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Ultrastructural Analysis of Rat Testes After Gossypol Acetic Acid (GAA) Treatment

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Summary. The present investigations were carried out to show the histological and ultrastructural alterations in rat testes 10 weeks after gossypol acetic acid treatment (dose: 30 mg gossypol acetic acid/kg/day). The morphological findings in the interstitial compartment were compared with the data from studies carried out to investigate the testosterone biosynthesis in gossypol acetic acid treated rats. No morphological changes in the epididymal and vasal epithelia were found; however, the germinal epithelial cells showed vacuolisation, pycnosis, disconnections of junctions, cytolysis and exfoliation of germ cells from the epithelium. The Sertoli cells were affected, too. Gossypol acetic acid seemed to stimulate the physiological activity pathologically; cellular organelles as mitochondria, endoplasmic reticulum, lysosomal vacuoles, pigment granules and nuclei were either enlarged in size and number or malformed in shape. The cellular contact was often restricted to spots or completely disconnected. If gossypol acetic acid was administered for a longer period of time some Sertoli cells were found to be unable to withstand the toxic stimulus, and the cells became necrotic too. In contrast to the toxic process in the germinal and Sertoli cells the Leydig cell compartment did not show any changes in fine structure, and therefore testosterone biosynthesis is presumed to be intact.

Key words: Gossypol acetic acid (GAA), Germinal ephithelium, Sertoli cell, Leydig cell.

Introduction

Considerable interest in gossypol, a phenolic compound isolated from cotton seeds, as a male antifertility agent has arisen around the world since the paper of Chinese scientists [18] was published.

In the recent numerous experiments have been carried out to establish the metabolical and physiological principles of this drug [1, 6, 13, 5, 14, 19, 14b, 20, 22, 26, 27].

In summary, the present literature confirms the capacity of gossypol acetic acid (GAA) to induce infertility in male animals; however, the precise mechanism of action is not clear. Antifertility efficacy of GAA has been reported to be dose and time dependent [18, 4, 22, 26]. According to reports of Hoffer [11], Bardin [2], Wang [25] and Xue [28] GAA did not interfere with the intact pituitary-gonadal hormonal axis. In contrast, disturbances of the hormonal axis were, however, found by Hadley [9] and Lin [16].

The present investigations were carried out to show the histological and ultrastructural alterations in rat testes 10 weeks after GAA-treatment (dose: 30 mg GAA/kg/day). The morphological findings in the interstitial compartment were compared with the data from studies carried out to investigate testosterone biosynthesis in GAA-treated rats. In one of our previous communications we have reported the proven infertility of GAA-treated rats [26].

Materials and Methods

GAA powder (supplied by courtesy of Dr. Sheldon Segal, Rockefeller Foundation, New York, USA) was suspended in 1% aequous carboxymethyl cellulose-NA solution and applied orally to 20 male Wistar rats (260–420 g body weight) by stomach intubation. Ten animals were fed with suspendant only (Placebo group) and nine animals were used as an untreated control group. The animals were caged singly under standard conditions (24 °C room temperature, 12:12 dark:light interval). A commercial diet (Altromin^{R}-H) and water were available ad libitum. GAA was kept in a brown bottle at 4 °C, and exposed to light for as short a time as possible because of its photosensitivity. The suspension was thoroughly agitated each time before filling the syringe. Fresh suspensions were prepared daily because there is evidence that gossypol might be unstable over an extended period of time.

At certain intervals the fertility rate of the experimental and control animals was proved by matings with females of proven fertility. The mating time was 14 days. For body-weight calculations the animals were weighed twice a week throughout the experimental period.

On the 43rd day of the experiment two animals of each group were vasectomized unilaterally under ether inhalation anaesthesia.

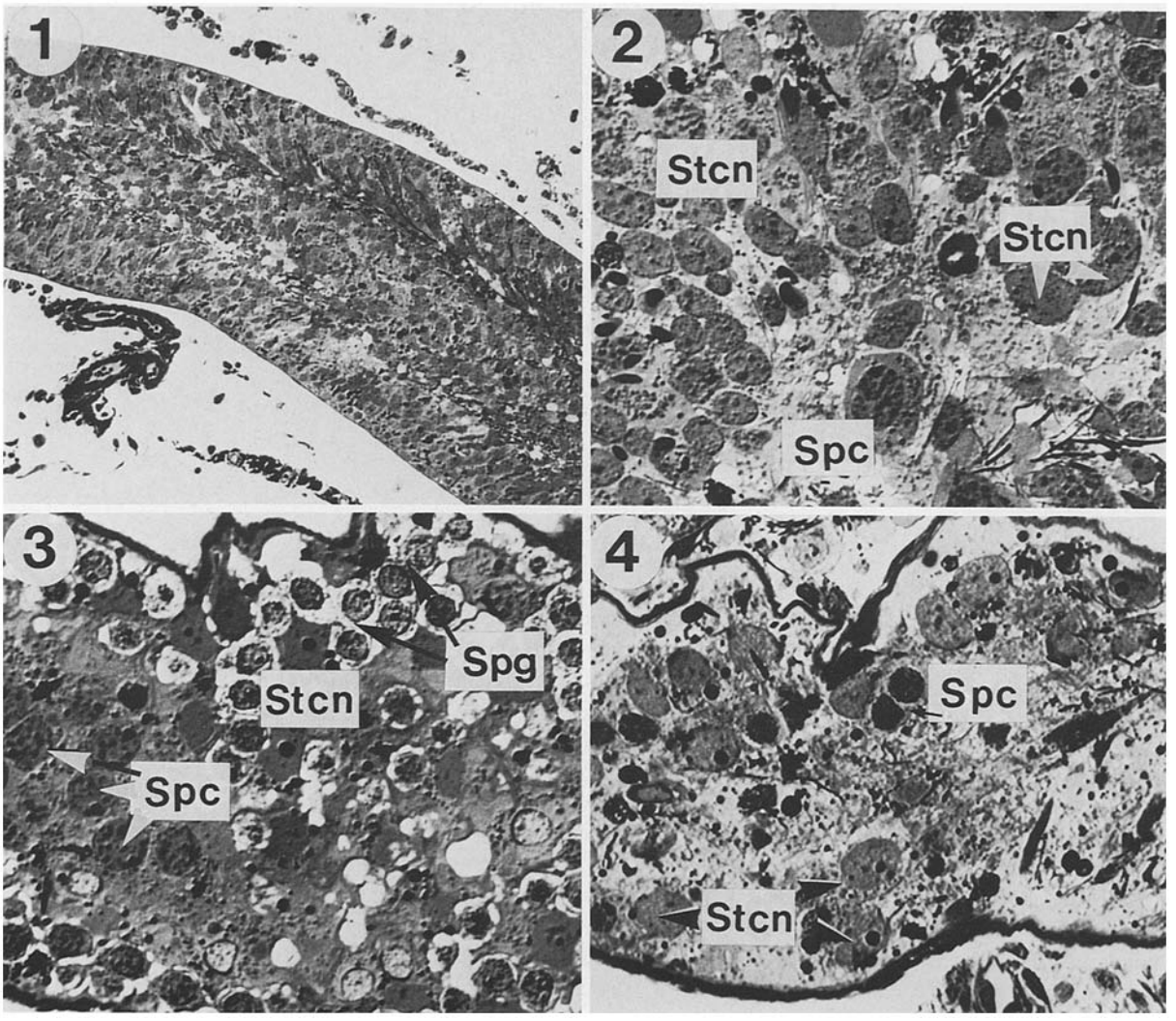


Fig. 1. A weakly GAA affected tubule showing the normal progress of spermatogenesis except spermiation. The luminal space is filled with detached germinal cells (magn.: 160:1)

Fig. 2–4. Progressive steps of epithelial disarrangement and tubular wall condensation (magn.: 800:1)

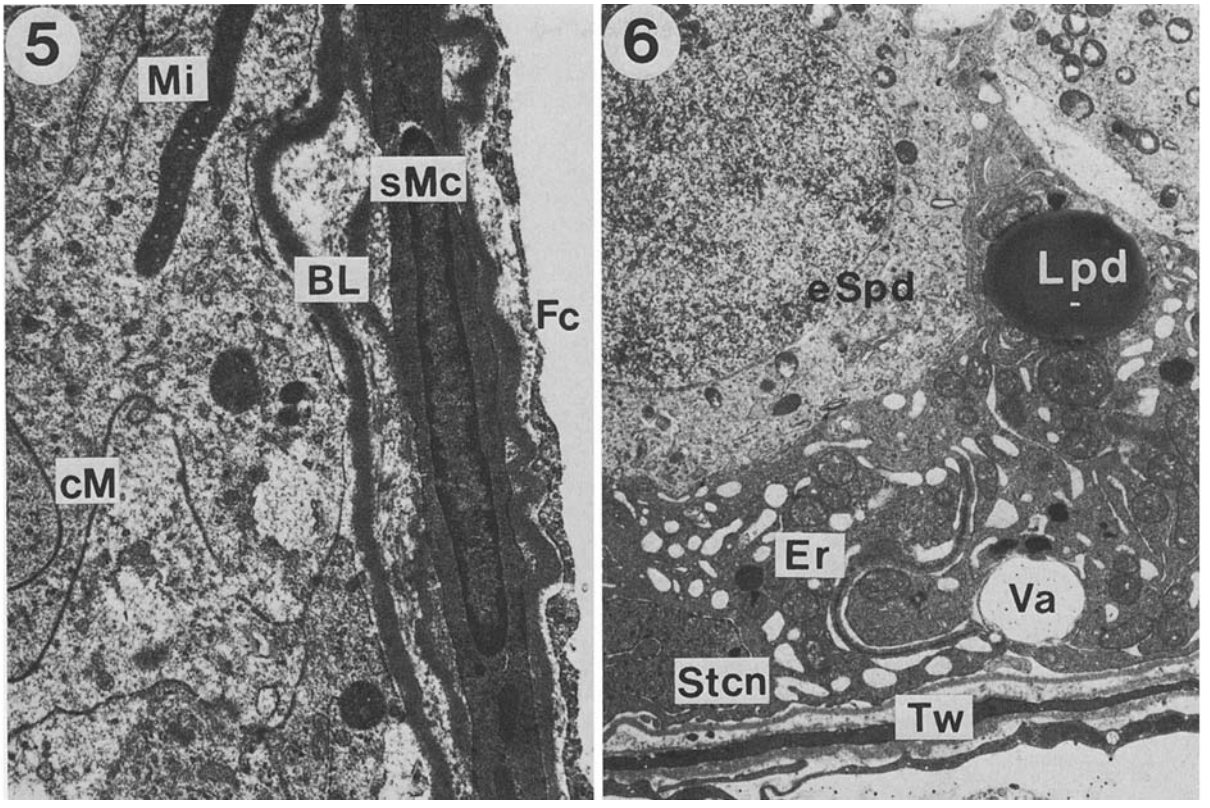
Abbreviations: *Ac* = Acrosomal complex, *AL* = Annulatae lamellae, *aL* = autophagic lysosome, *BL* = Basal lamina, *Cj* = Cell junction, *cM* = convoluted Membrane, *cMt* = condensed Material, *dCe* = distal Centriol, *Ep* = End piece, *Er* = Endoplasmic reticulum, *Erc* = Endoplasmic reticulum cysternae, *eSpd* = early Spermatid, *F1* = Flagellum 1, *F2* = Flagellum 2, *Fc* = Fibrocyte, *Gr* = Granule, *Gs* = Golgi system, *Ics* = Intercellular space, *Lpd* = Lipid droplet, *LSpd* = Later Spermatid, *Ly* = Lysosome, *Mi* = Mitochondria, *Mp* = Middle piece, *N* = Nucleus, *nSpc* = necrotic Spermatocyte, *pAr* = post Acrosomal ring, *pCe* = proximal Centriol, *PiGr* = Pigment Granule, *pNc* = post Nuclear cuff, *rEr* = rough Endoplasmic reticulum, *sMc* = smooth Muscle cell, *Spc* = Spermatocyte, *Spg* = Spermatogonium, *Stc* = Sertoli cell, *Stcn* = Sertoli cell nucleus, *Tw* = Tubular wall, *Va* = Vacuole

Vital and stained smears of the outdropping seminal fluid were prepared to evaluate the motility rate and the morphological appearance of the spermatozoa.

After cessation of treatment on day 70 the animals were anaesthesized by ether inhalation. Blood was taken by cardiac puncture, centrifuged and the serum was used for hormone analysis (testosterone, LH and FSH). The hormone analyses for testosterone and for LH and FSH were run in duplicate and in triplicate for the gonadotropins. The reproductive organs were carefully excised, weighed, and prepared for histological and electron microscopical investigations.

For light microscopy parts of the reproductive organs were fixed in Boulin's solution and after dehydration embedded in paraffin. The sections (6–8 μm) were stained either in Azan or in Hematoxylin-Eosin.

For electron microscopy small pieces were fixed in 1.5% glutardialdehyde, buffered in 175 mM cacodylate-NA solution (pH 7.3) for 1 h. Postfixation was done in a 1% aequous solution of OsO_4 for 2 h. After stepwise dehydration in ethanol the samples were embedded in Epon^R epoxy resin. Semithin and ultrathin sections were prepared on the Reichert microtome OM U3. The stained ultrathin



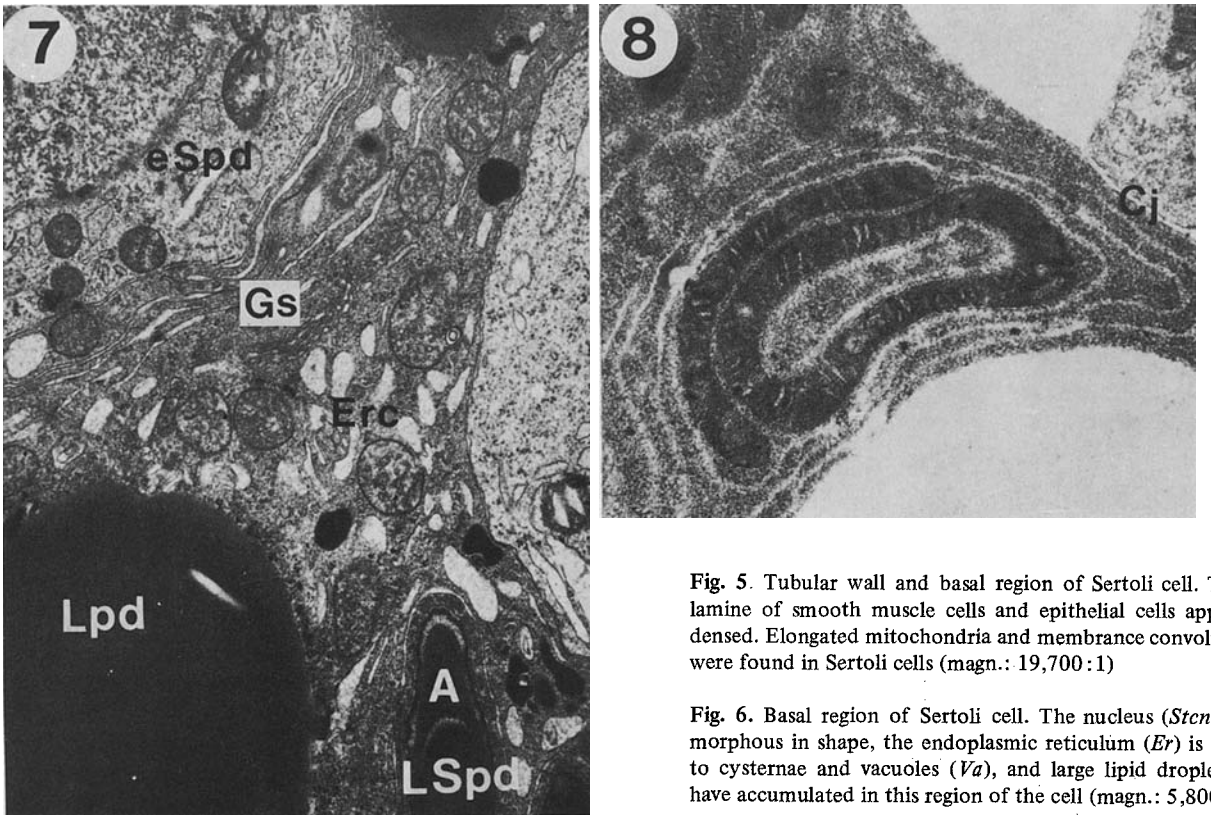
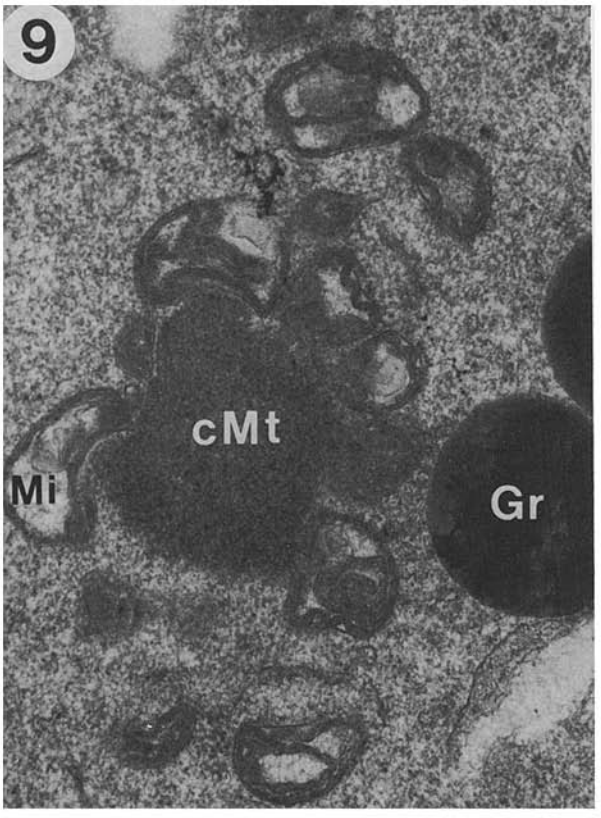


Fig. 5. Tubular wall and basal region of Sertoli cell. The basal lamine of smooth muscle cells and epithelial cells appear condensed. Elongated mitochondria and membrance convolutes (*cM*) were found in Sertoli cells (magn.: 19,700:1)

Fig. 6. Basal region of Sertoli cell. The nucleus (*Stcn*) is polymorphous in shape, the endoplasmic reticulum (*Er*) is extended to cysternae and vacuoles (*Va*), and large lipid droplets (*Lpd*) have accumulated in this region of the cell (magn.: 5,800:1)

Fig. 7. Middle part of Sertoli cell. Giant and electron dense lipid droplets (*Lpd*) are found in conjunction with numerous mitochondria. The endoplasmic reticulum (*Erc*) has disintegrated in single cysternae. The enclosed spermatid (*LSpd*) does not show malformations in its fine structure (magn.: 12,000:1)

Fig. 8. The formation of giant and characteristically arranged mitochondria is probably a consequence of an intensified lipoid synthesis (magn.: 12,000:1)


9
cMt
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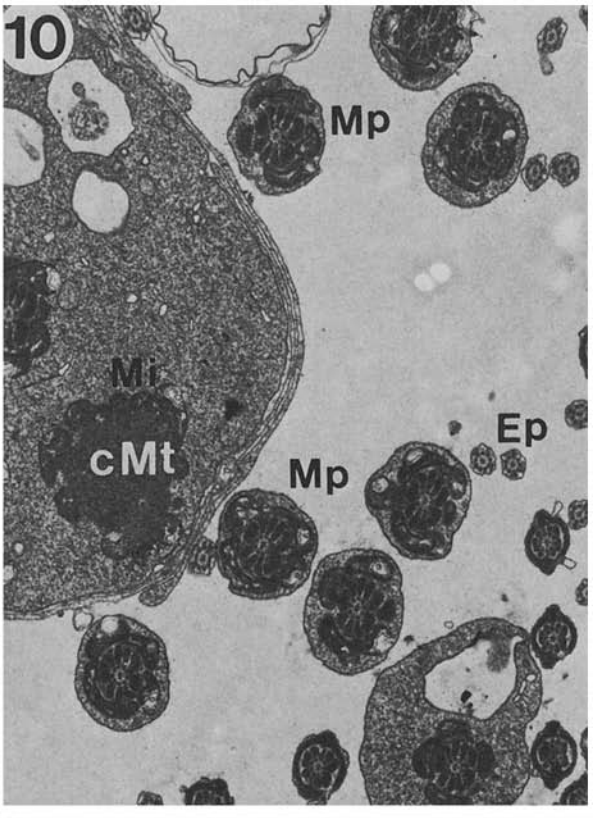
10
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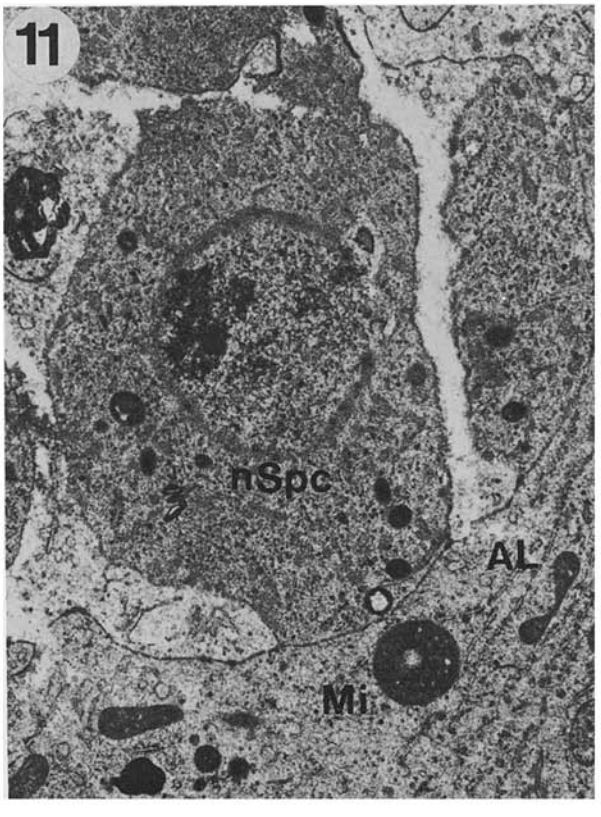
11
nSpc
AL
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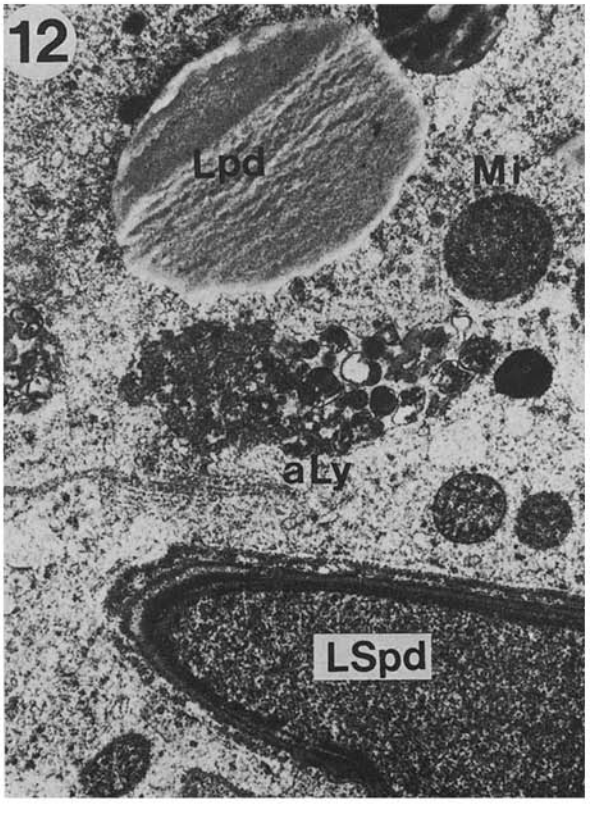

12
Lpd
Mi
aLy
LSpd

sections (uranyl acetate, lead citrate) were examined on a Philips 300 electron microscope.

Results

Histology of the Testis

Light Microscopical Findings. The histology of the testes of gossypol treated animals (30 mg GAA/kg/day for 70 days) appears quite variable. A large number of tubules show a histological arrangement comparable to controls. All stages of spermatogenesis are present, and normal spermatogenesis is indicated by convoluted spermatozoa in the lumen of the tubules. Close to these normal-shaped tubules, many tubules are characterised by a progressive necrosis up to the complete dissolution of the germinal epithelium (Fig. 1).

In weakly affected tubules spermatogenesis reaches the step of (or leads to) later spermatids. The processes of cell elongation, nuclear chromatin condensation, acrosome complex formation and the forming of the axial complex of flagella are similar to controls and do not show any malformations. Nevertheless, in these tubules spermatogenesis was never seen. No free spermatozoa were found in the luminal space. The tubular lumen is filled with cells detached from the germinal epithelium (Fig. 1).

In severely affected tubules the intercellular contact between Sertoli cells and germinal cells seems to be broken. Accumulations of dark and coarse granules were found both in the Sertoli cells and germinal cells. The single-lying spermatocytes of the pachytene stage appear either enlarged in size or show the typical state of cellular necrosis, indicated by their shrinkage and nuclear content condensation (Figs. 2 and 3).

The last step of epithelial necrosis is characterised by complete dissolution of germinal and Sertoli cells, and furthermore no distinct epithelial arrangements is still discernible (Fig. 4). At this stage of damage the tubular wall becomes fibrotic (Figs. 3 and 4).

Electron Microscopical Findings. Electron microscopical investigations were carried out on tubules previously described to be weakly affected. The tubules are surrounded by a smooth muscle layer, and one or two layers of fibrocytes (Fig. 5). The basal lamina appears to be condensed.

Tubular Epithelium

Sertoli Cells. Nearly all Sertoli cells show remarkable alterations in their fine structure. Primarily oval-shaped nuclei become polymorphous. Normally formed mitochondria of the tubular type are transformed to giant organelles with an irregular matrix structure. The organelles are arranged in a screwed and/or cyclic pattern (Figs. 5, 6 and 8).

The smooth endoplasmic reticulum is extended to wide cisternae, and demonstrates large vacuoles, largely accumulating in the basal and apical region of the cell (Figs. 6, 13 and 22). Frequently the stacks of lamellae are perforated similarly to the nuclear membrane. This special form of endoplasmic reticulum is referred to as "annulatae lamellae". Its distinct physiological function is not yet clear (Figs. 11 and 14). In close conjunction with the formation of giant mitochondria an intensified lipoid synthesis can be shown. Large lipid vesicles are irregularly scattered over the Sertoli cells. Their high osmophilic content (black-coloured) indicates the presence of unsaturated fatty acids (liquid lipids), the low osmophilic matrix points to siccated fats (deposit lipids: Figs. 6, 7 and 12). Obviously, the metabolic processes in the Sertoli cells are stressed. These facts imply an intensified abrasion of cellular organelles, and the formation of autophagic lysosomes (Figs. 9–11 and 21). Most of these lysosomes are stored as electron dense granules preferentially accumulating in the apical region of the cell (Figs. 9 and 15). These abnormalities are comparable to processes in ageing cells. Furthermore, the cellular contacts (desmosomes) are either restricted to a few and spotlike areas or completely dissolved.

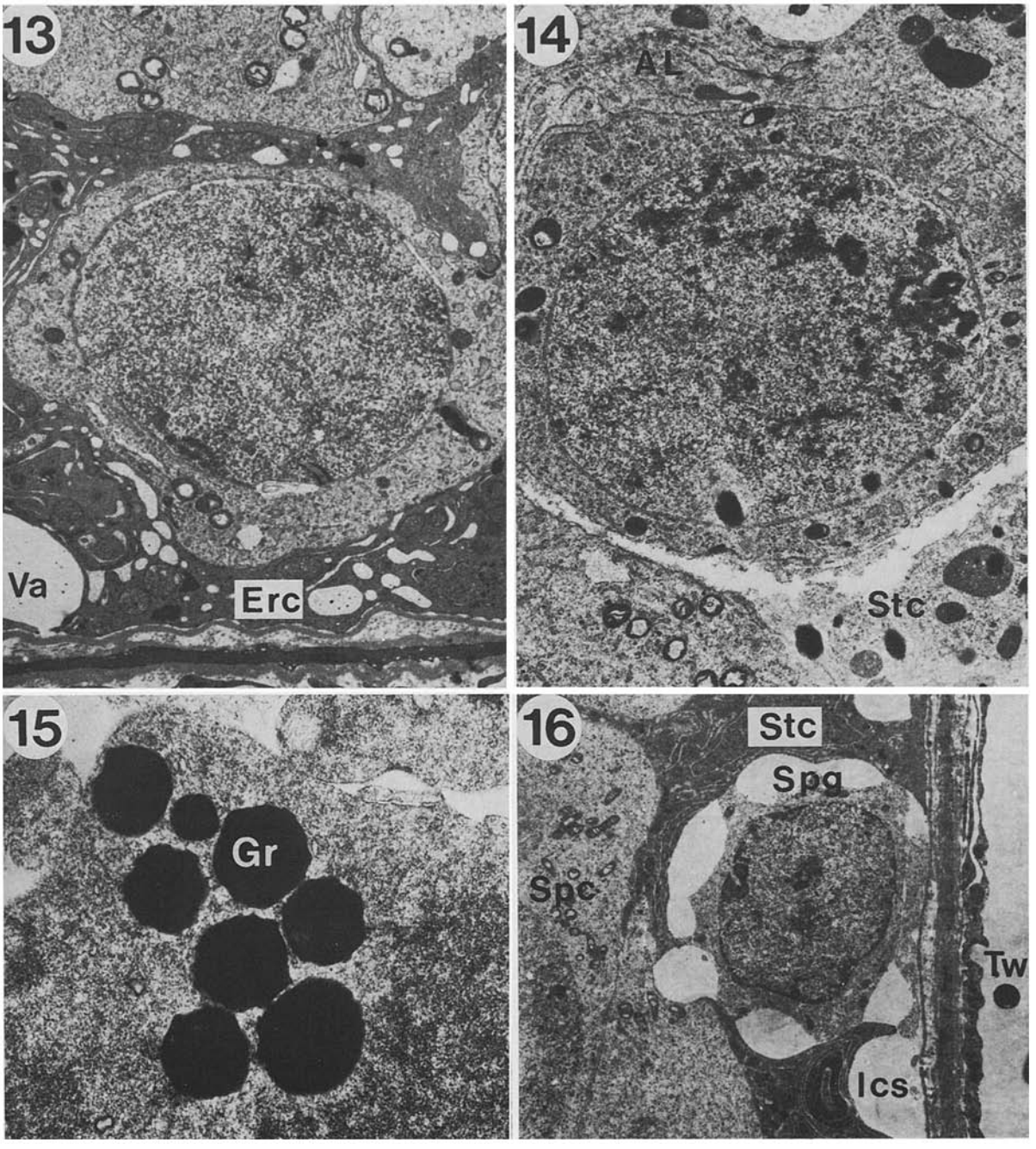
Germinal Cells. The morphological appearance of germinal cells depends on the extent of damage to the cell to cell junctions to Sertoli cells. In the case of more or less intact junctions the germinal cell fine structure does not differ from that of control sections (Figs. 13 and 14). If the cellular contact is confined to small desmosomic spots shrinkage processes, rudimentary nuclei, vacuolated endoplasmic reticulum, storage of granules and polynucleation in spermatids indicate the progressive necrosis in germinal cells. Most of these damaged cells are delivered to the luminal space and digested by heterophagic lysosomes, produced in Sertoli cells (Figs. 16–20 and 22).

Transformations are found particularly in later spermatids. The perinuclear microtubular cuff is sometimes formed asymetrically, while the acrosomal complex and the head membrane system do not show any malformations (Fig. 21). The mitochondrial chain of the middle piece is hardly affected. The matrix of the organelles appears vacuolated,

◀ **Fig. 9, 10.** Initial steps of electron dense granule formation. Dark granules are formed by enzymatic digestion of worn out cellular organelles as mitochondria (*Mi*) and endoplasmic reticulum lamellae. The granules are preferentialy stored in the apical and basal region of the Sertoli cell (magn.: 9 = 31,000:1; 10 = 12,00:1)

Fig. 11. Germinal cells like spermatocytes (*nSpc*) show a progressive necrosis of nuclei and lytic processes of cytoplasm. The cell to cell junctions are confined to small areas, and the intercellular spaces appear dilated. In Sertoli cells a variation of endoplasmic reticulum, the "Annulatae lamellae" (*AL*) was found (magn.: 4,600:1)

Fig. 12. Large lipid droplets (*Lpd*) and autophagic digestion of cellular organelles characterise the GAA affected Sertoli cell. In contrast, the later spermatids do not show alterations in their fine structure (magn.: 14,000:1)



Figs. 13 and 14. A considerable number of spermatogonia (13) and early spermatids (14) was found to be unaffected in the fine structure, but the beginning of cell junction dissolvement is already evident (magn.: 5,800:1)

Fig. 15. Depot granules in the apical region of Sertoli cells (magn.: 12,000:1)

Fig. 16. Shrinking processes of spermatogonial cytoplasm restrict cell junctions to small spots while the intercellular spaces appear more and more extended (magn.: 3,200:1)

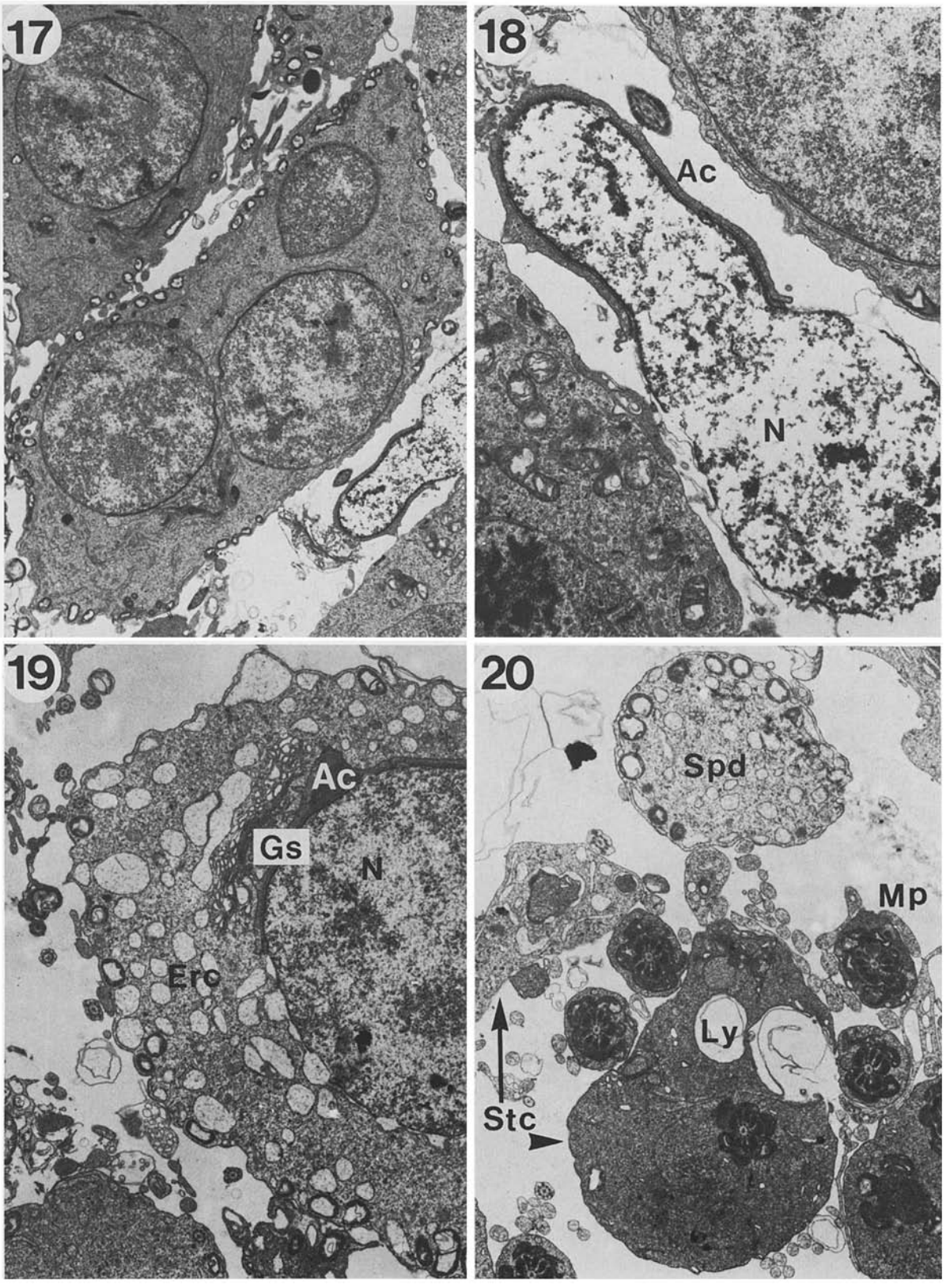


Fig. 17–20. Many germinal cells and portions of Sertoli cells are detached from the epithelia into the tubular lumen. Spermatids frequently show polynucleation (17). Spermatozoa (18), spermatids (19), and portions of Sertoli cells (20) undergo enzymatic cellolysis. The middle piece of flagellae show distinct necrotic alterations in mitochondria (magn.: 17 = 4,600:1; 18, 19, 20 = 12,000:1)

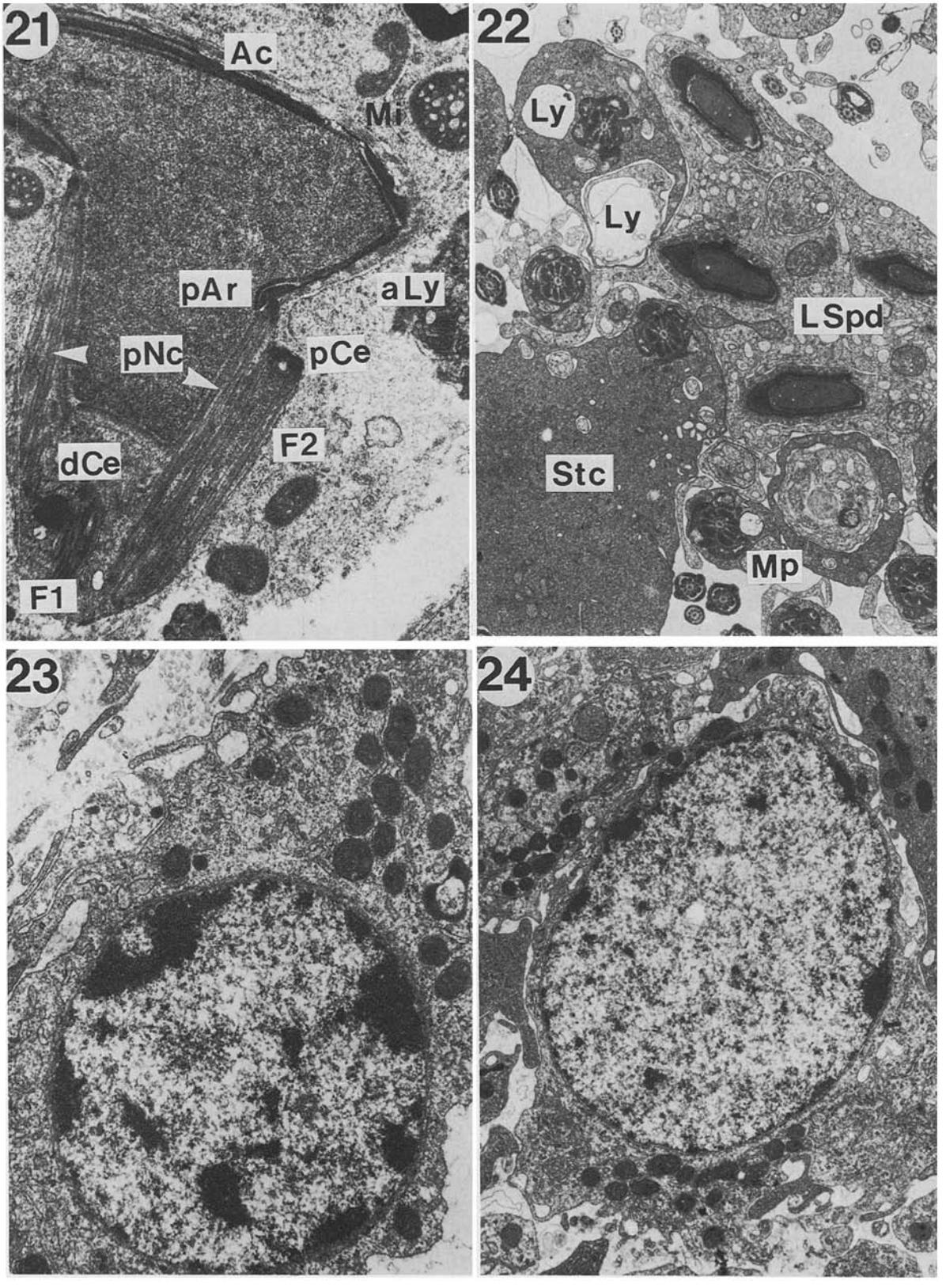
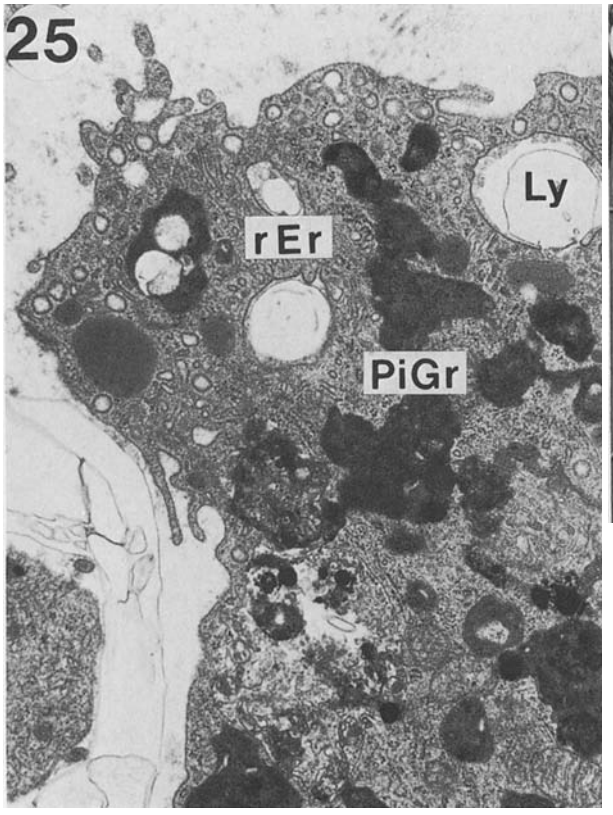


Fig. 21. In some cases GAA uptake induced an irregular development of later spermatids. The nuclear elongation, supported by the cuff of postnuclear fibrils (*pNc*), proceeds asymmetricaly, and sometimes two axial complexes (*F1* and *F2*) are formed (magn.: 14,000:1)

Fig. 22. The fine structure of sperm heads does not indicate a GAA effect on the acrosomal complex and head membrane system. In contrast, the mitochondrial shearth of the middle piece shows distinct malformations. The residual cell fragments of Sertoli cells are partially disintegrated by lysosomal enzymes (magn.: 12,000:1)

Figs. 23 and 24. The fine structure of Leydig cells is not altered by GAA uptake (magn.: 4,600:1)



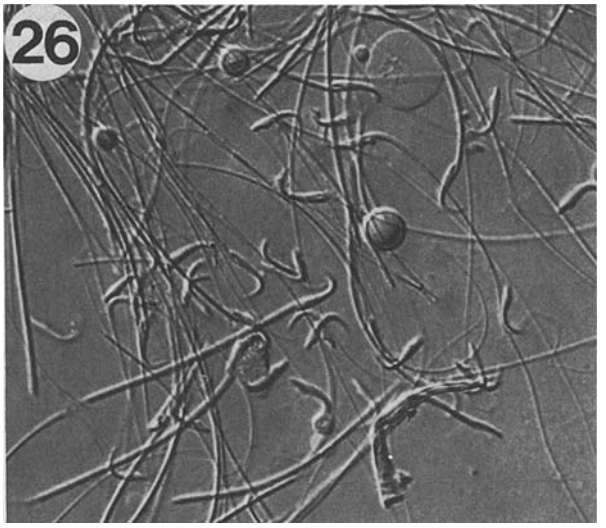


Fig. 25. Worn out Leydig cells with the typical pattern of pigment granules (PiGr) are the only "abnormal" cells which were found in GAA affected testes (magn.: 14,000:1)

Fig. 26. In a few cases head tail disconnections were observed in vas deferens exsudants (magn.: 800:1)

and the membranes are partially dissolved (Figs. 10, 20 and 22). In contrast, the tubular arrangement and the membrane system of flagellar main and end piece do not show alterations. In no case was a leukocyte infiltration found either in the epithelial compartment or in the luminal space of the tubules.

Interstitial Compartment

Gossypol administration seems to have no toxic effect on the morphology and cellular physiology of the interstitial compartment. The fine structure of Leydig cells and their physiological activities are not injured by GAA treatment (Figs. 23 and 24). Only in a few cells lipofuscin pigments as dark spots indicate evidence of damage (Fig. 25). The lymphoid spaces, the morphology of fibrocytes and the vascularisation appear normal. These findings are in accordance with the endocrine profiles obtained from GAA-treated rats in which the testosterone plasma levels do not differ significantly ($p > 0.05$) from untreated controls.

The morphology of the spermatozoa from the smear of the vas deferens at the 43rd day of GAA treatment was altered in at least half of the samples examined. In these instances head-tail disconnections were found (Fig. 26).

Discussion

The antifertility efficacy of GAA in male rats is reported to be dose, time, and strain dependent [3, 4, 18, 29, 22, 28, 28a, 26]. In male rats treated with 2.5 mg, 5.9 mg and 7.5 mg GAA/kg/day the fertility rate was not significantly lowered ($p > 0.05$) from approximately 90% to 50–70% [26]. In contrast, lower dosages in the range of 4 mg to 10 mg GAA/kg/day caused loss of fertility in male rats [7, 25]. Shi [22] postulates 12 mg GAA/kg/day as an effective antifertility dose. Zhou [30] found that administration of 7.5 mg GAA/kg/day for a period of 1 year led to infertility without gross degenerative changes in the testes and mating behaviour. In the present study 30 mg GAA/kg/day was administered to 20 male rats for 10 weeks. After 6 weeks mating results and semen smears from the vas deferens previously indicated a 100% infertility of the treated animals. The same effect in a similar period of time is referred to by Shi [22] and Xue [28, 28a].

According to information available in the literature orally administered GAA, given in a sufficient dosage for a sufficient period of time leads to complete immobilisation of epididymal and vasal spermatozoa in small animals [2–4, 7, 9, 11, 12, 17, 20]. Frequently, a significant decrease in sperm density and severe damage on sperm morphology up to head to tail disconnections were found. The present findings confirm these malformations, except the decrease of sperm number. Electron microscopical investigations define these abnormalities: swelling of membranes, dissociation and fragmentation of acrosomes, swelling and derangement of the mitochondrial sheath and axial complex [6–9, 11, 12, 17, 18, 28]. Despite this severe damage to sperm morphology no morphological deviations of the

epididymal and vasal epithelia were found [12]. Experiments involving ligation of the epididymis indicate that the site of action of GAA in inhibiting the sperm motility is not in the epididymis. A direct effect on the seminiferous epithelia may be assumed [6]. However, if GAA is directly brought into the fat pat of the rat epididymis alterations in sperm morphology and inhibition of sperm motility occured within 24 h [8]. In agreement with the reports of Hadley [9] and Hoffer [12] the germinal epithelial cells show vacuolisation, pycnosis, disconnections of junctions, cytolysis, and exfoliation of germ cells from the epithelium. According to the papers of Xue [28] the first cellular damage was found on spermatids after 2–3 weeks of GAA treatment. Pachytene, leptotene and zygotene spermatocytes and spermatogonia B showed malformations after 4–5 weeks. The affected seminiferous tubules became atrophic, and only a single layer of spermatogonia and Sertoli cells formed the epithelia.

Summarising the results of published papers, abnormalities which concern sperm morphology and progressive damage of germ cells are extensively discussed, but no detailed information is noted on the morphology and fine structure of Sertoli cells. Nevertheless, this investigation shows that the Sertoli cells are affected, too. GAA uptake seems to stimulate the physiological activity pathologically; cellular organelles such as mitochondria, endoplasmic reticulum, lysosomal vacuoles, pigment granules and nuclei are either enlarged in number and size or malformed in shape. The cellular contact is often restricted to spots or completely disconnected. The necrotic processes and exfoliation of germ cells are probably due to junction dissolvement. If GAA administration is continued for a longer period some Sertoli cells were found to be unable to compensate for the toxic stimmulus of GAA, and the cells became necrotic too. Large apical protrusions were delivered to luminal spaces. Irregulary formed membrane complexes of digested organelles, condensed granules, lipid droplets, and large vacuoles were found within the cytoplasma. In later spermatids, surrounded by necrotic Sertoli cells, remarkable damage on the mitochondrial sheath was found. The matrix of the organelles appears vacuolated, and the membrane compartment often dissolved.

In contrast to the report of Nadakavukaren [17] no changes were found in the axial complex except some irregular arrangements of perinuclear cuff, centrioles, and insertion of flagellum. However, in all seminiferous tubules showing these malformations a distinct number of unaffected Sertoli cells was also found.

These data indicate that GAA might have a primary effect on the Sertoli cells and secondly on germinal cells and on sperm motility. The inhibition of testicular ATP-ase [14, 14a] and sperm specific lactate dehydrogenase X [15] may be the initial source of the antifertility action of GAA. In vitro experiments, including the pronounced depression of spermatozoal fructose utilisation, confirm this assumption [14, 19, 23].

In contrast to toxic processes in germinal and Sertoli cells the Leydig cell compartment does not show any change in fine structure, and an intact testosterone biosynthesis is assumed. In accordance with our findings Hoffer [11] and Bardin [2] found no significant changes in serum LH, FHS and testosterone.

In contrast to a preliminary in vitro study Hadley [9] noted a significant decrease of testosterone biosynthesis in Leydig cells. It seems to be true that at the beginning of the in vitro experiment a lot of Leydig cells were killed by GAA so that the initial testosterone levels were lowered. Nevertheless, after a certain time the amount of testosterone stimmulated by HCG rose in controls and in GAA-treated samples.

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