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INHIBITORY EFFECTS OF GOSSYPOL ON CORTICOSTEROID 11-β-HYDROXYSTEROID DEHYDROGENASE FROM GUINEA PIG KIDNEY: A POSSIBLE MECHANISM FOR CAUSING HYPOKALEMIA

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Summary—Inhibition of 11-β-hydroxysteroid dehydrogenase (11-β-OHSD) in the kidney can cause excess mineralocorticoid effect and hypokalemia. To find out if gossypol, a potential oral contraceptive for men that has been associated with cases of hypokalemia, inhibits this enzyme, its effect on guinea pig kidney was studied. Working with microsomes from the kidney cortex, and using corticosterone as the substrate, racemic gossypol was found to be a competitive inhibitor of 11-β-OHSD with a Ki of 67 ± 5 μM. The (+) enantiomer was a little more potent than the (−) enantiomer. Microsomes from the kidneys of animals given gossypol for 2 weeks had lower enzyme activities than saline-treated animals. Microsomes from a strain of hairless guinea pigs had lower intrinsic enzyme activity than the normal animals. We conclude that there is genetic variation in the activity of this enzyme and that it can be inhibited by gossypol.

INTRODUCTION

Gossypol is a polyphenolic constituent of cotton seed which suppresses sperm formation and motility without affecting the hormone-producing cells of the testis [1–5]. Although it has been considered a promising agent to induce infertility in many laboratory animals and in humans [6, 7], hypokalemia has been observed in some men taking gossypol. This hypokalemia attributed to gossypol has inhibited further development of gossypol for use as a male oral contraceptive [8, 9]. Because the studies on hypokalemia have not yielded a satisfactory explanation of the mechanism underlying the development of hypokalemia during gossypol treatment [10–12], a reliable way to prevent gossypol-induced hypokalemia is not known.

Recently, it has been reported that the enzyme, 11-β-hydroxysteroid dehydrogenase (11-β-OHSD)'s inactivation of cortisol is responsible for the apparent selectivity of the mineralocorticoid receptor in the kidney for aldosterone despite the much higher circulating levels of glucocorticoid [13]. Inhibition or congenital deficiency of this enzyme results in a failure of the normal protective mechanism by which cortisol is converted to cortisone or corticosterone to 11-dehydrocorticosterone [14, 15]. As a result inhibition leads to apparent excessive mineralocorticoid effect in the kidney causing potassium loss and sodium retention. Hypokalemia can result from this. Gossypol has been found to inhibit many enzymes *in vitro*. If it inhibits 11-β-OHSD, this could be a mechanism for the hypokalemia.

The present paper reports *in vitro* and *in vivo* inhibitory effects of racemic gossypol acetic acid, (+) and (−) gossypol on corticosteroid 11-β-OHSD activity of the guinea pig kidney.

MATERIALS AND METHODS

Chemicals and solutions

Racemic gossypol acetic acid was a gift from Dr G. M. Waites (HRP/WHO, Geneva, Switzerland). Its purity was 99.9%. The (+) and (−) isomers of gossypol were a gift for Dr S. A. Matlin (City University, London, U.K.), these two enantiomers are chemically and optically pure and do not form acid complexes. Corticosterone, 11-dehydrocorticosterone, prednisone, NADP, glycyrrhizic acid and carbenoxolone were purchased from Sigma Co. (St Louis, MO, U.S.A.).

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Racemic gossypol acetic acid, (+) and (−) gossypol were dissolved in methanol to a concentration of 8.65 mM for the racemic and 9.65 mM for the (+) and (−) gossypol and kept at −10°C. For injection into guinea pigs, gossypol was dissolved in saline to a concentration of 1.73 mM by adding dropwise 10 N NaOH until it was in solution and then the pH value was rapidly brought back to 7.5 by dropwise addition of 5 N HCl. Glycyrrhizic acid and carbenoxolone were dissolved in distilled water.

In vitro effects of gossypol on 11-β-OHSD activity

Homogenate preparation and enzyme assay. Renal cortex was obtained from two strains of 1-month-old male Hartley guinea pigs: one with hair (haired) and one without hair (hairless). The tissue (0.5 g wet wt) was minced and homogenized in 10 ml Krebs–Henseleit buffer (NaCl 6.9 g, $NaHCO_3$ 2.1 g, D-glucose 2.0 g, KH_2PO_4 0.16 g, KCl 0.36 g, $MgSO_4 . 7 H_2O$ 0.29 g and $CaCl_2$ 0.37 g, in 1 l. distilled water, pH 7.2) with a Polytron tissue homogenizer. Throughout the procedure, the temperature was maintained in the range of 0–4°C. A 500 μl aliquot of homogenate from the pool was incubated at 37°C for 60 min with 100 μl NADP (5 mM) in Tris–HCl buffer (0.1 M, pH 8.0), 40 μl of 144 μM corticosterone, with or without gossypol, or glycyrrhizic acid. Incubations were run in duplicate. After incubation, steroids were extracted from the assay mixture. Concentrations of corticosterone and 11-dehydrocorticosterone were measured by HPLC, and the percent conversion of corticosterone to 11-dehydrocorticosterone by 11-β-OHSD and the yield of 11-dehydrocorticosterone were calculated to indicate the activity of 11-β-OHSD.

Preparation and microsomes and assay for 11-β-OHSD

A modification of the method of Monder and Lakshmi [16] was used. Male Hartley haired guinea pigs, weighing 200–250 g, were euthanized by CO_2. Kidneys were obtained and kidney cortex homogenized at 0°C in 4 vol 0.01 M sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose. All subsequent steps were performed at 0–4°C. The homogenate was centrifuged at 750 and 20,000 *g* in sequence for 30 min, saving the supernatant at each step. The last supernatant was centrifuged at 105,000 *g* for 1 h. The microsomal pellet was resuspended in sucrose–phosphate buffer, and centrifugation was repeated. The washed microsomal fraction was stored −70°C. The 11-β-OHSD activity of the microsomal pellet was stable for at least 6 weeks. Protein levels of microsome suspensions were measured with Sigma microprotein determination kits and adjusted to about 2 mg/ml.

Enzyme activities of 11-β-OHSD were determined by measuring the percent conversion of corticosterone to 11-dehydrocorticosterone and yield of 11-dehydrocorticosterone in the presence of NADP. The assay mixture contained 0.5 ml Krebs–Henseleit buffer (pH 7.2), 50 μl 5 mM NADP, 40 μl of 144 μM corticosterone in methanol and 40–80 μl of microsome suspension (2 mg/ml protein) in 0.01 M phosphate–sucrose buffer. Incubation was carried out in 13 × 100 mm glass tubes in a VWR model 1720 incubator at 37°C for 1 h. Enzyme reactions were terminated by adding methylene chloride. Steroids were extracted and measured by HPLC.

Extraction of steroids and HPLC measurement [17]

The basic method of Hofreiter *et al.* [17] was used. The glass tubes containing the assay mixture were put in an ice bath immediately after incubation, and 20 μl of 139 μM prednisone was added to each assay tube as internal standard. Extraction of corticosterone and its metabolite, 11-dehydrocorticosterone was performed using 3 ml methylene chloride. The tubes were vortexed for 1 min, then centrifuged at 750 *g* for 10 min. The aqueous layer was removed by aspiration. 300 μl Of 0.1 N NaOH was added to the organic phase followed by vortexing for 30 s. The mixture was centrifuged and the aqueous layer removed. A final wash of the organic phase was with 500 μl distilled water. 1.5 ml of the solvent phase was transferred to glass tubes and evaporated to dryness at 40–45°C water bath under nitrogen. For HPLC analysis, the dried extracts were dissolved in 100 μl methanol and 2 μl of the methanol solution was injected into the HPLC system. A standard curve for corticosterone and 11-dehydrocorticosterone was always determined concurrently with each assay using the same amount of microsome suspension after boiling to inactivate the enzyme. Peak heights of corticosterone or 11-dehydrocorticosterone divided by peak heights of the internal standard were plotted as functions of these steroidal

concentrations. All unknown concentrations of corticosterone and 11-dehydrocorticosterone were determined from these standard curves.

Chromatographic conditions

A Waters Assoc. (Milford, MA, U.S.A.) μ Bondapak C 18 column (300 × 4 mm, i.d.) was used at ambient temperature for all assays. Components of the HPLC assembly were made by Waters Associated Inc., and included a Model U 6K injector, Model 481 LC absorbance detector and a Model 6000 A solvent delivery system. Output from the absorbance unit was recorded with a BBC SE 120 chart recorder. A wavelength of 254 nm and a maximum sensitivity of 0.005 absorbance units, full scale, were used. The mobile phase consisted of methanol–water (60:40, v/v). At a flow rate of 1.0 ml/min, the retention times for corticosterone, 11-dehydrocorticosterone and prednisone were 11.1, 7.7 and 6.2 min, respectively. Assuming a minimal detectable peak height of 5 mm, then 1.7 pmol corticosterone, 1.97 pmol 11-dehydrocorticosterone and 1.1 pmol prednisone on the column can be detected. The coefficient of variation of the corticosterone assay was 1.3% using 6 simultaneous replicate samples at 5.8 nmol corticosterone added to the incubation mixture using boiled microsomes.

Corticosterone and 11-dehydrocorticosterone were added to guinea pig kidney microsomes to levels of 1.15, 3.46 and 11.5 μM corticosterone and 1.16, 3.48 and 11.6 μM 11-dehydrocorticosterone. The recovery rate by methylene chloride extraction were 96.8, 100 and 100.4% for corticosterone and 99.7, 94.5 and 95.3% for 11-dehydrocorticosterone, respectively.

Determination of kinetic constants [18]

The basic method of Monder *et al.* [18] was used. Reaction mixtures contained 0.5 ml Krebs–Henseleit buffer, 50 μl 5 mM NADP, 40 μl of 144 μM corticosterone as the substrate and 10–160 μg microsomal protein. The velocity of the reaction was directly proportional to the amount of microsomal protein added. For enzyme kinetic study, 40 μl of microsomal suspension (2 mg/ml protein) was chosen and different concentrations of corticosterone were added to the assay mixture. Each experimental point was determined in duplicate or triplicate. The data were plotted as double reciprocal plots and Dixon plots according to the Lineweaver–Burk linear transformation of the Michaelis–Menten equation [19].

Inhibition studies

Racemic gossypol acetic acid, (+), and (−) gossypol in various amounts from 35 to 722 μM were added to the incubation mixture. Steroid substrate was varied in the range of 2.3–11.5 μM. The IC_{50} values (concentrations inhibiting the reaction by 50%) were calculated by probit analysis from 5 concentrations of gossypol in the range of 35–691 μM. For the determination of K_m and V_{max} values, 4 concentrations of the substrate were used, while inhibition constants were determined with Dixon plots [19].

In vivo administration of gossypol acetic acid and its effect on 11-β-OHSD in microsomes of guinea pig kidney

To study the effect of gossypol acetic acid on 11-β-OHSD *in vivo*, two experiments using the same protocol were conducted at different times. Male Hartley haired and hairless guinea pigs, obtained from Charles River Labs, Inc. (Wilmington, MA, U.S.A.), weighing 340–350 g were housed three animals per cage. Water and Purina diet were allowed *ad libitum*. The results of the two experiments were similar and have been grouped together for analysis.

Each experiment included six groups. All drugs were given by s.c. injection daily for 14 days except group D. Group A, B, C and D were haired guinea pigs. Group A received saline 2 ml/day as control. Group B and C received gossypol acetic acid at 10.4 and 13.8 μmol/kg/day, separately. Group D were injected s.c. with 17.5 μmol of carbenoxolone 33 and 3 min before being sacrificed. Group E and F were hairless guinea pigs. Group E received a s.c. injection of saline 2 ml/day and group F received gossypol acetic acid 10.4 μmol/kg/day. All animals were euthanized by CO_2 48 h after the last dose of drug, with the exception of group D. Plasma samples were collected and gossypol levels were measured by HPLC by the method of Wu *et al.* [20]. Kidneys were obtained immediately after sacrificing the animals. Microsomes from the renal cortex were prepared from each individual animal and the enzyme activity of 11-β-OHSD was determined for each. The data were analyzed by group comparison using two sided unpaired student's *t*-tests.

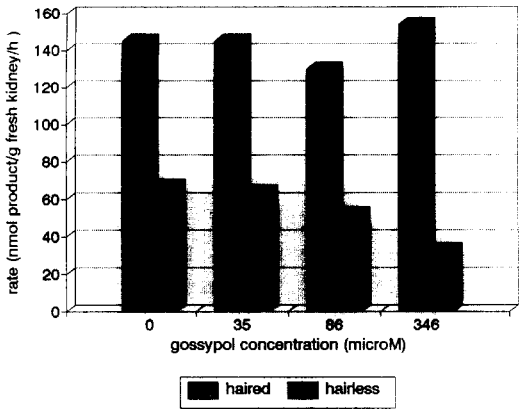

Fig. 1. *In vitro* effect of gossypol on 11-β-OHSD activity in the supernatant of kidney cortex homogenate of haired and hairless guinea pigs after 750 g × 30 min centrifugation. The rate is the amount (nmol) of 11-β-dehydrocorticosterone formed/h/g fresh kidney cortex.

RESULTS

In vitro studies with homogenate preparation

The renal cortex homogenate obtained from normal haired guinea pigs readily converted corticosterone to 11-dehydrocorticosterone. After 1 h incubation, the yield of 11-dehydrocorticosterone was 151 ± 4.7 nmol/g wet tissue/h, and the percent conversion of corticosterone to 11-dehydrocorticosterone was 64–67%. Glycyrrhizic acid inhibited the conversion in a dose-dependent manner. However, no significant inhibition was observed with up to 346 μM racemic gossypol acetic acid. More than 99% of the gossypol acetic acid added to the assay mixture was found to be protein bound after incubation, as determined by HPLC analysis.

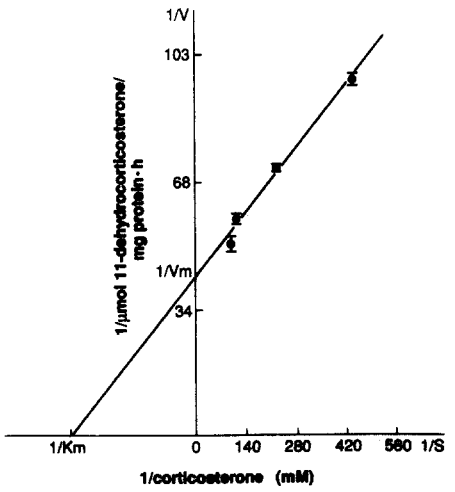

Fig. 2. Determination of K_m and V_m for 11-β-OHSD reaction in guinea pig kidney microsomal preparation. The system contained 0.5 mM NADP and 160 μg/ml microsomal protein at pH 7.2 (n = 3, microsomone batch number 900401).

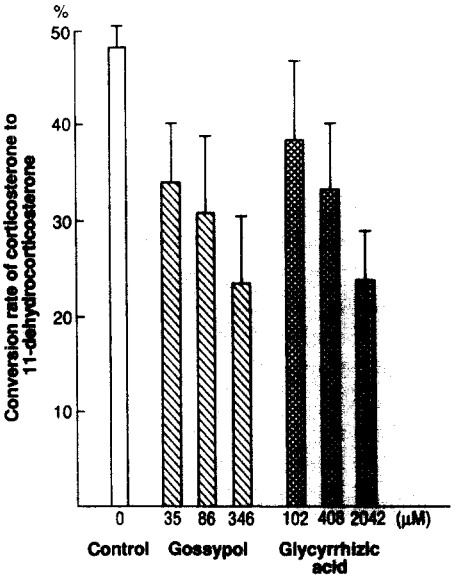

Fig. 3. *In vitro* effect of gossypol and glycyrrhizic acid on 11-β-OHSD activity in kidney microsomal preparation (haired animals).

Figure 1 shows the effect of gossypol acetic acid on 11-β-OHSD activity in the supernatant of kidney cortex homogenate of haired and hairless guinea pigs after 750 g × 30 min centrifugation. The 11-β-OHSD activity in the supernatant from hairless animals was much lower than that from haired. In addition, gossypol acetic acid showed some inhibitory effects on the 11-β-OHSD activity in the supernatant from hairless guinea pigs kidney homogenate, but no inhibition could be observed with the supernatant of haired guinea pigs kidney homogenate at the concentrations used.

Enzymic activity of 11-β-OHSD in haired guinea pig kidney microsomes

The velocity of the conversion of corticosterone to 11-dehydrocorticosterone was directly proportional to the amount of microsomal protein added to the assay mixture from 20–320 μg protein/ml. The velocity of the 11-β-OHSD reaction was measured by adding 80 μg microsomal protein to 0.5 ml assay mixture with different concentrations of corticosterone as substrate in the presence of 0.5 mM NADP. As shown in Fig. 2, the apparent K_m and V_{max} were calculated from the double reciprocal plots according to Dixon. In the absence of inhibitor, kinetic constants were: V_{max} = 38 nmol/mg.min, K_m (corticosterone) = 2.7 ± 0.2 μM.

Table 1. Effects of gossypol on 11-β-OHSD activity in kidney microsomes from guinea pigs

Gossypol concentration (μM)	Activity[a] Yield of 11-dehydrocorticosterone (μM)	%
0	24.97 ± 11.50	100
35	17.71 ± 5.43	77.9 ± 16.7
69	15.39 ± 7.29	62.4 ± 9.1
173	12.31 ± 5.26	52.3 ± 10.8
346	9.99 ± 5.17	39.4 ± 2.1

[a] $n = 3$ (three batches enzyme); ± SD.
IC_{50} = 141.4 μM with 95% confidence limit (64–314 μM). Working condition: 40 μl microsomal suspension (2 mg/ml protein); 11.5 nmol corticosterone in 0.5 ml assay system.

Inhibition of kidney microsomal 11-β-OHSD by gossypol acetic acid and glycyrrhizic acid in vitro

Figure 3 shows that microsomal 11-β-OHSD suspended in Krebs–Henseleit buffer was significantly inhibited by both gossypol acetic acid and glycyrrhizic acid. Both of these two compounds showed dose-dependent inhibition. When 160 μg microsomal protein and 11.5 nmol corticosterone were added in 0.5 ml assay mixture, the IC_{50}s were 297 and 1994 μM for gossypol acetic acid and glycyrrhizic acid, respectively. The relative inhibitory potency of gossypol acetic acid was about 6.7 times that for glycyrrhizic acid. As gossypol acetic acid methanol solution was used in the study, the possible effect of methanol on 11-β-OHSD activity was tested. A final concentration of methanol <6.5% in the assay system showed no effect on 11-β-OHSD activity.

Table 1 shows the detailed results for gossypol acetic acid inhibition of the enzyme activity of 11-β-OHSD when 80 μg microsome protein and 11.6 nmol corticosterone were used in 0.5 ml assay system. The inhibitory effect of gossypol acetic acid showed dose-dependence.

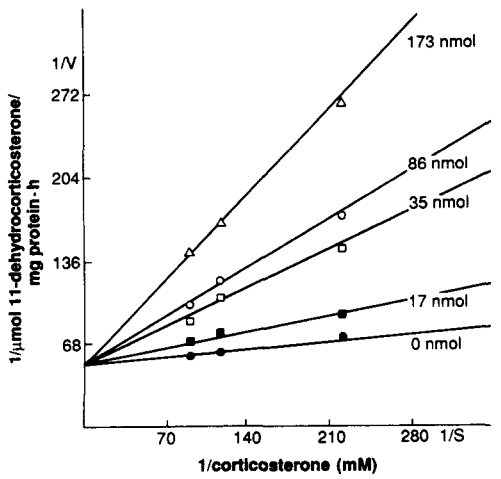

Fig. 4. Effect of gossypol on kidney microsomal 11-β-OHSD. Double reciprocal plot; assay system 0.5 ml, mean values from two batches assayed.

The IC_{50} of corticosterone to 11-dehydrocorticosterone was 237 μM with a 95% confidence limit (159–355 μM).

Kinetics of inhibition of kidney microsomal 11-β-OHSD by gossypol acetic acid

Figure 4 shows the inhibitory effects of varying concentrations of gossypol acetic acid on 11-β-OHSD at different corticosterone concentrations. The data plotted as double reciprocal plots converged on the ordinate, consistent with competitive inhibition. Another study of inhibition, plotted according to Dixon, confirmed the interpretation of competitive inhibition (Fig. 5). The inhibition constant was 67.4 ± 4.8 μM calculated from linearized plots of the data.

Inhibitory effects of (+) and (−) gossypol on 11-β-OHSD in the kidney microsome of the guinea pig

Chemically and optically purified (+) and (−) gossypol have been tested to observe their

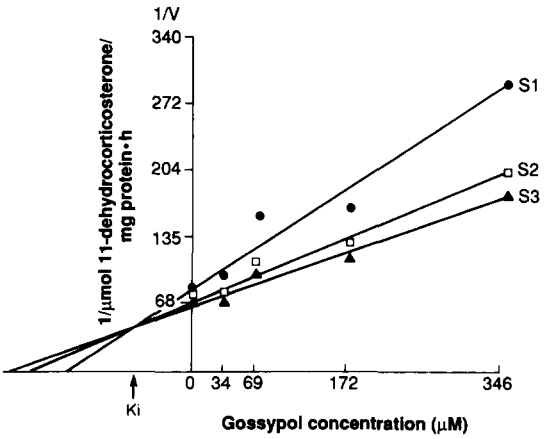

Fig. 5. Dixon plot of effect of gossypol on kidney microsomal 11-β-OHSD. Conditions were: NADP 500 μM, 40 μl microsomal suspension containing 80 μg protein, and volume of 0.5 ml V = rate of formation of 11-dehydrocorticosterone. S1: corticosterone 4.6 μM, $Y = 0.857 + 0.0059X$, $r = 0.96$; S2: corticosterone 9.2 μM, $Y = 0.0702 + 0.0038X$, $r = 0.98$; S3: corticosterone 11.5 μM, $Y = 0.0653 + 0.0032X$, $r = 0.99$; and K_i: 64.0 μ M (gossypol).

Table 2. Effects of (+) and (−) gossypol on 11-β-OHSD activity in kidney microsomes of guinea pig

Gossypol concentration (μM)	Percent inhibition of 11-β-OHSD activity			
	(+) gossypol		(−) gossypol	
	A	B	A	B
39	37.6	51.4	20.9	44.2
77	49.6	56.9	26.1	51.4
193	69.7	77.9	35.9	65.7
386	84.6	84.5	65.0	80.1
771	93.2	94.5	82.5	91.2
IC_{50}(μM)	71.5	44.9	206.0	65.0

A; adding 80 μg microsome protein in 0.5 ml assay system and B; adding 40 μg microsome protein in 0.5 ml assay system. Each value is the mean of duplicate determinations.

effects on guinea pig kidney microsomal 11-β-OHSD. As shown in Table 2, both (+) and (−) isomers of gossypol showed dose-dependent inhibition of kidney 11-β-OHSD. In contrast to their antifertility and other biological effects, the (+) isomer of gossypol appeared to be a more potent inhibitor than the (−) gossypol. These two isomers are not acetic acid complexes and dissolve more easily in methanol than racemic gossypol acetic acid.

Inhibitory effects of racemic gossypol acetic acid on kidney 11-β-OHSD of haired and hairless guinea pigs in vivo

The effects of racemic gossypol acetic acid administered s.c. on the 11-β-OHSD activity in the haired and hairless guinea pig kidney microsomes was observed in two experiments. For the 1st experiment, the animals weighed about 250 g and the dose of gossypol acetic acid of 10.4 μmol/kg/day given s.c. for 14 days was tolerated well and associated with weight gain, but 13.8 μmol/kg/day gossypol acetic acid caused impaired weight gain. For the 2nd experiment, guinea pigs weighing 460–520 g were used because the supplier did not have smaller animals at the time. Three haired guinea pigs on gossypol acetic acid 10.4 μmol/kg/day group and two on gossypol acetic acid 13.8 μmol/kg/day group died after 11–14 days of treatment. The hairless guinea pigs tolerated this dose of gossypol acetic acid well.

After grouping the data from two experiments (Table 3), the yield of 11-dehydrocorticosterone was 7.95 ± 1.74 nmol/mg microsomal protein/h in haired controls, and 4.26 ± 0.75 nmol/microsomal protein/h in haired guinea pigs receiving gossypol acetic acid 13.8 μmol/kg/day ($P < 0.01$). For the carbenoxolone group, the yield of 11-dehydrocorticosterone was 5.89 ± 1.89 nmol/mg microsomal protein/h ($P < 0.05$ compared to haired controls). Gossypol acetic acid 10.4 μmol/kg/day given to the haired group showed a small decrease (not significant) from control. The hairless control group with a value of 5.40 ± 0.78 nmol/mg microsomal protein/h was significantly lower ($P < 0.05$) than the haired control group. In addition, the yield of 11-dehydrocorticosterone was 3.60 ± 1.28 nmol/mg microsomal protein/h in hairless guinea pigs receiving 10.4 μmol/kg/day gossypol acetic acid which was significantly lower than hairless control group ($P < 0.05$). The plasma concentrations of gossypol at 48 h after last dose of racemic gossypol acetic acid were 23.2 ± 10.0 and 38.9 ± 19.4 nM for haired guinea pigs receiving 10.4 and 13.8 μmol/kg/day of gossypol acetic acid, respectively. For hairless animals receiving gossypol acetic acid 10.4 μmol/kg/day, the

Table 3. Results of *in vivo* study

Groups	1st study	2nd study	Combined
Haired control	9.03 ± 1.54	7.58 ± 1.77	7.96 ± 1.74
Haired gossypol			
10.4 μmol/kg/d	8.94 ± 4.70	4.56 ± 0.44	6.76 ± 3.77
13.8 μmol/kg/d	4.76 ± 0.15	3.86 ± 0.81	4.27 ± 0.75[a]
Haired carbonoxolone	7.08 ± 3.19	5.31 ± 0.35	5.89 ± 1.89[b]
Hairless control	4.30 (1)	5.75 ± 0.38(3)	5.40 ± 0.78(4)[b]
Hairless gossypol			
10.4 μmol/kg/d	2.53 ± 0.09(2)	4.30 ± 1.16(3)	3.60 ± 1.28[c]

Activity of 11-β-OHSD of guinea pig kidney cortex expressed as the rate of 11-dehydrocorticosterone production (nmol/mg microsomal protein/h). Results for each study are given separately and then combined [mean ± SD (N)].

[a] $P < 0.01$, compared with haired controls.

[b] $P < 0.05$, compared with haired controls.

[c] $P < 0.05$, compared with hairless controls.

plasma concentrations of gossypol were 14.5 ± 4.0 nM.

DISCUSSION

Gossypol is a potential oral contraceptive agent for men, [1–5] and a potential treatment of uterine myomata, endometriosis and dysfunctional uterine bleeding in women [21], and even a potential treatment for cancer [22]. The major problem with gossypol as a contraceptive was the occurrence of hypokalemia with the incidence of about 1–4.7% [8, 9]. Despite extensive investigation, the mechanism of gossypol-induced hypokalemia presumably due to renal potassium loss observed in some patients has not been explained [10–12].

The present observations demonstrate that gossypol has a potent competitive inhibitory effect on the enzyme activity of 11-β-OHSD *in vitro* using microsome preparations from guinea pig kidney. This inhibition was dose-dependent. The relative potency of gossypol acetic acid was about 6.7 times that of glycyrrhizic acid which is the glucuronide ester of glycyrrhetinic acid and is known to be an inhibitor of 11-β-OHSD both *in vitro* and *in vivo* [14, 23]. 11-β-OHSD oxidizes adrenal cortical hormones (cortisol and corticosterone) in the kidney to inactive compounds [24]. Inhibition or congenital absence of this enzyme leads to the impaired conversion of corticosterone to 11-dehydrocorticosterone or cortisol to cortisone and results in high intrarenal corticosteroid levels which can act on the mineralocorticoid receptor. Then, apparent excessive mineralocorticoid effect occurs in the kidney causing sodium retention and potassium loss. Our *in vitro* data indicate that the concentration of 35–69 μM gossypol showed significant inhibition of 11-β-OHSD *in vitro*, and the IC_{50} of corticosterone to 11-dehydrocorticosterone was 141.4 μM. Our preliminary *in vivo* experiment also suggested that 11-β-OHSD in guinea pig kidney could be inhibited by 14 daily s.c. doses of 13.8 μmol/kg/day of racemic gossypol acetic acid.

The enzyme activity of 11-β-OHSD in untreated hairless guinea pigs was lower than that of haired guinea pigs. The hairless guinea pig seemed more sensitive to the inhibition of 11-β-OHSD activity in kidney by gossypol, compared with haired animals (about 33% inhibition vs 15% inhibition at 10.4 μmol/kg dose). These data suggest that there are pharmacogenetic differences between the strains. Therefore, it would be very interesting to speculate that there may be genetic differences in 11-β-OHSD in different humans and in the sensitivity of different phenotypes to gossypol inhibition.

Both the (+) and (−) isomer of gossypol showed potent inhibition of 11-β-OHSD *in vitro*. The (+) isomer of gossypol appeared 1.5–2.8 times more potent than the (−) isomer as an inhibitor of 11-β-OHSD. This is interesting since the (−) isomer is the one with antifertility activity.

From these results, our hypothesis remains that gossypol inhibits 11-β-OHSD and causes glucocorticoid excess in the kidney leading to potassium loss and subsequent hypokalemia. Research is still needed to establish that the potency of gossypol given systemically is sufficient to inhibit 11-β-OHSD and cause hypokalemia, at least in some people. Recently, Lohiya *et al.* [25] reported the occurrence of hypokalemia in adult male longus monkeys after 120 day treatment with gossypol acetic acid at 8.6 μmol/day orally. Extensive renal potassium loss was evident and the renal excretion of sodium decreased markedly. This finding in longus monkeys is quite consistent with our hypothesis of the inhibition of 11-β-OHSD leading to gossypol-induced hypokalemia. Attempts to produce gossypol-induced hypokalemia in other species of animals, including dogs, rhesus monkeys, rats and guinea pigs have not been successful [26–28]. Therefore, we suggest that an important issue is the possible pharmacogenetic differences between species in the 11-β-OHSD and its sensitivity to gossypol. Pharmacogenetic differences in the enzyme may also account for why hypokalemia developed in some people taking doses of gossypol that did not cause hypokalemia in most people.

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REFERENCES

1. National Coordinating Group on Male Antifertility Agents. Gossypol, a new contraceptive for males. *Chin. Med. J.* **4** (1978) 417–428.
2. Wang Y. E., Luo Y. E. and Tang X. C.: Studies on the antifertility actions of cottonseed meal and gossypol. *Acta Pharmac. Sin.* **14** (1979) 663–669.
3. Xue S. P., Zong S. D., Su S. Y., Wu Y. W., Liu Y., Zhou Z. G. and Ma X. X.: Antifertility effect of gossypol on the germinal epithelium of the rat testis. A cytological, autoradiographical and ultrastructural observation. *Scientia Sin.* **23**(5) (1980) 642–657.

4. Wang N. G. and Lei H. P.: Antifertility effect of gossypol acetic acid on male rats. *Chin. Med. J.* **59** (1979) 402–405.
5. Sang G. W.: Effects of gossypol on male reproduction. In *Hormones in Normal and Abnormal Human Tissues.* (Edited by K. Fotherby and S. B. Pal) Walter de Gruyter and Co., Berlin, Vol. III (1983) pp. 215–249.
6. Prasad M. R. N. and Diczfalusy E.: Gossypol. *Int. J. Androl.* **5** (Suppl.) (1982) 53–70.
7. Xu D., Cai W. J., Zhu B. H., Dong C. J., Zheng Z. C. and Gao Z. Q.: Clinical safety and long-term administration of gossypol in 32 cases. *Contraception* **37** (1988) 129–136.
8. Wang C. and Yeung R. T. T.: Gossypol and hypokalemia. *Contraception* **32** (1985) 237–252.
9. Qian S. Z. and Wang Z. G.: A potential antifertility agent for males. *A. Rev. Pharmac. Toxic.* **24** (1984) 329–360.
10. Waller D. P., Niu X. Y. and Kim I.: Toxicology and mechanism of action of gossypol. In *Male Contraception: Advances and Future Prospects* (Edited by G. I. Zatuchni, A. Goldsmith, Z. M. Spieler and J. J. Sciarra) Harper and Row, Philadelphia (1986) pp. 183–200.
11. Wang Y. X. and Chang Z. X.: The mechanism of gossypol leads to hypokalemia. In *International Symposium on Gossypol Research for Male Fertility Regulation.* Wuhan, China (Abstr.) (1986) p. 8.
12. Ypersele C. V., Dieu J. P. and Marbaix E.: Effects of gossypol on the kidney with special emphasis on potassium (K) excretion. *Contraception* **37**(2) (1988) 173–178.
13. Edwards C. R. W., Burt D. and Stewart P. M.: The specificity of the human mineralocorticoid receptor: clinical clues to a biological conundrum. *J. Steroid Biochem.* **32** (1989) 213–216.
14. Stewart P. M., Wallace A. M., Valentino R., Burt D., Shackleton C. H. L. and Edwards C. R. W.: Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **10** (1987) 821–824.
15. Monder C., Shackleton C. H. L., Bradlow H. L., New M. I., Stoner E., Iohan F. and Lakshmi V.: The syndrome of apparent mineralocorticoid excess: its association with 11-β-dehydrogenase and 5β-reductase deficiency and some consequences for corticosteroid metabolism. *J. Clin. Endocr. Metab.* **63**(3) (1986) 550–557.
16. Monder C. and Lakshmi V.: Corticosteroid 11-β-hydroxysteroid dehydrogenase activities in vertebrate liver. *Steroids* **52** (1988) 515–528.
17. Hofreiter B. T., Allen J. P., Mizera A. C., Powers C. D. and Masi A. M.: High-performance liquid chromatography and radioimmunoassay of rat plasma corticosterone. *Steroids* **39** (1982) 547–555.
18. Monder C., Stewart P. M., Lakshmi V., Valentino R., Burt D. and Edwards C. R. W.: Licorice inhibits corticosteroid 11-β-dehydrogenase of rat kidney and liver: *in vivo* and *in vitro* studies. *Endocrinology* **125** (1989) 1046–1053.
19. Dixon M.: The determination of enzyme inhibitor constants. *Biochem. J.* **55** (1953) 170–171.
20. Wu D. F., Reidenberg M. M. and Drayer D. E.: Determination of gossypol enantiomers in plasma after administration of racemate using high-performance liquid chromatography with precolumn chemical derivation. *J. Chromat.* **433** (1988) 141–148.
21. Zhou S. W.: Clinical use of gossypol in gynecology. Chung Hua Fu Chan Ko Tsa Chih. **16** (1981) 137–141.
22. Flack M. R., Wu Y. W., Reidenberg M. M., Pyle R. G., Mullens N. M., Nisula B. C. and Loriaux D. L.: Treatment of adrenocortical carcinoma with gossypol. *AACR Abstr. Mtg* (1990).
23. Stewart P. M., Wallace A. M., Atherden S. M., Shearing C. H. and Edwards C. R. W.: Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11-β-hydroxysteroid dehydrogenase activity in man. *Clin. Sci.* **78** (1990) 49–54.
24. Edwards C. R. W., Stewart P. M., Burt D., Brett L., McIntyre M. A., Sutanto W. S., De Klowt E. R. and Monder C.: Localization of 11-β-hydroxysteroid dehydrogenase—tissue specific protector of the mineralocorticoid receptor. *Lancet* **10** (1988) 986–989.
25. Lohiya N. K., Sharma K., Kumar M. and Sharma S.: Limitations in developing gossypol acetic acid as a male contraceptive. *Contraception* 519–532.
26. Heywood R., Lloyd G. K., Majeed S. K. and Gopinath C.: The toxicity of gossypol to the male rat. *Toxicology* **40** (1986) 279–284.
27. Sang G. W., Zhang Y. G., Shi Q. X., Shen K. Y., Lu F. Y., *et al.*: Chronic toxicity of gossypol and the relationship to its metabolic fate in dogs and monkeys. *Acta Pharmac. Sin.* **1** (1980) 39–43.
28. Xue S. P., Luo H. Z., Zhu J. B., Li H. Z. and Zhao S. Y.: Evaluation of the renal tubulotoxic effect of gossypol by urinary protein analysis with SDS–polyacrylamide gel electrophoresis. *Contraception* **37** (1988) 163–172.