Effect of Gossypol Acetate on Guinea Pig Epididymal Spermatozoa *In Vivo* and Their Susceptibility to Capacitation *In Vitro*

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To determine the effects of gossypol acetate on guinea pig epididymal and vas deferens sperm maturity and *in vivo* susceptibility to *in vitro* capacitation and the acrosome reaction, we examined spermatozoa removed from 37 animals fed gossypol acetate (10–15 mg/kg/day) for 5 to 9 weeks, and 15 vegetable oil-fed, age-paired control animals. In gossypol-treated, reproductively immature guinea pigs, the number of spermatozoa in the epididymis was markedly reduced ($P < 0.01$) compared to controls, whereas the presence of spermatids and spermatocytes increased in the epididymis with the duration of gossypol administration. In sexually mature guinea pigs (given 15 mg/kg/day for 5 weeks), the epididymal sperm survival and forward motility were decreased significantly ($P < 0.025$ and $P < 0.01$, respectively), although the density of mature spermatozoa was the same as in control animals. The percentage of induced acrosome reactions (26.4 ± 12%) was almost three-fold lower than that of control animals (72.8 ± 4.6%). Also, in 31.5 ± 3.8% of spermatozoa from gossypol-treated animals, as compared to only 2.4 ± 0.7% of controls, the cytoplasmic droplet failed to migrate to its proper position in the midpiece and was retained in the neck region. With a few exceptions, spermatozoa from both experimental and control groups had comparable patterns of freeze-fractured membrane differentiations. Susceptibility to the induced acrosome reactions and the position of the retained cytoplasmic droplet reversed within 3 weeks after the end of gossypol feeding. This study helps establish the suitability of the guinea pig for studies on gossypol-induced infertility.

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The antifertility action of gossypol has been amply documented in male mice (Coulson et al, 1980), rats (Xue et al, 1980), hamsters (Chang et al, 1980; Saksena and Salmonsen, 1982), dogs (Sang et al, 1980), monkeys (Sang et al, 1980; Shandilya et al, 1982), and humans (National Coordinating Group on Male Antifertility Agents, 1978; Poso et al, 1980; Shi et al, 1981a) but not conclusively in guinea pigs (Pelletier and Friend, 1980; 1981). Although there is no question that gossypol inhibits fertility in the species mentioned, its mode of action is unknown. The National Coordinating Group on Male Fertility Agents (1978) reported that gossypol affected the development of the sperm nucleus and acrosome. Xue et al (1980) demonstrated that spermatids and spermatocytes were the most sensitive forms responding to gossypol treatment. Other studies consistently revealed specific tail lesions in the spermatozoa of infertile animals (Nadakavukaren et al, 1979; Hadley et al, 1981; Oko and Hrudka, 1982; Hoffer, 1982; Shandilya et al, 1982). Coulson et al (1980) also found that mice injected with 12.5 or 25 mg/kg body weight gossypol for 10 days exhibited statistically significant decreases in epididymal sperm count. By introducing gossypol into the epididymal fat-pads of rats, Hadley and Burgos (1982)

demonstrated that gossypol inhibits epididymal sperm motility as well as altering their morphology within 24 hours of administration. Likewise, Hadley et al (1981) further observed ultrastructural defects of rat epididymal sperm tails secondary to degeneration of the midpiece mitochondria in animals fed gossypol.

The present study was mainly undertaken to ascertain the effects of gossypol on the maturity of guinea pig epididymal spermatozoa, fertility, and *in vitro* susceptibility to capacitation and the acrosome reaction. We establish here that the guinea pig is a suitable animal model for studies on gossypol-induced infertility. Portions of this work have been previously presented (Shi and Friend, 1983a; 1983b).

Materials and Methods

Animals

Fifty-two male albino guinea pigs purchased from Charles River and Simenson laboratories were used in the studies. Animals referred to as adult or sexually mature were 30, 40, and 60 days old, while animals referred to as reproductively immature (neonatal and prepubertal) were 10 or 15 days of age: the time of spermatogenic arrest at the spermatocyte stage (Wagner and Manning, 1976). Use of age-matched controls compensated for the overlap in sexual maturity. Also, the ages only refer to the onset of treatment. All guinea pigs were housed in environmentally controlled rooms with 12-hour light/dark cycles, and maintained at approximately 21 ± 1 C. Food and water were provided *ad libitum*, and the animals were checked weekly for body weight.

Dosages and Treatment Schedules

Gossypol acetic acid (98.1%, with a melting point of 178–182 C) was obtained from the Zhejiang Institute of Experimental Medicine and Hygiene in China. Fresh gossypol acetic acid was suspended in pure vegetable oil (Wesson®) after being ground with a mortar and pestle. The mixture was then given daily *per os*. We divided the animals into four groups, the first consisting of 24 adult guinea pigs fed gossypol at a dose-level of 15/mg/kg/day for 2, 3, 4, and 5 weeks, respectively. Five of these animals were retained for testing recovery of fertility. The second group, serving as control animals, was comprised of 10 adult guinea pigs given Wesson oil only for 2, 3, 4, and 5 weeks, respectively. Thirteen younger guinea pigs constituted the third group and were fed gossypol acetic acid at a dose-level of 10 mg/kg/day for 9 weeks, while the fourth group consisted of five age-matched controls receiving oil alone for the same 9-week period. Our experiments were divided into two phases: a treatment phase of 2, 3, 4, 5, and 9 weeks, and a posttreatment phase of 1, 2, 3, 4, and 5 weeks after the end of gossypol feeding, following which we sacrificed the guinea pigs under ether anesthesia.

Medium

The acrosome reaction was carried out by the synchronous method of Yanagimachi and Usui (1974). The medium was according to Fleming and Yanagimachi (1981), but we used only one-half the normal concentration of glucose (2.78 mM/100 ml). Components of Ca^{2+}-free Minimal Capacitation Medium (MCM) were 111.67 mM NaCl, 2.70 mM KCl, 0.49 mM $MgCl_2 \cdot 6H_2O$, 2.78 mM D-Glucose, 10.00 mM Na-lactate, 1.00 mM Na-Pyruvate, Bovine Albumin Crystalline (300 mg/100 ml, Sigma), 100 μ penicillin G, and 5 mg streptomycin sulphate. All reagents were dissolved in 100 ml distilled water. We adjusted the *p*H of the medium to about 7.7, stored that solution at 4 C, and used it in experiments within 1 week after preparation.

Spermatozoa

The cauda epididymidis and vas deferens were incised, milked into 0.1 ml MCM, and diluted to 2.0 ml in both the control groups and the guinea pigs treated with gossypol. The pooled spermatozoa were then mixed (concentration, $1.2–2.4 \times 10^7$ cells/ml) with Ca^{2+}-free MCM and the mixture was treated as follows: 1) freshly removed spermatozoa mixed in Ca^{2+}-free MCM at room temperature were immediately fixed in 1.5% glutaraldehyde with 0.1 M Na-cacodylate buffer (*p*H 7.4) in preparation for thin sectioning and freeze-fracture. Subsequently, spermatozoa for electron microscopy and freeze-fracture techniques were processed as reported previously (Friend and Fawcett, 1974; Friend et al, 1977). 2) Another sample of the mixture was transferred to glass slides and checked for sperm survival and motility patterns. All viable spermatozoa were counted, and their modes of movement subjectively classified as forward progressive movement, circular movement, or weak flagellar beating but no progression. 3) To investigate the effect of gossypol on sperm capacitation *in vitro*, we incubated another sample (1.5 ml) at 37 C in Ca^{2+}-free MCM, using glass vials under sterile, air-tight capped mineral oil for 10 hours; 15 μl $CaCl_2$ (0.2 M) was next added to several of these preparations, so that the final concentration of $CaCl_2$ in the suspension was 2.0 mM. Incubation was continued for 30 minutes before fixation and freezing. In addition, the epididymides, both in groups treated with gossypol and in control animals, were stripped of adhering tissue and the weights recorded. By macerating each cauda epididymidis in 0.5 ml MCM, and then diluting to 20 ml with MCM, we determined the epididymal sperm density of one side of the cauda epididymidis with a LEVY hemocytometer. This procedure yields reliable data for comparing experimental and control animals, even though it may not reflect absolute sperm counts for each animal.

After incubation for 10 hours, Ca^{2+} was added to the sperm suspension, a drop was put on a glass slide and examined for sperm survival, patterns of movement,

and the acrosome reaction. Those spermatozoa which had lost their acrosomal caps without losing motility were recorded as acrosome-reacted. According to the method of Bearer and Friend (1982), we counted spermatozoa at random in a high-power field. Ten "fields" were counted in each droplet (a total of 500 spermatozoa), which was then allowed to air-dry on a glass coverslip. To prevent loss of spermatozoa during the staining procedure, we routinely made smears on coverslips previously coated with poly-l-lysine (Sigma) as an adhesive (Mazia et al, 1975). These smears were later stained with *erythrosin B* and *naphthyl yellow S* (Bryan and Akruk, 1977), and the percentage of cytoplasmic droplets retained in the neck region was assessed.

Fertility Testing

With termination of the gossypol-feeding period, five male guinea pigs (two given gossypol at a dosage of 15 mg/kg for 5 weeks and the other three given gossypol at a dosage of 10 mg/kg for 9 weeks), were paired weekly with two females of proven fertility in the proestrus phase of their cycle. Successful mating was determined by the presence of spermatozoa in the vaginal washings. These mated females were sacrificed 1 to 3 weeks after mating, and the number of implantation sites noted.

All statistical evaluations were performed by the Student's *t* test.

Results

Effect of Gossypol on Epididymal and Vas Deferens Sperm Density and Survival

The testes of half the sexually immature guinea pigs that had been fed gossypol (10 mg/kg/day for 9 weeks) did not fully descend into the scrotum but could be massaged into it. The concentration of cauda epididymal spermatozoa in gossypol-treated (10 mg/kg/day) neonatal and prepubertal guinea pigs ($2.83 \pm 0.59 \times 10^3$ cells) were markedly reduced ($P < 0.01$) at the end of treatment as compared with those of control animals ($4.49 \pm 0.45 \times 10^8$ cells) (Table 1). Sperm concentrations did not decrease in adults. In adults, spermatogenesis appeared normal, with the various stages of spermatogenic cells present in the testes. However, cauda epididymal and vas deferens sperm survival in gossypol-treated (15 mg/kg/day/5 weeks) adult guinea pigs had decreased significantly ($P < 0.01$) as compared with that of control animals (Table 2). Moreover, we observed that the number of spermatocytes and spermatids in the epididymal lumen (Figs. 5 and 6, Table 1) had increased with the duration of gossypol administration (approximately $1.6 \pm 0.2 \times 10^6$ cells/cauda and $2.1 \pm 0.5 \times 10^6$ cells/cauda by the 5th or 9th week, respectively, as compared with control animals ($0.005 \pm 0.006 \times 10^6$ and $0.023 \pm 0.013 \times 10^6$ cells/cauda, respectively). Because the results obtained from oral administration of 10 mg/kg/day of gossypol for 9 weeks were similar to 15 mg/kg/day of gossypol for 5 weeks with respect to induction of the acrosome reaction, cytoplasmic droplet migration, and cell morphology, we pooled the results and describe them together.

Forward Progressive Movement of Spermatozoa

Following oral administration of gossypol, the forward progressive movement of guinea pig spermatozoa had significantly decreased (67.9 ± 8.19 for 15 mg/kg/day/5 weeks and $70.5 \pm 6.7\%$ for 10 mg/kg/day/9 weeks, $P < 0.01$ and $P < 0.025$, respectively, Fig. 1) in contrast to such motility in vehicle-treated control animals ($87.9 \pm 8.5\%$ and $83.5 \pm 4.8\%$, respectively).

Effect of Gossypol on the Acrosome Reaction

More than 99% of the spermatozoa of animals not treated with gossypol and incubated in Ca^{2+}-free MCM at 37 C for 10 hours did not demonstrate

TABLE 1. Effect of Gossypol (10–15 mg/kg/day for 5–9 weeks) on Sperm Density in the Cauda Epididymidis

	Sperm Density* ($\times 10^8$ cells/cauda)		Germ Cell Density* ($\times 10^6$ cells/cauda)	
	Immature (10 mg/kg/day for 9 weeks)	Mature (15 mg/kg/day for 5 weeks)	Immature (10 mg/kg/day for 9 weeks)	Mature (15 mg/kg/day for 5 weeks)
Control	4.49 ± 0.45 (n=3)†	3.93 ± 0.79 (n=8)	0.005 ± 0.006 (n=3)	0.023 ± 0.013 (n=3)
Gossypol-treated	2.83 ± 0.59‡ (n=5)	3.66 ± 0.47 (n=7)	2.1 ± 0.5 (n=3)	1.6 ± 0.2 (n=3)

* Mean + SEM.
† Numbers of animals.
‡ Significantly decreased ($P < 0.01$) as compared with the control group.

TABLE 2. Effect of Gossypol on Guinea Pig Epididymal Sperm Survival

	Survival (%)*	
	Immature	Mature
Control	91.3 ± 2.3 (n=3)	93.6 ± 5.2 (n=8)
Gossypol-treated	87.5 ± 4.2 (n=8)	80.7 ± 11.4† (n=6)

* Mean ± SEM.
† $P < 0.01$ compared to controls.

the acrosome reaction: after that interval, however, the reaction was triggered in 72.8 ± 4.6% of the cells by the addition of calcium. The majority of the acrosome-reacted spermatozoa displayed "active motility." In comparison, the number of spermatozoa having undergone the acrosome reaction in gossypol-treated animals was gradually reduced as the duration of gossypol administration increased. In these treated animals, the reaction dropped significantly to 39.4 ± 17% ($P < 0.01$) and 26.4 ± 12.9% ($P < 0.001$) at 3 weeks and 5 weeks, respectively (Fig. 2). The vast majority of the acrosome-reacted spermatozoa displayed no "active motility" but exhibited weak flagellar beating with no progression. Induction of the acrosome reaction returned to control levels 5 weeks after gossypol feeding stopped (Fig. 4).

Effect on Cytoplasmic Droplet Migration

Most cytoplasmic droplets of epididymal spermatozoa from mature control animals of the same age were localized in the middle of the midpiece (Fig. 5) and only 2.4 ± 0.7% droplets resided in the neck region (Fig. 3). Spermatozoa from gossypol-treated animals demonstrated a failure of cytoplasmic droplet migration. Of these spermatozoa, 31.5 ± 3.8% still retained the cytoplasmic droplet in the neck region (Figs. 3, 6, and 7). The proper position was restored 3 weeks after stopping gossypol feeding.

Morphology

Light and electron microscopic examination revealed that about 25% of the acrosomes observed in spermatozoa from gossypol-fed guinea pigs were mottled, blunter in contour, and sometimes swollen, but freeze-fracture replicas of spermatozoa obtained from experimental and control animals generally had comparable membrane differentiations. Even blunt acrosomes had typical linear arrays of intramembranous particles (Fig. 8). Other specific intramembranous particle patterns looked for, ie, strands of particles in the midpiece, the particles of the annulus, the zipper, and aggregates of particles in front of the striated ring (Friend and Fawcett, 1974) could all be found in the spermatozoa of all groups of experimental as well as control animals (micrographs not shown). A few cells from gossypol-fed animals lacked large particles in the caudal contour of the nuclear envelope (the implantation fossa), and many lacked the plasmalemmal quilt pattern. But without collecting quantitative data on the incidence of these observations, we can attribute no special significance to the sporadic deviations from the expected freeze-fracture images.

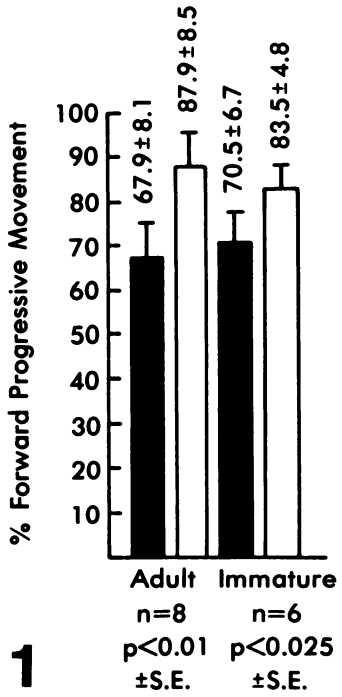
Recovery of Fertility

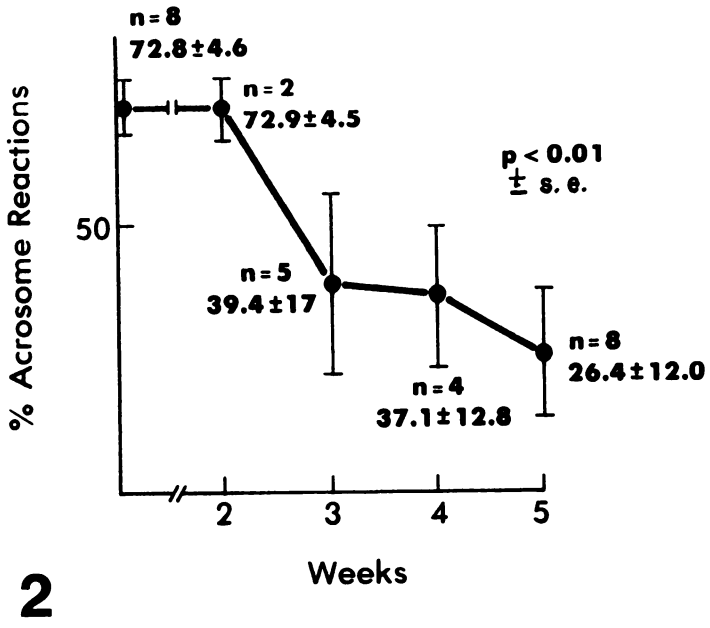
Fertility testing showed that guinea pigs became infertile when given 15 mg/kg gossypol for 5 weeks, or 10 mg/kg for 9 weeks, since no implantation sites in the mated females could be found. (Controls had several implantation sites per animal.) One or two weeks after the end of gossypol treatment, the guinea pigs remained infertile. Restoration of fertility in gossypol-fed guinea pigs (15 mg/kg/day) occurred 3 weeks after cessation of treatment. The number of implantation sites per animal in the mated females was then 2.8 ± 0.5. The usual range in untreated guinea pigs is 2.3 to 3.8 sites per animal (Wagner and Manning, 1976).

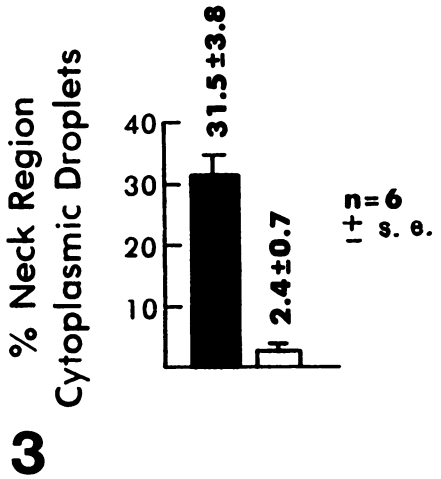
Discussion

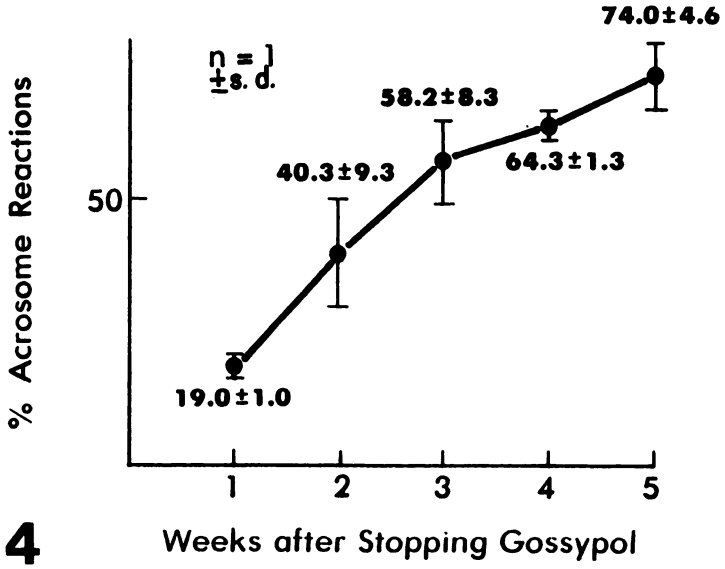
The results of our study show that the density of spermatozoa in the cauda epididymidis of gossypol-fed, sexually mature guinea pigs did not differ from those of control animals, suggesting that the kinetics of spermatogenesis proceeds normally in the gossypol-treated adult of this species. Nevertheless, guinea pigs fed gossypol at a dosage of 15 mg/kg for 5 weeks became infertile. Therefore, this paper adds the guinea pig to the list of animal models for exploring gossypol-induced infertility, and examines some of the parameters that contribute to it.

Epididymal sperm survival and the forward progressive movement of guinea pig spermatozoa were considerably decreased compared to controls, showing that the spermatozoa were indeed affected by the gossypol treatment despite the









Figs. 1–4. These graphs record in sequence: 1) Percentage forward progressive movement of guinea pig spermatozoa after 5 weeks of gossypol feeding (15 mg/kg/day); 2) Percentage guinea pig sperm acrosome reactions induced after 2–5 weeks of gossypol (10–15 mg/kg/day); 3) Percentage cytoplasmic droplets retained in the sperm neck region after 5–9 weeks of gossypol (10–15 mg/kg/day); 4) Acrosome reactions induced during the 5 weeks after stopping gossypol feeding (15 mg/kg/day).

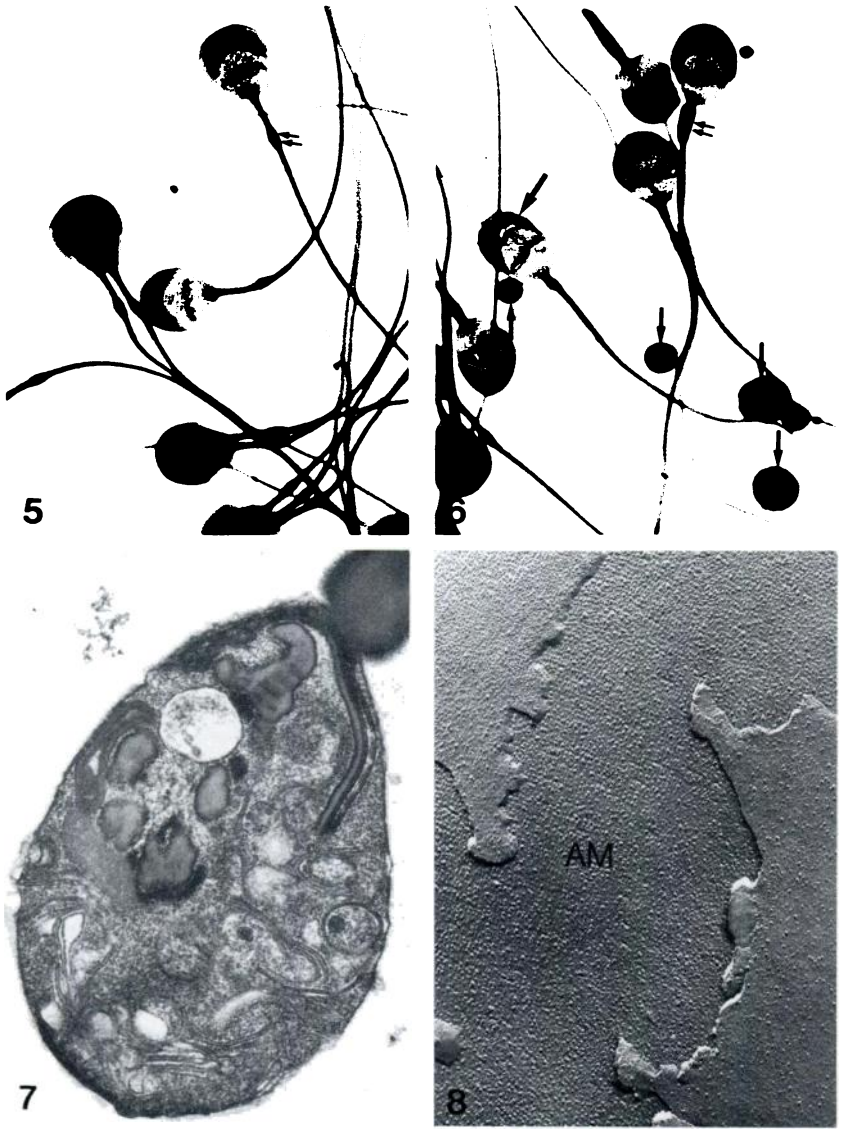


Fig. 5. Light micrograph of spermatozoa from the sexually mature guinea pig cauda epididymidis. Notice the position of the retained cytoplasmic droplet (double arrows) and the absence of spherical cells as compared to Fig. 6 (×1290). **Fig. 6.** Cauda epididymal spermatozoa from a guinea pig fed 10–15 mg/kg gossypol acetate for 5 weeks. Notice the mottled acrosome (large arrow), the exfoliated spherical cells, probable spermatids, and spermatocytes (arrows), and the rostral position of the retained cytoplasmic droplet (double arrows) (×1290). **Fig. 7.** Thin-sectional view of the cytoplasmic droplet retained in the neck region after 5 weeks of gossypol feeding. In this particular cell, the outer dense fibers may not be normally arranged. (×30,000). **Fig. 8.** Freeze-fracture through the acrosomal membrane (AM) generally reveal typical linear arrays of intramembranous particles despite the frequency of misshapen acrosomes. This acrosome was slightly blunter than normal. (×62,500).

lack of reduction in epididymal sperm numbers. Since sperm motility is a vital component in fertilization (Barros, 1974; Yanagimachi, 1981), the reduction of movement must be linked to gossypol's infertility effect. However, we observed no striking morphological alterations (Pelletier and Friend, 1983) in the tails of spermatozoa from the cauda epididymidis or the vas deferens. This finding is at variance with results in other species such as the rat (Oko and Hrudka, 1982; Shandilya et al, 1982; Hoffer, 1983), but it may represent differences in dose and duration of treatment, as well as differences in the animal. The possibility that the increased dosage or the prolonged length of treatment in our study may have produced morphologically visible defects cannot be excluded.

Light and qualitative electron microscopic examination revealed the failure of the retained cytoplasmic droplet to migrate caudally, implying that gossypol could have prevented epididymal spermatozoa from attaining full maturity. This effect may be related to an androgen defect in the cauda epididymidis (Blaquier et al, 1972), but no quantitative assessment of androgen levels in the sera or the cauda epididymidis was attempted in our investigation. Some workers have reported that gossypol decreased the levels of serum testosterone in rats (Hadley et al, 1981) and hamsters (Saksena and Salmonsen, 1982).

As the spermatozoa pass from the caput epididymidis to the cauda, there is normally a distal migration of the cytoplasmic droplet from the neck

region to the midpiece, where most of it is eventually shed (Bedford, 1975). In the present study, 31.5 ± 3.8% of gossypol-fed spermatozoa retained cytoplasmic droplets in the neck region. Similar retention of the cytoplasmic droplet at variable loci along the midpieces of rat spermatozoa after gossypol treatment have been observed (Bozek et al, 1981). Other investigators have shown that spermatozoa in which migration of the cytoplasmic droplet does not occur are generally incapable of fertilizing ova (Dott and Dingle, 1968), and midpiece retention of these droplets by spermatozoa has been associated with human sterility (Fujita et al, 1970).

Our observations also show that the guinea pig sperm acrosome reaction was considerably reduced ($P < 0.01$) as the duration of treatment increased, indicating that gossypol may have some effect on the "maturity" or conditioning of sperm membranes, similar to that of gossypol in other *in vitro* experiments (Shi and Friend, 1983a, 1983b). Since actively motile spermatozoa can fertilize eggs only after capacitation and the acrosome reaction (Yanagimachi and Noda, 1972, Yanagimachi, 1981), the abolition of the acrosome reaction deleted another essential component of fertility. Together, these observations intimate that the administration of gossypol does affect the maturity of spermatozoa present in the adult guinea pig epididymis.

In sexually immature guinea pigs, we observed that the numbers of mature epididymal spermatozoa were significantly decreased ($P < 0.01$), while the number of spermatids and spermatocytes in the epididymal lumen increased during gossypol administration. These results are in harmony with those reported by Shi et al, (1981b) regarding a decrease in both mature sperm production and motility in the rat after gossypol treatment. Russell and Clermont (1976) and Russell (1979), reported that the tubulobulbar complexes formed between Sertoli cells and late spermatids dissociated during normal spermiation, suggesting that the dissociation of the tubulobulbar complexes might be responsible for the final release of late spermatids. We speculate that gossypol might influence this release process, or influence the ectoplasmic specializations which form earlier between Sertoli cells and spermatocytes, with premature appearance of the cells in the epididymis. We have no concrete explanation of why fertility was restored in less time than it takes for a full spermatogenic cycle, other than that gossypol's effect may be exerted at the spermatid, rather than at the secondary spermatocyte stage.

While our studies do not provide firm conclusions about the exact site or mode of gossypol action in the guinea pig, the results do establish clearly that gossypol is an effective antifertility agent in this species and that the guinea pig is a reasonable animal model for studying its effects.

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