CLINICOPATHOLOGICAL EFFECTS OF GOSSYPOL IN MALE CYNOMOLGUS MONKEY[c]

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ABSTRACT

The effects of gossypol treatment on metabolic and key organ functions were studied in cynomolgus monkeys Macaca fascicularis. *Twelve male monkeys were treated intramuscularly with various doses of gossypol (0.5 mg/kg daily, weekly, or every 2 weeks) for 8 weeks. Hormonal and blood chemistry profiles were examined at 4 and 8 weeks after treatment. A high dose (0.5 mg/kg/day) of gossypol caused elevation of serum uric acid (UA), liver enzymes, aldolase (ALD), creatine phosphokinase (CPK) and lactic dehydrogenase (LDH_{4-5}), suggesting liver and skeletal muscle damage and some impairment of renal function. A moderate dose (0.5 mg/kg/week) of gossypol caused elevation of serum ALD, CPK and LDH_{4-5} and thus affected only skeletal muscle function, and a low dose (0.5 mg/kg/2 weeks) seemed to possess antifertility effects without toxicity. Various doses of gossypol given intramusculary thus had variable side effects on metabolic and key organ functions, and the maximum safe (nontoxic) dose was 0.5 mg/kg/2 weeks.*

INTRODUCTION

Administration of gossypol orally has been reported to produce no toxic effects in rats.[1-2] However, rats that survived gossypol treatment of 5-10 mg/kg had obviously depressed food intake and utilization, and weight again. Other workers have found gossypol toxicity at doses higher than 10 mg/kg in rats, but not at lower doses.[3-5] Moreover, gossypol is hepatotoxic to rats when given intraperitoneally at 5 mg/kg daily for 2 weeks.[6] In man, clinical chemistry findings in a few cases (number not specified) in China,[1,7] 8 cases in Brazil[8] and 6 cases in Austria[9] of men treated with gossypol (20 mg orally/day for 3 months), have demonstrated reduction in sperm motility and/or azoospermia without any changes in testosterone level, serum potassium (K) or other plasma parameters of vital organ function (heart, liver and kidney). The most serious side effect of gossypol is hypokalemic paralysis, which has been reported in 66 out of 8806 cases.[10]

Gossypol administration in a macaque at 10 mg/kg/day orally for 6 months also showed no adverse clinical findings, but reduction in sperm number and motility indicated positive antifertility effects.[11] In an attempt to develop gossypol as an injectable contraceptive, administration of the same dose intramuscularly in our previous study caused degeneration of the liver, kidney, spleen and even testis.[12] Further toxicological studies are therefore necessary because it has not been conclusively shown that all the side effects can be ascribed to either the dose of gossypol used or the animal model. Our studies were undertaken to evaluate the effect of various doses of intramuscular gossypol treatment on metabolic and key organ functions in a nonhuman primate *Macaca fascicularis.*

MATERIALS AND METHODS

Animals

Twelve adult male cynomolgus monkeys *(Macaca fascicularis)* used were 6-8 years in age and weighed 8-10 kg. The animals were supplied from two sources, the Primate Center of Chulalongkorn University and the Animal Center of Mahidol University. All animals were housed in individual cages and kept in a well ventilated room illuminated by daylight supplemented with artificial lighting for 12 h a day (0600-1800). The animals were fed daily in the morning with monkey chow containing 1.19% potassium (Pokphan Animal Feed Co., Ltd. Thailand) and in the afternoon with fresh fruits and vegetables. The animals were divided into one vehicle control and three treated groups, each with 3 animals.

Dosage and Treatment Schedule

Racemic (±) gossypol acetic acid (U.S. Department of Agriculture, 90% to 95% purity) was provided by Dr. Y. Thebtaranonth, Faculty of Science, Mahidol University. This gossypol was originally supplied as a gift by Dr. H.H.S. Fong of the University of Illinois at the Medical Center, Chicago, Illinois, U.S.A. Analytically pure gossypol was prepared by the acetic acid adduct method of Campbell *et al.*[13] Gossypol acetic acid was suspended in a small quantity of sesame oil (50 mg/ml) immediately before use. The drug was administered by intramuscular injection in the thigh. One group of monkeys was treated with gossypol at a dose of 0.5 mg/kg/day, a second group at 0.5 mg/kg/week, and a third at 0.5 mg/kg/2 weeks. Control animals were administered daily with sesame oil alone.

Blood Sampling

The animals health was evaluated regularly throughout the experiment. Blood samples (10-15 ml) were drawn from the femoral vein of the treated and vehicle control animals every 4 weeks. Each animal was fixed in a monkey chair and blood was drawn without anesthesia. The blood was allowed to clot in a refrigerator for 1-2 h and serum was separated by centrifugation at 3000 rpm for 15 min. Serum samples were then removed for analysis of blood chemistry profiles.

Blood Chemistry Profile Analysis

Serum concentrations of sodium (Na), potassium (K), carbon dioxide (CO_2),chloride (Cl), calcium (Ca), phosphorus (P), blood urea nitrogen (BUN), creatinine (CREA), uric acid (UA), cholesterol (CHOL), total bilirubin (T. Bili), direct bilirubin (D.Bili), total protein (TP), albumin (ALB), alkaline phosphatase (AP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), aldolase (ALD), lactic dehydrogenase (LDH), creatine phosphokinase (CPK), LDH isoenzymes and CPK isoenzymes were measured in the Clinical Pathology Laboratory at Ramathibodi Hospital, Mahidol University, according to the established procedures.[14] Normal range of these parameters were collected from normal male animals (N=50) in our colonies.

Statistical Analysis

Data were analysed by using Mann-Whitney U-Test.

RESULTS

Electrolytes

Table 1 lists the average values (mean ± SD) of serum electrolytes, Ca and P in each group of the animals. After 4 and 8 weeks of gossypol treatment, there were no significant changes in these parameters in any of the groups.

Blood Chemistry Profiles

Table 2 shows the average values (mean ± SD) of serum AP, GOT, GPT, ALD and CPK levels in each group of the animals. After 4 weeks of gossypol treatment, LDH increased significantly in group 1 animals whereas ALD and CPK increased significantly in group 1 and 2. And after 8 weeks of gossypol treatment, GPT increased significantly in group 1 while AP, ALD, LDH and CPK increased significantly in groups 1 and 2. These enzymes remained normal in group 3 and in the control group. LDH and CPK isoenzymes were measured as percent values in gossypol treated animals, including vehicle control animals (group 4) after 8 weeks of treatment, as shown in Table 3. In most of the gossypol treated animals (groups 1 and 2), percent of LDH_1 decreased significantly, percent of LDH_4 increased significantly, but percent of LDH_{2-3} were not significantly changed. Percent of LDH_5 increased significantly only in group 2. Percent of CPK_3 isoenzyme also increased significantly in groups 1 and 2, but remained normal in group 3 and in the vehicle controls. Percent of CPK_{1-2} were not significantly changed in any of the groups.

There were no changes in serum BUN, CREA, UA, T. Bili, D. Bili, TP, ALB and CHOL in any gossypol treated animal (data not shown), except that a significant increase in UA was found only in group 1 and 2 animals after 4 and 8 weeks of gossypol treatment (after 4 weeks : 3.8 ± 1.5 mg% in group 1, 3.1 ± 1.3 mg% in group 2; after 8 weeks : 4.0 ± 1.2 mg% in group 1, 3.3 ± 1.1 mg% in group 2; normal range 0–2.0 mg%; $p < 0.01$ *vs* before treatment).

DISCUSSION

The present study showed normokalemia in all of gossypol treated animals. Qian *et at.*,[15] however, reported that gossypol decreased the potassium content of myocardium in isolated rabbit heart. Later, these workers[16] found that gossypol reduced the intracellular K concentration in low-K-fed rats but not in normal-K-fed rats. Similar results were obtained for isolated skeletal muscles by Xu and Qian.[17] These were the first experimental studies showing a definite effect of gossypol on K metabolism.

Qian[10] and Qian *et al.*[18] reported that the incidence of hypokalemic paralysis was astonishingly higher among subjects taking gossypol than in the control population. Moreover, among gossypol-taking subjects, hypokalemic paralysis occurred only in those with a relatively low K intake, a phenomenon similar to that occurred in experimental rats.[15] Putting all these facts together, one might conclude that hypokalemic paralysis in gossypol-taking subjects is not merely a concomitant phenomenon, but is related to the effects of gossypol. However, the incidence of gossypol-induced hypokalemic paralysis has been low, and most subjects taking gossypol have not shown hypokalemia and their body K level was normal.[19]

The present study reveals evidence of apparent renal and liver malfunction in animals treated with high dose of gossypol (0.5 mg/kg/day), but not in those treated with lower dose (0.5 mg/kg/week). Significant increases in UA in those treated with high dose of gossypol may have resulted from massive tissue destruction of muscle, liver or red blood cell which could have caused renal malfunction. Significant increases in serum GPT in animals receiving high doses also indicated direct hepatotoxic effects. The increase in ALD which occurred in all treated animals is an indicator of skeletal muscle disease, though we cannot exclude a myocardial or liver origin. Study of ALD isoenzymes can help to identify the source of elevated ALD, but unfortunately, isoenzyme assay techniques are not available in our laboratory. However, the determination of other enzymes, e.g. LDH and CPK, is also useful, and these more easily differentiate skeletal muscle disease from other diseases.

Fractionation of the LDH activity sharpens its diagnostic value because LDH has a multitude of origins. In our studies, elevated LDH activity was due to marked increases in the relative amounts of LDH_4 and LDH_5 in all gossypol-treated animals, except those receiving a low dose (0.5 mg/kg/2 weeks) which were normal. Therefore, either liver and/or skeletal muscle, but not heart, was affected by gossypol.

Monkeys treated with gossypol orally at 4.0 mg/kg/day for 24 months had normal levels of serum Na, K, Mg, Cl, CREA, LDH, BUN, and GOT.[20] Only reduction of $LDH_{1,2}$ and elevation of LDH_{3-5} were shown in this group, which is consistent with our results. Higher doses of gossypol (5 or 10 mg/kg/day) for 6 months similarly showed no serious clinicopathological side effects.[11] Therefore, these results differed from ours for animals treated with high dose (0.5 mg/kg/day) of gossypol. The causes of these differences may be related to dose, duration, and probably route of administration which may affect the metabolism of the drug.

Another enzyme activity we measured in order to determine the precise site of gossypol toxicity was CPK. This enzyme is usually found in both skeletal and cardiac muscles as well as in the brain.[21] As only CPK_3 activity was elevated in our study, there is little doubt that gossypol toxicity originated from skeletal muscle in all of the treated animals. Moreover, skeletal muscle degeneration has also been confirmed by histopathological findings[12] in monkey receiving gossypol at high doses. Putting these facts together, we summarize the findings on toxicity of gossypol as follows : a high dose of gossypol (0.5 mg/kg/day) was very toxic and damaged the liver and skeletal muscle, a moderate dose (0.5 mg/kg/week) was toxic only to skeletal muscle, and a low dose of gossypol (0.5 mg/kg/2 weeks) was judged from blood chemistry profiles to be nontoxic. In our previous studies, the low dose of gossypol (0.5 mg/kg/2 weeks) appeared to be the most suitable antifertility dose from the marked reduction in sperm motility and velocity accompanied by an increase in the number of sperm with abnormal tails.[22] Therefore, this dose seems to be suitable for further experiments on long term treatment. However, whether such a dose, when used in long term treatment, produces toxicity or not remains to be elucidated.

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บทคัดย่อ

ได้ศึกษาผลของยาคุมกำเนิดกอสสิพอล ต่อการทำงานของอวัยวะระบบต่าง ๆ ในลิงหางยาวเพศผู้ โดยการฉีดกอสสิพอลขนาดต่าง ๆ กัน เข้าทางกล้ามเนื้อของลิงหางยาวเป็นเวลา 8 สัปดาห์ พบว่ากอสสิพอลขนาดสูง (0.5 มก /กก /วัน) ทำให้ระดับกรดยูริค (UA), เอ็มไซม์ต่าง ๆ ของตับ, อัลโดเลส (ALD), ครีอะทีนฟอสโฟไคเนส (CPK) และ แลคติกดีไฮโดรจีเนส (LDH_{4-5}) ในซีรัมสูงขึ้นแสดงว่ามีการเสื่อมสลายของตับ, กล้ามเนื้อลายและไตบางส่วนเกิดขึ้น กอสสิพอลขนาดกลาง (0.5 มก /กก /สัปดาห์) ทำให้ระดับ ALD, CPK และ LDH_{4-5} ในซีรัมสูงขึ้นแสดงว่ามีการเสื่อมของกล้ามเนื้อลายเท่านั้น และกอสสิพอลขนาดต่ำ (0.5 มก /กก /2 สัปดาห์) ไม่ทำให้เกิดผลข้างเคียงแต่ประการใด สรุปได้ว่ากอสสิพอลขนาดต่าง ๆ มีผลข้างเคียงต่อการทำงานของอวัยวะระบบต่าง ๆ และพบว่าขนาดที่ปลอดภัยที่สุดในลิงหางยาวคือ 0.5 มก /กก /2 สัปดาห์

TABLE 1 Serum levels ($\bar{X} \pm SD$) of electrolytes (Na, K, Cl and CO_2), calcium (Ca) and phosphorus (P) in control and gossypol-treated monkeys, compared to the normal range for monkeys in the colony

Group No.	Dose of Treatment	Duration of Treatment (wks)	(Electrolytes mmol/l) Na	K	Cl	CO_2	Ca (mg%)	P (mg %)
	–	0	145.5±5.1	3.7±1.5	104.1±1.4	21.6±3.3	9.1±1.2	5.1±1.4
1.	0.5	4	148.2±3.6	5.0±1.1	103.3±4.5	29.1±1.5	8.6±1.2	3.6±1.1
	mg/kg/d	8	154.1±3.3	4.7±3.3	102.1±3.3	19.2±2.3	10.2±2.2	8.0±2.4
2.	0.5	4	152.3±3.5	4.6±0.9	103.3±2.2	28.8±1.3	9.1±1.7	4.0±1.6
	mg/kg/wk	8	149.3±4.1	3.5±0.4	103.2±1.6	14.3±1.2	9.3±0.6	8.0±1.5
3.	0.5	4	157.1±4.0	5.1±1.1	101.2±5.2	12.0±1.3	10.1±2.1	4.2±1.3
	mg/kg/2wks	8	146.2±3.5	4.1±1.3	101.3±3.4	17.1±2.1	9.4±1.3	6.3±1.6
4	Vehicle control	4	144.1±3.4	3.6±0.8	106.4±2.5	21.3±1.1	9.5±1.1	5.4±1.4
		8	147.2±3.8	3.7±0.5	107.3±1.6	21.2±1.4	9.5±0.8	5.1±1.6
	Normal Range		135.0-145.0	3.5-5.0	95.0-105.0	14.0-22.0	8.5-10.0	3.3-5.6

TABLE 2 Serum levels ($\bar{X} \pm SD$) of alkaline phosphatase (AP), glutamic oxaloacetic transaminase (GOT), pyruvic transaminase (GPT), aldolase (ALD), lactate dehydrogenase (LDH) in control and gossypol-treated monkeys (*$p < 0.05$, **$p < 0.01$, ***$P < 0.005$), compared to the normal range for monkeys in the colony

Group No.	Dose of Treatment	Duration of Treatment (wks)	AP (u/l)	GOT (u/l)	GPT (u/l)	ALD (u/l)	LDH (u/l)	CPK (u/l)
	–	0	54.3±17.4	30.4±12.6	18.3±11.3	12.4±1.8	442.3±55.8	250.2±72.6
1	0.5	4	114.8±17.6	40.9±17.3	17.2±11.1	76.7±12.2***	1442.6±125.8***	421.6±53.8**
	mg/kg/d	8	116.2±13.5*	40.6±12.8	158.3± 29.7***	85.2±10.3***	1835.2±108.3***	520.9±82.2***
2	0.5	4	107.3±41.5	75.2±12.2	16.2±5.4	19.8±5.2*	532.4±131.3	367.4 ±64.6*
	mg/kg/wk	8	229.5±53.3*	55.1±13.4	19.6±7.5	21.3±4.5*	936.2±95.8***	387.5±53.8*
3	0.5	4	112.4±10.2	93.1±17.3	21.5±15.8	15.6±5.3	498.2±76.3	170.2±43.7
	mg/kg/2 wks	8	75.2±3.3	52.2±14.5	33.2±21.3	16.1±4.4	600.4±120.1	104.9±53.6
4	Vehicle control	4	107.4±71.5	30.1±13.3	18.2±5.4	14.3±3.7	432.5±48.3	95.6±14.6
		8	54.3±4.2	30.2±12.1	33.3±12.1	15.2±4.5	388.2±19.2	250.3±115.3
	Normal Range		30.0-115.0	7.0-40.0	7.0-40.0	0-15.0	140.0-510.0	50.0-275.0

TABLE 3 The average ($\bar{X}$ ± SD) percent of LDH isoenzymes and CPK isoenzymes in control and gossypol treated monkeys (*P< 0.05, **P< 0.01, ***P<0.005), compared to normal range for monkeys in the colony

Group No.	Dose of Treatment	LDH isoenzyme (%)					CPK isoenzyme (%)		
		LDH_1	LDH_2	LDH_3	LDH_4	LDH_5	CPK_1	CPK_2	CPK_3
–	0	17.3±3.1	21.1±4.2	21.8±4.3	15.1±1.5	24.7±2.5	2.2±0.4	NS	97.8±0.4
1.	0.5 mg/kg/d	8.5±2.4**	22.4±2.3	24.0±4.4	17.5±2.6*	27.3±1.5	1.8±0.6	NS	98.2±0.6
2.	0.5 mg/kg/wk	3.3±1.2**	12.1±2.0	22.8±4.1	29.8±3.4***	32.0±3.6	2.1±0.4	NS	97.9±0.4
3.	0.5 mg/kg/2wks	18.8±1.3	23.5±2.1	20.2±3.5	17.3±2.6	20.2±4.1	1.8±0.4	NS	98.2±0.4
4.	Vehicle Control	16.4±2.5	21.5±3.7	22.8±3.2	14.8±1.6	24.5±2.3	2.0±0.5	NS	98.0±0.5
	Normal Range	13-25	16-25	18-29	9-17	20-28	1-5	0-3	95-100