

Morphologic and Endocrine Changes in the Reproductive Organs in Rats Implanted with Gossypol Acetate Pellet in the Testis

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Gossypol acetate pellets in concentrations ranging from 0.5, 1.0, 5.0, 10.0, to 20.0 mg were implanted in the testis of rats for a period up to 7 weeks. The implant was made in one testis only. In animals with a 10.0-mg implant, the germinal epithelium showed degenerative changes following the 7-week period, but spermatogonia and spermatocytes were unaffected. The effect of gossypol acetate appeared to be at the spermatid level. The electron microscopic study showed that the acrosomal membranes were disrupted. Blood testosterone and LH, but not FSH, decreased significantly ($P < 0.01$). The fertility studies showed 100% infertility but no loss in libido in the 10-mg implant group. Up to the 7-week period, implants of less than 10 mg produced no significant effects on any of the parameters mentioned above. Animals with 20-mg implants had toxic symptoms. From the results in this study, it is concluded that 10.0 mg of gossypol acetate implanted in a single pellet in one testis causes antispermatogenic effects.

Key words: gossypol, testis, Sertoli cell, spermatozoa, hormones.

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Earlier studies report that gossypol, a phenolic extract from cotton seeds, has been used successfully as an effective male contraceptive (National Coordinating Group, 1978; Dai et al, 1978; Wang and Lei, 1979). These findings have led several laboratories around the world to confirm and extend this infor-

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mation (Chang et al, 1980; Saksena et al, 1981; Kalla and Vasudev, 1981). Although data are available regarding the optimum dose of gossypol acetate and the time period required to produce antispermatogenic effects (Wang and Lei, 1979; Chang et al, 1980; Kalla and Vasudev, 1981; Hoffer, 1982; Shandilya et al, 1982), the methods of administration reported do not allow us to determine the specific effects of gossypol acetate on spermatogenesis. To resolve this problem, a study was designed in which a gossypol acetate pellet was implanted directly in the testis for periods ranging from 2 to 7 weeks. The local implants offer several advantages over previously tried methods because they assure a consistent gossypol supply to the tissue without interference from metabolites produced elsewhere in the body. To evaluate the endocrine and morphologic effects of the implant, blood levels of T, LH, and FSH were determined. Testis morphology was studied using light and electron microscopy (EM). To compare the effects of the local implant versus general gossypol acetate effects, the implant was made only in one testis; the contralateral testis was left untreated. To determine whether gossypol acetate affects fertility, the gossypol-implanted males were mated with females in proestrus and the number of newborns were counted.

Materials and Methods

Materials

Gossypol was obtained from Dr. Sheldon Segal of the

Rockefeller Foundation (New York, NY), in the form of gossypol acetate. The purity of the compound was described as 99.9%; and the compound was kept in brown glass vials, covered with aluminum foil, and stored at -20°C . Gossypol acetate powder was mixed with cholesterol, methyl cellulose, alpha lactose, and stearic acid in equal ratios and manufactured into pellets (Innovative Research of America, Rockville, MD, Dr. S. Shafie). Each pellet had concentrations of 0.1, 1.0, 5.0, 10.0, or 20.0 mg. The purity of the gossypol acetate in sample pellets from each batch was reassayed using high-pressure liquid chromatography (HPLC) with reverse-phase chromatography and UV detector at 254 nm (Berardi and Goldblatt, 1969).

Animals

Adult male rats of the Sprague-Dawley strain, weighing between 260 and 300 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). A total of 150 rats were used in this study. The animals were divided equally into experimental and control groups. In the experimental group, there were five animals each for every implant period (2, 5, and 7 weeks), and for each pellet level (0.1, 1.0, 5.0, 10.0, and 20.0 mg). Equal numbers of controls were used in each group. Food and water were supplied *ad libitum*. Animals were maintained according to the University Animal Care Committee guidelines on the care and use of animals set forth by the National Institutes of Health.

Pellet Size

A 3-mm diameter pellet was made. The small size of the pellet is necessary to prevent injury to the implant area.

Rate of Gossypol Acetate Released from the Pellet

Although detailed studies were not conducted to determine the rate of gossypol released from a pellet after the implant was made, the preliminary data was obtained using a 5-mg gossypol implant and then determining the amount of gossypol acetate remaining in the pellet each week. The results showed approximately 200 to 250 μg /daily released from a pellet. The pellet size did not affect the rate of release.

Method of Pellet Implantation

A small incision was made in the scrotum in lightly ether-anesthetized rats. In one testis, a gossypol acetate pellet was implanted on the tunica albuginea. The contralateral testis was sham-operated and received the inert material containing no gossypol. A single suture was placed to prevent mobilization of the pellet. The scrotum wound was closed by a single wound clip.

Period of Implant

The experimental animals and the controls were kept for 2, 5, or 7 weeks. At the end of each implant period, four animals at each gossypol pellet level were sacrificed. Body and testis weight from animals with and without implant were recorded. No other test was done at the 2- and 5-week periods because observable differences in testes

and body weights were not found in any of the implant levels with the exception of the 20.0-mg implant. Animals with a 20.0-mg implant appeared to be in the toxic state, and for that reason histologic studies were not conducted at the 2- or 5-week period in any of the animals.

Radioimmunoassays

Serum LH and FSH levels were determined by the double-antibody RIA technique (Niswender et al, 1968). Standards were NIH-LH (biological activity $2.1 \times S-3$). Serum T levels were determined by RIA (Midgley et al, 1970; Midgley and Niswender, 1970). Radiolabeled T (43.5 Ci/mmol S.A.) and antiserum against testosterone-3-oxime-BSA were purchased from New England Nuclear Corporation (Boston, MA). All assays were performed in triplicate. The results were expressed in ng/dl serum.

Specimen Preparation for Electron Microscopic Studies

At the end of the 7-week period, three animals were selected randomly in each group and were used for morphologic studies. Animals were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) and were perfused through the left ventricle with saline followed by 5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 (Karnovsky, 1965). After 5 minutes of whole body perfusion, both testes were excised and dissected free. The spermatic cord was ligated twice and the cord between the ligatures was cut. The testes were transferred to a beaker containing saline, and the spermatic artery was cannulated with a 25-gauge blunted needle attached to polyethylene tubing (Clay-Adams Pe-60, Parsippany, NJ). Each testis was flushed with saline and then perfused with ice-cold 5% glutaraldehyde in 0.2 M S-collidine buffer at pH 7.4. Perfusion continued for 15 minutes until the testes were hard to the touch. The testes were sliced into 2 to 3-cm-thick sections with a razor blade and fixed for an additional 30 minutes by immersion in cold S-collidine buffer with 5% glutaraldehyde, postfixed for 60 minutes in cold 1% OsO_4 , dehydrated through a graded series of ethanol and embedded in Epon. The Epon-embedded tissues were sectioned into 1 to 2- μ -thick sections that were stained with 2% toluidine blue for light microscopic examination. Whole body perfusion plus local perfusion allowed excellent fixation of the testes. For light microscopy, 1 to 2- μ -thick sections were cut and stained with toluidine blue. At least 100 tubules in each group were examined to determine the percentage of normal seminiferous tubules. For EM studies, a Zeiss Em 10 electron microscope was used. Sections were cut with a diamond knife on a Porter-Blum MT-2 microtome and stained with uranyl acetate and lead citrate.

Fertility Study

At the end of the 7-week period, all males in each group were mated with females in proestrus. The females were of the same strain and were brought to the males and housed together. If lordosis and mounting were observed within 5 minutes of their meeting, the females were left with their mate overnight; otherwise, the females were

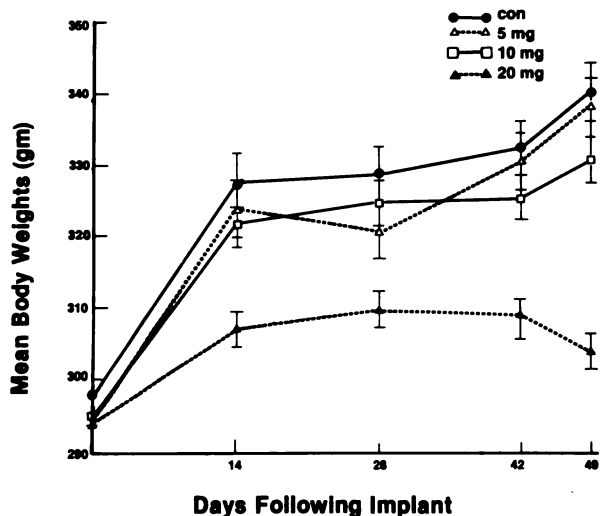


Fig. 1. Body weights of rats implanted with gossypol acetate pellet ranging in concentrations from 0.5 to 20.0 mg for a period varying up to 7 weeks. Body weight gain in rats receiving up to a 10.0-mg implant was not significantly different compared with controls. In rats with a 20-mg implant, the weight gain was less than 40% of the gain in other groups and these differences were significant from other groups ($P < 0.01$).

removed and a second proestrus female was paired with the male. The presence of a vaginal plug the following morning was an indication of the onset of pregnancy and was considered day 1 of pregnancy. Similarly, the controls were mated individually with proestrus females. Those who became pregnant were allowed to deliver normally and the number of pups born to each female was counted.

Body Weights

The animals were weighed at intervals throughout the study.

TABLE 1. Testis Weights with and without Gossypol Acetate (GA) Implant Following the 7-Week Period*

| Treatment GA/Pellet (mg) | GA - implanted | Non GA-Implanted |
|--------------------------------|----------------|------------------|
| None | 1.77 ± 0.205 | 1.60 ± 0.201 |
| 0.1 | 1.76 ± 0.018 | 1.58 ± 0.018 |
| 1.0 | 1.69 ± 0.100 | 1.41 ± 0.160 |
| 5.0 | 1.68 ± 0.070 | 1.60 ± 0.070 |
| 10.0 | 0.98 ± 0.205† | 1.57 ± 0.065 |
| 20.0 | 0.65 ± 0.060† | 1.40 ± 0.800 |

*Four animals in each group.

†Significantly decreased testis weight at 7 weeks, $P < 0.01$. Differences were not significant at 2 and 5 weeks. Although animals receiving the 20-mg implant had a further decrease in testis weights from those with 10 mg, the difference between the 10-mg and 20-mg groups was not significant. Animals with the 20-mg implant had significantly decreased testis weights at 5 weeks, and a further decrease by 7 weeks.

NOTE: The testis which received only the inert material pellet was not affected throughout the study.

Testes Weight

At each implant period, the testes from animals with and without the implant were weighed.

Statistical Analysis

The means and distribution of treatment groups were compared with controls using the Student-Newman Keuls test (Sokal and Rohlf, 1969).

Results

Body Weights

No significant differences in body weights were found with a gossypol acetate implant of 10 mg or less throughout the study. Animals with a 20-mg implant had significantly decreased body weights starting at the 2-week period ($P < 0.01$, see Fig. 1).

Testis Weight

Implants of up to 5 mg of gossypol acetate had no significant effect on testis weights. Testis weights decreased significantly with 10- and 20-mg implants at the 7-week period ($P < 0.01$). While testis weights were not significantly affected after 2 and 5 weeks at the 10-mg implant level, significant decreases in testis weights were seen at the 5-week period in the 20-mg implant group ($P < 0.01$). No significant effect was found in the testis weights of nongossypol-implanted animals (Table 1).

Fertility Study

Animals with up to 5 mg of gossypol implants were normal in their copulatory behavior and retained their fertility. Significant differences were found with 10- or 20-mg gossypol implants. Following the 7-week period, 100% of the males with 10-mg implants were infertile. Their copulatory behavior and libido, however, were not affected. With 20-mg implants, all of the males were infertile following the 7-week period, and their libido was decreased (Table 2). The major differences between the rats with 10- and 20-mg implants were in their copulatory behavior, since both groups were infertile after 7 weeks.

Histologic Studies

No changes in germinal epithelial morphology were observed with up to 5-mg gossypol implants. At the 10-mg implant level (Fig. 2) after 2 weeks, the seminiferous tubules showed few degenerative changes, and there was minimal disruption of the germinal epithelium. Shown in Fig. 3 is an electron micrograph of seminiferous tubules that revealed normal Sertoli-Sertoli cell junctions. Shown in Fig. 4

is the result of treatment with 10 mg of gossypol after 7 weeks, when many seminiferous tubules had disrupted germinal epithelium and some contained only Sertoli cells and spermatogonia. Shown in Figs. 5 through 8 are electron micrographs illustrating the effect of 10-mg gossypol implants on spermatids following the 7-week period. Figure 5 shows that the coarse fibers of the midpiece are disorganized. Figure 6 shows that the acrosomes are also disorganized and have disrupted membranes. In Figs. 5 to 8, the smooth endoplasmic reticulum (SER) are dilated. Figure 9 shows that with 20-mg gossypol implants degeneration of testis morphology is more advanced, since some seminiferous tubules contain no germ cells other than spermatogonia, while the rest were completely denuded and mostly Sertoli cells remained. The electron microscope study showed that there were severe alterations in the fine structure of the Sertoli cells. There were overall decreases in cytoplasmic ground substances and a marked increase in whorls of SER. Several damaged seminiferous tubules exhibited changes in the basal lamina that included thickening and irregularity. Despite the severe damage to the Sertoli cells, significant numbers of spermatogonia were present.

The Leydig cell structure, size and number appear normal in all of the studies.

Hormonal Levels

Up to 5 mg of implant, T and LH levels were not significantly affected. Significant differences were observed with 10 and 20 mg of implant ($P < 0.01$). The differences between 10 and 20 mg of implant

TABLE 2. Percent of Pregnancies and Number of Pups Born to Normal Females Who Were Mated with Males that had been Implanted with Gossypol Acetate (GA) Pellets for 7 Weeks*

| Treatment GA/Pellet (mg) | Percent of Female Pregnancies | Number of Pups Born |
|--------------------------------|----------------------------------|------------------------|
| None | 85 ± 5 | 14 ± 2 |
| 0.1 | 95 ± 3 | 12 ± 3 |
| 0.1 | 85 ± 5 | 10 ± 5 |
| 5.0 | 90 ± 6 | 10 ± 2 |
| 10.0 | — | — |
| 20.0 | — | — |

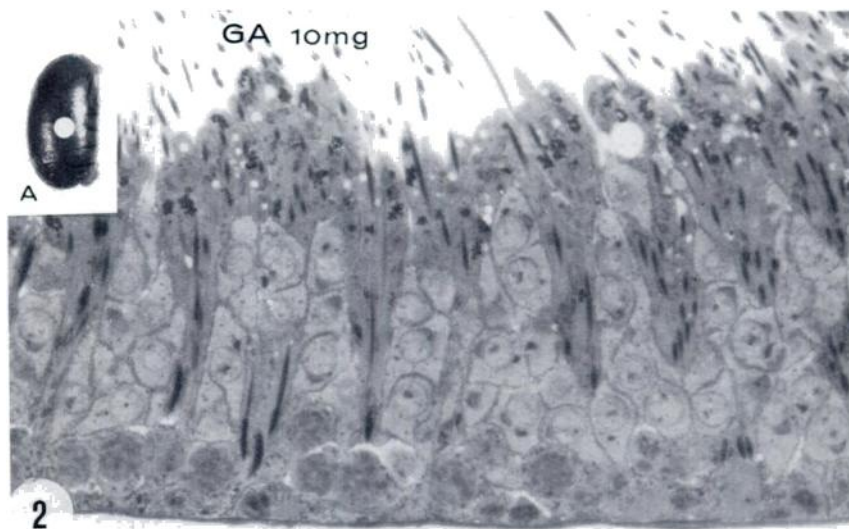
*Five males in each treatment group. The treated males had one testis receiving the implant, the second testis was implanted with a pellet containing inert material and no GA. Implants of up to 5 mg of gossypol acetate caused no significant differences in the number of pregnancies and number of pups. Animals receiving 10- and 20-mg implants were infertile.

were not significant. FSH levels remained unchanged in all animals throughout the study (see Table 3).

Discussion

The antispermatogenic effect of gossypol acetate shown in this research supports numerous studies reported in the literature and the local implant used in this study extends knowledge regarding the mechanism of gossypol effect on the germinal epithelium. The results of this study demonstrate that the anti-spermatogenic effect of gossypol acetate is mediated within the germinal epithelium rather than elsewhere, such as via the hypothalamic-hypophyseal axis. Other investigators have reported similar conclusions using indirect evidence (Nadakavukaren et

Fig. 2. Light micrograph of rat testis with 10 mg of gossypol acetate (GA) pellet and contralateral testis with implant (inset A) containing inert material only (controls) following the 2-week period. Although gossypol-implanted testes appear normal morphologically, there were fewer mature spermatozoa in the seminiferous tubule lumen. The Sertoli cells appear normal ($\times 100$). The control (inset A) shows no diminution in size or obvious damage in morphology. Morphology picture is omitted because of normal structure.



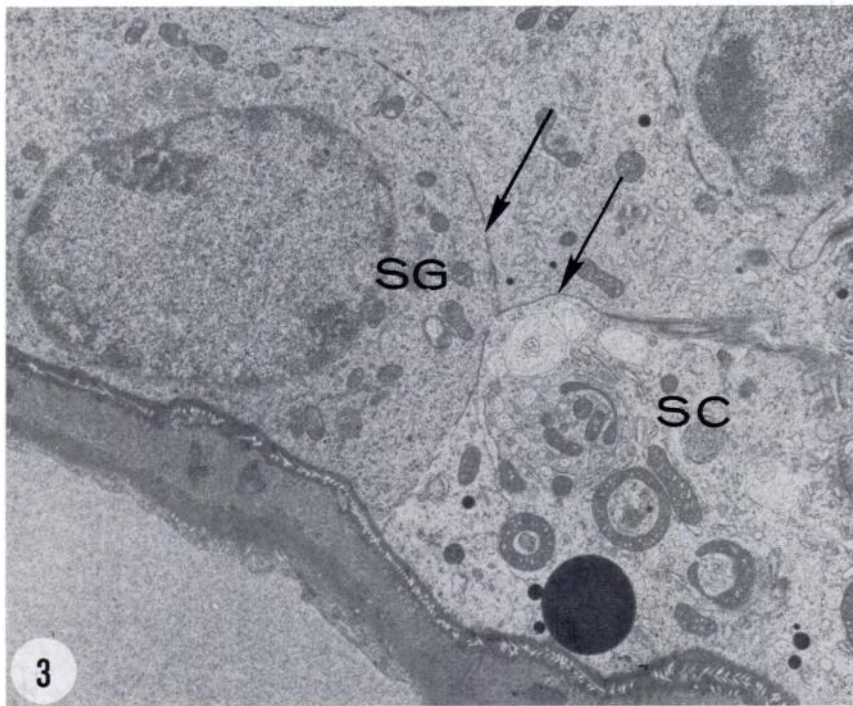


Fig. 3. The electron microscopic examination through a basal region of seminiferous tubules of testis treated with 20-mg gossypol acetate implant. Sertoli cell (SC) and intercellular junctions (arrows) appear normal. Spermatogonia (SG) nestled between the Sertoli cells seem to be undamaged. The myoid cell appears normal ($\times 12,000$).



Fig. 4. Light micrograph of seminiferous tubules of testis treated with a 10-mg gossypol acetate pellet for 7 weeks. The inset shows the gross morphology of the contralateral testis (which received no implant) (inset A) compared with a testis treated with 10 mg of gossypol acetate (inset B). In the gossypol-treated testes, seminiferous tubules have disrupted germinal epithelium and spermatids are sloughed into the lumen ($\times 100$).

Fig. 5. Electron micrograph showing coarse fibers (CF) from the sperm middle piece in the cytoplasm. 10 mg gossypol implanted for 7 weeks ($\times 14,000$).

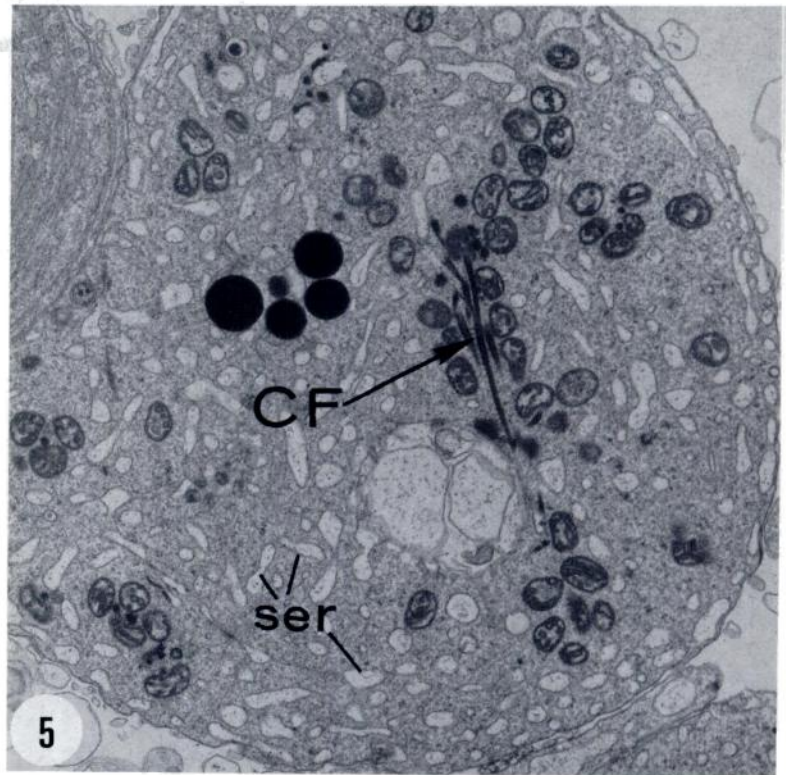
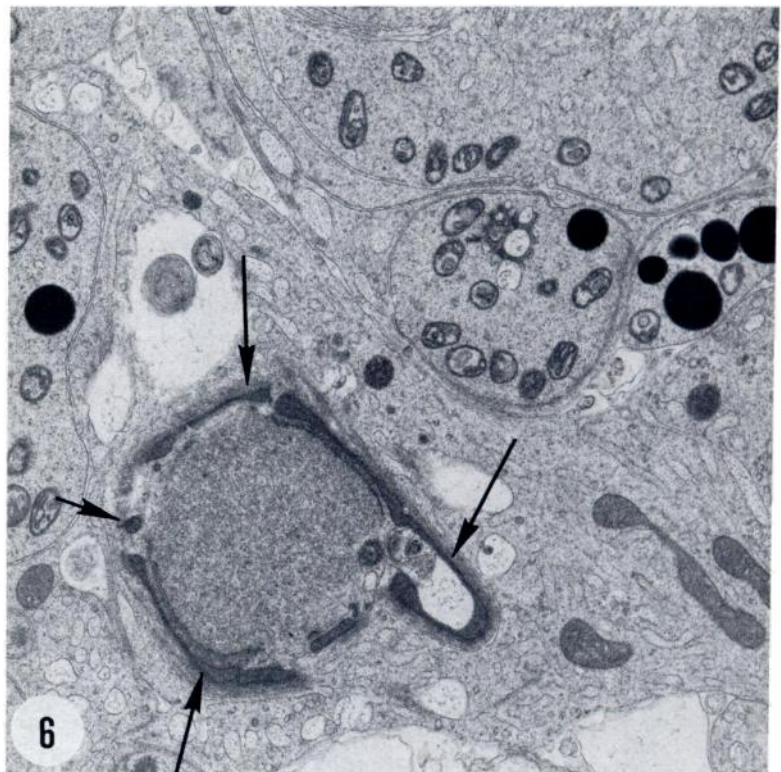


Fig. 6. Electron micrograph showing that acrosomes are fragmented after 10-mg gossypol implants for 7 weeks (see arrows) ($\times 14,000$).



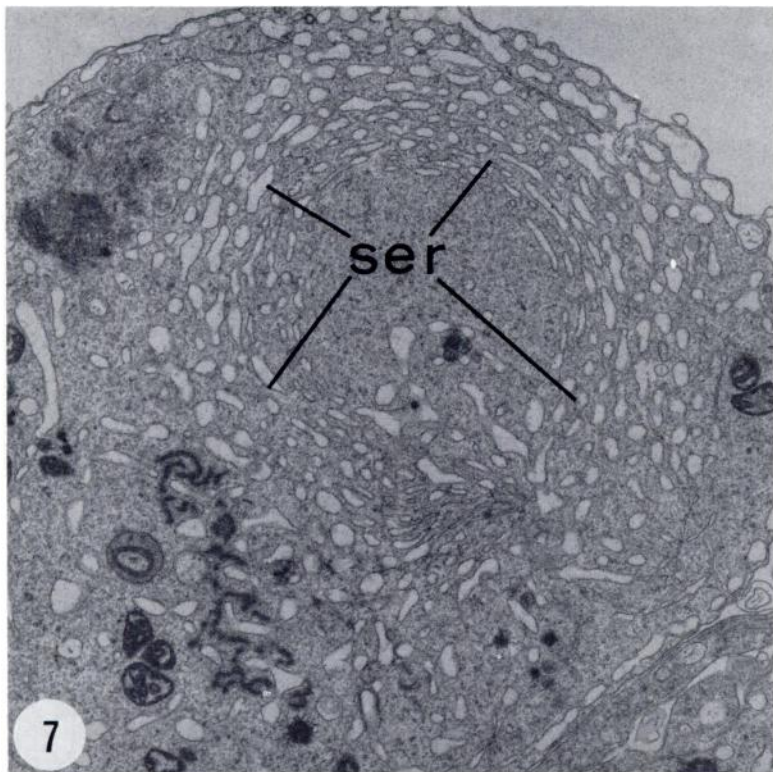


Fig. 7. Electron micrograph showing that the SER in the spermatid cytoplasm is highly dilated after implantation of 10 mg of gossypol for 7 weeks ($\times 14,000$).

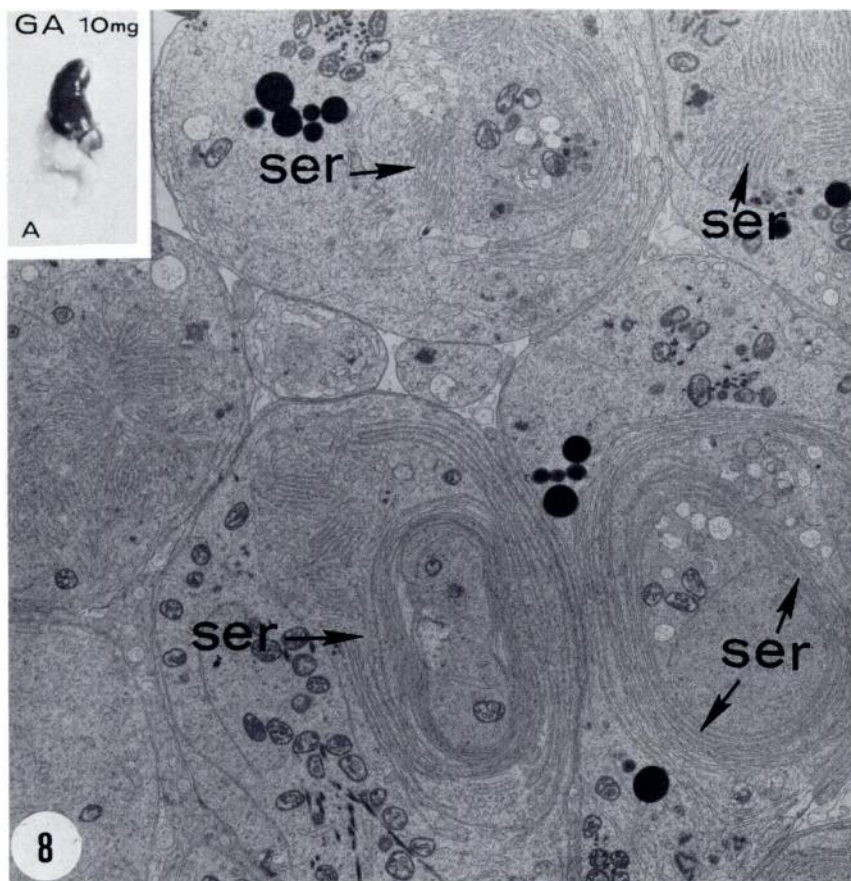


Fig. 8. Electron micrograph showing rat testis implanted with 10-mg gossypol pellet at the 7-week period. The spermatid cytoplasm contains large whorls of SER. The inset (A) shows gross morphology of GA-implanted testis ($\times 14,000$).

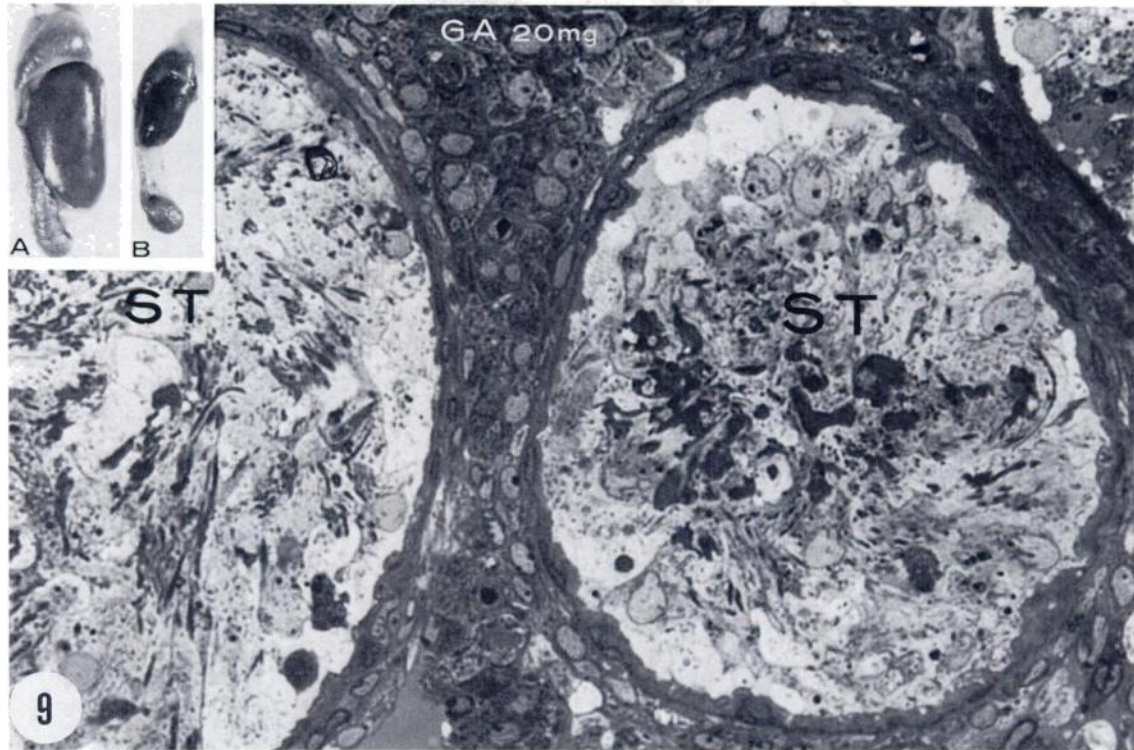


Fig. 9. Light micrograph of rat testis implanted with a 20-mg pellet for the 7-week period. The seminiferous tubules are filled with Sertoli cells and debris from the germinal epithelium. Some tubules are completely free of germinal epithelium but some have significant numbers of spermatogonia beneath the Sertoli cells. No spermatocytes or spermatids could be found. The insets (A and B) show a comparison of GA-implanted and contralateral testes (nongossypol-implanted) after the 7-week implant period ($\times 100$).

al, 1979; Chang et al, 1980; Hoffer, 1981; Shandilya et al, 1982). Our conclusion is based on the fact that the contralateral testis, which was left unimplanted, was not affected in size, in weight, or morphologically. There are two possibilities. First, the amount of gossypol released from the implanted testis was too small to reach the contralateral testis, and second, gossypol acetate is metabolized to an inactive compound within the testis. Both these possibilities are under study at present.

The decrease in T and LH levels with 10- or 20-mg implants appears to suggest that the hypothalamic-hypophyseal-gonadal axis is affected. However, the normal size and morphology of the contralateral testis (nonimplanted) casts doubt as to whether degenerative changes in the implanted testis are induced because of low T and LH levels. The possibility that gossypol may decrease the binding sites and/or affinity of LH to LH receptors in the testis is not ruled out.

To determine whether the lack of T is the cause of germinal epithelium degeneration in gossypol-treated rats, preliminary studies were conducted using im-

plants containing various levels of T together with gossypol acetate. The results showed that T was ineffective in preventing gossypol-induced germinal degeneration. Because of preliminary data, T was ineffective in restoring gossypol-induced changes;

TABLE 3. Blood Levels of T, LH, and FSH in Gossypol Acetate (GA) Pellet-implanted Male Rats (ng/dl)*

| Treatment† GA/Pellet (mg) | T | LH | FSH |
|---------------------------------|---------------|---------------|----------------|
| None | 98 \pm 2.4 | 66 \pm 6.7 | 590 \pm 25.2 |
| 0.1 | 96 \pm 1.8 | 63 \pm 5.6 | 587 \pm 24.2 |
| 1.0 | 84 \pm 2.4 | 82 \pm 9.8 | 553 \pm 66.0 |
| 5.0 | 109 \pm 2.5 | 72 \pm 7.2 | 472 \pm 55.0 |
| 10.0 | 49 \pm 1.8‡ | 55 \pm 2.1‡ | 626 \pm 29.0 |
| 20.0 | 37 \pm 0.7‡ | 46 \pm 2.5‡ | 545 \pm 65.0 |

*Four animals in each group.

†Results shown here are following 7 weeks implant \pm SE.

‡Significantly reduced, $P < 0.01$, compared to controls. Differences between the 10-mg and 20-mg groups were not significantly different although the 20-mg group had larger decreases in LH and to FSH levels were unchanged in all animals throughout the study.

the results of this study are not reported here. Furthermore, Leydig cell number and size were not affected in gossypol-treated animals, which suggests that the lack of T is not the cause of germinal epithelium degeneration in treated rats. Shandilya et al (1982) also reported a normal response of Leydig cells to LHRH in gossypol-treated monkeys. Similarly, Kalla et al (1983) reported that gossypol acetate had no effect on T production when added *in vitro* at concentrations of 3.5×10^{-5} M to 3.5×10^{-4} M. Hadley et al (1981), on the other hand, reported decreased T production after LH stimulation of gossypol-treated Leydig cells. It is difficult to compare our data with others because of differences in methods of administration and the levels of gossypol acetate used in these studies.

As far as the site of gossypol effects within the germinal epithelium is concerned, our data suggest that it is at the later stages of spermatogenesis, during the formation of spermatids. Similar data have been reported by Dai et al (1978), Bozek et al (1981), Kennedy et al (1983), and Hoffer (1983). Shandilya et al (1982) reported the direct effect of gossypol acetate on sperm motility and alterations in the axial filament complex.

The effect of gossypol acetate on sperm maturation within the epididymis has been reported. Bozek et al (1981) found that spermatozoa from the cauda epididymidis exhibited wrinkled and disorganized cell membranes in the head and tail regions, malformed sperm heads, and retention of the cytoplasmic droplet at various locations along the tail midpiece. Similarly, Hadley et al (1981) reported degeneration in the tail region, particularly in the mitochondrial sheath. Although we did not examine epididymal spermatozoa in detail, the electron microscopic study showed accumulation of very few spermatozoa in the lumen, which was filled largely with immature germ cells and cell debris, suggesting a direct effect of gossypol on sperm maturation processes. These data will be published elsewhere. Nadakavukaren (1979) and Chang et al (1980) suggest that gossypol impairs cytoplasmic droplet migration. Midpiece retention of the cytoplasmic droplet by spermatozoa has been associated with human sterility by Fujita et al (1970), Ensult et al (1975), Bedford (1975) and Okagaki et al (1980).

The effect of gossypol acetate on Sertoli cell morphology is controversial (Pelletier and Friend, 1980; Hoffer, 1983). Our data showed that Sertoli-Sertoli cell junctions were intact and the blood-testis barrier was not interrupted. Although Sertoli cells showed

more lysosomal and lipid storage activities, the effects appear to be nonspecific. The normal FSH levels throughout our study support the morphologic characteristics found in the Sertoli cells. Several studies have linked FSH levels to Sertoli cell function (Krueger et al, 1974; Steinberger and Steinberger, 1976; Means et al, 1976; Russell, 1980).

Our data provide direct evidence that the effects of gossypol acetate are mediated locally within the testis and spermatids appear to be specifically affected. A local implant of 10 mg of gossypol acetate appears to be the ideal level for antispermatogenic effects without impairing the libido. Pellets containing more than 10 mg produced toxic symptoms.

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New Dosages and Packaging for Pergonal® and Serophene®

Serono Laboratories, Inc., announced that their infertility product line has been expanded with an additional dosage strength and new packing. Serophene® (clomiphene citrate tablets, USP) now is available in a new 10-tablet box. The tablets are blister-packed in fives for unit-dispensing convenience. There will be new packaging for the standard 75-IU dosage of Pergonal® (Menotropins for injection, USP). In addition to the single 75-IU package size, Pergonal® will now be packaged in a convenient 10 pack. In addition, a new strength is now available—a 150-IU ampule. The higher strength is designed for more convenient administration, as this is the most commonly prescribed dose.

The First Symposium on Morphological Basis of Human Reproductive Function

The first symposium on the "Morphological Basis of Human Reproductive Function" (C. Conti, President) will be held in Fiuggi Terme on September 11 and 12, 1986. The program will feature lectures on the testis given by A. Fabrini, D. M. de Kretser, M. L. Dufau, and N. E. Skakkebaek. G. C. Balboni, A. Ianniruberto, and A. R. Midgley will speak about the ovary, B. Baccetti, M. C. Oregebin-Crist, R. Yanagimachi, S. Metafora, F. Menchini-Fabris will give lectures on the epididymis and spermatozoa. The local organizing committee is headed by D. Conte, Coordinator, Cattedra de Andrologia, V Clinica Medica.

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