Antifertility mechanisms of gossypol acetic acid in female rats

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Summary. Gossypol acetic acid was administered orally (30, 60, 90 and 120 mg/kg/day) on Days 1–5 *post coitum* to mature female rats. At autopsy on Day 10, pregnancy in most treated animals (6/7 and 6/8) was blocked at high doses (90 and 120 mg/kg/day respectively). As the daily dose decreased to 60 mg/kg/day half (4/8) were not pregnant. However, at a lower dose (30 mg/kg/day), or at a single dose of 200 mg/kg at Day 1 *p.c.*, pregnancy was not blocked. The concentrations of progesterone in the serum of these females were significantly decreased except at the low dose. The numbers of implantation sites in the treated females that did remain pregnant were similar to those in control females except at the dose of 120 mg/kg/day. Gossypol did not retard the development of the preimplantation embryo or cavitation. The Pontamine Blue test revealed that the drug did not interfere with the initiation of implantation. We suggest that gossypol has an antifertility effect in the female rat because it is luteolytic and disrupts post-implantation development.

Introduction

Since Liu (1957) proposed the possible effects of cotton seed oil on fertility in men and women, studies of the effects of gossypol on male fertility have been extensive. In contrast, investigation of its effects in females has been limited. Gossypol has been reported to inhibit implantation and decrease the concentration of progesterone in circulating blood of the female rat (Murthy *et al.*, 1981; Hahn *et al.*, 1981; Yuan *et al.*, 1983; Lin *et al.*, 1985). Several reports have been published on endometrial changes after treatment with gossypol in women. Liu *et al.* (1981) found a marked reduction of progesterone concentration and steroid receptor content in human endometrium after gossypol treatment and suggested that the gossypol might have a direct inhibitory action on endometrial receptors. After gossypol treatment three different characteristics of the endometrium were noted: irregular secretory activity, proliferative activity and atrophy (Zhu *et al.*, 1984). In addition, the Department of Obstetrics and Gynecology, 1st Teaching Hospital (1979) also found marked atrophy in the endometrium of women after ingestion of crude cotton seed oil. However, the exact mechanism of action of gossypol in its antifertility effect in the female remains to be explored.

In the present study, the antifertility mechanisms of gossypol acetic acid in female rats were studied with special reference to the embryo–maternal interaction.

Materials and Methods

Animals. The Sprague–Dawley rats were mature virgins (230–250 g) provided by the animal house of our Institute and were housed in a temperature- (15–20°C) and light- (06:30–18:30 h) controlled room. Food and water were available *ad libitum*. The female rats were caged overnight with the male (2 females:1 male). The finding of spermatozoa in the vaginal smear on the following morning was taken as evidence of mating, and this day was designated as Day 1 *post coitum* (*p.c.*).

Antifertility studies. Mated female rats were given gossypol acetic acid by gavage at various daily doses (30, 60, 90 and 120 mg/kg) on Days 1–5 *p.c.* Only vehicle (10 ml/kg/day) was given to the control females. The gossypol acetic acid was purchased from Wuxi College of Light Industry (China) and its purity was 99%. It was suspended in a steroid-suspending vehicle, 5% solution of *Tragacanthae pulvis*, i.e. gum tragacanth (purchased from Shanghai Medicine Company). At Day 10 *p.c.* autopsy was performed under pentobarbitone anaesthesia. The numbers of corpora lutea and implantation sites were counted and recorded. The initiation of implantation was determined at Day 6 *p.c.* when 0.6 ml 1% Pontamine Blue was injected intravenously and 20 min later autopsy was carried out and the number of dye sites determined. Blood samples were withdrawn from the abdominal aorta, and the serum was kept at -30°C until radioimmunoassay of progesterone.

Recovery of blastocysts. At autopsy on Day 5 of pregnancy, the ovary and the genital tract were removed and the uterus and oviduct were separated at the tubo-uterine junction. Under a stereomicroscope embryos were flushed from the oviduct and the uterus with saline (9 g NaCl/l), collected and examined promptly.

Radioimmunoassay of progesterone. Serum samples were diluted with phosphate buffer (pH 7.2) and 200 μl aliquants were extracted twice with 5 ml diethyl ether. After extraction the ether phase was evaporated in a water bath (40°C) to dryness. The dried extract was redissolved in 2.2 ml phosphate buffer, and two 0.5 ml aliquants of the redissolved extract were transferred to assay tubes. The matched assay reagents used in the present study were provided by the WHO Programme, and the assay procedure was in accordance with the description of the WHO Method Manual (Sufi *et al.*, 1982). In brief, 100 μl [^3H]progesterone working solution and 100 μl antiserum were added to each sample or standard in duplicate. The tubes were vortex-mixed and incubated at 4°C overnight ($> 18\text{ h}$); the total volume of the incubate was 700 μl . After incubation, 0.2 ml of the dextran-coated charcoal suspension was added to each tube, vortex-mixed and stood at 4°C for 15 min, and centrifuged at 1700 *g* for 15 min. The supernatant was then decanted into scintillation vials and counted with an LKB-type β counter. The sensitivity of the assays, defined as twice the standard deviation of the blank values, was 0.19 pmol per assay tube. The recovery of [^3H]progesterone added to the rat serum was $88.5 \pm 7.6\%$ (mean $\pm$ s.d.). Intra-assay coefficient of variation was $9.3 \pm 3.6\%$. Interassay coefficient of variation for a serum sample containing 19 ± 3 nmol progesterone/l was 15.8%.

Data analysis. Student's *t* test was used for data analysis.

Results

Ability of gossypol acetic acid to block early pregnancy

Five successive doses of gossypol acetic acid were administered orally at Days 1–5 *p.c.* The daily dose used in different groups was 30, 60, 90 or 120 mg/kg. At autopsy on Day 10, pregnancy in most treated animals (6/7 and 6/8) was blocked at high doses (90 and 120 mg/kg/day respectively). As the daily dose decreased to 60 mg/kg half (4/8) were not pregnant. However, at a lower dose (30 mg/kg/day), or at a single dose of 200 mg/kg at Day 1 *p.c.*, pregnancy was not blocked. The numbers of implantation sites in the treated females that did remain pregnant were similar to those in control females except at the dose of 120 mg/kg (Table 1).

When progesterone (4 mg) was subcutaneously injected immediately after the oral treatment of gossypol (90 mg/kg on Days 1–5) until autopsy at Day 10 *p.c.*, the antifertility effect was partly reversed: 8 out of 12 rats became pregnant, although the number of implantation sites (5.8 ± 1.0) was lower than that in the control animals (10.9 ± 0.7).

Effects on serum progesterone concentrations

Serum concentrations of progesterone at Day 10 *p.c.* in the gossypol-treated females were significantly lower than those in the control females except in Group 1 (Table 1). Values in non-pregnant animals in Groups 2–4 (41.1–71.3 nmol/l) were lower than those in pregnant rats (75.9–130.8 nmol/l).

To determine when the progesterone concentrations decreased, values in the blood samples collected at Day 5 and Day 6 were also measured. The results (Table 2 & 3) indicated that even as early as Days 5–6 of pregnancy the serum progesterone concentration had begun to decrease.

Table 1. Effects of gossypol acetic acid on pregnancy and serum progesterone concentrations in the rat at Day 10 *p.c.*

Group	Oral dose (mg/kg/day)	No. of animals			No. of implantation sites	No. of CL	No. of implantation sites	Progesterone conc. (nmol/l)	
		Total	With implantation sites					Non-pregnant	Pregnant
1	5 × 30	8	8		8.6 ± 1.5	11.9 ± 1.5		—	219.7 ± 25.1
2	5 × 60	8	4*		12.0 ± 0.7	12.8 ± 0.8		41.1 ± 5.4***	130.8 ± 4.8*
3	5 × 90	7	1*		11.1 ± 0.9	13.0		71.3 ± 14.5***	96.3
4	5 × 120	8	2*		10.1 ± 0.8	4.5 ± 2.5**		45.0 ± 4.0***	75.9*
5	1 × 200	7	7		19.6 ± 0.6	12.4 ± 0.9		—	—
6	Vehicle (10 ml/kg)	9	9		11.6 ± 0.6	10.9 ± 0.7		—	215.3 ± 24.1

Values are mean ± s.e.m.

P* < 0.05, *P* < 0.01 compared with control value (Group 6).****P* < 0.001 compared with pregnancy control value.**Table 2.** Effects of gossypol on the development of preimplantation embryos and serum progesterone concentrations in rats killed on Day 5 *p.c.*

Treatment	No. of rats	No. and location of embryos (%)				Stage of development (%)				Progesterone conc. (nmol/l)
		Total	Oviduct	Uterus		2-cell	3-cell	Morula	Blastocyst	
Gossypol (4 × 90 mg/kg/day)	13	105	5 (5)	100 (95)		4 (4)	3 (3)	6 (6)	88 (83)	4 (4)
Vehicle (10 ml/kg)	9	89	0	89 (100)		0	0	0	68 (76)	21 (24)

Values for progesterone concentration are mean ± s.e.m.

**P* < 0.001 compared with control (vehicle) group.

Table 3. Effects of gossypol on the initiation of implantation and serum progesterone concentration in rats killed on Day 6 *p.c.*

Treatment	No. of animals		No. of CL	No. of dye sites	Progesterone conc. (nmol/l)	
	Total	With dye sites			Non-implanted	Implanted
Gossypol (5 × 90 mg/kg/day)	15	9	15.5 ± 1.5	10 ± 1.3	98.9 ± 14.3*	157.6 ± 14.0**
Vehicle (10 ml/kg)	13	11	14.5 ± 0.7	12.5 ± 0.8	191.8 ± 58.0	257.0 ± 15.2

Values are mean ± s.e.m.

* $P < 0.05$, ** $P < 0.001$ compared with control value.

Development of preimplantation embryos

Table 2 shows the effects of gossypol on embryo development. Embryos recovered in the morning (09:00–11:00 h) on Day 5 *p.c.* were mainly located in the uterus. After gossypol treatment most embryos recovered were at the blastocyst stage. The blastocysts recovered from the gossypol-treated females appeared similar to those of control females. Therefore, it is concluded that the gossypol treatment did not retard the preimplantation development of embryo in the rat.

Initiation of implantation

In mated females given gossypol acetic acid at a dose of 90 mg/kg/day on Days 1–5 *p.c.*, the uterine Pontamine Blue reaction was examined at Day 6 *p.c.* to determine whether implantation had begun. The number of dye sites in the treated group was not significantly different from that in the control group (Table 3). Therefore, gossypol did not interfere with the initiation of implantation.

Discussion

The present study showed that the antifertility efficacy of gossypol acetic acid in female rats is dose-related. Large oral doses (90 and 120 mg/kg/day) given for 5 successive days (Days 1–5 *p.c.*) resulted in failure to become pregnant. A lower dose of 30 mg/kg/day did not affect the pregnancy when examined on Day 10 *p.c.*, and results for a dose of 60 mg/kg/day were intermediate. Gossypol exhibited a luteolytic action; progesterone concentrations were decreased in females that did not become pregnant and in treated females that remained pregnant. This study also indicated that the progesterone concentrations in the treated females had fallen by Day 5–6 *p.c.*

Since an increase in vascular permeability occurs at the endometrial implantation sites at or before apposition of the trophoblast and the endometrium, a phenomenon which can be taken to define the beginning of implantation (Kennedy, 1983; Findlay, 1983), we carried out the Pontamine Blue test to explore whether implantation was initiated after gossypol treatment. The results showed that the dye sites in the treated group and control group were not significantly different, indicating that the initiation of implantation was not retarded by the gossypol treatment. Pre-implantation development was not retarded and blastocysts recovered from the uterus appeared normal. The results in Table 1 indicate that post-implantation development of embryos was blocked since 16 out of 23 mated females in Groups 2–4 were not pregnant at Day 10 *p.c.* Zhou & Lei (1984) reported that treatment of female rats with gossypol acetic acid caused marked atrophy of the endometrium. Zhu *et al.* (1984) analysed the ultrastructural changes of the endometrium

induced by gossypol and suggested that a cytotoxic effect of gossypol on the endometrium in women could not be excluded. Gossypol could, however, affect blood flow through the endometrium or some other factor, since the successful initiation of implantation indicates that the endometrium was competent at that stage.

At the dosage of 90 or 120 mg/kg/day for 5 days gossypol exerted a toxic effect as there was a decrease in body weight of about 20 g after 10 days. However, the development of the preimplantation embryos was not retarded under these conditions, suggesting that the early embryo may be more resistant to the toxic action of gossypol.

This study received financial support from the Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization. We thank Dr R. B. Heap for help in the preparation of this manuscript.

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Received 9 September 1986