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Effect of Methoxychlor on Reproductive Systems of the Rat (41861)

HARPAL S. BAL

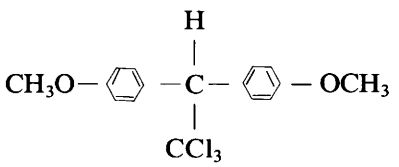
Department of Veterinary Anatomy, College of Veterinary Medicine, Iowa State University, Ames, Iowa

Abstract. Methoxychlor (MeOCL), a chlorinated hydrocarbon pesticide, was administered by oral gavage for 70 days to male and 14 days to female rats at dose levels of 100 and 200 mg/kg/day of body weight. Methoxychlor inhibited spermatogenesis in the males and folliculogenesis in female rats. The sustentacular (Sertoli) cells showed degenerative fatty changes as a result of which blood–testis barrier could have been affected. Degenerative changes of the spermatogonia and the spermatocytes and transformation of some of these cells into polynucleated and binucleate cells were observed. Myelin figures and degenerate mitochondria were observed in the binucleate cells at the ultrastructural level. Some of the seminiferous tubules were devoid of all cellular elements with the exception of spermatogonia. The epithelium of the ductus epididymis manifested conspicuous cytoplasmic vacuolations, and near the tail, the duct was distended with fluid which compressed the lining epithelium. Atresia of the ovarian follicles was evident with pyknosis and karyorhexis of the granulosa cells.

The original objective of this research project was to study the mutagenic effect of methoxychlor on the rat. The experiment was designed to carry out at least three generation studies on the effect of methoxychlor following the protocols suggested by the U.S. Food and Drug Administration (1). Unfortunately, as a result of the experiment, the treated female rats bred to the treated males did not bear any progeny except the controls.

The histopathological evaluation of testes, epididymis, and ovaries of rats treated orally with 100 and 200 mg/kg of methoxychlor is reported in this study. Although the previous investigators have studied the toxicity effects of methoxychlor, none has reported histopathological changes of the reproductive organs of the animals studied.

Methoxychlor, a chlorinated hydrocarbon insecticide with the chemical structure



has a composition of 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane. This compound is closely related to DDT. Since 1960, the World Health Organization (WHO) has indicated a need for a suitable replacement for DDT because of insect resistance and toxicity to animals (2). Methoxychlor is less toxic than DDT. It has a broad range of effectiveness and long residual action compared to other approved insecticides (3, 4).

The acute LD_{50} of methoxychlor in rats is 6000 mg/kg body weight (4, 5). At dose levels lower than 6000 mg/kg body weight, methoxychlor affects the testes. Weanling rats fed with 1% methoxychlor exhibited histopathological changes in the testes, accessory sex glands, and kidneys (6). In male rats, *exposure* to 1% or more of methoxychlor in feed resulted in pathological changes of the rat testes. No pathological changes in the ovaries of female rats were produced by doses less than 100 mg/kg/day (6–9). Methoxychlor fed 3% in diet for 45 days to adult male rats caused apparent suppression of spermatogenesis, although sustentacular (Sertoli) cells and spermatogonia appeared normal (5). However, methoxychlor fed to rats mixed with Purina Fox Chow meal at a concentration of 0.0025 to 0.16% over a 2-year period had no abnormal effects on the testes.

Osborne–Mendel female rats were fed with 1% methoxychlor in corn oil incorporated in ground commercial laboratory meal at a level of 100 to 500 ppm for 104 weeks. Fifty percent of the female rats developed carcinoma of the ovaries (10).

BALB/c and C3H male mice of 3-weeks age ingested 750 ppm of technical methoxychlor mixed in ground laboratory meal for a

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period of 2 years. Fifty-three percent of the methoxychlor-treated males and 11% of the controls developed carcinoma of the testes. Tumors of mice treated with methoxychlor were more malignant than they were in the controls (10). Other effects of methoxychlor such as carcinoma of the liver in mice and dogs, atrophy of the testes in the rabbit and hyperplasia of the mammary glands and uterus in swine were also reported by the same investigator (10).

Estrogenic activity of methoxychlor *in vivo* and a rare case of anemia in a human after methoxychlor exposure have recently been reported (11–13). Methoxychlor was a recommended pesticide since the 1950s for the control of ectoparasites in dairy cattle, although its excretion in milk did not represent any threat to human health (14). The objective of this investigation was to determine the effect of methoxychlor on the testes and ovaries of the rat.

Materials and Methods. Forty specific-pathogen-free (SPF) rats (10 weeks old), 15 males and 25 females, of Wistar strain (Cesco Lab Co., Omaha, Nebr.) were used in these studies.

Males. The male rats were divided into two treatment groups and a control group of five each. Two dose levels of technical methoxychlor[1] were tested in this investigation. One group of five male rats were given 100 mg/kg/day of methoxychlor (1% solution) suspended in gum tragacanth, and the other group received 200 mg/kg/day (2% solution) of methoxychlor in Mazola corn oil[2] by intubation. Controls were dosed with the vehicle solutions at similar dose levels, respectively. These are the nontoxic vehicles in which methoxychlor is either soluble (corn oil) or can remain suspended as emulsion in water with gum tragacanth. The dosing was continued for a period of 70 days to cover the entire spermatogenic cycle of the male rats.

Females. The female rats were divided into two groups of 10, and each group was administered methoxychlor by intubation at the rate of 100 mg/kg/day in gum tragacanth suspension and 200 mg/kg/day in Mazola corn oil for a period of 3 weeks (four estrous cycles) before mating. Female control rats were divided into two groups of five and each group was given gum tragacanth suspension in water and corn oil at the same dose levels as the treatment groups for the same period of four estrous cycles. The rats were caged individually and provided *ad libitum* with Teklad[3] mouse and rat diet containing 4% fat. Water was available 24 hr/day.

After the treatment period, the male and female groups treated at similar dose levels of 100 and 200 mg/kg/day, respectively, were mated. Similarly the female controls were mated with the male controls. Two females were caged with a male during estrus in the evenings. Vaginal smears were taken the next morning and examined for the presence of sperm under the light microscope. Females with positive sperm in the smear or vaginal plug were considered to have conceived. The day on which coagulated semen (with/without visible sperm) or vaginal plugs were present in the vagina of the female rats was considered as Day 1 of gestation. The matings were continued for a period of 15 days.

Twenty females which were observed to have conceived were put in separate cages. They were dosed daily (100 and 200 mg/kg/day to the respective groups) during the entire period of gestation. Half of the females treated with each respective dose level were killed on Day 13 of gestation in order to examine for the number and distribution of embryos in each uterine horn. The rest of the females were killed after the expiration of the gestation period since they did not litter. Neither viable nor dead embryos and fetuses were observed in the female rats killed on Day 13 of gestation nor in those after the expiration of the gestation period. Viable embryos were present in half the control group of female rats killed on Day 13 of gestation.

Since the methoxychlor-treated females turned out to be barren, it was appropriate to examine the male reproductive organs, especially the testes. The testes (including the epididymis) of half the methoxychlor-treated

[1] Obtained from E. I. Dupont Nemours and Company, Inc., 5725 East River Road, Chicago, Ill. 60631.

[2] Best Foods, Englewood Cliffs, N.J.

[3] Teklad Mills, Winfield, Iowa.

rats, controls and the ovaries of females were fixed in 10% buffered neutral Formalin solution. The parts of the ovaries that needed to be examined were the corpora lutea and the follicles. It was not deemed necessary to study the histopathological changes of the ovaries at the fine structure level since the degenerative changes of the follicles were quite obvious. Two male rats which were unlikely to survive after 9 weeks of treatment with methoxychlor were killed and their tissues were collected for histological evaluation. The ovaries and testes of the rats were embedded in paraffin, sectioned at 6 μm in thickness, and stained with Harris' hematoxylin and eosin.

Electron microscopic technique. The male rats were anesthetized and perfused through the descending aorta with 2% glutaraldehyde solution. The tissues were excised and cut in 1-mm blocks and fixed in 1% OsO_4 (osmium tetroxide) in buffer for at least 2 hrs, washed overnight in cold buffer, dehydrated in ascending grades of ethanol, and embedded in Epon. Poststaining after osmification with 0 to 5% uranyl acetate in acetate buffer was done *en bloc.* Ultrathin sections were cut and stained with uranyl acetate and lead citrate. They were examined and photographed under the Hitachi HU 12A transmission electron microscope at 50 kv.

Results. *Light Microscope Changes. Testes.* There were degenerative changes in the seminiferous tubules of all the treated rats. There was so much deviation in the tubular morphological manifestation from one animal to another that the whole phenomenon can be described as a disorganized picture of the seminiferous tubules. The following histopathological changes were observed in the seminiferous tubules.

Sustentacular (Sertoli) cells. Degeneration of these cells was observed. Large vacuoles appeared in the cytoplasm toward the adluminal compartment of the seminiferous tubules (Fig. 1). In the center of the tubular lumina, fragments of sustentacular cell cytoplasm and pyknotic nuclei of germ cells were observed.

Spermatogonia. The cellular relations within the seminiferous tubules varied between animals and within the same testis. Spermatogonia of A type were observed in mitotic divisions along the periphery of some tubules. Intermediate and B-type spermatogonia were also observed. Seminiferous tubules with advanced degenerative changes were devoid of all cells except a few spermatogonia at the periphery and disfigured sperm (Fig. 2).

Spermatocytes. Primary spermatocytes were disorganized, manifesting a variety of morphological changes. Some of these cells retained their intercellular connections but were displaced. A few appeared to be in the initial stage of the first maturation division. No further advancement toward complete maturation was evident.

Other changes in the seminiferous tubules. Multinucleated and binucleated cells were observed (Figs. 1, 7). Some of these cells appeared in degenerate forms. Degenerated cells and some calcified bodies were seen. A few spermatids were seen scattered and disoriented in some tubules (Fig. 6). Hyaline degenerate bodies, probably of multinucleated cells, were present in tubules exhibiting such changes. Large vacuoles toward the center of the tubules were not uncommon (Fig. 1).

Ductus epididymidis. Ballooning and vacuolation of epithelial cells of the ductus epididymidis were observed (Fig. 4). At about the region of the tail of the ductus epididymidis, there appeared to be some blockage of the flow of the seminal fluid. Here the ductus epididymidis was distended with seminal fluid resulting in compression of the lining epithelium. There were desquamated degenerate epithelial cells in the lumen of the duct.

Ovaries. The ovarian follicles of the female rats treated with methoxychlor were atretic. The granulosa cells of the ovarian follicles exhibited all the initial forms of degenerative changes characterized by pyknosis and karyorhexis of their nuclei. Desquamation of these granulosa cells was also evident (Fig. 3). These degenerative changes in the ovarian follicles probably contributed to failure of ovulation and conception in the females.

Ultrastructural changes in the seminiferous tubules. In sections of the seminiferous tubules of the control rats, the mitochondria in the spermatids were lined along the periphery close to the plasma membrane (Fig. 5). These mitochondria do not have the characteristic cristae but appear as rounded vesicles with a

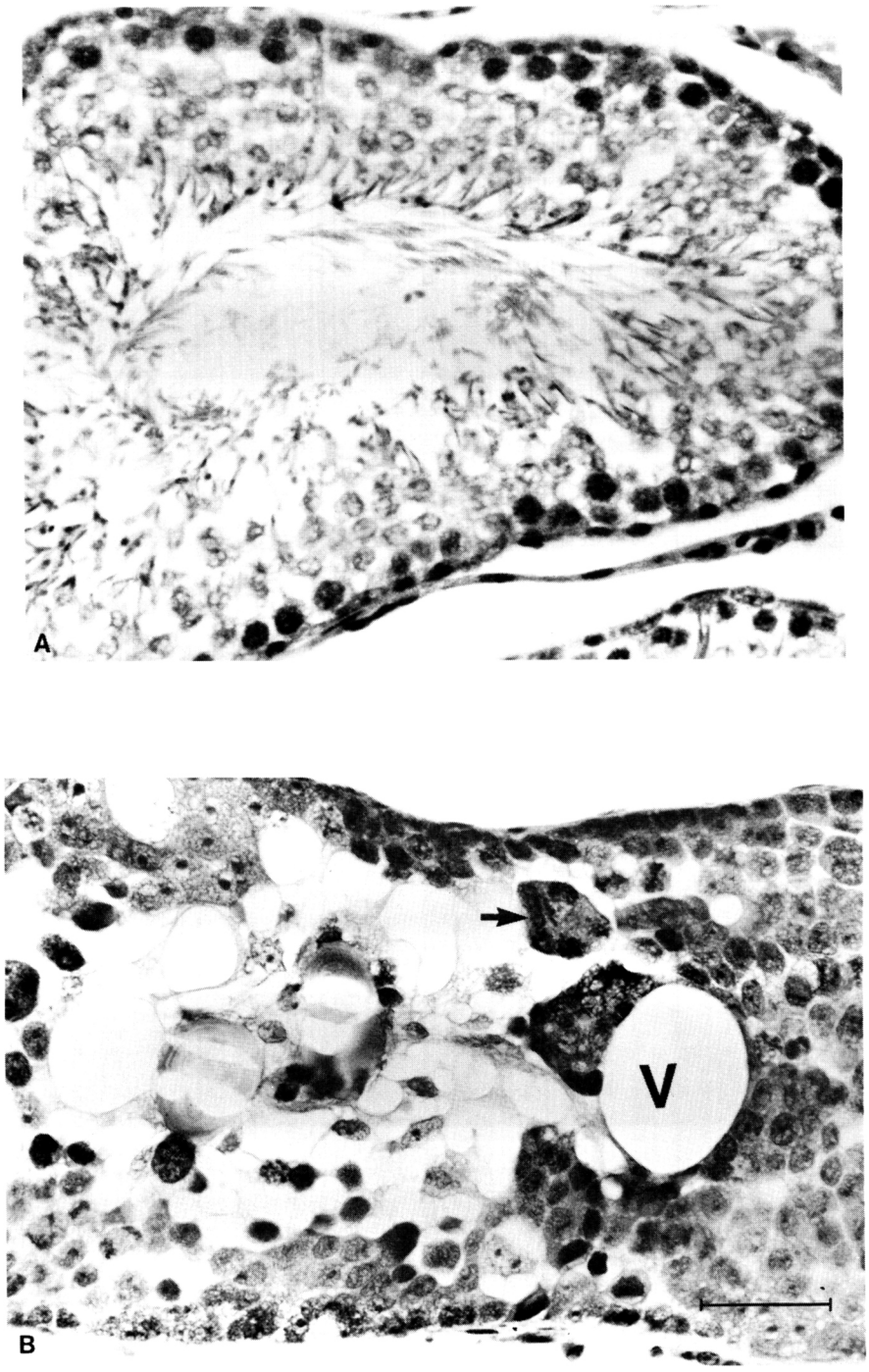

FIG. 1. (A) A section of seminiferous tubule of a control rat showing normal process of spermatogenesis. (B) Section of a seminiferous tubule of methoxychlor-treated rat showing vacuoles (v), multinucleated cells (arrows), and overall disorganization. H & E, ×570.

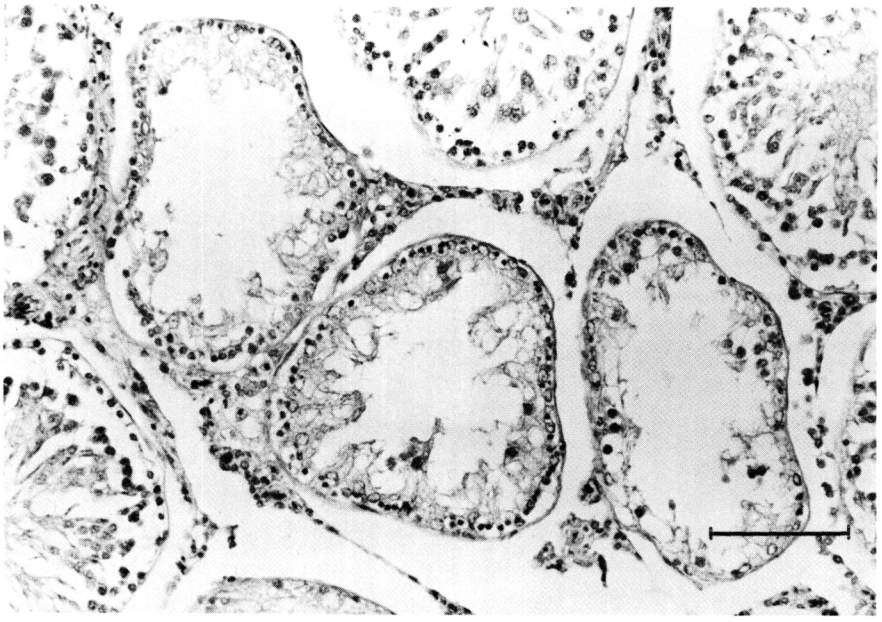

FIG. 2. Section of the testis of methoxychlor-treated rat showing lack of normal spermatogenesis. It appeared that all cell types except spermatogonia have degenerated. H & E, ×143.

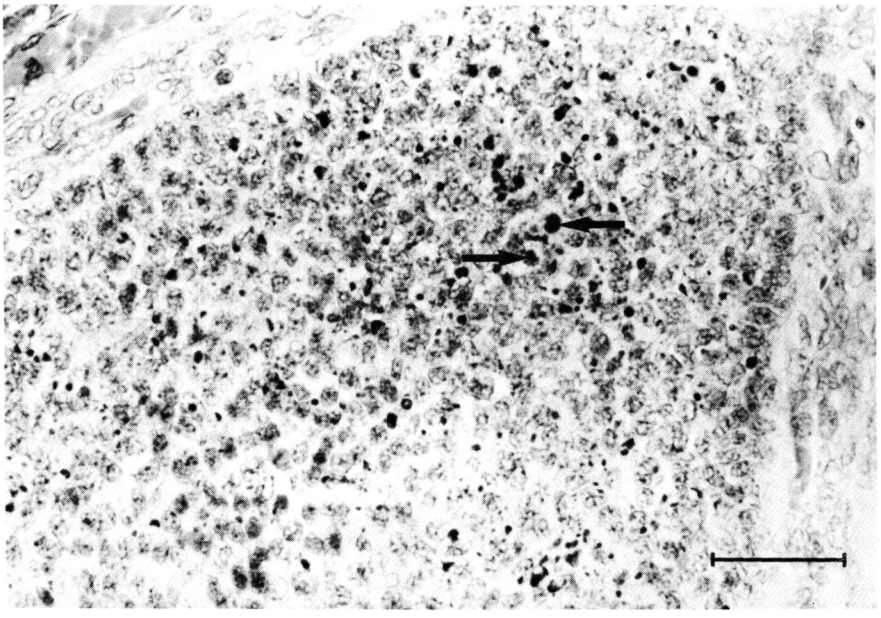

FIG. 3. Section of an ovarian follicle of methoxychlor-treated rat showing degenerative changes (atresia) of the granulosa cells (arrows). H & E, ×360.

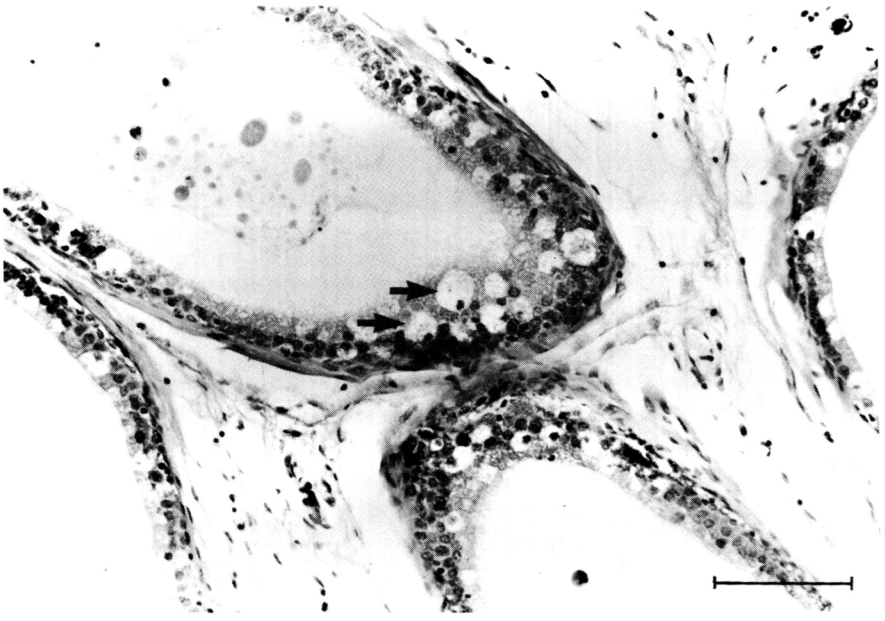

FIG. 4. Section of the ductus epididymidis of methoxychlor-treated rat showing vacuolation and ballooning of the epithelium (arrows). H & E, ×143.

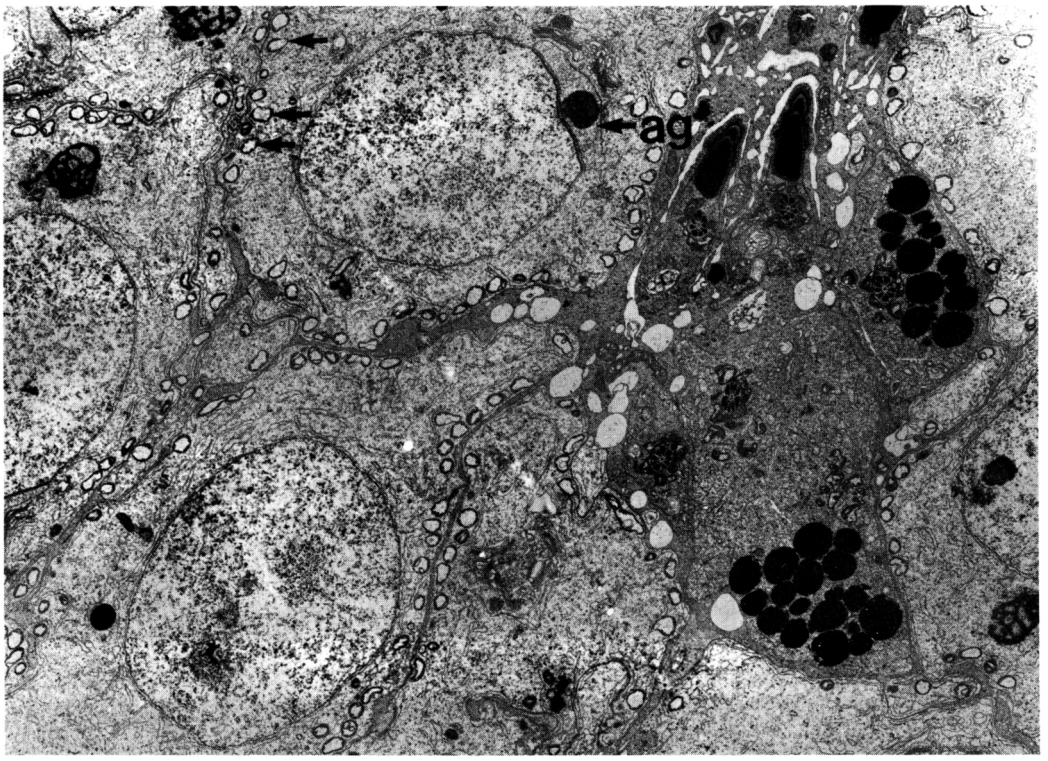

FIG. 5. Section of the spermatids of the control rat showing energized mitochondria (arrows) at the periphery and acrosome with granule (ag). ×4675.

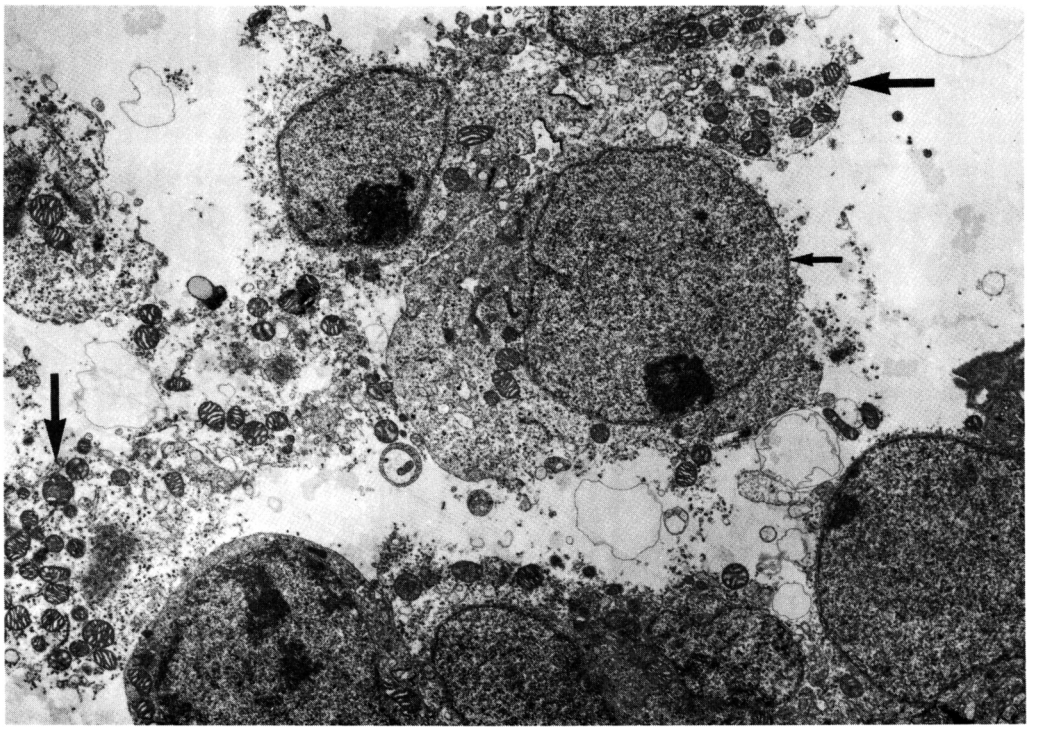

FIG. 6. Section of the seminiferous tubule of methoxychlor-treated rat showing cytoplasmic debris (large arrows) and scattered spermatids (small arrow). ×4675.

double membrane. Such a type of mitochondria manifesting a special functional state has been referred to as energized mitochondria. In those spermatids which had almost completed the process of spermiogenesis, the mitochondria appeared to be reforming their cristae around the flagellum.

In the methoxychlor-treated animals, multinucleated and binucleated cells were seen as in the light microscopic sections of the seminiferous tubules. In these cells, the mitochondria appeared to show degenerative changes with vacuoles around them. A variety of degenerative morphological manifestations of mitochondrial colonies within the binucleated cells was observed (Fig. 7).

In the lighter-appearing multinucleated cells, larger than normal-appearing nucleoli were observed. The multinucleated cells appeared to be the deformed spermatocytes which were fused probably as a result of abnormal cytokinesis. In the normal process of cytokinesis, intercellular bridges are formed in these germ cells and persist up to the spermatid stage.

Degenerating cells were either multinucleated or were surrounded by sustentacular cell cytoplasm. The sustentacular cell cytoplasm had a number of membrane-bound vesicles and other inclusions.

In the sections of the nuclei of some sustentacular cells, there were more than six nucleoli. There appeared some desquamated spermatids and some extracellular organelles in the lumen of the tubules. Occasional macrophages with inclusions and secondary lysosomes were observed. The synchrony of the spermatogenic cycle appeared to be disrupted.

Discussion. From the histopathological changes in the reproductive organs studied in this investigation (testes, epididymis, and ovaries), it is evident that methoxychlor impairs their normal reproductive processes. Previous investigators have used lower doses of methoxychlor in feed (less than 1%) without reporting detailed histopathological changes.

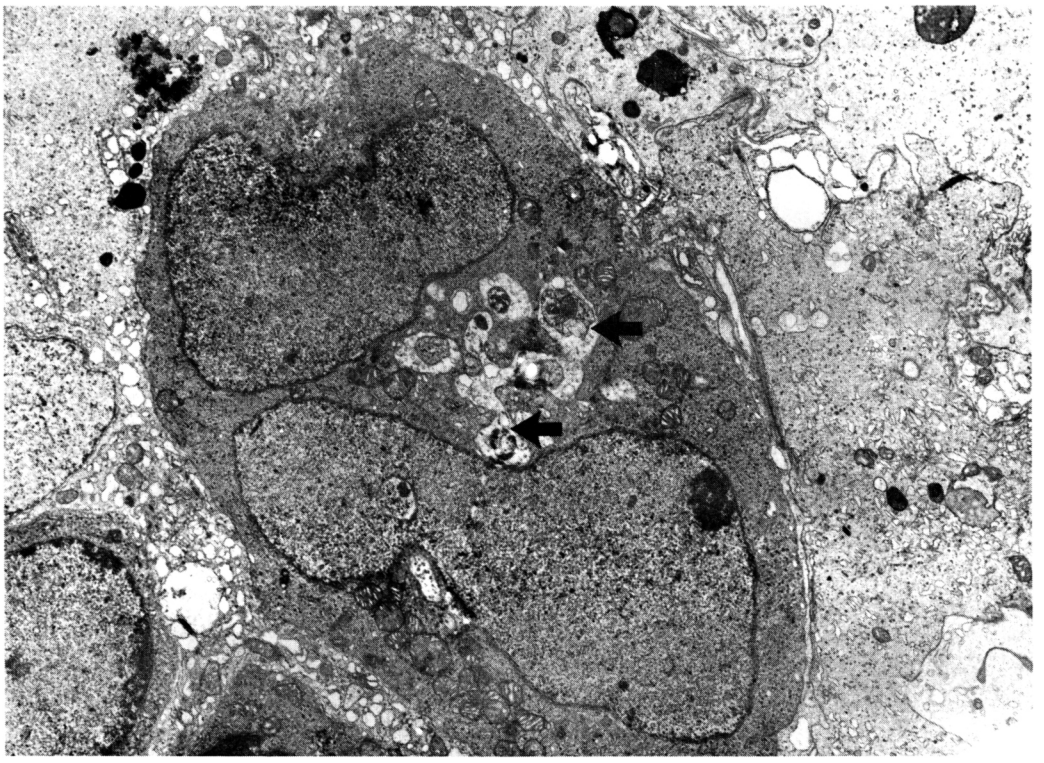

FIG. 7. Section of the seminiferous tubule of methoxychlor-treated rat showing a binucleated cell with degenerating mitochondria (arrows). This cell is almost surrounded by sustentacular cell cytoplasm with many clear membrane bound vesicles. ×5170.

Some have used 3% methoxychlor in diets which did not cover the entire spermatogenic cycle in the male rats (5, 9). It was, therefore, considered appropriate to study the effects of methoxychlor at the dose levels used in this investigation.

The main regulatory cell of the normal process of spermatogenesis is the sustentacular cell with multiple functions (15, 16). In the sections of the methoxychlor-treated rats, large vacuoles in the cytoplasm of the sustentacular cells were observed and manifestations of advanced degenerative changes were also evident. The vacuoles resulting from the removal of lipid during the processing of the tissue from the sustentacular cell cytoplasm could have been from the phagocytosed residual bodies of the germ cells or intracellular fatty changes. The phagocytic ability of the sustentacular cells is established (17). The germ cells which cannot differentiate into sperm would either be phagocytosed by the sustentacular cells or would undergo autolysis. Residual bodies containing lipid and RNA cast off during spermiogenesis are normally phagocytosed by the sustentacular cells (16). As a result of the degenerative changes of the sustentacular cells, the presence of their intercellular junctions which maintain the blood–testis barrier was not observed. Normally the blood–testis barrier is able to develop without the presence of germ cells and in the absence of gonadotrophic hormone stimulation (18). One of the main functions of the blood–testis barrier is to cordon off the primary spermatocytes with a changed genetic composition from the body fluids so that they do not elicit an immune response from the body. Presence of monocyte-like cells have been correlated with the fragmentation of the sustentacular cell junctions (19). Occasional macrophages, which are of monocytic origin, were observed in the

seminiferous tubules of the methoxychlor-treated rats.

The binucleated or the multinucleated cells probably resulted due to fusion of the germ cells (primary and secondary spermatocytes, spermatids). The germ cells probably fused because of incomplete cytokinesis during the process of spermatogenesis. Binucleate and multinucleate giant forms are sometimes present among spermatogonia and spermatocytes as a consequence of a peculiar mode of cytokinesis (20). The specific manner in which methoxychlor could have affected the formation of multinucleated cells cannot be explained.

The peripheral distribution of energized mitochondria in relation to the cell membrane of the spermatids of the control rats was observed. Similar morphology and disposition of energized mitochondria in the rat spermatids have been reported (21). Energized mitochondria are seen when energy is being generated much faster than it is dissipated (22). In the experimental rats where the ultimate fate of the germ cells appeared to be death, the energy requirements of the spermatids would be reduced. Therefore, in the methoxychlor-treated rats, the absence of energized mitochondria could be a logical explanation. Degenerate mitochondria were observed in the distorted (binucleate) germ cells.

In this study, the epithelium of the ductus epididymidis exhibited many vesicles and vacuoles. Similar changes in the epididymal epithelium of the rat treated with clomiphene citrate up to 12 weeks were observed (23). Toward the tail of the corpus epididymidis, the lumen of the duct was distended with fluid containing dead cells and disfigured sperm. This distension could have been caused by either obstruction of the duct or stenosis. The epithelial lining of the duct appeared compressed. The blood flow per unit weight through this part of the epididymis is higher than it is for the rest of the epididymis (24).

The sterility of the methoxychlor-treated females was due to the fact that the normal process of folliculogenesis in the ovaries was interrupted, leading to atresia of the ovarian follicles. The granulosa cells exhibited early necrotic changes manifested by pyknosis and karyorhexis of their nuclei. These changes in the ovaries as well as the arrest of normal process of spermatogenesis in the methoxychlor-treated rats could have been due to the estrogenic properties of methoxychlor. Recent studies indicate that metabolites of methoxychlor account for its estrogenic properties *in vivo* (11). Methoxychlor has also been designated as a proestrogen because it requires metabolic activation to achieve its activities (10).

The precise manner in which methoxychlor affects the seminiferous epithelium or the ovarian follicles is not clear. It has been shown that 0.1 μg of diethylstilbestrol has the same estrogenic activity as 1 mg of technical methoxychlor in the rat (25). The estrogenic properties of methoxychlor could surpress the testosterone secretion by the interstitial (Leydig) cells of the testes by inhibiting the release of follicular stimulating hormone (FSH). FSH facilitates the ability of the testes of rats to respond to luteinizing hormone (LH) and secrete testosterone (26). In conclusion, technical methoxychlor does hamper the normal process of spermatogenesis and folliculogenesis. If the effects of methoxychlor on the reproductive systems of the rat are due to its estrogenic properties, further investigations are needed to determine its precise mode of action.

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