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STUDIES ON THE EFFECTS OF GOSSYPOL IN MALE CYNOMOLGUS MONKEY. I. SEMEN ANALYSIS

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Abstract

To investigate the effectiveness of the antifertility action of gossypol in the subhuman primate, various doses of gossypol were administered intramuscularly to 12 male cynomolgus monkeys. The doses ranged from 0.25 to 1.25 mg/kg/day or week, for 8 weeks. The semen was examined weekly for sperm concentration, motility, velocity and morphological characteristics. The total sperm count per ejaculation fluctuated in both treated and control groups. The percent sperm motility and the average sperm velocity (μm/sec) decreased gradually and significantly with duration and dose of treatment in all treated animals. The major abnormalities observed in sperm were coiled and broken tails as well as decapitation. The present study suggested that the antifertility effect of gossypol may be mediated through its suppressive effect on sperm motility which appears to be related to both the dose and the tail lesion. Doses of 0.25 mg/kg/week and 0.5 mg/kg/2 weeks for 8 weeks showed significant suppression of sperm motility without causing any toxicity.

Introduction

Gossypol has been used as an antifertility agent in China, where after gossypol administration at a dose of 20 mg/day for about 2 months, more than 99% of the men became oligospermic with less than 4 million spermatozoa per ml of semen[1]. More

recent studies have demonstrated that gossypol also exhibits antifertility activity in male laboratory animals, but there are marked species differences in response to gossypol. The effective doses to reduce sperm concentration and motility ranged from 10 to 30 mg/kg/day for 2 weeks in the rat[1-3] and from 5 to 10 mg/kg/day for 6-12 weeks in the hamster[4-7]. By contrast, gossypol did not induce infertility in the rabbit, either in animals treated with 10 mg/kg/day given for 14 weeks which showed little change in sperm concentration and motility[4] or in animals treated for up to 250 days resulting severe toxicity and eventual death[8]. In mice, an oral dose of 15-30 mg/kg/day did not significantly affect the motility of epididymal and vasal spermatozoa[9], but a reduction of sperm count[10,11] was reported after subcutaneous injections of gossypol.

Although man is sensitive to the antifertility effect of gossypol, some higher mammals such as subhuman primates were reported to be more resistant than humans, but there is some conflict in the data. In cynomolgus monkeys, gossypol at a dose of 10 mg/kg/day given orally for as long as 6 months only decreased the sperm count and motility in the ejaculates[12]. However, we have found toxicity after intramuscular administration of this dose in the same species[13]. Marked reduction in sperm count per ejaculate and sperm motility were reported in the bonnet monkey[14] but not in rhesus monkey[15] given gossypol orally at 4 mg/kg/day for 3 months. These discrepancies may attributed to the different doses of gossypol administered as well as to the animals studied. Therefore, the present studies were designed to obtain more information about the antifertility effects of gossypol, especially the effects on sperm count, motility and morphology, in the male subhuman primate *(Macaca fascicularis)* under various doses of treatment.

Materials and Methods

Animals

Twelve adult male cynomolgus monkeys *(Macaca fascicularis)* were used ranging in age between 6-8 years and in weight between 8-10 kg. The animals were supplied from 2 sources, the Primate Center of Chulalongkorn University and the Animal Center of Mahidol University. All animals were housed in individual cages. All cages were kept in a well-ventilated room illuminated by daylight and supplemented by artificial lighting for 12 h a day (0600-1800 h). The animals were fed daily in the morning with monkey chow (Pokphan Animal Feed Co. Ltd., Thailand) and in the afternoon with fresh fruits and vegetables as supplements. The animals were divided into one vehicle control and six treated groups. Each group had 3 animals. A few animals were treated again after recovery to normal physiologicial states.

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, USA. Analytically pure gossypol was prepared from the acetic acid adduct method of Campbell *et al.*[16]. 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 at the thigh muscle. The doses of gossypol ranged from 0.25-1.25 mg/kg/day or week. Two groups of animals were treated with gossypol at doses of 1.25 and 0.5 mg/kg/day and three groups at 1.25, 0.5 and 0.25 mg/kg/week, respectively. The other group was treated at a dose of 0.5 mg/kg/2 weeks. Control animals were administered with sesame oil alone.

Semen Evaluation

Semen specimens were collected by eletroejaculation using rectal probe[17]. The semen samples were collected in 5 ml sterile glass beakers. Because of the solid plug coagulum and very tiny volume, the semen was made up to 1 ml with Baker's solution (pH 7.4) and stirred well. The liquid was removed from the solid plug particles with a pasteur pipette and then put into a tube for semen analysis. The sperm motility and velocity were evaluated by multiexposure photography (MEP) techniques adapted from Makler *et al.*[18]. A drop of well-mixed raw semen, approximately 2-3 mm in diameter, was placed on a clear glass slide and covered with a small cover slip (22 × 22 mm). The slide was put on the stage of a phase-contrast microscope. A still camera loaded with 100 ASA black-and-white film (Kodak Plus X Pan film, Px 135-36) was attached to the microscope with the aid of bellows or extention rings. A slotted black disc was placed between the light source and the condenser of the microscope and was rotated by an electronic motor. Samples were exposed for 3 times in a second in each frame.

Statistical Analysis

Data were analysed by Student's *t*-test.

Results

The total sperm count per ejaculation, sperm motility and velocity in gossypol-treated and control animals are summarized in Fig. 1. In vehicle control and pretreatment animals, the total sperm count per ejaculate ranged from 20 to 2000×10^6 cells. In treated animals, the sperm count per ejaculate also fluctuated throughout the period of treatment. Among groups 1,2, and 5 a clear gradual decrease in sperm concentration was seen after 2 to 8 weeks of treatment, but a rebound in sperm production was noted after 8 weeks of treatment in group 1.

Fig. 2 shows multiexposure photographs of sperm from control (A) and gossypol (1.25 mg/kg/day)–treated animals (B). The motile (M) sperm are seen as

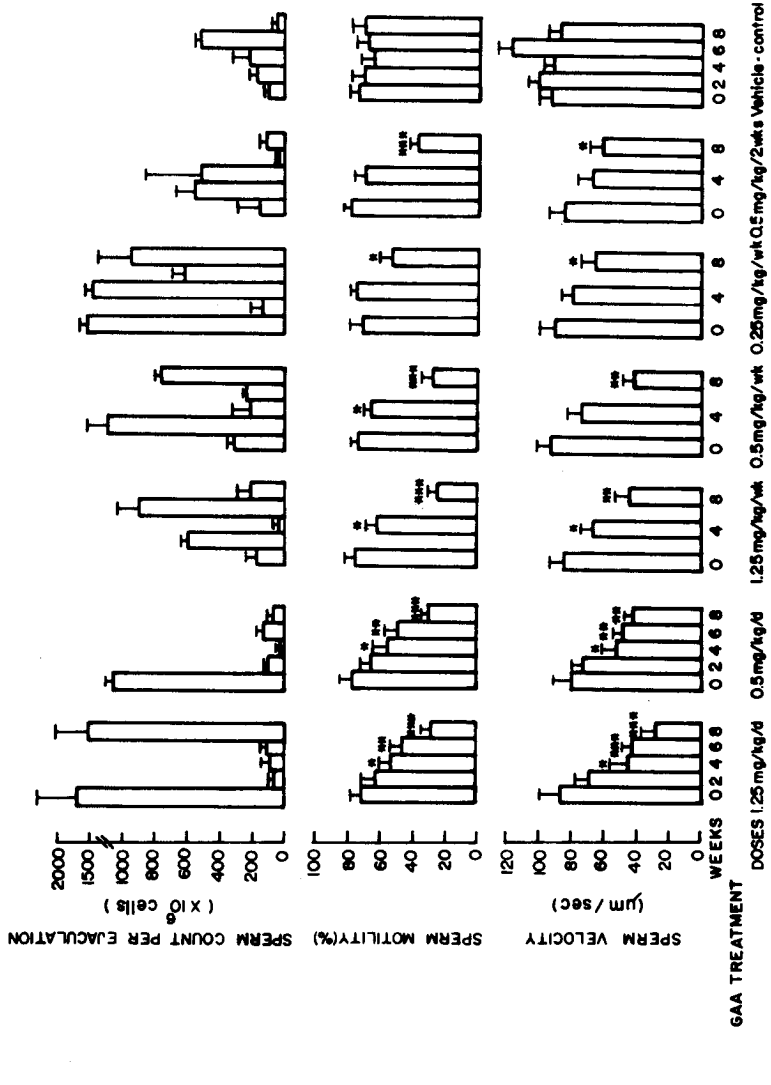

Fig. 1. The average ($\bar{X}\pm$ SD) number of sperm per ejaculation (x 10^6 cells) (upper), percent motility (middle) and velocity (μm/sec) (lower) in control and gossypol treated monkeys. The group numbers are shown circled. Weeks after treatment are shown under each bar. The doses of treatment are also shown under the bars. (*, $p<0.05$;**, $p<0.01$;***, $p<0.005$).

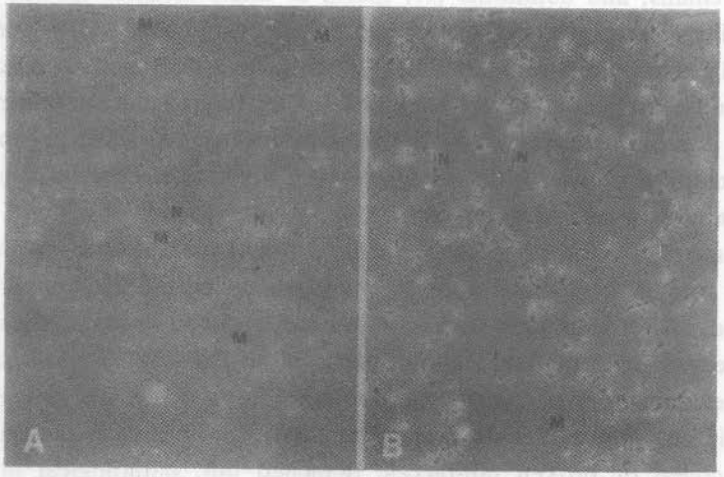

Fig. 2. Multiple exposure photographs of normal *(A)* and gossypol (8 weeks after 1.25 mg/kg/day)-treated *(B)* monkey sperm. The motile sperm *(M)* are seen as 3-dotted chains and nonmotile sperm *(N)* as single bright dots. Some sperm are labelled as *M* and *N* as examples. The percent motilities were 72.5 *(A)* and 11.1 *(B)*, respectively. × 200.

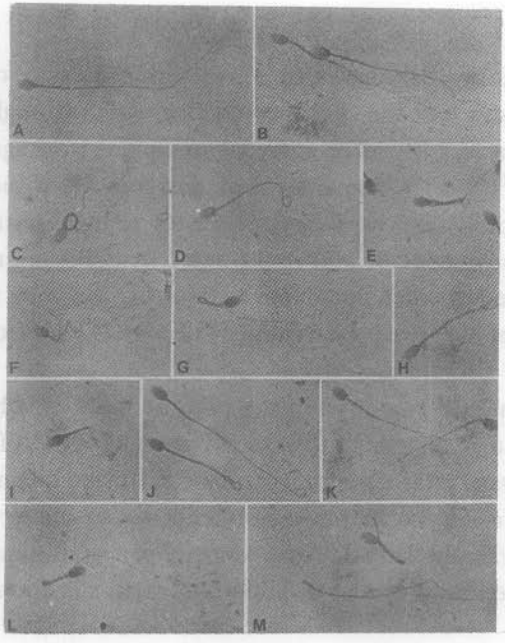

Fig. 3. Photomicrographs of sperms in control and gossypol-treated monkeys: *A* and *B* : normal; *C* : coiled flagellum; *D* : coiled flagellum tip; *E* : bent and loop flagellum; *F* and *G* : bent midpiece; *H* : bent flagellum; *I* : cytoplasmic droplet and detached tail; *J* and *K* : pathological midpiece;*L* : pathological midpiece and bent tail; *M* : decapitation and detached tail. × 1000

three-dotted chains, and nonmotile (N) sperm as single brighter spotted heads. The percent motility in each gossypol-treated group is shown in Fig. 1. The sperm motility in the control group ranged between 50-90% during the 8-week period. After treatment with various doses of gossypol for 2,4,6 and 8 weeks, the percentage sperm motility of most treated groups decreased gradually and significantly. However in group 5 and 6, the percent motility showed little change after 4 weeks of treatment, but decreased significantly after 8 weeks of treatment.

The average value of sperm velocity (μm/sec) measured from multiexposure photographs among the control animals ranged between 80-120 μm/sec during the 8-week period. After treatment with gossypol for 2,4,6 and 8 weeks, the mean sperm velocity decreased gradually and significantly in all of the treated animals.

On examination of sperm morphology, there were no changes in head morphology in any group of the animals. But there was an increase in the number of sperm with coiled or broken tail-pieces, detached tail, pathological midpiece and decapitation (Fig. 3). The different abnormal tail structures are summarized in Table 1. The percentage of bent, coiled and detached tails and of decapitated sperm increased significantly with the dose and duration of treatment, but not the percentage of cytoplasmic droplets or pathological midpieces (data not shown). The most common abnormal tail structures found, even at the lowest doses of gossypol were bent and coiled tails.

Discussion

The present study demonstrates a marked reduction of sperm motility in gossypol-treated monkeys, but the sperm count fluctuated throughout the experiment. The reduction of sperm motility after gossypol treatment is in agreement with reports by other investigators[6,12,14,19]. The effects of gossypol were more pronounced and consistent on sperm motility and were directly related to the duration of treatment. The forward progressive movement of gossypol-treated cynomolgus monkey spermatozoa was considerably decreased when compared to controls, showing that the spermatozoa were indeed affected by the gossypol treatment despite the lack of reduction in sperm number.

The MEP technique was used in the present study for objective evaluation of sperm motility because it has a number of advantages. One of the most important advantages is that forward motile sperm are seen clearly and distinctly and all necessary details can be defined easily, so that measurements can be made with a high degree of accuracy. Another advantage is that sperm velocity can be estimated, and the velocity of spermatozoa may be one of the most important factors in the assessment of fertility of semen samples[20].

In man, subjects with higher fertility have higher numbers of spermatozoa with

Table 1. The average ($\bar{x} \pm SD$) percent of abnormal morphology of sperm tail in control and gossypol-treated monkeys.

Group No.	Dose of Treatment	Duration of Treatment (Wk)	Abnormal Tail Differentiation (%)				Total Abnormal Tail Sperm (%)
			Bent Tail	Coiled Tail	Detached Tail	Decapitation	
1	1.25	0	7.0±1.5	5.5±1.1	0.6±0.1	2.0±0.5	16.4±1.1
	mg/kg/day	2	10.4±2.1	10.1±1.3•	0.5±0.1	5.4±0.9•	26.3±3.3•
		4	18.5±2.5••	15.3±2.3•	1.1±0.2	6.7±1.0••	44.4±3.1•••
		6	27.4±3.3••	16.1±2.5•	1.5±0.2••	7.1±1.2•	55.6±4.3•••
		8	25.8±3.4•••	24.1±3.3•••	2.1±0.3••	8.5±1.4••	62.3±5.5•••
2	0.5	0	6.2±1.3	6.1±1.2	0.7±0.1	2.1±0.7	5.5±4.2
	mg/kg/day	2	11.2±1.5•	10.3±1.8	0.3±0.1•	6.1±1.3•	27.6±3.1•
		4	18.1±2.5••	14.0±3.1•	1.0±0.2	7.3±1.4•	41.6±5.4••
		6	25.5±3.4•••	16.6±2.3••	0.6±0.1	7.1±1.4•	51.1±7.6•••
		8	26.7±3.3•••	24.0±2.1•••	2.3±0.2•••	10.4±1.8••	63.4±8.3•••
3	1.25	0	6.1±1.3	6.3±1.5	0.8±0.2	3.0±0.3	16.5±1.5
	mg/kg/wk	2	11.8±2.2	9.0±1.7	0.7±0.1	3.0±0.2	24.7±2.5•
		4	14.0±2.3•	12.0±2.1•	0.5±0.1	4.1±0.5	30.7±3.3••
		6	16.1±2.1••	14.1±1.4••	1.0±0.3	5.3±0.6•	37.2±2.2•••
		8	20.5±2.6•••	14.3±1.0•••	1.5±0.4	6.2±0.5•••	46.5±4.4•••
4	0.5	0	5.0±2.0	5.7±1.4	0.6±0.1	4.9±0.9	16.6±2.2
	mg/kg/wk	2	11.6±2.3•	8.2±1.7	0.1±0.0	3.5±0.4	23.2±2.3•
		4	13.2±2.5•	9.1±1.6	1.5±0.2••	3.1±1.0	29.5±2.7••
		6	14.1±2.3•	9.3±1.8	1.8±0.2••	4.6±0.4	30.2±2.5••
		8	18.5±3.4•	12.3±2.1•	3.2±0.8••	5.2±0.3	41.4±3.5•••
5	0.25	0	8.1±2.3	6.9±1.0	0.2±0.0	2.1±0.4	17.5±2.9
	mg/kg/wk	2	8.4±1.6	7.5±0.1	0.7±0.1	3.6±0.3•	20.7±1.6
		4	10.7±2.4	9.3±1.4	0.1±0.0	3.0±1.1	23.3±2.8
		6	15.4±2.7	12.1±1.5•	2.0±0.1•••	4.6±0.8•	36.5±2.9•••
		8	16.3±4.3••	13.5±2.2•	1.4±0.2•••	4.4±0.7•	37.4±2.3•••
6	0.5	0	7.1±1.4	7.9±0.3	0.3±0.0	1.0±0.1	16.5±0.7
	mg/kg/2wks	2	12.4±1.3•	9.1±1.3	1.0±0.1•••	3.0±0.1•••	25.2±2.5•
		4	13.3±1.2••	11.1±1.6	1.0±0.1•••	2.1±0.1•••	29.9±2.6•
		6	17.1±1.3•••	15.3±1.9••	–	2.3±0.2•••	35.7±3.0•••
		8	12.7±1.3•••	15.4±1.4••	1.0±0.1•••	2.5±0.7•••	36.6±2.8•••
7	Vehicle	0	5.1±0.2	5.9±0.8	0.5±0.0	4.8±1.2	16.7±1.5
	control	2	5.3±1.3	6.5±0.9	1.4±0.2	3.9±1.1	17.6±2.1
		4	4.9±0.6	4.7±1.0	0.1±0.0	3.1±0.7	14.3±1.7
		6	3.3±1.1	4.1±0.4	0.2±0.0	2.5±0.2	10.2±1.5
		8	2.2±0.3	3.4±0.5	0.1±0.0	2.2±0.4	7.7±1.4

•, $p < 0.05$; ••, $p < 0.01$; •••, $p < 0.005$

speeds between 40 and 50 μm/sec than do subjects of lower fertility.[21] In the present study, sperm velocity is reduced in correlation with sperm motility (for example, the sperm velocity decreases to 20-40 μm/sec when the sperm motility is 20-30%). The reduction of sperm velocity and motility are also correlated with the increase in the number of sperm with abnormal tails. Forward motility is poor or absent in sperm with coiled or broken tails.

In our studies, the reduction in sperm motility observed with gossypol treatment appears to result from abnormality of the tail structure. The presence of abnormal sperm contributes important information about semen quality and potential infertility of the male.[22]. In the present investigation, gossypol treatment produces a detrimental effect on semen characteristics with regard to sperm abnormalities. We have noticed a definite trend toward higher abnormalities with higher dosages. Most of the abnormal forms in the ejaculates of treated animals consisted of sperm cells with looped and coiled tails.

As seen in the present study, the suppression of sperm motility caused by gossypol appears to be dose-related, so that the higher the dose of gossypol, the sooner the sperm motility decreases. Therefore, in terms of suppression of sperm motility, gossypol doses of 1.25 and 0.5 mg/kg/day appeared to be the most effective. However, these doses were very toxic to the animals. We have found evidence of damage to various organs e.g. liver, lung, kidney and skeletal muscle[23]. So to reduce the toxicity and the quantity of the drug accumulated in the body, the regimen of administration was changed from one injection per day to one injection per week. The effectiveness of gossypol in suppression of sperm motility is reduced by changing the regimen of administration, so that longer periods of treatment are preferred. However, the doses ranging from 0.5-1.25 mg/kg/week are still toxic to the monkeys, particularly to liver and skeletal muscle[23]. As shown in our previous experiments[23], only the last two doses 0.25 mg/kg/week and 0.5 mg/kg/2 weeks were sufficiently safe to produce no toxicity to any organ. Although these doses of gossypol did not suppress the sperm motility after 4 weeks of treatment, the sperm motility was reduced markedly after 8 weeks of treatment, but the inhibition is not as much as those suppressed by the toxic doses.

Since sperm motility is a vital component in fertilization[24,25], the reduction in movement may be linked to gossypol's infertility effect. In rats, the occurrence of immotile sperm was virtually restricted to gossypol-treated rats. This finding has been well corroborated by a number of studies[1,4,26-28] and it is conceivable that gossypol provokes immotility of spermatozoa. By introducing gossypol into the epididymal fat pads of rats, Hadley and Burgos[29] demonstrated that gossypol inhibits epididymal motility as well as altering their morphology within 24 h of administration. Reduced sperm motility is also a major cause of infertility[30] and in other species (humans and

bulls), sperm motility below 50% has often been associated with low conception rate and poor fertility.[31,32]

A preliminary fertility test was also performed by mating a male monkey treated with gossypol for eight weeks to at least 3 fertile females at mid-cycle stage. The results revealed the presence of sperm in the vagina of all mated females, but these sperm failed to induce conception. These preliminary results therefore suggest that these gossypol-treated animals are infertile. By the criteria of inhibition of sperm motility without toxicity, gossypol doses of 0.25 mg/kg/week and 0.5 mg/kg/2 weeks appeared to be the most suitable antifertility doses. The lower dose of 0.5 mg/kg/2 weeks seems to be the best for long term treatment, but whether this dose produces toxicity or not remains to be elucidated.

The present study demonstrates a marked reduction of sperm motility in gossypol-treated monkeys, but the sperm number fluctuated throughout the experiment. This fluctuation in sperm number may be because the volume of monkey semen obtained was very small and the semen has a solid plug coagulum which does not liquefy. Therefore, only spermatozoa which could be mechanically separated from the coagulum were studied, so that the sperm number obtained depends on the size of the solid plug coagulum. However, in terms of sperm production, the present results are different from others[12], who reported azoospermia in the same species after oral gossypol treatment for 6 months. A significant decrease in sperm count per ejaculate after gossypol treatment for 75 days has also been observed in bonnet monkey given 4 mg gossypol/animal orally[14]. The discrepancy may result from different duration of treatment and routes of administration. However, administration of gossypol (50 and 100 mg/kg/day) orally to langurs for 90 days also caused no changes in sperm concentration[19]. Similarly, Bardin *et al.*[3] did not observe any significant changes in the sperm count per ejaculate in rhesus monkeys treated with gossypol at doses of 5,20 and 80 mg/monkey for 10-12 week.[3]. In conclusion, the present study clearly demonstrates that gossypol did not induce oligo- or azoo-spermia in treated animals, but did cause reduction in sperm motility and velocity accompanied by an increase in the number of sperm with abnormal tails.

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บทคัดย่อ

การศึกษานี้แสดงว่ากอสสิพอลมีผลในการต้านกำเนิด โดยไปกดการเคลื่อนไหวของสเปอร์มซึ่งขึ้นอยู่กับขนาดของยาที่ให้ และความผิดปกติของหางด้วย ขนาดของยาที่น้อยที่สุดที่ใช้ในการศึกษานี้ คือ 0.25 ม.ก./ก.ก./สัปดาห์ หรือ 0.5 ม.ก./ก.ก./2 สัปดาห์ มีผลในการต้านกำเนิดเนื่องจากสามารถลดการเคลื่อนไหวของสเปอร์มลงอย่างชัดเจน หลังจากให้ยาแล้วเป็นเวลา 8 สัปดาห์