Effect of (+)-Gossypol on Fertility in Male Hamsters

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(+)-Gossypol was isolated from the bark of *Thespesia populnea* and tested for its ability to inhibit the fertility of male hamsters. Male hamsters of proven fertility were treated orally for 54 days with 40 mg/kg of (+)-gossypol, 40 mg/kg of racemic gossypol, or 5% gum acacia (vehicle control) and were mated with estrous female hamsters during the fourth and seventh weeks of treatment. Both the control and the (+)-gossypol-treated animals exhibited normal fertility throughout the experiment. The racemic gossypol-treated animals were infertile when evaluated during both the fourth and seventh weeks of treatment. Morphologic examination of the testicular tissue could not explain the loss of fertility. These data demonstrate the inability of (+)gossypol to decrease male fertility and suggest that the activity of racemic gossypol may be due primarily to the presence of the (−) optical isomer.

Key words: gossypol, male antifertility, (+)-gossypol, male contraceptive, hamster.

The ability of gossypol, a bis-esquiterpene isolated from the cotton plant (genus *Gossypium*), to inhibit male fertility has been demonstrated in several species (National Coordinating Group on Male Antifertility Agents, 1978; Chang et al, 1980; Hadley et al, 1981; Waller et al, 1980). The gossypol utilized in all of these experiments probably was the optically inactive, racemic mixture.

The (+) optical isomer of gossypol can be isolated from the plant *Thespesia populnea* (King and de Silva, 1968). This optical isomerism exists due to the restricted rotation around the carbon–carbon bond connecting the two symmetric sesquiterpene structures. No (−) optical isomer of gossypol has yet been reported.

Wang et al (1979) reported that (+)-gossypol did not affect fertility and was nontoxic when administered to rats at a dose of 12 mg/kg/day for 6 weeks. The authors' laboratory has extended the investigation of the effects of the specific (+) optical isomer to hamsters in the following experiments.

Materials and Methods

Preparation of Gossypol

(+)-Gossypol was isolated from the bark of *Thespesia populnea* Soland. ex. Correa (Malvaceae) by exhaustive extraction with petroleum ether. Purified (+)-gossypol (mp 184 C, $[\alpha]_D$ + 454°, c 1.0 $CHCl_3$) was obtained after repeated recrystallization from benzene–petroleum ether. These physical data are similar to the values previously reported by King and de Silva (1968) for (+)-gossypol (mp 181–184 C, $[\alpha]_D$ + 445° ± 10, c 0.15, $CHCl_3$). The infrared, proton magnetic resonance and mass spectrometry (ms) spectra were consistent with (+)-gossypol.

The purity of the (+)-gossypol was determined to be greater than 98% utilizing ms and high-performance liquid chromatography (HPLC) (reverse phase μ-Bondapak C_{18} column, acetonitrile–water–acetic acid [7:2:1]). Both ms and HPLC indicated the presence of a contaminant (<2%) which could not be completely removed from the sample utilizing preparative thin-layer chromatography, preparative HPLC, or reverse-phase column chromatography.

The racemic gossypol used in this study was gener-

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ated from gossypol acetic acid by the procedure of Campbell et al (1937). Repeated crystallization from ether–petroleum ether resulted in analytically pure gossypol according to ms and HPLC analyses.

Animals

Male and female golden (Syrian) hamsters 8 weeks of age (80–100 g) were purchased from a local supplier. On arrival, the males were housed one per cage in an environmentally controlled room (21 ± 1 C, relative humidity 55 ± 5%, lights on 0700–2100 hours). Food and water were provided *ad libitum* throughout the experiment.

Female hamsters utilized to prove the initial (pretreatment) fertility of males and to test the fertility of males during treatment arrived about 2 weeks before scheduled pairing. After 1 week of acclimatization, the estrous cycle in each female was determined according to the method of Orsini (1961). A female was paired with a male only after a minimum of two estrous cycles was observed.

Pairing

A proestrous female was placed in the cage with a male between 2100 and 2300 hours and observed under red light for lordosis and mounting by the male. The female remained with the male overnight, and the mating was considered positive only if lordosis and at least one copulatory cycle were observed. This was considered day 0 of gestation. On the morning following a positive mating, the female was removed from the male's cage, and a vaginal smear was obtained and examined for the presence of spermatozoa. Subsequently, the male was allowed a minimum of one night for recovery and was paired with a second female. On day 14 of gestation the females were killed, and the number of normal fetuses and implantation sites was counted and recorded.

Males were mated as described above to prove initial fertility as well as to evaluate fertility during treatment (days 23–29 and days 49–54).

Dosing

Twenty-seven males of proven fertility were randomly assigned to three treatment groups: 1) vehicle control (5% gum acacia); 2) (+)-gossypol (40 mg/kg); and 3) racemic gossypol (40 mg/kg). All doses were administered in a volume of 0.1 ml/10 g of body weight. Doses were prepared daily by triturating the test substance with gum acacia and slowly adding water to form a suspension. Every day between 1100 and 1300 hours, the animals were weighed and treated by oral gavage using a stainless-steel feeding needle. The treatment was continued for 54 days.

Histology

Males were killed 3 days after the final administration of gossypol. They were perfused-fixed through the heart with a 5% buffered (0.15 M caccodylate; pH 7.4) glutaraldehyde solution for 30 minutes. The testis and caput epididymis were diced and fixed by immersion. After three washes in buffer the tissue was postfixed in an osmium:ferrocyanide mixture (Russell and Burquet, 1977), dehydrated in ascending concentrations of ethanol, infiltrated with propylene oxide, and embedded in Araldite (502). Thick sections ranging from 0.5–1.0 microns in thickness were obtained on an ultramicrotome, stained with toluidine blue, and examined under the light microscope. The classification of Clermont (1954) was used to describe stage-related events.

Results

No gross signs of toxicity were observed in any of the groups during the treatment period. Two animals died in the group treated with racemic gossypol, but autopsy did not reveal a definite cause of death, in that the animals appeared normal on gross observation. Reduced body weight was observed after 54 days of treatment with (+)-gossypol and after 21 days of treatment with racemic gossypol, when compared with control animals (Table 1). A significant difference in body weight between the (+)-gossypol-treated animals and racemic gossypol-treated animals also occurred after 42 days of treatment.

Spermatozoa were observed in almost all vaginal smears in all three groups throughout the experiment. In only two instances, the females did not have sperm in their smears. Each of these females

TABLE 1. Effects of Gossypol on Body Weight of Male Hamsters (grams)

	Days of Treatment			
Treatment Group	1	21	42	54
Control	127 ± 9	133 ± 11.3	137.2 ± 10.6	141.0 ± 11.4
(+)-Gossypol	124 ± 9	127.7 ± 6.8	127.6 ± 7.7	128.1 ± 9.3*
Racemic gossypol	124 ± 11	123.7 ± 11.1†	118.7 ± 12.8†	113.4 ± 13.9‡

* Significantly different from control at $P < 0.01$.
† Significantly different from control at $P < 0.05$.
‡ Significantly different from control at $P < 0.005$ and (+)-gossypol at $P < 0.02$.

had been mated with different racemic gossypol-treated males during the 7th week.

Fertility was determined during treatment days 23–29 and 49–54 (Table 2). No decrease in fertility was observed at any time in the (+)-gossypol group when compared with controls. However, during weeks 5 and 7, the racemic gossypol-treated group exhibited a complete inhibition of fertility. No effect on the number of implantation sites or the number of fetuses was observed in the (+)-gossypol-treated group when compared with control animals (Table 2).

The mean combined testicular weights were not significantly different among control, racemic-, and (+)-gossypol-treated hamsters, as determined by analysis of variance. Examination of testis sections revealed complete spermatogenesis in both control and experimental groups, ie, all animals produced morphologically normal sperm, which were also seen within the epididymis. Three animals in the racemic gossypol-treated group showed a partial disruption of spermatogenesis, which was quantitative in nature. Of these three, the pattern of degeneration of spermatogenic cells was more prominent in one of the hamsters due to a relatively large number of degenerating cells. It was readily apparent that stage-specific degenerating cells were present among numerous healthy spermatogenic cells. Specific degenerating cells were as follows: 1) pachytene spermatocytes in stage VII; 2) step 7 spermatids in stage VII; and 3) step 7 spermatids in stages VII–VIII. The latter were retained after the normal time of sperm release and phagocytosed by the Sertoli cell in stages IX and XII. Meiotic degenerations were noted with approximately equal frequency in both experimental and control animals. Other than these stage-specific features, no apparent defects were noted.

Discussion

Recent experiments demonstrating the ability of gossypol to inhibit male fertility have utilized gossypol of unknown optical activity. The gossypol used in those experiments probably was the racemic mixture of gossypol obtained from plants of the genus *Gossypium*.

In the present experiments the (+) optical isomer was tested for antifertility activity in male hamsters. A high dose (40 mg/kg) was chosen to ensure that even a weak antifertility response would be detected. The authors previously demonstrated that a dose of 40 mg/kg of racemic gossypol is capable of causing infertility in male hamsters after 6 weeks of treatment (Waller et al, 1981). Other reports have demonstrated that doses as low as 5 mg/kg are able to decrease male fertility in hamsters when administered for 8 weeks (Chang et al, 1980). The dose and duration of treatment utilized by Wang et al (1979) to study the effects of (+)-gossypol were close to the minimum requirements for antifertility activity in rats (12 mg/kg for 10 weeks) with racemic gossypol (Chang et al, 1980). Thus, the lack of activity of (+)-gossypol in the rat could have been due to inadequate dose or length of treatment.

The present results, however, demonstrate conclusively that no antifertility activity is observed in male hamsters treated with a high dose of (+)-gossypol during a relatively long time.

The ability of racemic gossypol to cause infertility in males and the total lack of effect of (+)-gossypol on male fertility can be seen clearly in Table 2. These results support the previous claim of Wang et al (1979) that (+)-gossypol has no effect on male fertility.

Gravimetric measurements of testicular weights

TABLE 2. Effects of Gossypol on Fertility of Male Hamsters

Treatment (No. of males treated)	Days of Treatment					
	23–29			49–54		
	No. Females Pregnant/ Mated	Mean No. Implant Sites ± SD	Mean No. Normal Fetuses ± SD	No. Females Pregnant/ Mated	Mean No. Implant Sites ± SD	Mean No. Normal Fetuses ± SD
Control (9)	18/18	12.5 ± 2.2	11.8 ± 2.4	18/18	10.8 ± 2.0	10.2 ± 1.9
(+)-Gossypol (9)	17/18	12.3 ± 2.2	11.6 ± 2.3	17/18	11.5 ± 1.8	10.9 ± 2.0
Racemic gossypol (7)	0/14	0	0	0/14	0	0

and histologic examination of the testes indicated that the primary cause of infertility in racemic gossypol-treated hamsters was not due to changes in the testes, as shown from the results of microscopic studies. Major histologic changes were seen in only one animal, with two others showing the same pattern but to a much lesser extent. Other racemic gossypol-treated animals showed no histologic changes.

The morphologic pattern of response was similar to that produced in rats (Russell and Clermont, 1977) and hamsters (Russell, unpublished) after hypophysectomy, and in rats after a variety of treatments that interfere with hormonal stimulation of the testis (Russell et al, 1981). Therefore, it appears that racemic gossypol is capable of inhibiting spermatogenesis by this route and does so only to a minor extent, but the precise site of actions (pituitary, Leydig cells, spermatogenic cells, etc) is unknown.

The inhibition of increase in body weight in animals treated with (+)-gossypol was not as great as that observed in animals treated with racemic gossypol. There was a significant difference between the body weights of control *versus* (+)-gossypol-treated animals after 54 days. In addition, at 54 days the body weight of the (+)-gossypol group was also significantly different from that of the racemic gossypol group. These data indicate that (+)-gossypol is slightly less toxic than racemic gossypol. Since the effect on body weight of equivalent doses of (+)-gossypol and racemic gossypol was not the same, it appears that the negative isomer may possess toxicity greater than that of (+)-gossypol. This is somewhat discouraging, but if the (−) isomer can be obtained, it may be much more efficacious in producing male infertility. Thus, a decreased dose of the (−) isomer may have equal, or even less, toxicity than the racemic mixture at the doses required for male antifertility effects.

These data indicate that the (−) optical isomer may inhibit male fertility in a stereospecific manner, while toxicity may be a result of nonstereospecific interactions. It is of great importance to obtain the (−) optical isomer of gossypol and to determine whether, indeed, it is a more potent male antifertility agent as well as less toxic than the racemic gossypol.

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