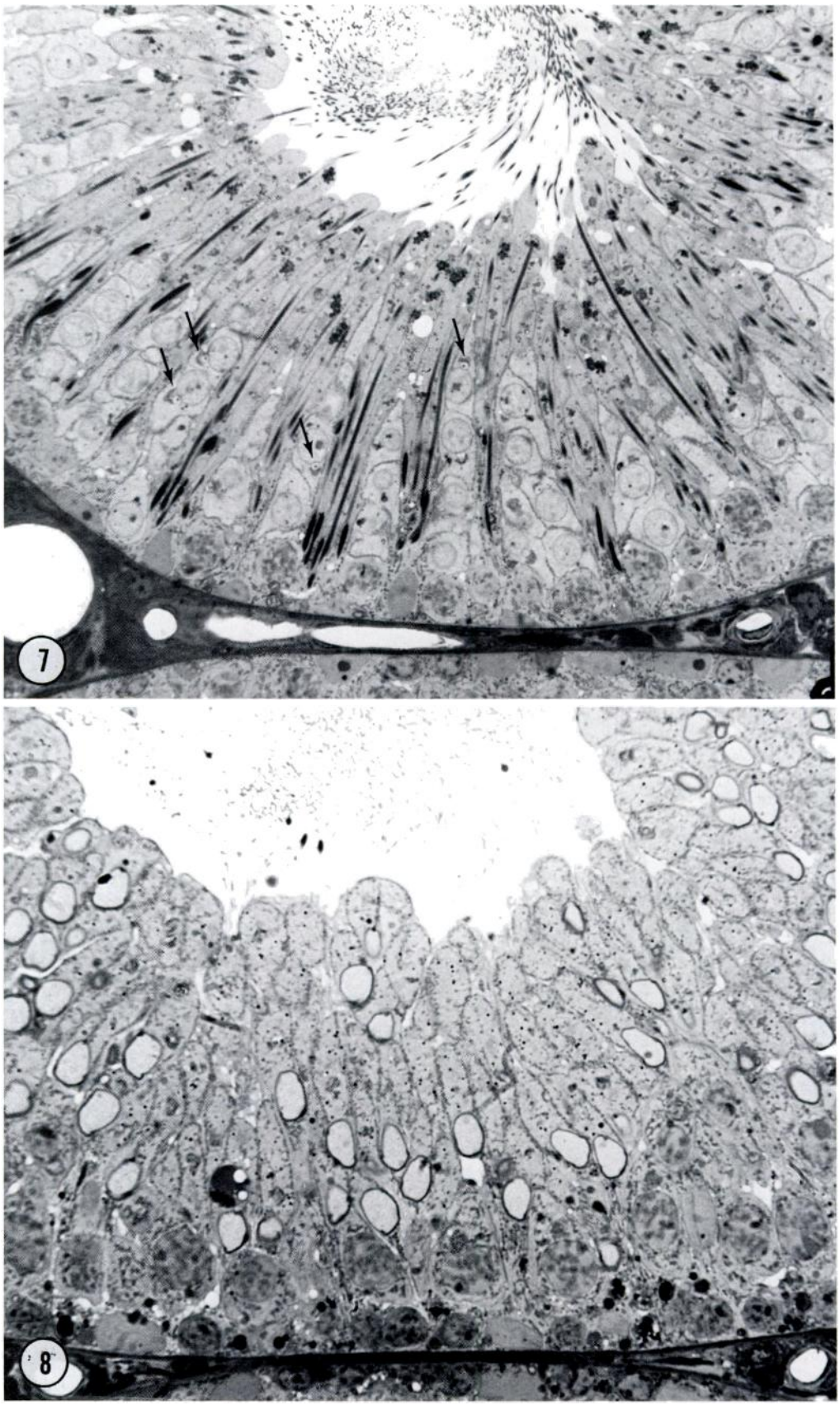

Fig. 7. Spermatids in the Golgi phase of spermiogenesis. The spherical acrosomal granules (arrows) are clearly visible in this section. **Fig. 8.** Light micrograph of a stage X cross-section of a seminiferous tubule from an isolated perfused testis. Pachytene spermatocytes and elongated spermatids are present in the interstices of Sertoli cells. Note the lipid droplets near the basement membrane.

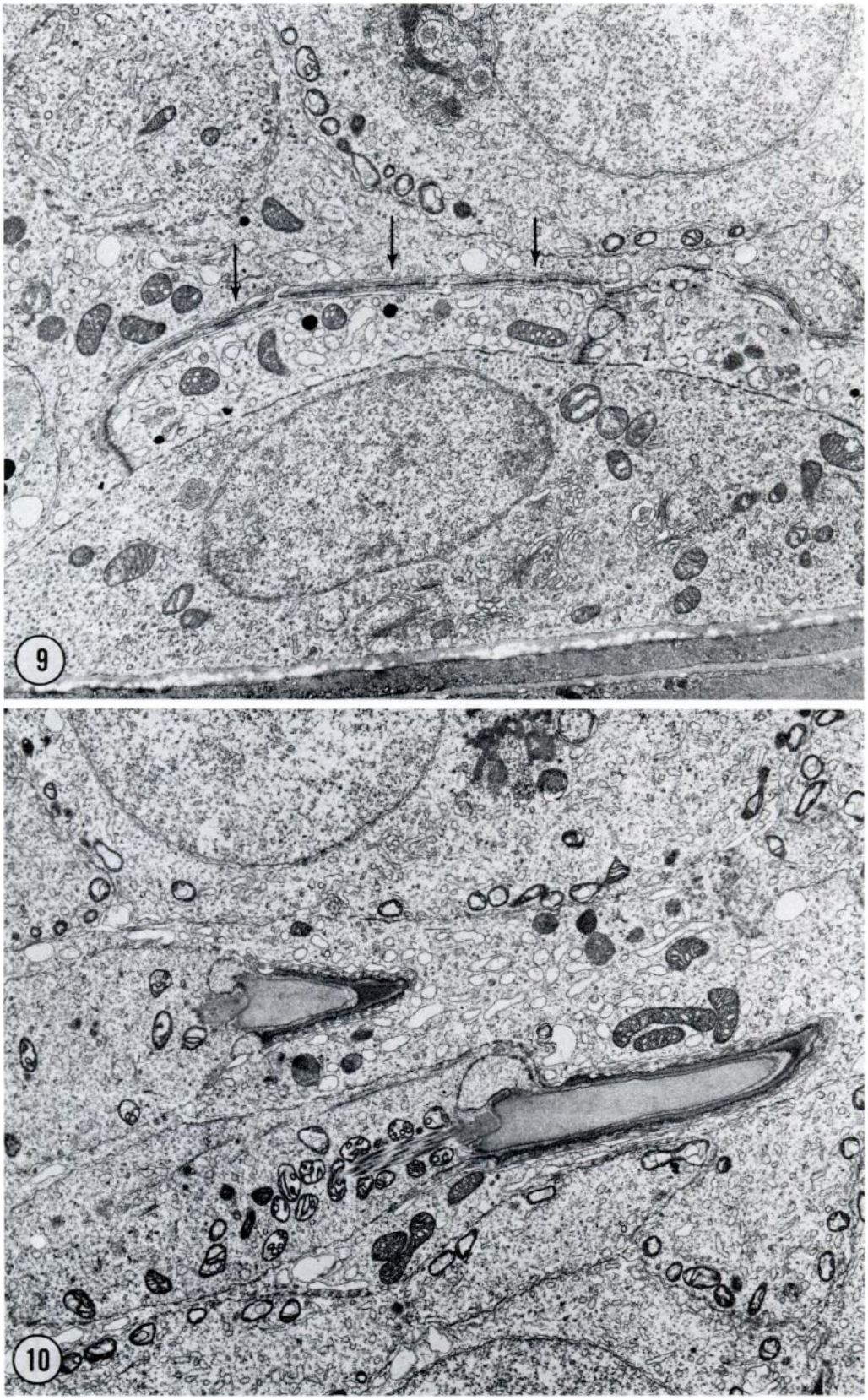

Fig. 9. Electron micrograph showing the normal appearance of a spermatogonium in the isolated perfused testis. Portions of a round spermatid and three Sertoli cells can also be observed. Notice that Sertoli cell–Sertoli cell junctions (arrows) are well-preserved and intercellular spaces are minimal. **Fig. 10.** As shown in this electron micrograph of an isolated perfused testis, the nuclei of older spermatids elongate markedly, and the bulk of the cytoplasm shifts away from the nucleus toward the lumen, leaving the heads of the differentiating spermatozoa deeply embedded in the Sertoli cells. Portions of round spermatids and spermatocytes are also visible in this section. The ultrastructure of all the cells shown here is normal.

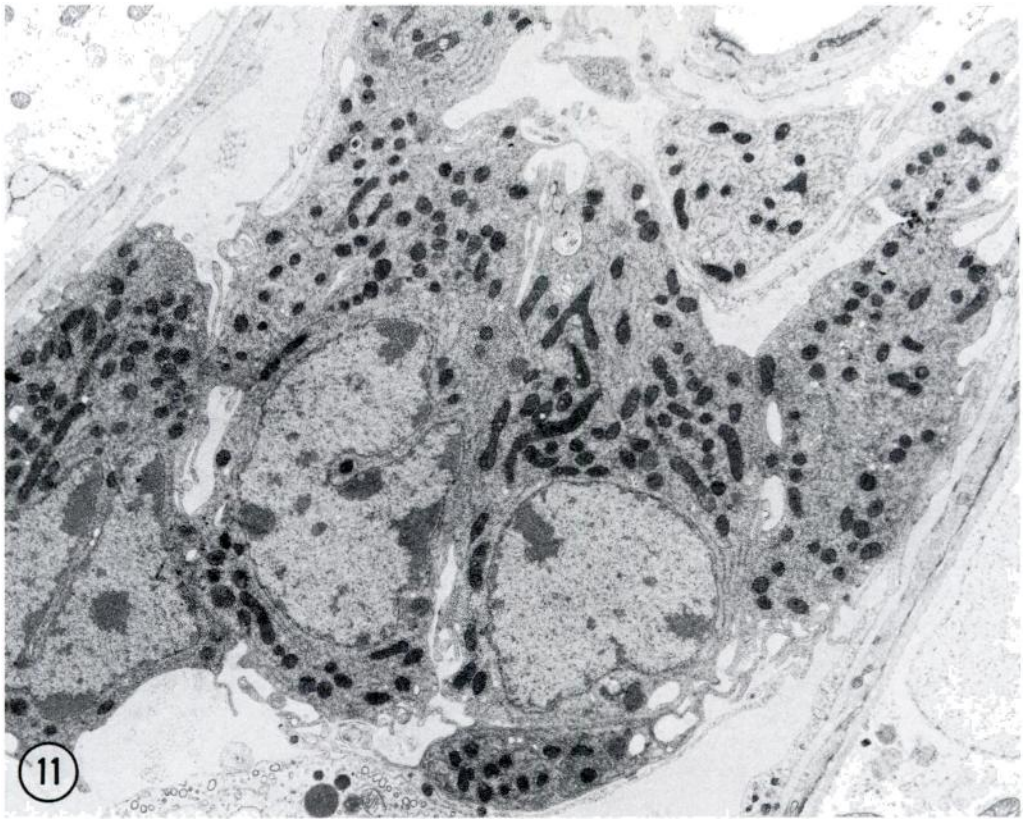

Fig. 11. This electron micrograph shows Leydig cells from an isolated perfused testis that are similar to control Leydig cells in all respects. Well-developed smooth endoplasmic reticulum and numerous mitochondria are easily recognized.

Discussion

The investigations presented here demonstrate that the isolated rat testis is comparable morphologically to the control organ following a 1-hour recirculating perfusion with albuminated Krebs-Ringer bicarbonate buffer containing either 2 mM glucose or 15 mM glucose and 100 mU insulin/ml. All germ, Sertoli, and Leydig cells show the same histologic and ultrastructural characteristics as the controls. The degree of intercellular vacuolization is not significantly increased by the perfusion (Table 1). While some investigators believe that vacuoles are unavoidable artifacts of fixation, others contend that occasional intercellular vacuoles are normally present in seminiferous tubules. The significance of these vacuoles is not understood at the present time. It is noteworthy to observe that even under optimal conditions of fixation, only 57% of the tubular profiles in control testes are virtually free of intercellular vacuolization. The fact that such an unexpectedly high percentage of seminiferous tubules in control testes (43%) exhibits moderate or marked intercellular vacuolization must be taken into account in studies designed to evaluate the effects of various experimental conditions on testicular morphology.

The structural integrity of the perfused testis was not affected by switching from a perfusate containing 15 mM glucose and 100 mU insulin per ml to a medium containing 2 mM glucose and no insulin. These two conditions represent extreme levels of substrate supply that might be used for metabolic studies.

The perfusate used in the present study does not contain erythrocytes. Instead, oxygenation of the testes is assured by oxygen dissolved in perfusate water. In the absence of red blood cells, the viscosity of the perfusate is considerably lower than that of blood. Therefore, an increase in the rate of perfusate flowing through the organ compensates for its smaller capacity to transport oxygen. Thus, the rat testis, as previously demonstrated in the liver, kidney, lung, and diaphragm of small animals, can be successfully perfused with a medium

lacking erythrocytes (Scholz, 1965; Bowman, 1970; Deleaunois, 1964; Bierkamper and Goldberg, 1978). A medium free of erythrocytes is considered preferable because the weight of the erythrocytes in the perfusate usually exceeds that of the perfused organ (Brunengraber et al, 1975). As a result, erythrocyte metabolism becomes superimposed on the metabolism of the organ under study, and one is left to deal with a dual tissue perfusion.

Cobb et al (1980) perfused isolated rat testes in open circuit with protein-free Eagles minimum essential medium (MEM) enriched with 25 mM $NaHCO_3$. These authors demonstrated appropriate synthesis, but low secretion, of testosterone and estradiol by the model after stimulation with chorionic gonadotrophin. The impaired secretion of hormones was ascribed to the use of protein-free perfusate. Although Cobb et al do not describe the microscopic anatomy of their model, their report is indicative of some degree of tissue edema since their isolated perfused testes had an 11% increase in water content over that of controls. In addition, it has previously been shown that the isolated perfused liver does not synthesize fatty acids and cholesterol when perfused with a protein-free medium (Brunengraber et al, 1975). In the isolated perfused rat kidney, metabolic performance is impaired severely when albumin is omitted from the perfusate (Little and Cohen, 1974). We therefore conclude that in order to avoid tissue edema, low testosterone secretion, and/or possible impairment of lipid metabolism, rat testis perfusion should be conducted with albuminated medium. Under these conditions, the model appears histologically and ultrastructurally identical to control testes. It can therefore be used to study the early influence of toxic compounds on the microscopic anatomy of the male gonad.

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