

STUDIES ON THE EFFECTS OF GOSSYPOL IN MALE CYNOMOLGUS MONKEY. IV. HISTOPATHOLOGICAL FINDINGS

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Abstract

To investigate the toxicity of gossypol in subhuman primate, gossypol acetic acid (10 mg/kg/day) was administered intramuscularly to male cynomolgus monkeys, Macaca fascicularis for 2 weeks. After autopsy and complete postmortem examinations of 28 organs, histopathological changes were found in several vital organs. The liver and lungs showed congestion and hydropic degeneration. The spleen was congested and showed evidence of extramedullary hematopoiesis. The stomach and intestines demonstrated mucosal degeneration with accumulated brown pigments accompanied by macrophage infiltration in intestinal lamina propria. The kidneys showed mild hydropic degeneration of the renal tubules. Degenerative changes were also noted in the seminiferous tubules. Lysis of skeletal muscle fibers and erythrocytes was marked. It is concluded that gossypol, which is an effective antifertility agent in several mammalian species, exhibits a wide spectrum of toxicity in cynomolgus monkeys.

Introduction

Gossypol, an undesired component in cottonseed meal, was found to be toxic to nonruminant animals taking cottonseed¹⁻². As early as 1915, gossypol was reported to be responsible for the toxic effects observed in pigs after prolonged ingestion of cottonseed diet³. The usual manifestations of gossypol toxicity are depressed appetite, loss of body weight and inefficient protein utilization⁴⁻⁶. Many organs were found to be damaged such as the lungs, the liver, and the kidneys. Circulation failure, often followed by death, was

also reported^{1,7}. Presently, several findings suggest that gossypol can show toxic activity in laboratory animals at the required antifertility dosage, but there appear to be species differences in the response.

In China, gossypol has been used successfully as a male contraceptive for approximately 10,000 men⁸. It has been claimed that by ingesting gossypol at much lower levels than those causing toxicity, i.e. 20 mg daily for about 2 months, more than 99% of the men became oligospermic with less than 4 million spermatozoa per ml of semen. Gossypol does not cause any drastic acute side effect although about 1-12% of these people complained of fatigue, decrease in male libido, dizziness, gastrointestinal symptoms and hypokalemia⁸. However, the details of its physiological effects, mode of action, and reversibility are still not well understood. Several studies suggest that the laboratory rat is a satisfactory model for such studies⁹⁻¹². In this species, the effect of gossypol on testis and epididymis had been well documented, but its effect on somatic tissue has been less well studied¹³. However, because of species differences in response to gossypol, the physiological effects of gossypol in rats, including toxicity, cannot be extrapolated to humans. Moreover, there have been few studies of the effects of gossypol in subhuman primates¹⁴⁻¹⁷. Accordingly, the present investigations were designed to establish the antifertility potential of gossypol in a subhuman primate model, *Macaca fascicularis*, with the hope that these observations would provide better baseline data for the development of gossypol as a suitable male contraceptive in humans. In this paper, we report the effects of intramuscular administration of gossypol at high doses on the histopathological changes of various organs of male cynomolgus monkey.

Materials and Methods

Animals

Six 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 maintained in the Primate Center of Chulalongkorn 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 vehicle control and treated groups. Each group had 3 animals.

Dosage and Treatment Schedule

Racemic (\pm) gossypol acetic acid (U.S. Department of Agriculture 90% to 95% purity) was provided by Dr. Y. Thebtaranont, 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 from the acetic acid adduct method of Campbell *et al.*¹⁸. 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 at a dose of 10 mg/kg/day for 2 weeks. Control animals were administered with sesame oil alone.

Autopsy of the Animals

Animals were autopsied and complete postmortem examinations were performed. All tissues were fixed by immersion in 10% neutral-buffered formalin for 48 h. Following dehydration in a series of graded ethanol solutions, the tissues were embedded in paraffin. Sections were cut at 5 μ , stained with haematoxylin and eosin (H&E), and examined with a light microscope. Slides from the control group were examined to provide a baseline for assessing the degree of change of tissues from treated animals.

Results

Gross Pathology

Skin was slightly edematous. Subcutaneous adipose tissue was swollen and very deep yellowish fat was noted. On gross inspection, we did not find any significant changes in the vital organs, except spotted hemorrhage along the mucosa of small and large intestines. No fluid was found in the thoracic and abdominal cavities.

Histopathology

Complete tissue sets from the control and gossypol-treated animals were examined under light microscope and included 28 organs : brain, pituitary, heart, lungs, trachea, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, liver, pancreas, spleen, kidneys, ureters, urinary bladder, prostate, seminal vesicles, epididymis, testes, adrenal, thyroid, parathyroid, thymus, skeletal muscles and skin. No evidence of histological differences could be detected by light microscopy between control and experimental animals in the brain, pituitary, heart, trachea, esophagus, pancreas, ureters, urinary bladder, prostate, seminal vesicles, epididymis, thyroid, parathyroid, thymus and skin. Representative sections of the normal appearance and histopathology of the major visceral organs are illustrated in Plates 1-5.

Heart. The histology of both atria and ventricles in gossypol-treated animals was essentially similar to those observed in normal control. Plate 1-A demonstrates normal myocardial fibers cut in cross-section from the treated animals.

Plate 1. Photographs of heart (A), lung (B and C), spleen (D) and kidney (E) in gossypol treated monkey.

- A. High power of myocardium in cross section. Each muscle cell (MC) is intact. $\times 1000$.
- B. Low power of alveolar wall. The alveolar wall is congested. A large number of red blood cells (RBC) accumulate in the alveolar capillaries. $\times 200$.
- C. High power of pneumocytes with vacuolation in their cytoplasm and brown pigmented macrophages. $\times 1000$.
- D. High power of splenic red pulp. There is evidence of splenic congestion. A large number of red blood cells (RBC) are packed in the splenic sinuses and cords. Numerous nucleated red blood cells (nRBC) are observed. Erythroblasts (E) are scattered in the cords. Lymphocytes (L) and reticulocytes (R) are seen. Numerous brown pigmented macrophages (arrows) are present surrounding the arteries. $\times 1000$.
- E. Low power of renal cortex. Marked hydropic degeneration of the renal tubules, particularly proximal convoluted tubules (P) can be seen. A number of epithelial cells become foamy and lose their nuclei. Distal convoluted tubules (D) also show degeneration but of a less severe nature.

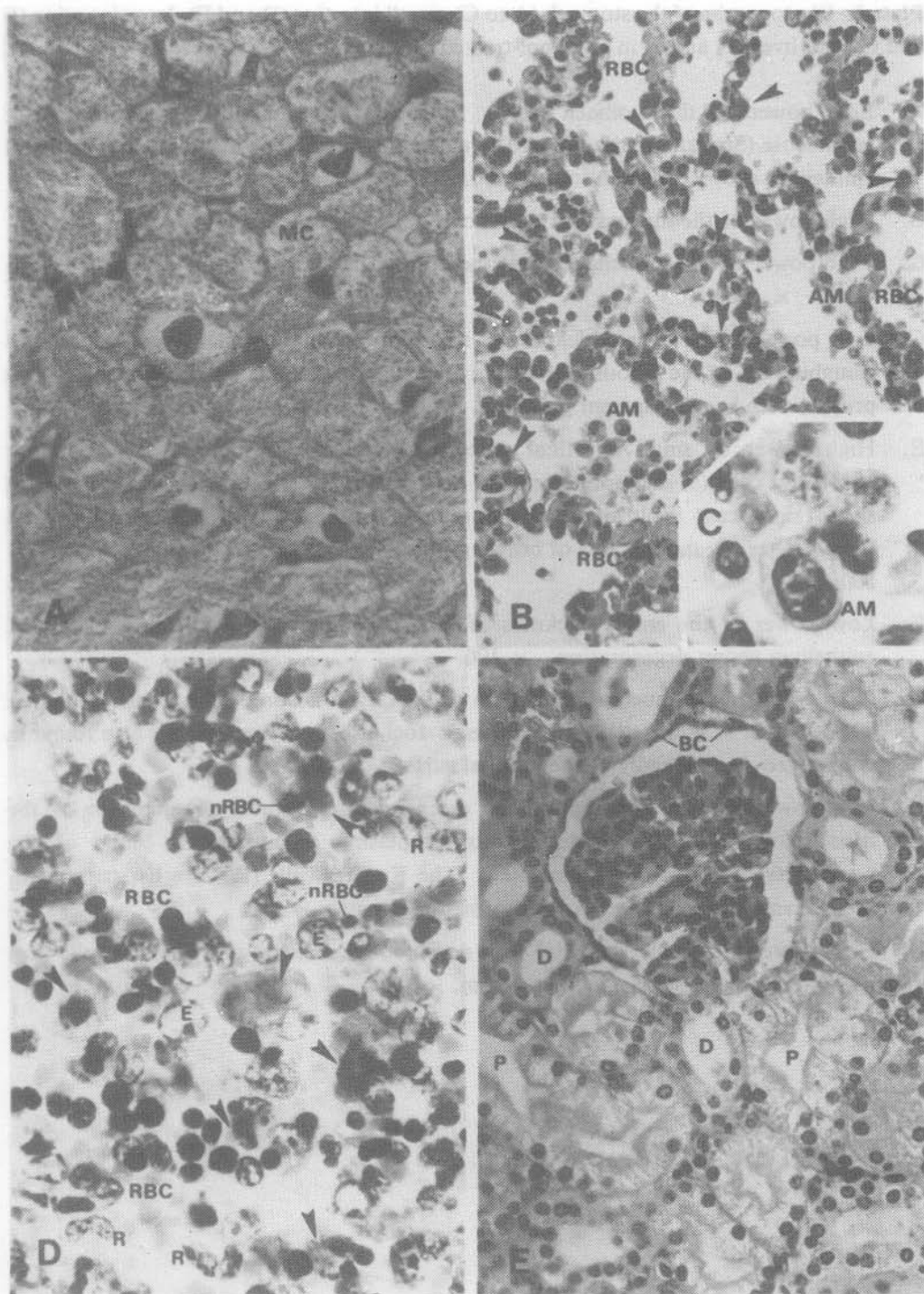
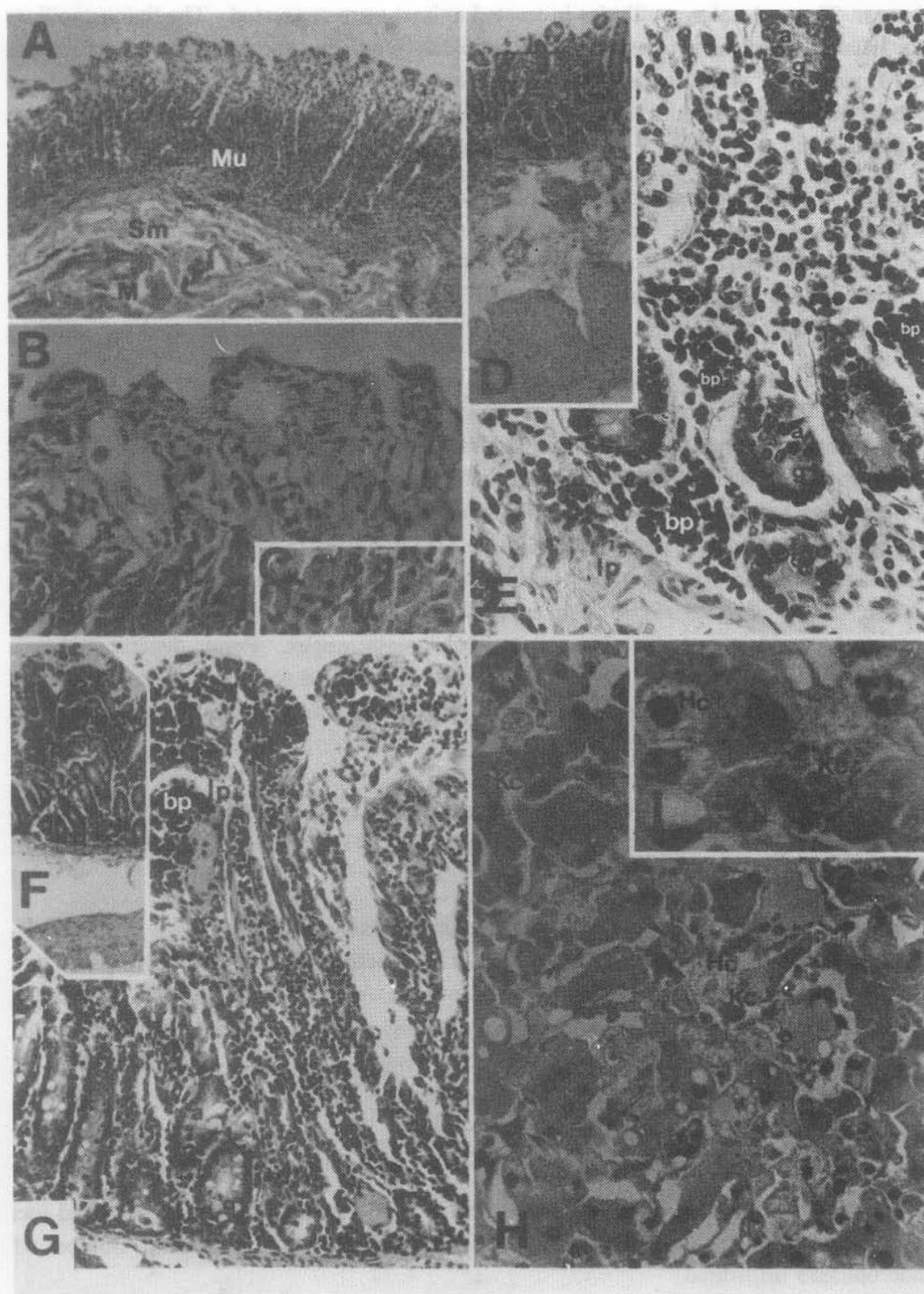


Plate 2. Photographs of the stomach (A to C), small intestine (D and E), large intestine (F and G) and liver (H and I) in gossypol-treated monkey.

- A. Low power of the stomach wall. Mucosa (Mu), muscularis mucosa (MM), submucosa (Sm) and muscularis (M) are demonstrated. The upper layer of mucosal epithelium shows degeneration. $\times 100$.
- B. High power of the mucosal surface shows degenerative changes. $\times 1000$.
- C. High power of the epithelial cells at the bottom of the gastric glands which remain intact. $\times 1000$.
- C. Low power of the entire thickness of the small intestinal wall. Degeneration and sloughing off of the villous surface are noted. Scattering is observed in the lamina propria with brown pigment granules. $\times 100$.
- E. High power of small intestinal mucosa in rectangular area of D. Columnar absorptive cells (a) and goblet cells (g) are noted in the villi. The area in the circle shows significant infiltrations of the mucosa with plasma cells, eosinophils and lymphocytes. Scattered foci of brown pigments (bp) are noted in lamina propia. $\times 800$.
- F. Low power of the entire thickness of large intestinal wall. The upper layer of epithelium shows degeneration. $\times 100$.
- G. High power of the mucosal epithelium of the large intestine. The glands contain a large number of goblet cells (g). Scattered foci of brown pigments are also found in lamina propia, markedly at the luminal surface. $\times 800$.
- H. Low power of liver showing marked extensive centrolobular degeneration of the hepatocytes (Hc) with moderate intracytoplasmic vacuolation. Many of the hepatocytes are severely degenerated. The Kupffer cells (Kc) are hypertrophied, became rounded cells, and contain brown pigments. $\times 200$.
- I. High power of hepatocytes (Hc) with degenerative changes and Kupffer cells (Kc) with brown pigments in their cytoplasm. $\times 1000$.



Lungs. These alveolar capillaries in treated animal were congested. The lining capillary epithelial cells became hypertrophied and cuboidal. Numerous foamy alveolar macrophages (AM) were seen within the alveoli. A number of alveolar lining macrophages contained brown pigmented granules in their cytoplasm (arrows). A number of lymphocytes also infiltrated the alveolar wall. A few pneumocytes showed vacuolated cytoplasm.

Spleen. On the examination of the spleen sections, two major territories, red pulp and white pulp, were evident. In white pulp, there were no obvious abnormalities. The red pulp was examined at higher magnification in Plate 1-D. However, there was evidence of extramedullary hematopoiesis. The most distinctive observation in gossypol-treated spleen was the presence of numerous brown pigment macrophages (arrows) surrounding the arteries.

Kidneys. The cortex of the kidney is shown in Plate 1-E. The renal corpuscles were morphologically unremarkable. In contrast, the kidney showed marked hydropic degeneration of the renal tubules especially the proximal convoluted tubules. Parts of the tubules were scattering, abnormally dilated and lined by flattened epithelium instead of cuboidal cells. Tubular epithelial cells were locally absent in some areas, but the basal lamina appeared intact. A number of tubular epithelial cells became foamy and lose their nuclei. Some parts of the tubular epithelial cells still had a cuboidal shape but had increased cytoplasmic basophilia and hyperchromatic nuclei. Fewer degenerated cells were found in the distal tubules. The interstitium revealed focal interstitial edema and fibrosis associated with lymphocyte infiltration. There was mild endothelial swelling in the arteriole.

Stomach. There were numerous sites where the upper layers of epithelium were sloughing off (Plate 2-A, B). The epithelial cells at the bottom of the gastric glands were still intact (Plate 2-C).

Small Intestine. The entire thickness of the small intestinal wall is shown in Plate 2-D. Significant pathological change was observed in the mucosal layer, particularly degeneration of the villi. A number of round, intensely staining nuclei of lymphocytes were scattered at different levels of the epithelial layer. The remaining nuclei belonged to goblet cells which were located at different levels of the epithelial layer, just basal to the mucous cup. In gossypol-treated animals, there was significant interstitial infiltration of the mucosa with plasma cells, eosinophils and lymphocytes (circle). There was scattered foci of brown pigments (bp) in the lamina propria with the increased number of macrophages.

Large intestine. Similar to the small intestine, in gossypol-treated animals, scattered foci of the brown pigments (bp) were found in lamina propria with a large number of macrophages infiltrated.

Liver. In gossypol-treated animals, marked extensive centrilobular degeneration of the hepatocytes (Hc) was observed (Plate 2-H). Hepatic cells showed variable amounts of cytoplasm and have a flocculent appearance or are uniformly stained, suggesting loss of nuclei. Some underwent severe degeneration. Moderate intracytoplasmic vacuolation was generally observed. The sinusoidal lining cells, Kupffer cells (Kc), have irregular shape, often amoeboid. They were markedly hypertrophied and altered in shape to be rounded cells. Their cytoplasm contained prominent brown pigments (Plate 2-I). The portal zones revealed normal bile ducts, hepatic arteries and portal veins with minimal mononuclear cell infiltrations. The findings also included a fair amount of extramedullary erythropoietic cells in the dilated sinusoids.

Adrenal glands. Plate 3 demonstrates the parenchymal cells in adrenal cortex (A to C) and medulla (D). The parenchymal cells of the outer part of the cortex, zona glomerulosa (A), showed mild degeneration. The nuclei are spherical, the cells possess a small amount of lightly staining cytoplasm. The rest of adrenal cortex, zona fascicularis (B) and zona reticularis (C), as well as medulla (D) showed normal appearance.

Epididymis. A section through epididymis is shown in Plate 4-A. The epithelium which lines the epididymal ducts contained two types of cells, tall columnar cells (c) and basal cells (b) (Plate 4-B). The lumen contained few spermatozoa. The morphology observed was not remarkably different from the normal.

Testes. Sections of testis (Plate 4-C and D) show that the spermatogenesis still occurred but with marked disruption. Maturing spermatids (Sd) and spermatozoa were missing in most of the tubules. Many of early spermatids and spermatocytes were found in the lumen of the tubule. Few spermatozoa which had small, darkly staining ovoid heads, were found to be in contact with Sertoli cell (S) cytoplasm. Early spermatids which underwent degeneration were also found in groups near the lumen of the tubule. Secondary spermatocytes were rarely found but did not show any significant changes. Primary spermatocytes, especially pachytene spermatocytes (P), which typically showed chromatin in the process of being organized into chromosomal form, were markedly degenerated. At the periphery of tubule were the spermatogonia (Sg) and these showed marked degenerative changes. Both the Sertoli cells (S) in the wall of the tubule and Leydig cells (L) between the tubules, unlike the germ cells, were unaffected by gossypol treatment.

Skeletal muscles. A section through the gastrocnemius muscle of gossypol-treated animals is shown in Plate 5-A to C. The abnormally different sizes of the fibers were noted. Some of the muscle cells were degenerated (dMC) and showed only traces of the periphery of the cell and nuclei without cytoplasm. Infiltration of macrophages (arrows) was commonly present in several bundles of them.

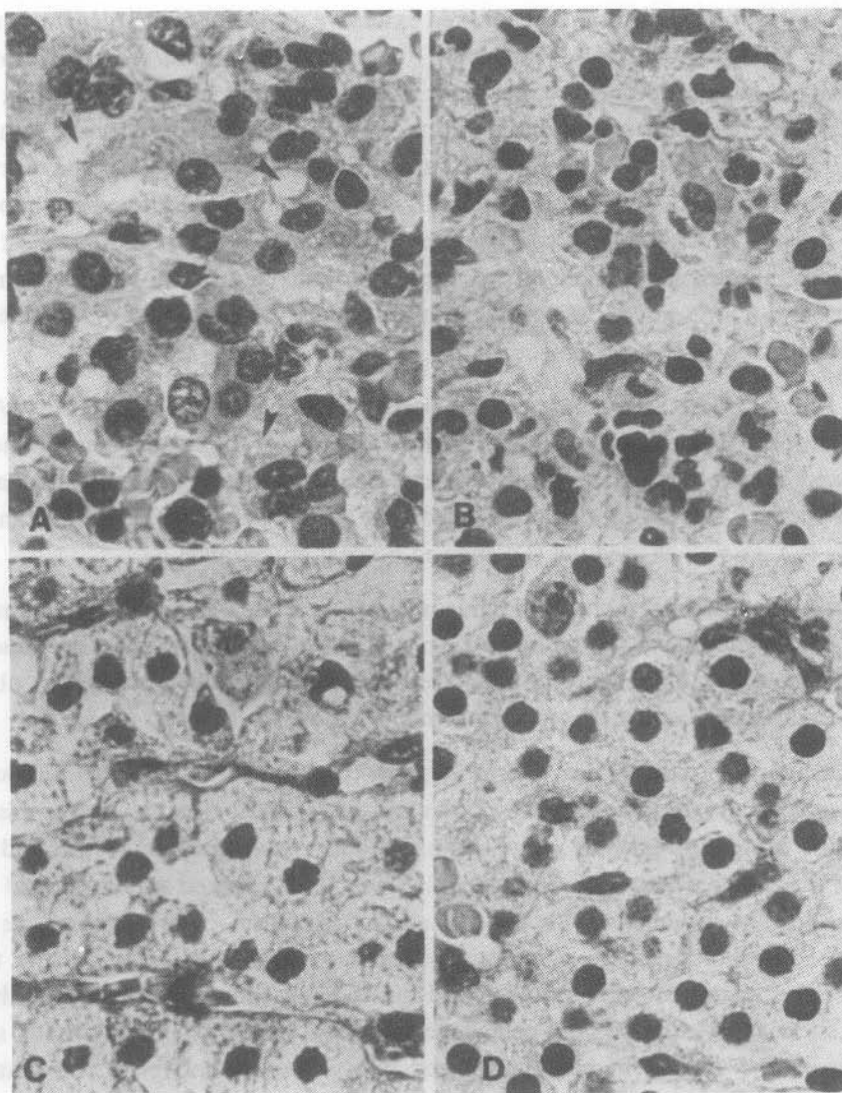


Plate 3. Photographs of adrenal cortex (A to C) and medulla (D) in gossypol treated monkey.

A. High power of cells in zona glomerulosa showing mild degeneration and vacuolation (arrows). $\times 1000$.

B-D. High power of cells in zona fasciculata (B), zona reticularis (C) and adrenal medulla (D) showing normal characteristics. $\times 1000$.

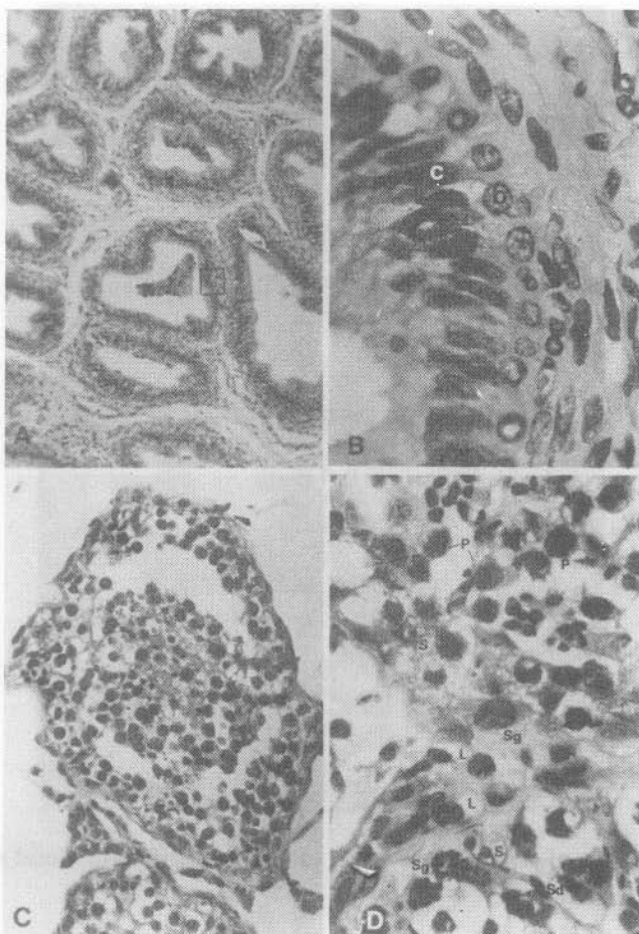


Plate 4. Photographs of epididymis (A and B) and testis (C and D) in gossypol treated monkey.

- A. Low power of epididymis showing normal characteristics. $\times 100$.
- B. High power of epididymal epithelium from the rectangular area in A. The two types of epithelial cells lining epididymal ducts, columnar cells (c) and basal cells (b) are demonstrated. $\times 1000$.
- C. Low power of seminiferous tubules showing severe degenerative changes. $\times 200$.
- D. High power of seminiferous tubules. Degenerative spermatids (Sd) and pachytene spermatocytes (P) are found near the lumen of the tubule. The spermatogonia (Sg) which are at the periphery of the tubules also show degenerative changes. Sertoli cells (S) and Leydig cells (L) show normal characteristics. $\times 1000$.

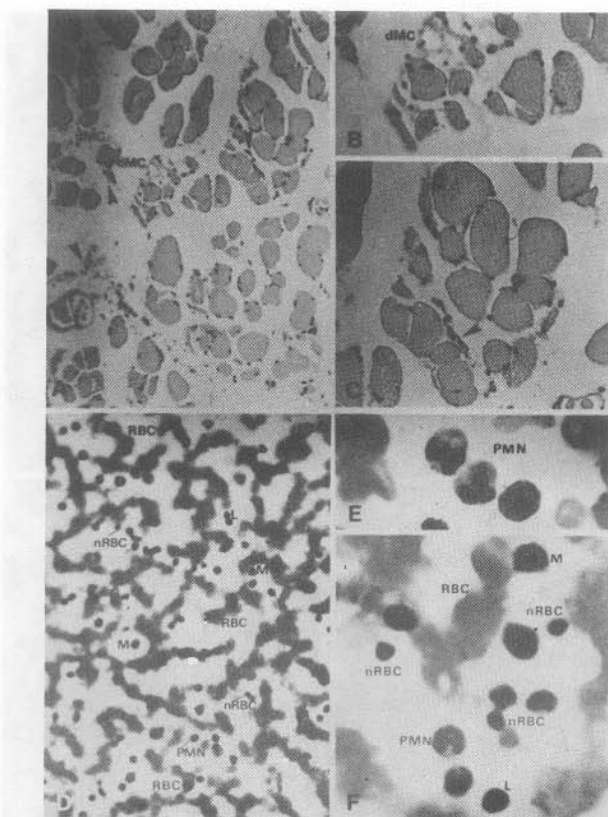


Plate 5. Photographs of skeletal muscle (A to C) and peripheral blood smear (D to F) in gossypol treated monkey.

- A. Low power of skeletal muscle in cross section showing abnormal variable sizes of the fibers. Degenerated muscle cells (dMC) and leukocytic infiltration (arrows) are demonstrated. $\times 100$.
- B. High power of a muscular bundle showing degenerated muscle cell (dMC). $\times 400$.
- C. High power of a muscular bundle showing leucocytic infiltration (arrow). $\times 400$.
- D. Low power of peripheral blood smear showing red blood cells (RBC) with the increased number of nucleated red blood cell (nRBC), polymorphonuclear neutrophils (PMN), lymphocytes (L) and monocytes (M). $\times 100$
- E. High power of peripheral blood smear showing polymorphonuclear neutrophils (PMN). $\times 1000$.
- F. High power of peripheral blood smear showing nucleated red blood cells (nRBC), polymorphonuclear neutrophils (PMN), lymphocytes (L) and monocytes (M). $\times 1000$.

Peripheral blood smear. Plate 5-D demonstrates a low power view of a smear with blood cells well distributed. Most of the cells were erythrocytes (RBC). Abnormally, in gossypol-treated animals, there were a number of nucleated red blood cells (nRBC), which can be distinguished from white blood cells because of their smaller size. These nucleated red blood cells were present after 2 weeks of gossypol treatment in large numbers, almost 60% of the total white blood cells. Hemolysis was marked. The white blood cells including lymphocytes (L), monocytes (M), polymorphonuclear neutrophils (PMN), eosinophils and basophils were seen in markedly increased numbers.

Discussion

Intramuscular injection of a high dose (10 mg/kg/d-daily for 2 wks) of gossypol to monkeys as used here can decrease motility significantly without altering sperm concentration (unpublished data). Despite the lack of change in the number of ejaculated sperm, the present paper indicates that histopathological changes are found in the testis of all gossypol-treated animals, but no alterations were observed in the histology of epididymis. There have been no previous reports of any effects of gossypol on monkey testes¹⁴⁻¹⁷. In the present study, the most striking effect of gossypol was the severe damage to seminiferous tubules, which contrasts with our previous studies of gossypol-treated monkeys at lower doses (0.5-1.25 mg/kg/d) which had normal seminiferous tubules¹⁹. However, the present results are similar to those found in rats treated with gossypol at 30 mg/kg/d orally^{20,21}, where testicular damage was evident after three or more weeks of treatment, with only Leydig cells, Sertoli cells and spermatogonia remaining undamaged after chronic treatment^{8,20,21}. The normal appearance of Sertoli cells and Leydig cells in gossypol-treated animals contrasts with reports that gossypol decreases cell proliferation in primary cultured Sertoli cells and transformed cell lines originating from Sertoli cell tumors^{22,23}. However, our *in vitro* studies on dispersed mouse Leydig cells suggests that gossypol may decrease Leydig cell functions without causing morphological damage (submitted for publication).

The pattern of gossypol distribution in mice, rats, rabbits, dogs and monkeys appears to be similar, with the highest concentrations of gossypol being found in the liver, followed by spleen, lungs, kidneys, heart, testes and fat⁸. Some reports have shown that repeated doses of gossypol given orally to the rat (7.5-30 mg/kg/d for 10-52 wks) suppressed fertility and damaged germinal epithelium but had no histopathological effects in somatic tissue^{12,13,20,24-27}. Other investigators have observed hemorrhages in liver, lung and spleen in rats treated at similar doses^{14,28}. There have also been reports of intestinal dilatation and impaction, hemorrhagic congestion of gastro-intestinal tract, pulmonary and renal congestion, and degeneration of seminiferous tubules⁴. The

hepatotoxicity observed in our studies consisted of generalized centrilobular necrosis, which was often so extensive that it involved the entire hepatic lobule, confirming that the liver is the other major site of toxic injury²⁹. In rabbits treated with gossypol (10-80 mg/kg/d), sudden death with congested liver and lung indicated that these organs are the primary site of action³⁰. Similar findings have been reported in other species including dogs, cats and poultry^{20,31-33}, suggesting a common toxic mechanism in various species. Even in rhesus monkeys fed with gossypol (4-8 mg/kg/d for 4-24 months), fatty infiltration and focal inflammation were observed^{9,34-37}. However, in cynomolgus monkeys, Shandilya *et al.*¹⁸ failed to find any serious toxicity when gossypol was given orally (10 mg/kg/d). The reasons for these differences are still not clear but could be related to different degrees of drug accumulation in the liver.

Our results on the effects of gossypol on kidney tubules confirm previous reports in rats⁴, dogs^{28,33} and rhesus monkeys^{9, 34-37}. It has been suggested that gossypol might interfere with the availability of free iron by binding to it, and this might promote nitrogen loss via urinary excretion, leading to edema and eventually to death³⁸. Additional studies with poultry and sheep red blood cells indicated that gossypol prevents release of oxygen from oxyhemoglobin and caused *in vitro* lysis of red blood cells¹. The higher rate of red blood cell hemolysis observed in our studies could cause increased serum K⁺ levels. Significantly increased number of macrophages with prominent brown pigments in their cytoplasm were accumulated in various organs, more prominently in liver, intestine and spleen. These macrophages phagocytosed the dead red blood cells, accumulating the residues as brown pigments in the cytoplasm. In peripheral blood smears, the presence of nucleated red blood cells supports the idea that the rate of erythropoiesis is increased. The net effect of hemolysis would be anemia and reduced oxygen carrying capacity of the blood, leading to necrosis of various organs. The sloughing off of epithelial cells, especially those in the gastrointestinal tract which have a high rate of turnover, might also be caused by anemia.

In the present study degeneration of skeletal muscle, although less prominent, suggests that gossypol shows toxicity to muscle cells. The recognition of a toxic metabolic or endocrine myopathy as seen in hyperthyroidism is also difficult³⁸. However, the present study, as well as our previous report³⁹ showing abnormal increases in serum muscular enzymes after gossypol treatment confirm that gossypol is a drug causing myopathy. Some direct effects of gossypol on skeletal muscle have been reported, eg. on mitochondrial degeneration^{40, 41}, depression of catechol-o-methyl-transferase activity⁴², blocking of neuromuscular transmission, and lowering of the cholinergic responses *in vitro*⁴³⁻⁴⁵.

In conclusion, the doses of gossypol used in the present study cause histopathological changes in various tissues. The dose may be too high for cynomolgus monkeys which may

be a species that is sensitive to the toxic effect of the drug. In addition, the parenteral route of treatment may cause more accumulation of the drug than other routes, so that a cumulative toxic effect was reached. Further work on gossypol as a potential human contraceptive is needed, especially in subhuman primates and in clinical trials, before the safety and reversibility of gossypol as an antifertility agent can be established with certainty.

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บทคัดย่อ

ได้ศึกษาผลของการเป็นพิษของยาคุมกำเนิดกอสสิพอลในลิงหางยาวเพศผู้ โดยทำการฉีดกอสสิพอลอะซิติกแอซิด ขนาด 10 ม.ก./ ก.ก./ วัน เข้ากล้ามเนื้อต้นขา เป็นเวลา 2 สัปดาห์ ทำการตรวจชิ้นเนื้อทั้งหมด 28 อวัยวะทางกล้องจุลทัศน์ พบการเปลี่ยนแปลงทางพยาธิวิทยา คือ มีการทำลายของเซลล์ในอวัยวะที่สำคัญหลายอวัยวะ โดยเฉพาะอย่างยิ่งตับ, ปอด, ม้าม, ไต, อัณฑะ, กระเพาะ, ลำไส้, กล้ามเนื้อ และการแตกของเม็ดเลือดแดง จากการศึกษาครั้งนี้สรุปได้ว่า แม้ว่าการกอสสิพอล จะถูกนำมาใช้เป็นยาคุมกำเนิดได้ผลในสัตว์หลายจำพวกก็ตาม แต่ก็ยังให้ผลที่เป็นพิษโดยเฉพาะในลิงหางยาวเพศผู้