Effect of Gossypol on Testicular Testosterone Production *In Vitro*

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Testicular minces were utilized to study the effect of gossypol on testosterone production. Testosterone production was assessed in both control and gossypol treated groups after 0 to 4 hours incubation in the presence of hCG. Media testosterone was measured by radioimmunoassay. Gossypol did not alter testosterone production when present in incubates at the concentrations of 3.5×10^{-5}M, 7×10^{-5}M and 3.5×10^{-4}M. Preincubation of testis mince with gossypol (7×10^{-6}M, 7×10^{-5}M, 3.5×10^{-4}M) for 1 to 4 hours did not alter subsequent hCG induced testosterone production in mature rats. Testosterone production however, was inhibited in immature rat testis when the whole testis was incubated for 4 hours with different concentrations of gossypol (7×10^{-6}M, 7×10^{-5}M, 3.5×10^{-4}M). *In vivo* testosterone production was not inhibited in the immature rat testis 24 hours after oral administration of gossypol (100 mg/kg).

Key words: gossypol, Leydig cell, testosterone.

Gossypol, a polyphenolic compound isolated from seeds, stems, and roots of the cotton plant *Gossypium sp.* has been considered an effective male antifertility agent by Chinese workers (National Coordinating Group, 1978). A series of experiments conducted on laboratory animals confirmed the antifertility potential of gossypol (Nadakavukaren et al, 1979; Chang et al, 1980; Kalla et al, 1982; Weinbauer et al, 1982). Although the mechanism of gossypol action in inducing infertility in some animal species, including humans, is far from clear, it is well known that the drug inhibits spermatogenesis and motility of spermatozoa in the excurrent ducts. The weights of the accessory sex organs, the levels of citric acid and fructose in seminal plasma, and plasma levels of LH and testosterone remain normal in rats, monkeys, and humans after gossypol treatment (National Coordinating Group, 1978; Wang et al, 1979; Sundaram, 1980; Frick et al, 1981; Coutinho et al, 1981; Kalla and Vasudev, 1981; Kalla et al, 1981, 1982). On the other hand, Hadley and coworkers (1981) have reported an inhibitory effect of gossypol on testosterone biosynthesis *in vitro*. Lin et al (1981) have also reported reduction in testosterone biosynthesis after gossypol treatment.

In view of the diversified results obtained on testosterone biosynthesis after gossypol treatment, the present studies were designed to investigate the effect of gossypol on testosterone biosynthesis in both mature and immature animals.

Material and Methods

Animals

Male Wistar rats (90 days old) of proven fertility were purchased from Chemie Linz AG. Animals were caged singly and kept under controlled light and temperature. All the animals were fed commercial diet and water *ad libitum*. Male and female rats were allowed to mate and their progeny was used when 21 to 25 days old.

Chemicals

Gossypol acetic acid (98.4%) obtained from USDA and Rockefeller Foundation, New York, was used without further purification. Human chorionic gonadotropin (hCG) was obtained from Serono Laboratory, Italy.

These investigations were supported by the Rockefeller Foundation, New York, Fonds zur Förderung der wissenschaftlichen Forschung, Proj. 3380, Stiftungs-und Förderungsgesellschaft der Paris-Lodron, Universität Salzburg and Institutional grants from the University of Salzburg.

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Submitted for publication July 22, 1982; accepted for publication April 15, 1983.

0196-3635/83/0900/0331/$01.05 © American Society of Andrology
(J Androl 1983; 4:331–335)

Preparation of the media and condition of the incubation

Gossypol acetic acid was dissolved in absolute alcohol to make 0.02 M stock solution, which was then serially diluted with Hanks solution containing 100 IU hCG/ml. The control samples contained only ethanol in Hanks solution. The concentration of alcohol (0.01%) was the same in both experimental and control samples. For each point determination separate samples were run.

The adult male rats were anesthesized with ether. The testes were removed weighed, decapsulated, and pooled. A 200 mg aliquot of the tissue was taken and transferred to a 10 ml conical flask containing 3 ml of incubation media. Incubation was carried out at 35 to 37 C for various time intervals under 5% CO_2 in oxygen in a Dubnoff metabolic shaker. Following incubations, the contents of each conical flask were transferred to centrifuge tubes (the conical flask was washed three times with 0.5 ml Hanks solution) and centrifuged for 10 min at 1500 × g. Aliquots of supernatant (2 ml) were stored in plastic tubes at −20 C until assayed for testosterone concentration by radioimmunoassay.

Controls were run to show that the two methods, whole decapsulated testis and testis minces, did not cause significantly different results. Testicular minces and decapsulated testis from both mature and immature animals were used. In the case of decapsulated testis, a 8.5- and seven-fold increase in testosterone production was obtained from the testes of immature and mature rats, respectively. The testicular minces of immature animals showed a nine-fold increase and the testicular minces of mature animals a six-fold increase in testosterone production. In both cases, the incubation was made with and without hCG. To estimate the testosterone production after gossypol treatment *in vitro*, a zero time point determination was made with all the concentrations used in the present investigations. A zero time point was calculated from the initiation of incubation and did not include the preparation time, which was 30 to 40 minutes. The stimulation obtained with 50 IU of hCG was not significantly higher than that obtained with 5 or 10 IU of hCG; the low doses gave a single fold stimulation. The values of testosterone concentration in media before and after extraction were comparable. The basal value of testosterone (not shown in the text) was also measured.

Experiment 1

Testes were removed from mature Wistar rats, weighed, and pooled. A 200 mg aliquot was taken from the pooled tissue and transferred to a 10 ml conical flask containing 3 ml of incubating media. Incubation was carried out from 1 to 4 hours with 50 μg (3.5×10^{-5}M) of gossypol/flask by the method described above.

Experiment 2

Testes were removed from mature Wistar rats, weighed, and pooled. A 200 mg aliquot was taken from the pooled material and incubated for 2 and 4 hours in the presence of 100 μg (7×10^{-5}M) or 500 μg (3.5×10^{-4}M) of gossypol/flask by the method described above.

Experiment 3

Testes were removed from mature Wistar rats, weighed, and pooled. A 200 mg aliquot was taken from the pooled tissue and preincubated for 1 to 4 hours in the presence of 10 μg (7×10^{-6}M), 100 μg (7×10^{-5}M) or 500 μg (3.5×10^{-4}M) of gossypol/flask. After preincubation, 3 ml buffer with 100 IU of hCG/ml was added and the incubation continued for an additional 4 hours. The rest of the procedure was the same as described above.

Experiment 4

Testes were removed from 21 to 25-day-old rats. The testes (60 to 80 gm) from all the animals (n = 14) were pooled and distributed randomly among treatment and control groups; each testis was weighed and the tunica removed. The incubation was carried out for 4 hours in the presence of 100 IU hCG/ml, and 10 μg (7×10^{-6}M), 100 μg (7×10^{-5}M) and 500 μg (3.5×10^{-4}M) of gossypol/flask. The rest of the procedure was the same as described above.

Experiment 5

Immature four-week-old male Wistar rats were exposed to a single oral dose of gossypol, 100 mg/kg body weight, by oral intubation. Control animals received the ethanol vehicle alone. Twenty-four hours after the drug administration, animals were sacrificed, and the testes were removed and weighed. The whole testis was incubated in the presence of hCG (100 IU of hCG/ml) for four hours. The rest of the procedure was the same as described above.

Testosterone Assay

The supernatant obtained after *in vitro* incubation was assayed for testosterone concentration by radioimmunoassay (Bartke et al, 1973).

Statistical analysis

Statistical analysis was done by one-way and two-way analysis of variance, Mann and Whitney-U-test (Ryan et al, 1976) and by Kruskal and Wallis' selected comparisons (Langley, 1979). In the two-way analysis of variance, both the factors (time and treatment) were also tested for their interaction. The chosen probability of error was 5%.

Results

Figure 1 summarizes changes in the testosterone (T) production by the testicular mince. Under the experimental conditions used, maximum stimulation in terms of T production was obtained 4 hours after incubation. However, no significant differ-

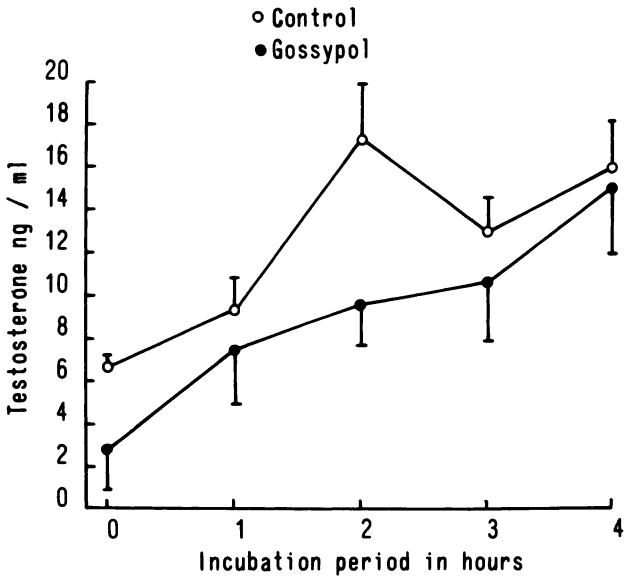

Fig. 1. Time-response curve to demonstrate maximal hCG-induced (100 IU/ml) testosterone production by testis minces after 4 hours of incubation. Significant differences in the testosterone production were observed after 4 hours for both the control ($p < .005$) and gossypol-treated (7×10^{-6}M) group ($p < 0.005$). However, no significant difference was observed between the control and the gossypol treatment group ($p > 0.05$). Each value illustrates the mean (±SE) of triplicate observations.

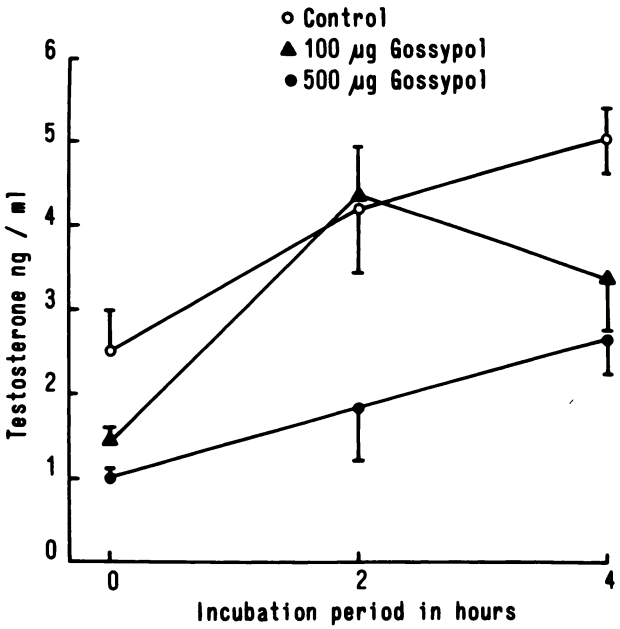

Fig. 2. Effect of gossypol on hCG-induced testosterone production by testis minces. Two hundred mg of testes minces were incubated with 100 µg (7×10^{-5}M) and 500 µg (3.5×10^{-4}M) of gossypol for 2 and 4 hours [In the testis minces incubated without hCG, the T concentration in the media was 0.96 ng/ml (±0.15)]. After incubation media testosterone was measured by radioimmunoassay. Each point represents the mean (±SE) of five observations. Differences in testosterone production along with the time and treatment were observed, but the interaction was not significant ($p > 0.05$). The level of significance was the same when the experiment was repeated.

ences were observed between control and gossypol treatment groups ($p > 0.05$) (Figs. 1, 2). The preincubation of testis mince with gossypol for 1 to 4 hours did not inhibit testosterone production at any of the three concentrations used (Fig. 3). No significant differences between the treatments ($p > 0.05$) and no significant interaction between time and treatments ($p > 0.05$) were observed. After 8 hours of incubation (4 hours preincubation with gossypol, followed by 4 hours incubation with hCG) marked reduction in the testosterone concentration was observed in all the groups, including the control (Fig. 3).

When whole testes from immature rats were incubated with different concentrations of gossypol, 10 µg (7×10^{-6} M), 100 µg (7×10^{-5} M), 500 µg (3.5×10^{-4} M), marked inhibition in testosterone production was observed (Fig. 4); the inhibition was significant at 100 µg ($p < 0.01$) and 500 µg ($p < 0.005$) of gossypol (Fig. 4). However, no inhibition in testosterone production was observed when the whole testis from the gossypol treated rats (100 mg/kg) was incubated in the presence of hCG; the testosterone production in the treated group was not different from that of the control (Fig. 5).

Discussion

In our investigations, we have used Bartke's method with little modification for *in vitro* testosterone production (Bartke and Lackritz, 1981). Essentially, there are three different methods employed for *in vitro* testosterone production: use of 1) isolated Leydig cells, 2) testicular mince or suspension, and, 3) whole testis. The results obtained by various workers for *in vitro* testosterone production in the presence of gossypol using different methods are in agreement (Hadley et al, 1981; Lin et al, 1981; Saksena et al, 1981). In the present investigations however, we have not observed inhibition in testosterone production when testis minces from adult rat were incubated with different concentrations of gossypol for varied time periods. Our results do not agree with those of Hadley et al, 1981 and Lin et al, 1981. This discrepancy in the results may be due to different concentrations of gossypol. It should be mentioned that gossypol used by these workers is different from that used in our studies. Quite inter-

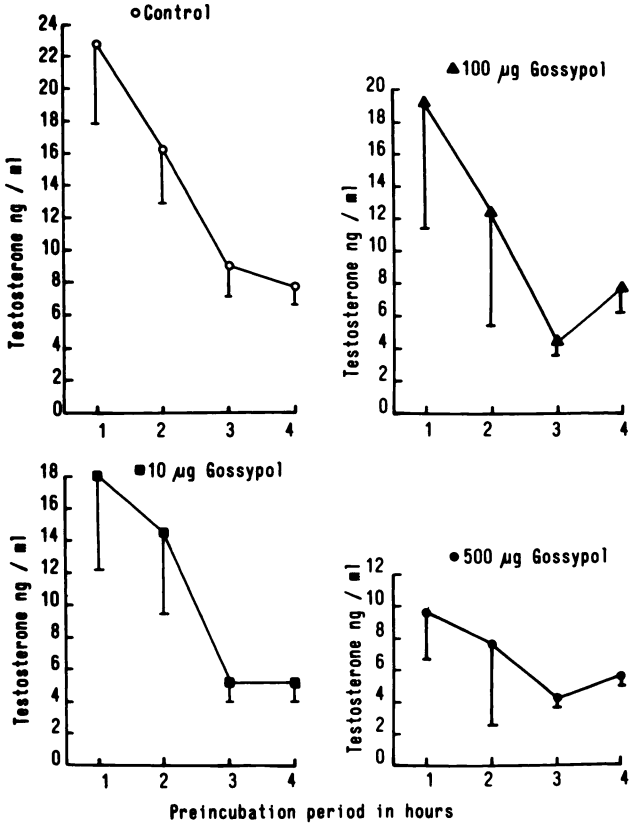

Fig. 3. The effect of preincubation with gossypol, 10 μg (7 × 10^{-6}M), 100 μg (7 × 10^{-5}M), 500 μg (3.5 × 10^{-4}M), on testosterone production by testis minces. Two hundred mg of testes minces were preincubated with gossypol for 1 to 4 hours. After preincubation, buffer with 100 IU hCG/ml was added and the incubation continued for an additional 4 hours. Media testosterone was measured by radioimmunoassay. At zero time the T values were 2.5 ng/ml (±0.95), 2.4 ng/ml (±0.83), and 2.0 ng/ml (±0.74) after 10 μg, 100 μg, and 500 μg of gossypol, respectively. Each point represents the mean (±SE) of five observations. No significant difference in testosterone production was observed.

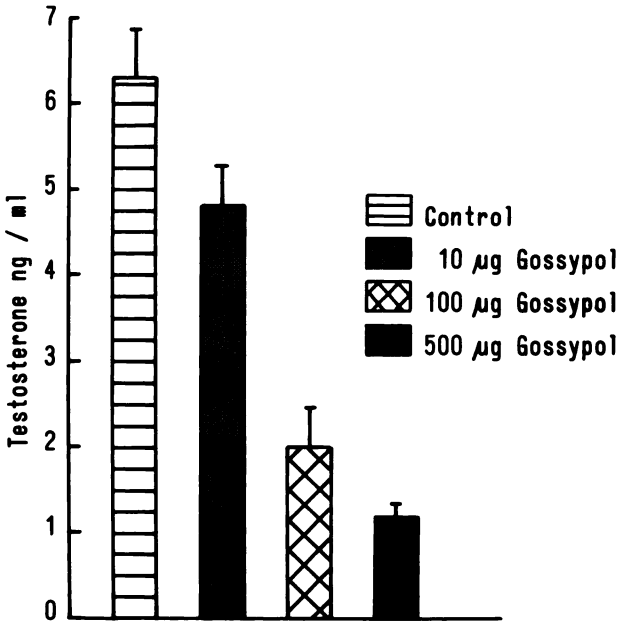

Fig. 4. Effect of gossypol on hCG-induced testosterone production by whole testis of immature rat.The whole testes were incubated with different concentrations of gossypol, 10 μg (7 × 10^{-6}M), 100 μg (7 × 10^{-5}M), 500 μg (3.5 × 10^{-4}M) for 4 hours. After incubation, media testosterone was measured by radioimmunoassay. [In the testis incubated without hCG, the T concentration in the media was 0.66 ng/ml (±0.05)]. Each bar represents the mean (±SE) of five observations. Significant differences in testosterone production were observed after 100 μg of gossypol treatment ($p < 0.05$) and 500 μg of gossypol treatment ($p < 0.01$). The level of significance was the same when the experiment was repeated.

estingly, Saksena et al, 1981, have reported that the effect on testosterone production of gossypol from different sources was not uniform even at the same molar concentrations.

The discrepancy in testosterone production obtained with different batches of gossypol is difficult to explain. Earlier it was thought that the impurity of gossypol plays an important role in its biological effects. Recently, Waller and his associates (1981) have, however, shown that the impurities present in the gossypol samples do not alter the biological efficacy of the compound. It has been reported that gossypol exists in two isomeric forms, i.e., dextrorotatory and levorotatory. It is quite reasonable to believe that the levorotatory to dextrorotatory ratio of gossypol might be the deciding factor. Waller and his associates have shown that the biological activity of gossypol is associated with the levorotatory form (Waller et al, 1982). It is quite possible that the ratio of dextrorotatory to levorotatory isomers is different in gossypol batches isolated and purified by different groups, and therefore exerts different biological effects.

We have observed marked inhibition in testosterone production in immature rat testis after gossypol treatment; the observations are very different from that of the mature testis. The inhibition in testosterone production in the testis of immature rats after gossypol treatment may be due to 1. high concentration of gossypol/gram tissue weight or gossypol/Leydig cell; the difference in the number of Leydig cell/testis from 21-day-old rats (the juvenile phase) and from adult rats is almost twenty-fold (Paz et al, 1980a), 2. the metabolism of Leydig cells in terms of testosterone production is different in the early juvenile phase (the

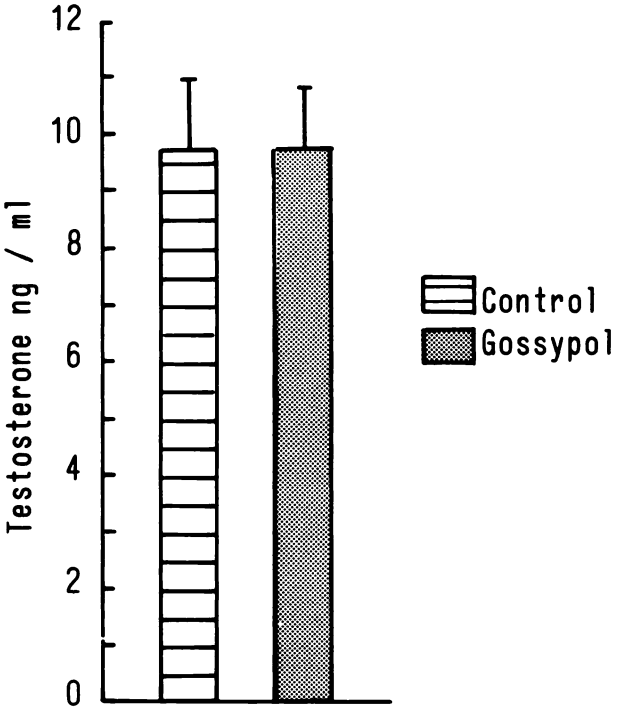

Fig. 5. Effect of gossypol on testosterone production by immature rat testis *in vivo*. Immature rats (three weeks old) exposed to a single oral dose (100 mg/kg body weight) were sacrificed after 24 hours and the whole testis was incubated in the presence of hCG for 4 hours. After incubation, the media testosterone was measured by radioimmunoassay. Each bar illustrates the mean (±SE) of six observations. No significant difference was observed in the two groups.

main androgens produced are 5 κ-reduced metabolites) while in the late juvenile, early puberty, and adult stages, testosterone is the major androgen product (Paz et al, 1980b) 3. the metabolism and biological response of gossypol is different in immature rats.

We have not observed any changes in the serum level of testosterone 24 hours after administration of a single high dose of gossypol, (Weinbauer et al, 1983) nor have we found any effect on testosterone production 24 hours after gossypol treatment. It is quite possible that the duration of gossypol treatment in the immature rats was not sufficient to induce any effect on T production.

In conclusion, our studies suggest that gossypol does not inhibit T production in testes minces from the mature rat testis. However, at high concentrations, gossypol inhibits testosterone biosynthesis in the immature rat testis, the inhibition possibly being due to a decrease in the number of viable Leydig cells and/or the direct effect of gossypol on T biosynthesis.

Acknowledgment

The authors wish to thank Professor H. Adam for helpful discussion, Dr. G. Bernroider for kindly providing a working facility on a PDP-11 computer (Digital Equipments Ltd.), Ms. G. Hölzl, University of Innsbruck, and Ms. V. Nussbaumer, University of Salzburg, for excellent technical assistance, and Ms. P. Köhle for typing the manuscript.

References

Bartke A, Steele RE, Musto N, Caldwell BV. Fluctuations in plasma testosterone levels in adult male rats and mice. Endocrinology 1973; 92:1223–1228.

Bartke A, Lackritz RM. Bromocriptine stimulates testosterone production by mouse testis *in vitro*. Fertil Steril 1981; 35:473–476.

Chang MC, Gu Z, Saksena SK. Effects of gossypol on fertility of male rats, hamsters and rabbits. Contraception 1980; 21:461–469.

Coutinho E, Melo JF, Segal S, Barbosa I. Suppression of spermatogenesis in men by gossypol. Isr J Med Sci 1981; 17:741.

Frick J, Danner CH, Köhle R, Kunit G. Male fertility regulation. In: Cortes-Prieto J, Campos da Paz I, Neves-e-Castro M, eds. Research in fertility and sterility. Lancaster: MPT, 1981:291–304.

Hadley MA, Lin YC, Dym M. Effects of gossypol on the reproductive system of male rats. J Androl 1981; 2:190–199.

Kalla NR, Vasudev M. Studies on the male antifertility agent–gossypol acetic acid. III. Effect of gossypol on rat testis. Andrologia 1981; 217–226.

Kalla NR, Foo JTW, Sheth AR. Studies on the male antifertility agent—gossypol acetic acid. V. Effect of gossypol acetic acid on the fertility of male rats. Andrologia 1982 (in press).

Langley R. Practical statistics. London: Pan Books, 1979.

Lin T, Murono EP, Osterman J, Nankin HR, Couson PB. Gossypol inhibits testicular steroidogenesis. Fertil Steril 1981; 35:563–566.

Nadakavukaren MJ, Sorensen RH, Tone JN. Effect of gossypol on the ultrastructure of rat spermatozoa. Cell Tiss Res 1979; 204:293–296.

National Coordinating Group on Male Antifertility Agents. Gossypol—a new antifertility agent for males. Chin Med J 1978; 4:417–428.

Paz GF, Winter JSD, Reyes FI, Faiman C. Development pattern of $\Delta^5$3β-hydroxysteroid dehydrogenase activity in isolated rat Leydig cells. Steroids 1980a; 36:661–674.

Paz GR, Winter JSD, Reyes FI, Faiman C. Development pattern of testosterone production by the rat testis. Steroids 1980b; 36:675–688.

Ryan TA, Joiner BL, Ryan BF. Minitab-student handbook. Boston, Duxbury Press, 1976.

Saksena SK, Salmonsen R, Lau IF, Chang MC. Gossypol: its toxicological and endocrinological effects in male rabbits. Contraception 1981; 24:203–213.

Sundaram K. Effect of gossypol on rhesus monkeys. Chicago: PARFR Workshop on Gossypol, 1980.

Waller DP, Fong HHS, Cordel GA, Soejarto DD. Antifertility effects of gossypol and its impurities on male hamsters. Contraception 1981; 23:653–660.

Waller DP, Bunyapraphatsara N, Fong HHS. Effect of (+)−gossypol on fertility in male hamsters. J Androl 1982; 3:38.

Wang YE, Lou TG, Tang XC. Studies on the antifertility actions of cottonseed meal and gossypol. Acta Pharmacol Sinic 1979; 14:663–669.

Weinbauer GF, Rovan E, Frick J. Antifertility efficacy of gossypol acetic acid in male rats. Andrologia 1982; 14:270–275.

Weinbauer GF, Rovan E, Frick J, Adam H. The endocrine status of gossypol-treated rats. Andrologia 1983; (in press).