

The ultrastructure of normal fetal and neonatal pig testis germ cells and the influence of fetal decapitation on the germ cell development

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Summary

The development of germ cells in the male pig was investigated ultrastructurally in normal and decapitated fetuses. The age ranged respectively from 30 days *p.c.* till one month after birth and from 52 days *p.c.* until birth. The ultrastructural organization of the germ cells changes dramatically between 30 days *p.c.* and 52 days *p.c.* which coincides with the formation of 'true' sex cords. From 52 days *p.c.* onwards the morphology is rather stable: cells show a 'hydrated' appearance and typical cell bridges. There is

no obvious difference in the ultrastructure of germ cells in decapitated animals, their normal littermates and control animals. Therefore germ cell development in the pig is likely to be insensitive to gonadotropins during the fetal period. The development of pig germ cells follows closely the pattern described for several species. Quantitatively there is an increase in the ratio of germ cell/Sertoli cell per cross sectional diameter in the decapitated animals.

Key words: ultrastructure, germ cells, testis, fetal, pig.

Introduction

Deprivation of gonadotropins by experimental techniques such as hypophysectomy or decapitation *in utero* or by a natural condition such as anencephalia results in a sharp drop in the number of Leydig cells in the testis as a common feature for several species (Ch'in, 1938; Tseng, Alexander & Kittinger, 1975; Gulyas, Tullner & Hodgen, 1977; Colenbrander, van Rossum-Kok, van Staaten & Wensing, 1979; Baker & Scrimgeour, 1980; van Vorstenbosch, Colenbrander & Wensing, 1982, 1984a).

The effect of deprivation of gonadotropins on the development of the sex cords is not so evident and the available data are conflicting. On the one hand no obvious qualitative or quantitative differences are reported: the main effect is some decrease in the number of gonocytes and a decrease of the sex cord diameter (Ch'in, 1938; Creasy & Jost, 1966; Tseng *et al.* 1975; Baker & Scrimgeour, 1980). On the other hand experimental reduction of FSH levels in the perinatal period in the rat causes a significant reduction in the number of Sertoli cells (Orth, 1984).

During normal development in the pig a sharp increase in the number of germ cells per cord cross section can be observed during sex cord formation,

thereafter the increase is much less and after birth the number of germ cells is even temporarily reduced (van Vorstenbosch *et al.* 1984a). However, if calculated for the whole of the testis, an exponential growth continues during the fetal and neonatal period (van Straaten & Wensing, 1977). After decapitation a significant increase in the number of germ cells per cross section can be observed from approximately 80 days *p.c.* onward (when LH and FSH become occasionally detectable in the peripheral circulation of the normal fetus) while the total number per testis is the same as in control animals (Colenbrander *et al.* 1979). Also the number of Sertoli cells per cross section in decapitated animals increases significantly from 80 days onward. However, for Sertoli cells no data for the testis as a whole are available (van Vorstenbosch *et al.* 1984a). Qualitatively no differences could be observed between the ultrastructure of Sertoli cells of controls and of decapitated animals (van Vorstenbosch, Spek, Colenbrander & Wensing, 1984b).

Data on the structural development of germ cells in the fetal and neonatal period in the pig are absent apart from the very early period ranging from the indifferent gonad until the early testicular differentiation (Pelliniemi, 1975).

The purpose of this study was (1) to provide a description of the development of fetal and neonatal germ cells from the period of sex cord formation until one month after birth in control animals and from 52 days *p.c.* until birth in the decapitated animals and (2) to investigate the influence of gonadotrophins on some aspects of sex cord development.

Materials and methods

Electron microscopy

Yorkshire and Dutch Landrace cross-bred fetuses and neonates were used. Additionally several such fetuses were decapitated at 42 days *p.c.* (the earliest possible age) according to the method of Stryker & Dziuk (1975); littermates served as controls. Fetuses and neonates of normal litters were also used. Data on the animals used, as well as sampling data and the number of litters, are shown in Table 1. Materials were handled and processed as reported elsewhere (van Vorstenbosch *et al.* 1982). In brief, a double fixation in 2.5% glutaraldehyde followed by 1% OsO₄ according to the method of Sabatini, Bensch & Barnett (1963) was succeeded by block staining in 2% uranyl acetate prior to dehydration in graded series of acetone. Materials were cleared in propylene oxide and embedded in either DER-I mixture (Lockwood, 1964) or in Durcupan ACM (Fluka). Semithin sections were stained with either paragon or toluidine blue. Thin sections were stained with lead citrate (Venable & Coggeshall, 1965). A Philips 201G electron microscope was used at 60 kV with a 30 µm objective aperture.

Light microscopical morphometry

Morphometry was carried out on 1 µm thick Araldite sections of the testes of five control and five decapitated animals at the age of 110 days *p.c.* The sections were toluidine-blue stained. 110 days *p.c.* was chosen as at that age a clear difference in the sex-cord diameter between the two groups can be observed. Only true cross sections of the cords were used. A point raster was calibrated on an object micrometer, objective ×40 and ocular magnification ×10, resulting in a point equivalence of 40.5 µm² (= raster-

surface factor). The surface of the cords and the surface of the germ cells were estimated by counting the overlying raster points, which were multiplied by the raster-surface factor.

For each cord cross section the proportion of Sertoli cell surface was calculated by subtracting the total germ cell surface per cross-sectioned cord from the surface of that cord. Also for each cross-sectioned cord the number of germ cell nuclei and of Sertoli cell nuclei was counted.

Individual germ and Sertoli cells were 'normalized' by dividing their proportional surface by the number of their respective nuclei. The ratio of germ cells/Sertoli cells per cross diameter was estimated by dividing the number of germ cell nuclei by the number of Sertoli cell nuclei.

For technical reasons collecting data on testicular weight was not possible, so calculations on the total number of Sertoli cells per testis could not be made.

Since there was only little variation in the sex-cord diameters between the control animals and also between the diameters of the sex cords of the decapitated animals the data were pooled as if they came from one control and one decapitated.

Results

Light microscopy

The results are summarized in Table 2.

There is a sharp increase in the average surface of cross-sectioned cords in the decapitated animals if compared with controls; a substantial part of this increase is accounted for by the germ cells. There is no difference between 'normalized' individual germ cells and Sertoli cell surface in decapitated and in control animals. However, if one calculates the ratio of germ cells/Sertoli cells per cross-sectioned cord for controls and decapitated animals, there is an increase in this ratio in decapitated animals.

Electron microscopy

At 30 and 35 days *p.c.* 'sex cords' consist of cell plates and cord-like structures, which contain numerous

Table 1. Experimental design: the number of animals observed in each group

	Age in days*											
	post coitum						after birth†					
	35	52	62	75	80	90	110‡	term	10	20	30	
Decapitated animals§		5	5	5	3	3	5	7				
Controls		10	10	5	6	6	5	12				
Untreated animals	9	2	2	2		3		3	5	5	5¶	
Litter	2	6	4	6	3	4	3	3				

* ±1 day variation in the sampling date, the age of the animals was precisely known.

† Term at 114 days *post coitum*.

‡ Animals used for morphometry.

§ Decapitation at 42 days *post coitum*.

¶ Litter origin of the neonatal piglets unknown.

Table 2. Morphometric data

N	Cord		Germ cells			Sertoli cells		Normalized surface: total surface/number of nuclei \pm S.E.M.		
	Total of overlaying points	Surface ($\mu\text{m}^2 \pm$ S.E.M.)	% surface of cord	Surface ($\mu\text{m}^2 \pm$ S.E.M.)	% surface of cord	Surface ($\mu\text{m}^2 \pm$ S.E.M.) (cord surf. - germ cell surf.)	Number of nuclei per cord diam. \pm S.E.M.	Germ cells	Sertoli cells	
(C) 166	8255	1840.6 \pm 202	27.56	507 \pm 29.6	72.44	1199 \pm 70.5	4.3 \pm 0.17	22.4 \pm 0.39	117.9 \pm 9.90	55.2 \pm 3.9
(D) 143	8721	2470.2 \pm 144	38.04	940 \pm 60.7	61.95	1514 \pm 93	7.3 \pm 0.32	27.4 \pm 0.73	128.7 \pm 8.30	53.2 \pm 3.3

Age of the animals used: 110 days *post coitum*.

5 control and 5 decapitated animals.

N, total number of cross sectioned sexcords.

Ratio germ cells/Sertoli cells/cross diam. - control animals 0.192 \pm 0.08
- decapitated animals 0.266 \pm 0.01

C, controls.

D, decapitated.

Point raster used: 1 point corresponds to 40.5 μm^2 .

Magnification, $\times 400$.

early Sertoli cells surrounding a relatively small number of germ cells. Usually these germ cells are solitary; pairs or clusters are rare. Most germ cells are piriform, but ovoid forms occur regularly. The piriform germ cells have their tapered pole directed towards the basal lamina of the cord, but direct contacts with this lamina are seldom seen.

The germ cells frequently show small cytoplasmic extensions in direct contact with the Sertoli cells. The latter loosely surround the germ cells. The width of the intercellular space varies considerably. Pinoscytosis was not observed. The nucleus of a germ cell is generally large and ovoid or circular and chromatin is finely dispersed. One or more eccentrically placed and elaborate nucleoli can be seen. Each nucleolus is composed of a very well-developed 'pars nucleonema' with electron-dense material attached to it which rests upon the surface of a less electron-dense 'pars amorpha'. The 'pars nucleonema' sometimes approaches but never contacts the nuclear membrane. In the cytoplasm, numerous ribosomes either free or clustered into polysomes and a few microtubules can be observed. The mitochondria are large and ovoid, displaying a modest electron density and a finely dispersed matrix. Their cristae are mostly tubular or vesicular. Electron-dense 'cement' is sometimes present; when present it ties the mitochondria into a cluster. The Golgi apparatus is not very elaborate and consists of some stacks with a few dictyosomes. The rough endoplasmic reticulum (RER) displays several long profiles having a few ribosomes (transitional form) attached and short profiles having relatively densely packed ribosomes. Endoplasmic reticulum completely devoid of ribosomes was not observed. One or several chromatoid bodies (nuages) consisting of fine granular material are present (Figs 1, 2).

At 52 days *post coitum* the early anastomosing plate-like 'cords' have developed into real cords which are circular in cross section. The scattered germ cells are surrounded by Sertoli cells. In comparison with the situation at 35 days *p.c.* the number of germ cells per cross section has increased considerably. However, mitotic figures are seldom seen. Most germ cells are arranged in pairs or clusters. Within a cluster cell processes of Sertoli cells cannot be observed, indicating a very close contact between these germ cells. Cellular bridges covered with sleeves of very electron-dense material occur between germ cells from 52 days *p.c.* onwards. The cytoplasmic content of the bridges hardly differs from that in the rest of these interconnected cells. Sometimes they are filled with microtubules. The germ cells are spherical, each with a large round nucleus. The intercellular spaces between Sertoli cells and germ cells are now narrow and regular (Fig. 3). Coated pits between germ cells and Sertoli cells and electron-dense cell junctions are regularly observed (Fig. 4H,I). A number of germ cells has migrated to the periphery of the cord and rest upon a well-developed basal lamina and can now be called spermatogonia. Morphologically, however, they do not differ from the other germ cells. These spermatogonia are mostly piriform which is indicative of their recent migration. They may be covered by very thin processes of the Sertoli cells except at the basal lamina. The large ovoid nucleus still shows well-developed nucleoli, although the 'pars nucleonema' is less developed. However, from now onwards the 'pars amorpha' is enlarged if compared to that of 35 days *p.c.* Between the inner and outer nuclear membranes vacuole-like structures are a regular observation (Fig. 4A-C). The packing of the organelles in the cytoplasm is less dense than at 35 days *p.c.* Single cilia are sometimes seen. Some

long profiles of endoplasmic reticulum free of ribosomes can be seen spatially coupled to mitochondria. Smaller profiles with or without ribosomes are also observed. Regularly these profiles show a close contact to the cell membrane and are frequently radially directed (Fig. 3). This pattern can be found also in the later stages of the observation period. The morphology of the organelles does not differ markedly from that at 35 days *p.c.* Typical dense-cored organelles embedded in some cytoplasm and enveloped by a cistern of ER begin to appear and become quite

common (Fig. 4E–G). These structures do not show any topographical relationship to other organelles. Between decapitated animals, their normal littermates and control animals, no obvious difference in ultrastructure could be observed.

At 62 days *p.c.* there are more germ cells in contact with the basal lamina. Paired spermatogonia are frequently seen, but the number of clusters has decreased. Intercellular bridges are commonly present. The nuclei are large and round. The nucleoli show the same organization as mentioned above;



Fig. 1. 35 days *p.c.*, part of sex cord. Nuage (asterisk). Basal lamina (arrowheads). Sertoli cells (s). Germ cell (g). $\times 7500$.

however, the 'pars nucleonema' tends to be less developed. Sometimes the nucleonema contacts the inner nuclear membrane (Fig. 4J). The cytoplasm is remarkably clear. The mitochondria are large, round or ovoid. They show a tendency to form clusters, especially near the nuclei. In such chains mitochondria are 'cemented' by strong electron-dense material. The mitochondrial cristae are mostly vesicular or tubular (Fig. 4D). These vesicular cristae can become quite large, resulting in a large central vacuole. Polysomes or free ribosomes are present in

low numbers. Microtubules are regularly seen. The Golgi apparatus is not very elaborate.

During later development the ultrastructure does not change markedly. Only the nucleolus becomes smaller, usually showing its characteristic organization of a central amorphous part with spokes of electron-dense material. The 'pars nucleonema', which is attached to these spokes, seems to diminish during development. The mitochondria tend to elongate and show mostly lamellar cristae and frequently a central vacuole (Fig. 5). At approximately

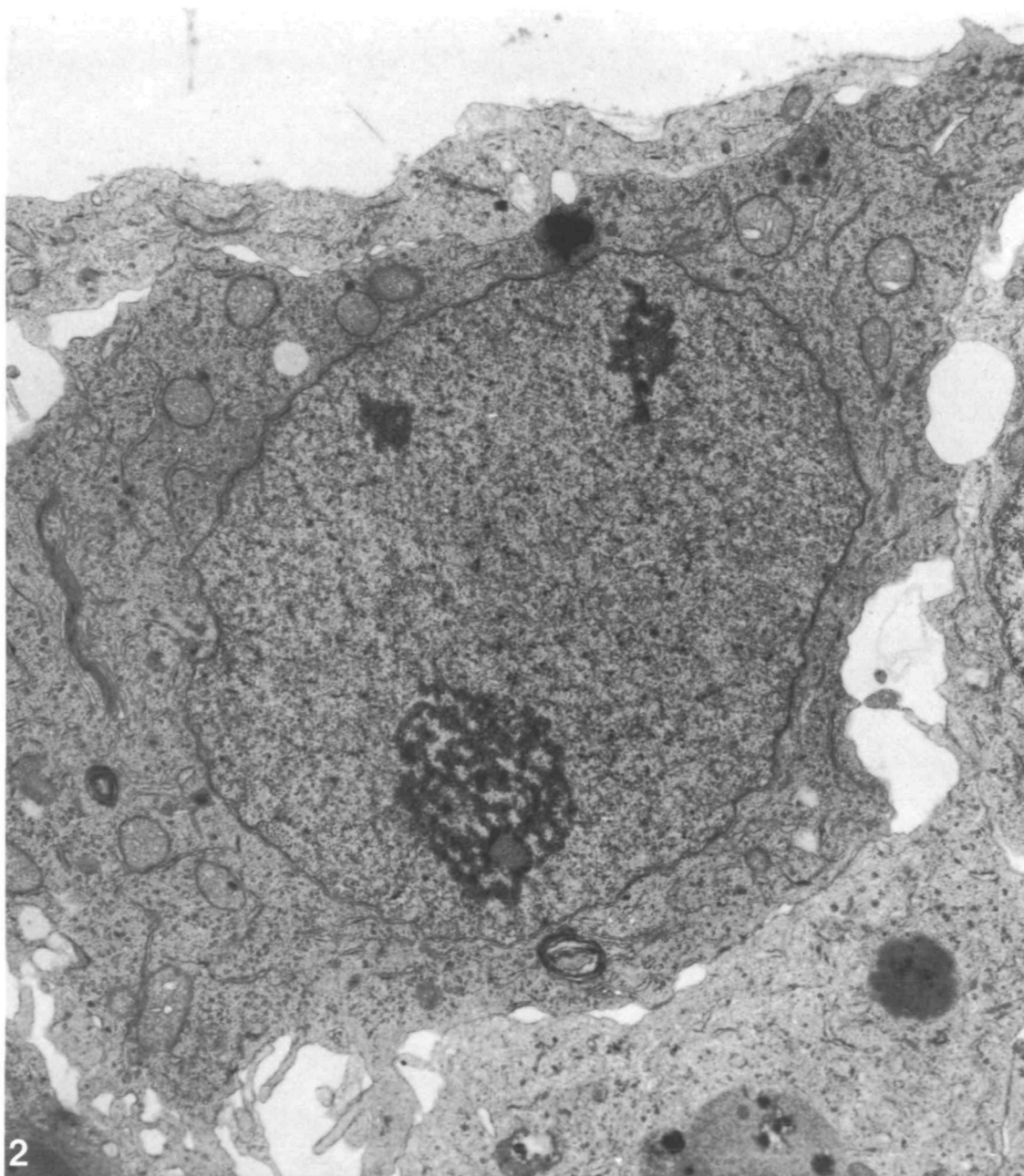


Fig. 2. 35 days *p.c.*, germ cell piriform. Note the very well-developed nucleonema part of the nucleolus and the relatively small amorphous part. Compare the matrix of this cell with that of Fig. 3. $\times 18000$.

90 days *p.c.* most of the germ cells make contact with the basal lamina. Degenerating germ cells are frequently seen, especially in the centres of the cord. During the whole of the observation period no synaptonemal complexes can be observed, indicating that meiosis has not started.

Between decapitated and control animals there is no obvious difference in germ cell architecture during the entire observation period.

In all developmental stages some germ cells and spermatogonia show signs of early degeneration. Large vacuoles in the cytoplasm, autophagosomes in

various stages, can be observed. Sometimes myelin figures can be seen, but no obvious nuclear condensation towards pycnosis is observed (Fig. 6).

Discussion

In this study the ultrastructural development of the germ cell compartment in the fetal and neonatal porcine testis has been described and it can be concluded that this development closely follows the pattern already described for other mammalian

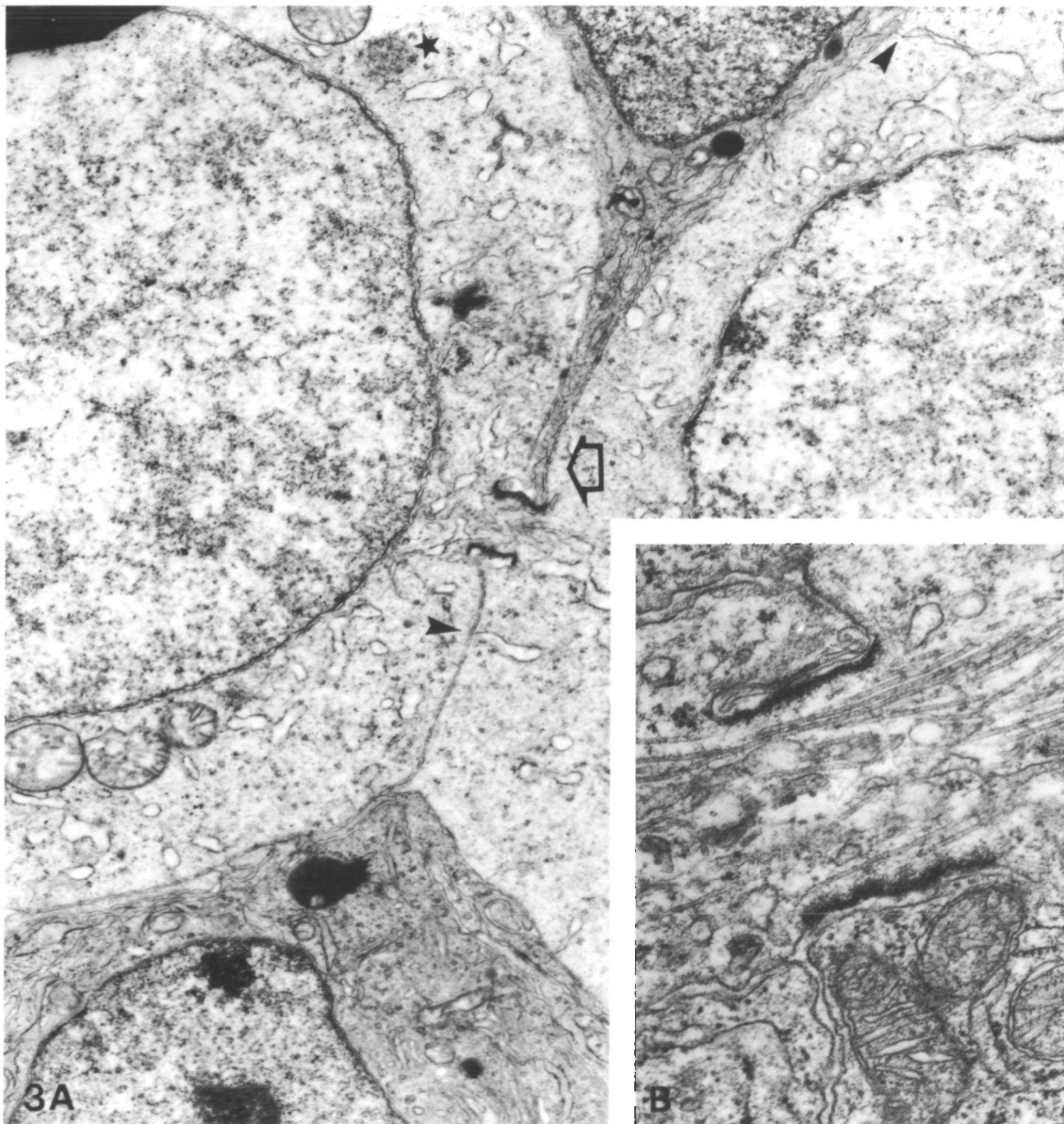


Fig. 3. (A) 52 days *p.c.* decapitated fetus. Two germ cells interconnected by a cell bridge. A thin process of a Sertoli cell penetrates the intercellular space between both germ cells (open arrow). Nuage or chromatoid body (asterisk). ER profiles perpendicular to the cell membrane and approaching it very closely (arrowheads). Note the electron-dense sleeve. Note also the relative 'dilution' of the cell matrix if compared with Figs 2 and 5. $\times 16\,000$. (B) 30 days *post partum*. Higher magnification of a cell bridge. In this case the bridge is filled with microtubules, indicating that mitosis has just finished. $\times 63\,000$.

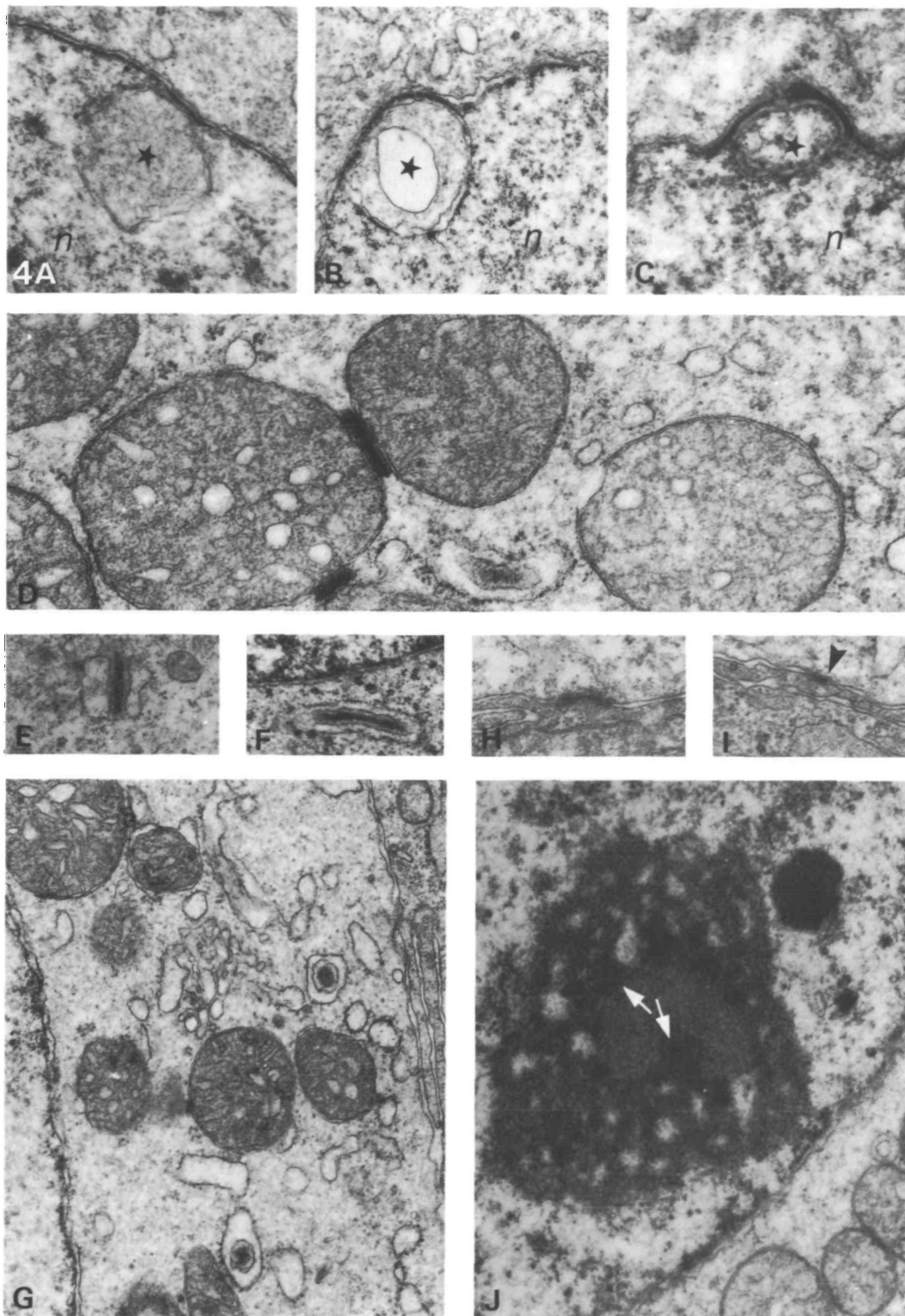


Fig. 4. (A–C) Some examples of unusual structures (*) associated with the nuclear envelope. *n*, nuclear plasma. Age of animals, 52 days *p.c.* – controls. (D) Strongly electron-dense ‘cement’ coupling mitochondria into a cluster. 35 days *p.c.* (E–G) Sections through the dense-cored structure. (E) 52 days *p.c.* decapitated animal; (F) 90 days *p.c.* control animal; (G) 52 days *p.c.* control animal. (H) Electron-dense cell connection, sometimes accompanied by microfilaments. The extension of the type of cell connection can vary considerably. 75 days *p.c.* decapitated animal. (I) ‘Rivet-type’ electron-dense cell connections are very often seen. 52 days *p.c.* control animal. (J) Nucleolus. The nucleonemar part is connected by electron-dense spokes (arrows) to the amorphous part. 80 days *p.c.* control animal. (A–C, H–J) $\times 30\,000$; (D, G) $\times 40\,000$; (E, F) $\times 27\,000$.

species (Wartenberg, Holstein & Vossmeier, 1971; Gondos & Conner, 1973; Fukada, Hedinger & Grosscurth, 1975; Hadziselimovic, 1977; Franchi & Mandl, 1964). From our data and the data presented by Gondos & Hobel (1971) and Pelliniemi (1975) it appears that the early germ cells display a pattern suggesting amoeboid movement and gradual morphological differentiation. After formation of the cords the morphology of the cells is more or less stable until the start of meiosis. A comparable qualitative development was also observed in the germ cells of the decapitated fetuses suggesting that this developmental process is gonadotropin independent. This observation is in agreement with earlier findings

of decapitated rats (Creasy & Jost, 1966) and with observations on anencephalics and hypophysectomized animals (Ch'in, 1938; Creasy & Jost, 1966; Tseng *et al.* 1975; Baker & Scrimgeour, 1980).

In an earlier study we reported that the qualitative development of porcine Sertoli cells in decapitated animals was also undisturbed (van Vorstenbosch *et al.* 1984a). This means that the sex cords are not affected qualitatively by gonadotropin deprivation. The number of germ cells per testis in the decapitated pig does not differ markedly from that in the normal testis of the pig fetus (Colenbrander *et al.* 1979). In this study it was found that the germ cell/Sertoli cell ratio per cross-sectioned cord increased in the decapitated

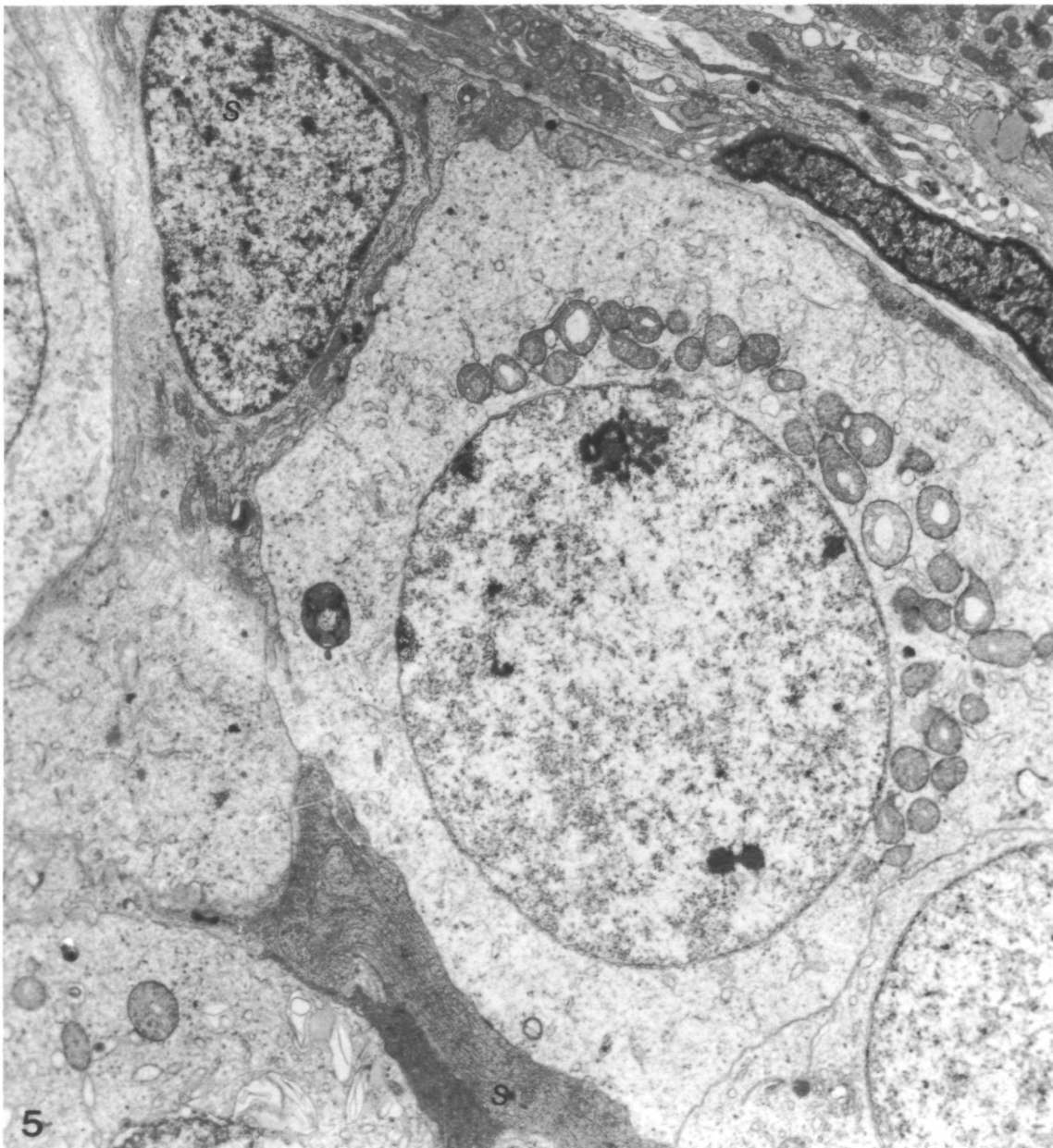


Fig. 5. Decapitated animal at term. Note the close arrangement around the nucleus and the central vacuoles in many mitochondria. s, Sertoli cell. $\times 7500$.

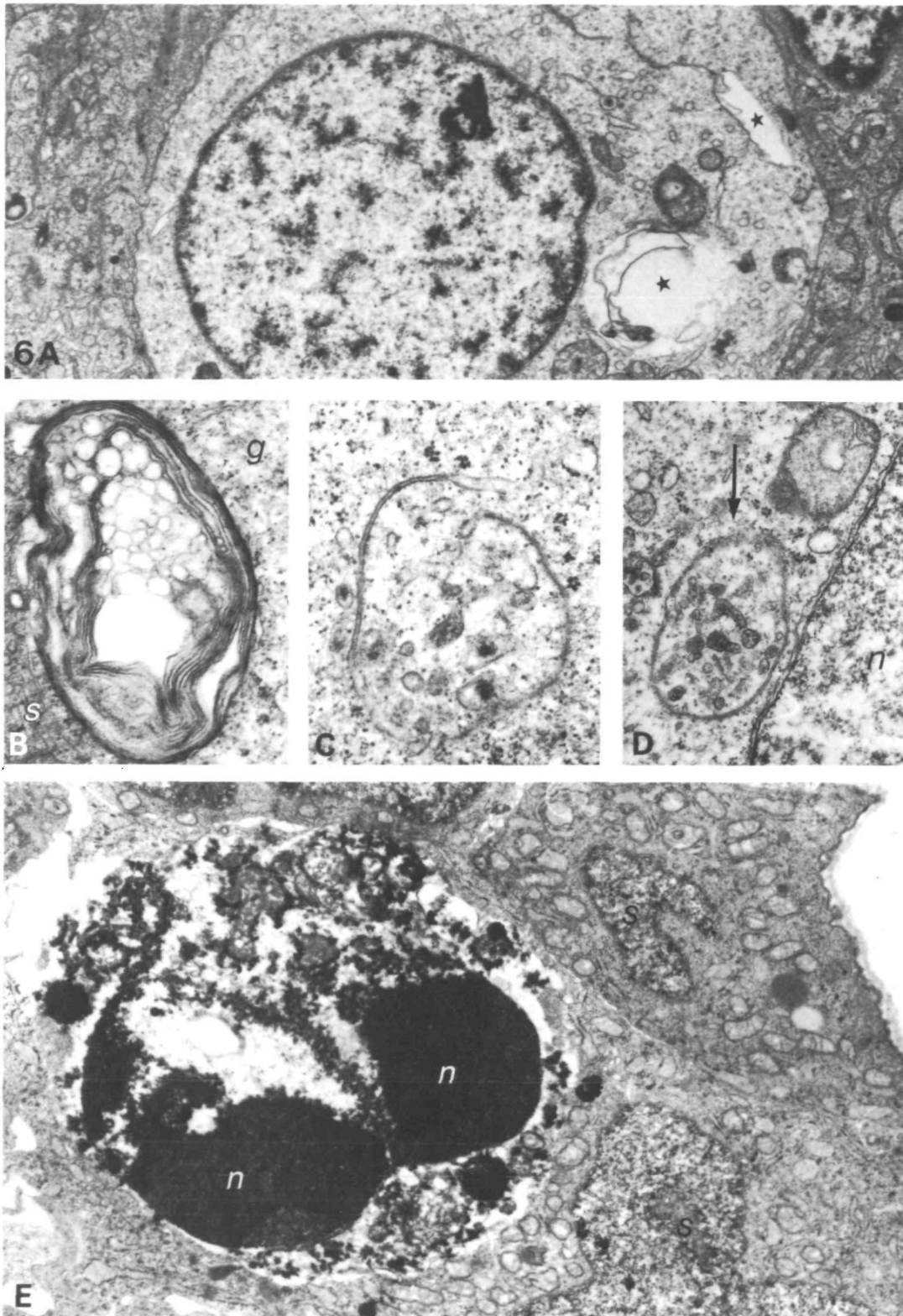


Fig. 6. Some aspects of germ cell degeneration. (A) Decapitated animal at term – focal cytoplasmic degeneration (asterisk). (B) Lamellar body including vacuolized cytoplasm. *g*, germ cell cytoplasm. *s*, Sertoli cell cytoplasm. Control animal – 52 days *p.c.* (C) Starting formation of autophagosome. Control animal – 52 days *p.c.* (D) Autophagosome (arrow). *n*, nucleus. Decapitated animal – 62 days *p.c.* (E) Example of totally degenerated germ cell with pyknotic nucleus. *s*, Sertoli cell. *n*, nucleus. Control animal – 52 days *p.c.*

fetuses. Although not measured directly, this implies a decrease in the number of Sertoli cells per testis in the decapitated animals. This apparent quantitative difference between control and decapitated animals coincides with the appearance or the absence of FSH in the peripheral circulation (Liwska, 1975; Colenbrander, van de Wiel, van Rossum-Kok & Wensing, 1982; Ponzilius, Parvizi, Elsaesser & Ellendorf, 1986). Apparently the deprivation of gonadotropins (most likely FSH) results in a normal qualitative but abnormal quantitative development of Sertoli cells, while no effect on germ cell development is noted. These findings are in agreement with the effect of FSH deprivation in the neonatal rat (Orth, 1984).

A rather peculiar finding was that, although degenerating germ cells can be observed in any of the stages studied, no signs of phagocytizing Sertoli cells could be observed. However, phagocytizing activity is described for several other species (Franchi & Mandl, 1964; Gondos & Hobel, 1971; Haziselimovic, 1977; Gondos & Conner, 1973). How the cell debris in the pig fetal testis is phagocytized has still to be explained.

The nuclear membrane anomalies are observed regularly in both decapitated and control animals. Their variable appearance might reflect different stages of a single developmental process. Similar 'anomalies' have been described in neoplastic germ cells of adult (Akhtar & Sidike, 1979) and in fetal and neonatal man (Hadziselimovic, 1977; Fukuda *et al.* 1975; Chemes, Fawcett & Dym, 1978).

The dense-cored bodies are typical of the germ cells. We never observed them in any other cell type in the testes. Similar bodies have been reported previously as dense-cored vesicles for the rat (Eddy, 1974), but their nature remains unknown.

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