THE ACTIONS OF GOSSYPOL ON THE PHYSIOLOGIC ANTIOXIDANT DEFENSE SYSTEM

by

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(ABSTRACT)

Gossypol, a yellow polyphenolic pigment found in cottonseeds, is known to promote the production of reactive species of oxygen in vitro, and has toxic actions on spermatogenic epithelium, hepatocytes and cardiac myocytes in vivo. Species vary in tissue sensitivity to the toxic effects of gossypol. The spermatogenic epithelium is the most sensitive tissue to gossypol in rats, followed by the liver. Toxic effects to the rat heart are found only after prolonged administration of gossypol. The antioxidant defense system that protects cells from injury by reactive species of oxygen was examined in the present study to determine a possible pathogenesis for gossypol associated tissue damage.

The concentrations of several hepatic antioxidants including catalase, glutathione peroxidase, ascorbate and copper-zinc superoxide dismutase were decreased in gossypol treated rats. Catalase, glutathione peroxidase, ascorbate and glucose-6-phosphate dehydrogenase were decreased in the testis. In contrast, antioxidants including catalase and glutathione reductase were increased in the hearts of gossypol treated rats. The selective inhibition of testis and hepatic

antioxidants may account for the greater sensitivity of these organs to reactive oxygen species generated by gossypol. The rat heart may adapt to oxidative insult by inducing the production of antioxidants.

Glucose-6-phosphate dehydrogenase activity was decreased in the testis but not liver or heart of gossypol treated rats. This important enzyme is known to produce NADPH reducing equivalents for testosterone biosynthesis and the glutathione antioxidant system. In the present study, micromolar concentrations of gossypol inhibited glucose-6-phosphate dehydrogenase in a competitive manner with respect to glucose-6-phosphate. This may explain the degeneration of spermatogenic epithelium as well as decreases in serum testosterone concentrations in gossypol treated rats.

Gossypol is known to cause antifertility in women and female rats. The present study found irregularities in the estrous cycles and ultrastructural changes in endometrial macula adherentes of gossypol treated female rats.

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A Review of the Literature

History

Gossypol is a yellow, polyphenolic pigment (MW518.54) found in cotton plants of the genus Gossypium (Fig. 1). The cottonseed contains the highest concentrations of gossypol, but it is also found in the stem and roots (Adams et al., 1960; Merck Index, 1976).

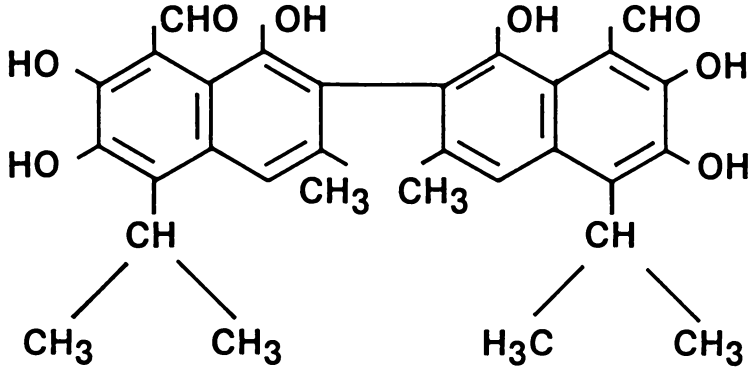

Figure 1. The Structure of Gossypol.

The use of gossypol as a contraceptive was first suggested in the 1950's when people in several regions of China showed high rates of infertility. Epidemiologic studies linked the infertility to the use

of cooking oil extracted from cottonseeds by a new method. Oil was traditionally pressed from cottonseeds after heating, but newer technology allowed extraction without heat. This new crude cottonseed oil caused infertility in men and interrupted menstrual cycles in women. On withdrawal of the oil, many of the women cycled normally and men gradually regained fertility (Zatuchni and Osborn, 1981). Recovery generally depended on the dose and length of time that the individual consumed gossypol (Liu, 1985). Animal experiments of the 1970's implicated gossypol as the antifertility agent in cottonseeds. Heat used in the original oil processing technique destroyed much of the gossypol, but the new method allowed high concentrations of gossypol to remain in the extracted oil (National Coordinating Group on Male Antifertility Agents, 1978). Extensive clinical trials involving 8806 Chinese men from 1972 through 1980, examined the use of purified gossypol and its derivatives gossypol acetic acid and gossypol formic acid, as reversible male contraceptive agents. These trials established optimal dosage rates to achieve over 99% antifertility efficacy, while producing minimal side effects (Liu et al., 1981).

Other gossypol containing parts of the cotton plant are used in the western world. Extract of cotton root bark is a herbal medicine historically used in the Southwestern United States for induction of menstruation and abortion in women (Conway and Slocumb, 1979). However, large scale clinical trials using purified gossypol in women have not been reported.

The Effects of Gossypol on Male Fertility

Administration of low oral doses of gossypol (20 mg/day) to men for several weeks causes decreased motility and numbers of spermatozoa. These effects are generally reversible within two to three months after withdrawal of gossypol, especially if treatment is confined to two years (Liu et al., 1981). Similar effects are found in rats (Hadley et al., 1981; Zhou and Lei, 1981), monkeys (Shandilya et al., 1982), hamsters (Waller et al., 1981; Chang et al, 1980), and mice (Coulson et al., 1980).

Studies in rats demonstrated damage to testis germinal epithelium that increased in severity with the length of time on daily gossypol treatment (Xue, 1981). Ultrastructural examination of epididymal spermatozoa and testicular spermatids in gossypol treated rats, demonstrated marked damage to the tail region, especially pronounced in the mitochondrial sheath of the midpiece. A high incidence of segmental aplasia of the mitochondrial sheath was found in both epididymal and testicular sperm (Oko and Hrundka, 1982). Decreased epididymal sperm counts were also found (Nadakavukaren et al., 1979). Sperm were immotile and sperm counts were decreased in the ejaculate of treated rats, but libido was not affected (Hadley et al., 1981). The rate of testicular fluid secretion was not affected by treatment with gossypol (Wong, et al., 1984).

Gossypol is also effective in decreasing sperm motility in vitro (Chongthammakun et al., 1986) and was investigated as a spermicide for use as a vaginal contraceptive in women (Ratsula, et al.). These

spermicidal actions were enhanced when gossypol was co-precipitated with polyvinyl pyrrolidine (Waller et al., 1980).

Some studies suggest that gossypol disturbs the normal reproductive endocrine balance in male rats. Gossypol treatment decreased serum LH and testosterone in male rats but serum FSH remained constant. Leydig cells from gossypol treated rats produced less testosterone than control cells when incubated in vitro with LH. LH stimulated control Leydig cells demonstrated decreased testosterone production when incubated with increasing amounts of gossypol (Hadley et al, 1981). These findings may indicate that gossypol causes a disruption of the hypothalamic pituitary axis as well as a direct effect on Leydig cell testosterone production. Other studies failed to confirm an antiandrogenic effect of gossypol in rats (Wang et al., 1984).

The antifertility actions of gossypol are attributed by some investigators to the inhibition of testicular enzymes. Experiments using ejaculated spermatozoa from monkeys demonstrated that glycolysis was inhibited by gossypol and that the site of inhibition was lactate dehydrogenase-X (Stephens et al., 1983). LDH-X is an isoenzyme with a unique electrophoretic mobility found only in mature testicular epithelium and spermatozoa. The role of this enzyme is unclear, but decreased LDH-X activity is found in humans and laboratory animals in some cases of infertility (Blanco, 1980; Maugh, 1981).

The Effects of Gossypol on Female Fertility

Most gossypol studies concentrate on male mammals, but several recent reports examine effects on females. Intramuscular injections of gossypol in female rats with normal estrous cycles caused irregular cycling for the duration of the treatment (Lin et al., 1985). Gossypol treatment of pseudopregnant rats decreased the decidual reaction (Lin et al., 1984). Gossypol administration did not inhibit ovulation in other studies (Hahn, et al., 1981). Treatment of rats in early pregnancy caused abnormal changes in the endometrium (Rikihisa et al., 1985). Gossypol may cause these changes by inhibiting reproductive hormone production because treated animals had lower levels of serum estradiol and progesterone than controls (Lin et al. 1985).

Administration of gossypol to female rats in early pregnancy reduced ovum implantation and decreased the number of implanted embryos that were carried to term. Lower levels of serum estradiol and progesterone were also found in gossypol treated pregnant rats compared to pregnant controls. When gossypol treated pregnant rats were supplemented with estradiol and progesterone, implantation and litter sizes increased to control values (Lin et al., 1985). These experiments add support to the suggestion that gossypol inhibits steroidogenesis and thereby inhibits normal reproductive function in the female rat.

Pharmacokinetics of Gossypol

Dogs, monkeys, mice, rats, and rabbits were orally dosed with radioactively labeled gossypol to study its pharmacokinetics. Gossypol accumulated mainly in the liver in these species with significant amounts also found in muscle, kidney, and blood (National Coordinating Group on Male Antifertility Agents, 1978; Graham et al., 1985). In swine, gossypol tends to accumulate within muscles (Abou-Donia and Dieckert, 1975). The germinal epithelium of the testis appears highly sensitive to gossypol because only small concentrations were found in testicular tissue.

The amount of gossypol absorbed by the gastrointestinal tract is dependent on the amount of free gossypol that is fed. Gossypol that is bound to proteins (bound gossypol) is largely unabsorbed and passes in the feces. In pigs, ingestion of high protein rations with excess lysine increases tolerance to gossypol. It is proposed that the carbonyl groups of gossypol (Fig. 1) bind to lysine residues (Abou-Donia, 1976).

Supplementation of iron salts in feed also decreases gossypol toxicity. It is thought that iron forms insoluble chelates with gossypol that inhibit absorption by the gastrointestinal tract (Abou-Donia, et al., 1969). The lower toxicity of iron supplemented rations may also be explained by the proposal that gossypol is decarbonylated in the gastrointestinal tract by an autoxidation catalyzed by iron (Abou-Donia, 1976). Decarbonylation of ^{14}C-gossypol in rats was quantitated by measuring $^{14}CO_2$ in expired air (Abou-Donia

et al., 1969). Since $^{14}CO_2$ was detected in expired air of rats only one hour after ingestion of ^{14}C-gossypol, it was suggested that this process takes place in the digestive tract (Abou-Donia et al., 1969). The decarbonylation reaction is thought to cause the formation of apogossypol, an unstable and less toxic metabolite. Even without iron supplementation, species differ in the amount of gossypol that is metabolized by this pathway. Decarbonylation is an important metabolic pathway in the rat as 12% of the ingested radioactivity was detected in expired air by 13 days after administration of ^{14}C-gossypol. In a similar experiment, only 2.1% of the dose was recovered in expired air of pigs by 20 days after administration of ^{14}C-gossypol (Abou-Donia, Diekert, 1975) and 3.3% of the radioactive carbon was found in the expired air of chickens 16 days after ^{14}C-gossypol administration (Abou-Donia and Lyman, 1970).

These species differences are paralleled by differences in the amounts of gossypol absorbed by the tissues. The peak of radioactive gossypol deposited in tissues was only 12.5% of the oral dose in rats (Abou-Donia et al., 1969) compared with 32.9% in pigs (Abou-Donia and Dieckert, 1975) and 16.8% in chickens (Abou-Donia and Lyman, 1970). Some investigators propose that less gossypol is absorbed by the gastrointestinal tract in the rat than the pig and chicken due to differences in enteric decarbonylation (Abou-Donia, 1976). This may account for the higher tolerance to the toxic effects of gossypol by

the rat than the pig. The oral LD_{50} is 550 mg/kg in pigs compared to 2400-3340 mg/kg in rats (Qian and Wang, 1984).

Elimination of gossypol from the body is slow. The biological half-life ($t_{\frac{1}{2}}$) of a single dose of gossypol was 48 hours in the rat on a basal diet and 23 hours with iron supplemented rations (Abou-Donia et al., 1969). In the same study, 97% of the gossypol dose was eliminated by 19 days. In laying hens, the biological half-life of a single dose of gossypol was 30 hours (Abou-Donia and Lyman, 1970), compared to 78 hours in swine (Abou-Donia and Dieckert, 1975).

The liver is thought to metabolize gossypol by decarbonylation, oxidation, and conjugation with glucuronic acid (Abou-Donia, 1976). The metabolites are more water soluble than gossypol and more easily passed in the bile. After excretion into the intestine, bacterial enzymes may cleave the glucuronide and allow enterohepatic cycling of gossypol metabolites (Abou-Donia, 1976). In all species tested, the majority of ingested or injected gossypol was eliminated via the feces (National Coordinating Group on Male Antifertility Agents, 1978; Abou-Donia, 1976).

Unlike most xenobiotics, little gossypol is eliminated via the urine. In a study of rats given a single dose of ^{14}C-gossypol and monitored for excretion of radioactivity over 19 days, 83% was eliminated in the feces, 11.73% in exhaled CO_2 while only 2.5% was detected in urine (Abou-Donia, 1969). Similar results were found in Rhesus monkeys and pigs (Abou-Donia, 1976) even though significant amounts of

gossypol were detected in the kidneys. Gossypol is thought to be filtered and secreted by the classic organic acid system. The urinary pH of most carnivores is acidic which would cause the majority of gossypol (pk = 7.2) to exist in a nonionized form in the renal tubules. The distal convoluted tubule is permeable to nonionized lipid soluble compounds such as gossypol. Reabsorption in the distal convoluted tubule is proposed to account for the low urinary excretion of gossypol (Goldinger et al., 1985).

Toxicity of Gossypol

Toxic side effects of gossypol administration in human clinical trials include fatigue, gastrointestinal upset, decreased libido, dizziness, mouth dryness, and hypokalemia. These symptoms are largely eliminated as the dose of gossypol is decreased (Liu et al, 1981). Symptoms of hypokalemia and fatigue are generally found in people on low potassium diets and potassium supplementation usually eliminates the problem. Evidence suggests that gossypol increases urinary potassium loss by enhancing renal prostaglandin E synthesis and inhibiting sodium potassium ATPase (Quian, 1981).

The particular sensitivity of swine to the toxic effects of gossypol was studied in the 1950's (Smith, 1957). Pigs fed diets high in cottonseed meal showed clinical signs of dyspnea, panting, weakness and anorexia for several days prior to death. Postmortem examination revealed congestive heart failure with dilatation of both ventricles. The livers of these pigs were congested and necrotic. Microscopic

examination of the liver revealed extensive centrilobular necrosis that was so severe in some cases that almost no viable parenchyma remained. The necrotic spaces around central veins were filled with pooled erythrocytes. Skeletal muscles appeared pale, but no microscopic lesions were identified.

Dogs on cottonseed meal rations are reported to die of a similar congestive heart failure with hypertrophic cardiomyopathy (West, 1940; Patton et al., 1985). Microscopic examination revealed many cardiac myofibers with perinuclear vacuoles and others with large hyperchromatic nuclei. Cardiac edema and atrophied myofibers were also reported.

Adult ruminants are much less sensitive to the toxic effects of gossypol than monogastrics. Some investigators propose that gossypol binds to proteins in the ruminal contents, rendering it relatively nontoxic. However, young calves are very susceptible to gossypol poisoning (Rogers and Henaghan, 1975; Orgad-Klopfer and Adler, 1986). The digestive system of young calves behaves much like that of a monogastric animal until the rumen is fully developed. Gossypol associated lesions in young calves are similar to the congestive heart failure described in pigs and dogs and the toxic hepatitis described in pigs. Gastroenteritis was also reported in calves fed cottonseed meal.

Toxic effects of gossypol vary with the dose. Chronic administration of low doses of gossypol commonly causes anorexia and weight loss in pigs (Abou-Donia and Dieckert, 1975) and rats (Heywood et al., 1986).

Use of Cottonseed Meal as a Protein Supplement

The cottonseed, once a useless byproduct of cotton fiber production, is now a valuable commodity in a major industry. An estimated 4.47 million tons of cottonseeds were produced in the U.S. alone during 1980-81 according to the USDA (US Fats and Oil Statistics, 1980). Production of 2.05 million tons of cottonseed meal worth $256 million and 0.71 million tons of cottonseed oil worth $343 million were estimated for that time period. Cottonseed oil is used in production of prepared foods, cooking and salad oils, shortening, and margarine. Cottonseed meal is extensively used in animal feeds due to its high protein content. Cottonseed meal is used in rations of ruminants, pigs, chickens, horses, dogs, cats, and catfish (Personal communication with Mr. Tom Wedegaertner of the National Cottonseed Products Association, Inc. Memphis, Tenn.). High protein flour formulated with cottonseed meal is used in developing countries to prevent malnutrition in people (Zatuchni and Osborn, 1981).

Justification

Cottonseed meal is used in ever increasing quantities in animal feed. The ubiquity of cottonseed oil in products for human consumption becomes obvious if one reads ingredient labels on a multitude of cookies, potato and corn chips, breads and other prepared foods. In 1978, the FDA established a limit of 450 ppm on the free gossypol content in cottonseed products for human and animal consumption (Food and Drug Cosmetic Law, 1978). These guidelines were established due to

the absence of overt toxic effects at these concentrations. In several species including humans, infertility is achieved in the male without significant effects on other organs at low levels of gossypol intake. Few reports of the effects of chronic low doses of gossypol on female reproductive functions in animals and humans are found in the literature. There are no published reports indicating allowable limits of gossypol residues in animal tissues for human consumption.

Many reports describing the overt toxic effects of high doses of gossypol on the heart and liver as well as the more subtle effects of chronic low dose treatment on the male reproductive system are found in the literature. The mechanism of action of gossypol toxicity on a subcellular level is controversial and largely unknown. DePeyster et al. recently proposed that the wide array of biological actions of gossypol may be explained by the common underlying mechanism of free radical damage (DePeyster et al., 1984). This proposal was supported by *in vitro* experiments with rat liver microsomes and human sperm. These experiments suggested the formation of reactive oxygen species including superoxide radical and hydrogen peroxide when gossypol acetic acid was incubated with microsomes and sperm. Reactive species of oxygen are implicated as a cause of a multitude of pathological changes. Free radical reactions with proteins and lipids often change the molecular configuration and may lead to cell death (Freeman and Crapo, 1982). The interaction of free radicals with DNA may cause damage to the molecular structure and induce strand breakage. Therefore, the promotion of free radicals by gossypol warranted further investigation.

Molecular Oxygen and its Reactive Intermediates

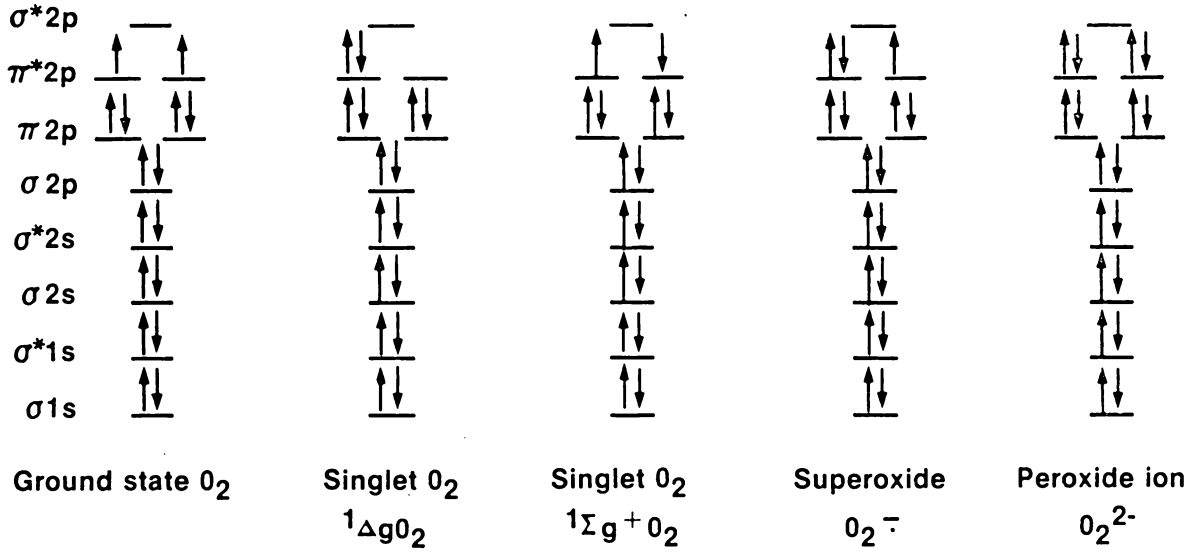

Figure 2. Molecular orbitals of different species of dioxygen (redrawn from Halliwell and Gutteridge, 1985).

In an attempt to explain the behavior of oxygen, it is necessary to examine the unique characteristics which place it on the end of the respiratory chain as the terminal electron acceptor in aerobic life forms. The thermodynamic properties of oxygen make it an excellent acceptor of electrons. This is clearly illustrated by the violent redox reaction that occurs when gasoline is ignited in the presence of

oxygen. Here, a sharp burst of energy is emitted as oxygen accepts electrons from the reduced organic compound, gasoline.

This feature of oxygen is exploited in a similar but much more controlled manner by animals which depend on a supply of reduced organic nutrients such as glucose for energy. When pairs of electrons are transferred from these reduced nutrient to oxygen through a series of redox reactions, energy is released due to the large difference in electrochemical potential between the two molecules. The reactions ultimately result in the reduction of oxygen to water and the simultaneous oxidation of the organic nutrient to carbon dioxide. The energy obtained from these reactions is used to drive the metabolic processes of animals (McCord, 1979).

If oxygen were as unstable as it appears thermodynamically, it would become a scarce molecule. However, there is a feature that lends stability to oxygen and thus allows it to exist in a molecular form (Hassan and Fridovich, 1979). Kinetically, oxygen cannot readily accept electron pairs (McCord, 1979; Fridovich, 1979). This can be explained by examining the molecular orbital configurations of oxygen (Fig. 2).

The electrons in the outer shell of an oxygen atom are shared with those of another oxygen atom to form the covalent bond of molecular oxygen, O_2. The orbitals more central to the molecule, such as the 2s orbitals as well as the first three 2p orbitals, are completely filled by electrons. According to the physical rules that govern electron configuration, only two electrons may occupy any atomic orbital, and to

do so they must have opposite spins. These electrons are said to be spin-paired. It is actually the opposite spins on the two electrons which counteract their tendency to repel each other and permit them to coexist in an orbital. However, the outer two orbitals (π*2p) are incompletely filled. The molecule attains maximal stability by spreading out the outer two electrons across these two orbitals in an unpaired arangement with parallel spins (Hassan and Fridovich, 1979; Fee and Valentine, 1977; Brady and Humiston, 1975). During metabolic redox reactions, electrons are generally donated to the outer orbitals of oxygen in a spin-paired arrangement. These oppositely spinning electrons cannot enter the available p orbitals of molecular oxygen without one of the outer electrons undergoining an inversion of spin. A noncatalyzed spin reversal is slow and unlikely to occur at temperatures which sustain life; thus molecular oxygen is kinetically more stable due to its electronic configuration than it would appear on a thermodynamic basis (McCord, 1979; Fridovich, 1979).

This spin reversal is avoided if oxygen accepts a single electron (a univalent reaction), thereby forming the free radical, superoxide (Fridovich, 1979). During nonenzymatic oxidations, organic compounds will more readily transfer an electron pair to two oxygen molecules in two successive univalent reactions than transfer the pair in a single divalent reaction (McCord, 1979). Through a series of nonenzymatic univalent reactions, a molecule of oxygen can ultimately accept four electrons to form two water molecules (Fridovich, 1978). Although oxygen and water are fairly unreactive, the intermediate products

(superoxide, hydrogen peroxide and the hydroxyl radical) formed in the intervening reduction reactions and the spin altered species (singlet oxygen) are thought to be responsible for oxygen toxicity (Fridovich, 1976; Fridovich, 1978).

Therapeutic administration of oxygen has found success for nearly 200 years; however, prolonged deliverance of high oxygen concentrations proves deleterious (McCord, 1979; Pfenning, 1979). Administration of 100% oxygen to adult rats for 60-72 hours causes diffuse pulmonary capillary endothelial swelling, degeneration and necrosis, followed by pulmonary edema, hypoxemia and death (Crapo, 1977). The toxic effects of oxygen prove to be more acute under increased pressure. The delivery of 100% oxygen to rats in a chamber pressurized to five atmospheres causes convulsions and death in less than 30 minutes (McCord, 1979; West, 1979). In humans, the toxicity of oxygen is exacerbated by the high pressures of deep sea diving. On extremely deep dives, oxygen concentrations must be reduced to under one percent of the inspired gas mixture to avoid deleterious effects (West, 1979).

To prevent pathological changes at normal oxygen concentrations, aerobic organisms have adapted enzymatically to avoid the whole series of univalent reduction reactions and prevent the formation of superoxide, hydrogen peroxide and the hydroxyl radical. Oxidative enzymes such as cytochrome oxidase catalyze the tetravalent reduction of oxygen to water without the release of detectable intermediates (Hassan and Fridovich, 1979; Fridovich, 1976). Although aerobic cells use cytochrome oxidase pathways for most of their oxygen consumption,

many other biological oxidations result in the formation of reactive intermediates.

Hydroxyl Radical

Ionizing radiation of tissues causes the hydrolysis of body water and formation of reactive intermediates such as hydroxyl radical ($OH^{\bullet}$), hydrated electron ($\bar{e}_{aq}$) and atomic hydrogen ($H^{\bullet}$). These species can subsequently react with dissolved O_2 or various cell components. Most of the DNA and membrane damage due to ionizing radiation is attributed to $OH^{\bullet}$. Hydrated electrons and $H^{\bullet}$ react with dissolved O_2 to form superoxide anion ($O_2^{\bar{\bullet}}$).

$$\bar{e}_{aq} + O_2 \rightarrow O_2^{\bar{\bullet}}$$

$$H^{\bullet} + O_2 \rightarrow HO_2 \rightarrow O_2^{\bar{\bullet}} + H^{+}$$

Hydroxyl radical is one of the most reactive radicals known. It reacts very quickly and indiscriminately with all classes of molecules found in biological systems including sugars, amino acids, phospholipids, nucleotides and organic acids. Hydroxyl radical reacts in three major ways: hydrogen abstraction, addition, and electron transfer. $OH^{\bullet}$ reacts with ethanol by hydrogen abstraction or by removing a hydrogen atom to form water and ethanol radical. The ethanol radical thus contains an unpaired electron on one of the carbons and tends to react with surrounding compounds. Hydroxyl radical tends to act by addition to aromatic rings. Hydroxyl radical reacts with the aromatic nucleotide bases of DNA and RNA by adding on to a double bond. This causes damage to the molecular structure and

may induce strand breakage. Cells often die after this form of damage or those that survive may undergo mutations. Hydroxyl radical transfers electrons to inorganic compounds such as the chloride ion, causing the formation of chloride radicals that are capable of propagating the chain reaction (Halliwell and Gutteridge, 1985).

Singlet Oxygens

Two other forms of molecular oxygen, the singlet oxygens ($^1O_2$), are much less stable than ground state oxygen. Singlet oxygen ($^1\Delta gO_2$) is less stable because the two outer electrons are not spread across the two outer orbitals, but are spin-paired in a single π*2p orbital (Fig. 2). $^1\Delta gO_2$ is 22.4 kcal above ground state oxygen. The second form of $^1O_2$, ($^1\Sigma g^+O_2$) has an energy of 37.5 kcal above ground stage O_2 because the two single electrons in the outer orbitals are spinning in opposite directions (Fig. 2). The highly energetic $^1\Sigma g^+O_2$ tends to decay to the $^1\Delta gO_2$ form which is the only significant species of $^1O_2$ in biological systems. These oxygen species do not have the spin restriction found in ground state O_2 and therefore are kinetically much less stable and more ready to accept electron pairs from nonradicals.

$^1O_2$ is not itself a free radical, but is a product of some reactions with radicals and tends to form radicals. $^1O_2$ may be formed when hypochlorite ion, a product of phagocyte myeloperoxidase, reacts with hydrogen peroxide. It may also be generated by photosensitization reactions where a photosensitizer molecule absorbs certain wavelengths of light, raising it to an excited state. This energy is transferred

to molecular oxygen to form $^1O_2$. Riboflavin, flavin mononucleotide, flavin adenine dinucleotide, chlorophylls a and b, bilirubin, retinal and porphyrins may act as photosensitizers. Singlet oxygen may react with surrounding molecules or it may react with and damage the photosensitizer.

Singlet oxygen may either combine with molecules or transfer its energy to molecules. Singlet oxygen is responsible for peroxidizing lipids or damaging proteins by these methods (Halliwell and Gutteridge, 1985).

Superoxide Radical

The inability of ground state oxygen to readily accept electron pairs is avoided if oxygen accepts electrons one at a time. The addition of an electron to molecular oxygen (Fig. 2) causes the formation of the free radical, superoxide ($O_2^{\cdot-}$). Superoxide may be produced by the autoxidation of epinephrine, hemoglobin, flavins and quinones or enzymatically by xanthine oxidase (Misra and Fridovich, 1971; Misra and Fridovich, 1972a; Misra and Fridovich, 1972b; Misra and Fridovich 1972c; Misra, 1974; Fridovich, 1979). Superoxide radical has been implicated in the killing of bacteria and protozoa (Hassan and Fridovich, 1977a; Murray et al., 1980), inactivation of viruses (Lavelle et al., 1973), lysis of erythrocytes (Weiss, 1980; Weiss and LeBuglio, 1980), destruction of granulocytes (Salin and McCord, 1975; Salin and McCord, 1974; Salin and McCord, 1977), depolymerization of hyaluronate (Greenwald and Moy, 1980; Greenwald, 1980), inactivation of

enzymes (Salin and McCord, 1975; Salin and McCord, 1974; Salin and McCord, 1977), peroxidation of lipids (Halliwell, 1978), and damage to DNA (Fridovich, 1976; Brawn and Fridovich, 1980).

Superoxide may not be the precise culprit causing all of these damages. Superoxide generated in physiological processes may dismute spontaneously or via a reaction catalyzed by superoxide dismutase, to form hydrogen peroxide (H_2O_2) (Fridovich, 1979; Salin and McCord, 1977; Brawn and Fridovich, 1980; Hassan, 1980; Halliwell, 1980;Fee, 1981). H_2O_2 may react with an additional $O_2^{\cdot-}$ radical in the presence of iron to form the more reactive hydroxyl radical by the Haber-Weiss reaction (Hassan Fridovich, 1976; Fridovich, 1978; Johnson and Lehmeyer, 1977; Brawn and Fridovich, 1980; Hassan, 1980; Halliwell, 1980; Babior, 1978; Fridovich, 1981; Cohen, 1977; Gabig and Babior, 1979; Johnston et al., 1975a).

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + {}^1O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

$$\underline{\text{Sum}} \qquad O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe} {}^1O_2 + OH^{\cdot} + OH^-$$

The ubiquity of iron salts in biological systems promotes the formation of $OH^{\cdot}$ from $O_2^{\cdot-}$.

Phagocytic cells such as neutrophils and macrophages play an important role in tissue defense mechanisms. Neutrophils undergo a sharp increase in oxygen consumption when phagocytizing bacteria. This oxygen is enzymatically reduced by a membrane bound NAD(P)H dependent oxidase to $O_2^{\cdot-}$. The newly formed $O_2^{\cdot-}$ is released into phagosomes.

This subjects the bacteria inside phagosomes to toxic concentrations of $O_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$, and is essential for bacterial killing. Unfortunately, the activity of the respiratory burst is not limited to the portions of the plasma membranes that invaginate into phagosomes, as the production of oxygen intermediates appears to be a generalized reaction over the entire plasma membrane. Many intermediates escape into the extracellular fluids where there is little superoxide dismutase to protect the tissues (Johnston et al., 1975b; Klebanoff, 1975; Rosen and Klebanoff, 1977; Fee, 1980; Foote et al., 1980).

Removal of superoxide radicals involves a dismutation reaction, which denotes a simultaneous oxidation and reduction. When two superoxide molecules react in such a reaction, one is oxidized to molecular oxygen while the other is reduced to hydrogen peroxide. Spontaneous dismutation of superoxide will occur, but it is believed to be slow compared to the enzymatic reaction.

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen Peroxide

The significance of the superoxide radical has only been investigated over the past two decades (McCord et al., 1971) but hydrogen peroxide, also a reduction intermediate has been documented as a bacteriocidal agent since the early 1900's. Superoxide is formed by the addition of one electron to molecular oxygen (Fig. 2). A second electron (Fig. 2) added to oxygen forms the peroxide ion (O_2^{-2}). A common peroxide found in biological systems is the protonated form,

hydrogen peroxide (H_2O_2). Hydrogen peroxide (Fig. 2) is not a radical, but is a good oxidizing agent. H_2O_2 may be produced by dismutation of $O_2^{\cdot-}$, or by reactions catalyzed by D-amino acid oxidase, and urate oxidase. H_2O_2 can inactivate some enzymes by oxidizing essential thiol groups. It can easily cross cell membranes unlike $OH^{\cdot}$ and may then react with Fe^{+2} or Cu^{+1} ions to form the hydroxyl radical (Halliwell and Gutteridge, 1985).

To avoid the toxic effects of large accumulations of reactive oxygen species in cells, most life forms have evolved enzymes to scavenge these intermediates (Brawn and Fridovich, 1980; Hassan, 1980; Halliwell, 1980).

Biochemical Defense Mechanism Against Free Radical Injury

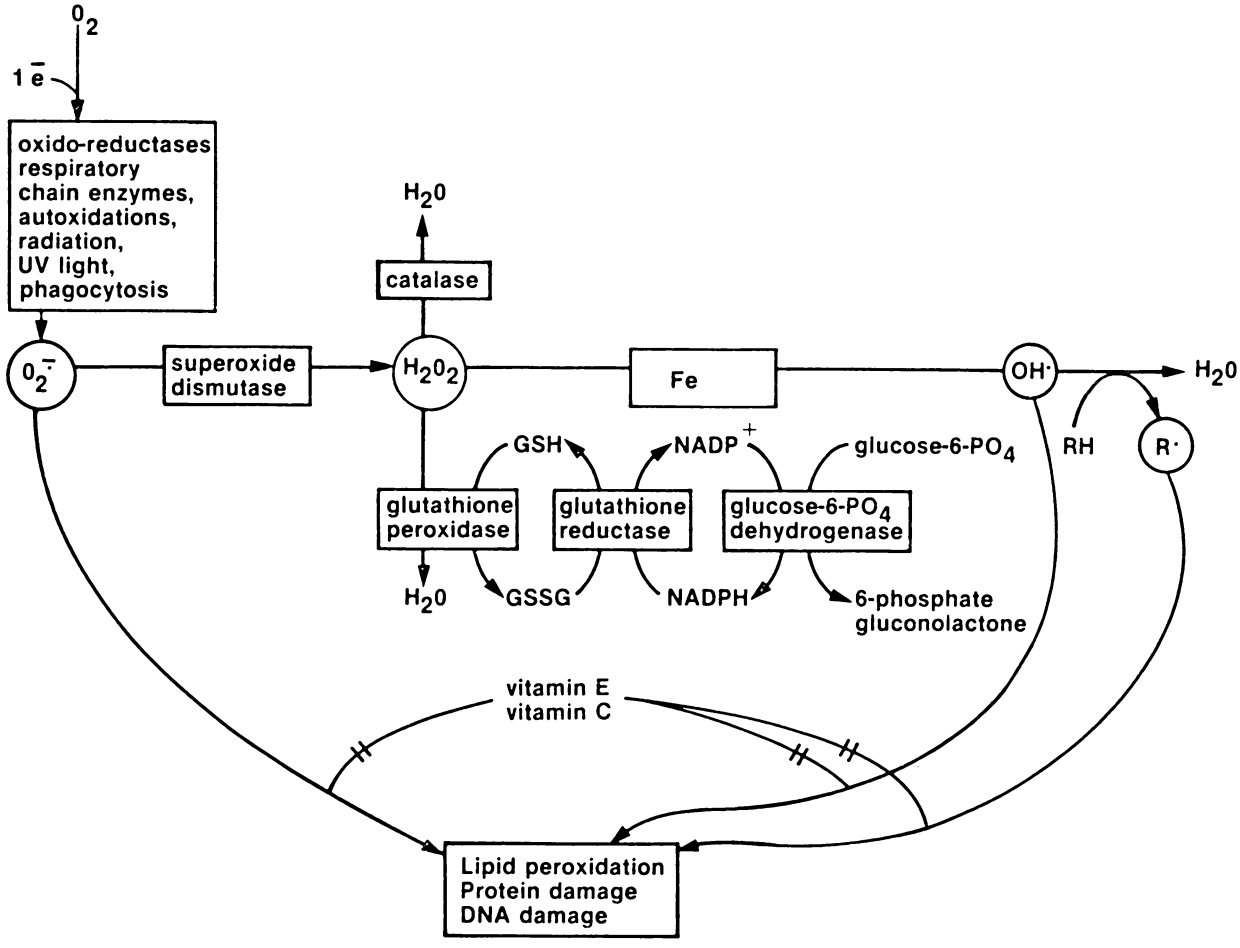

Figure 3. Free radical generation and cellular defense mechanism summary (adapted from Clark et al., 1985).

A free radical is generally defined as any molecule possessing an unpaired electron in its outer orbital. The presence of an unpaired electron creates instability in these species, causing them to react

rather nonspecifically with surrounding molecules. Superoxide radicals may undergo oxidation or reduction reactions, but free radical reactions in general are often oxidation reactions whereby an electron is removed from adjacent molecules to satisfy the requirements of the free radical, giving it stability, but creating a new radical on the adjacent molecule. Now the original molecule is no longer a radical, but the newly formed radical may react in a second redox reaction with a neighboring compound (Slater, 1984). This process could go on indefinitely in a chain reaction if no defense mechanisms were present to break the chain reaction. Free radical reactions with proteins and lipids often change the molecular conformation and may ultimately lead to the disruption of metabolic processes and eventually to the death of the cell (Freeman and Crapo, 1982). The amount of damage depends on the quantity and quality of the free radicals as well as the defense mechanisms present. Free radical injuries are not always fatal to cells. Cell death due to free radical injury is likely to be some combination of an overwhelming production of free radicals and a compromised defense system.

Defense mechanisms for free radicals are present in all cells that survive in aerobic conditions. The evolution of free radical scavengers is thought to parallel the increase in atmospheric oxygen two to three billion years ago with the advent of photosynthetic organisms (Fridovich, 1975).

Cellular defense systems against injury from reactive species of oxygen are divided into two major categories (Freeman and Crapo, 1982).

The first category is composed of the low molecular weight free radical scavengers such as vitamin E, which protects against lipid phase oxidations, as well as ascorbate and glutathione, which protect from aqueous phase oxidations. The second category is composed of the enzymatic free radical scavengers such as superoxide dismutase (SOD), which protects the cells from the effects of superoxide radical ($O_2^{\cdot-}$), and glutathione peroxidase, glutathione reductase and catalase, which protect the cell from hydrogen peroxide.

Vitamin E, vitamin C, β-carotene and uric acid are low molecular weight free radical scavengers acting to reduce lipid peroxidation (Halliwell, 1985). Vitamin E, a series of isomers of a lipid soluble phenol called tocopherol, tends to localize in the center of membranes and is also found in plasma. Vitamin E acts as a strong antioxidant, by reducing reactive species of oxygen and organic radicals, such as lipid peroxy radicals, thus breaking free radical chain reactions. It is present in higher concentrations in membranes that receive the greatest numbers of free radical insults, such as the outer segment membranes of the retinal rods. Vitamin E is found in high concentrations in plasma lipoproteins and is thought to be the only lipid soluble antioxidant present in plasma. Vitamin E acts as a chain terminator because it reacts with lipid peroxy radicals to form a vitamin E free radical. This radical is resonance-stabilized as the unpaired electron is delocalized on the aromatic ring, giving it much greater stability. Vitamin C may subsequently scavenge the vitamin E radical by reducing it back to the original form. Vitamin C probably

acts as an antioxidant for vitamin E at the surface of the membrane due to its hydrophilic nature (Barton et al., 1983).

In addition to its action on vitamin E, vitamin C also scavenges $O_2^{\cdot-}$, $OH^{\cdot}$, and $^1O_2$. In the lung, ascorbate is thought to act as an extracellular antioxidant in contrast to SOD which acts almost exclusively as an intracellular scavenger. Vitamin C is a water soluble vitamin found in aqueous solutions such as the cytosol and aqueous humor. The effects of vitamin C may not all be protective to the cell as it can also reduce Fe^{3+} to Fe^{2+} which may in turn promote the Haber-Weiss reaction and the formation of highly reactive hydroxyl radicals (Bendich et al., 1986).

β-carotene may also inhibit lipid peroxidation as it acts to scavenge singlet oxygen. Other compounds scavenge reactive species of oxygen, such as polyunsaturated fatty acids, sugars, sulfur containing amino acids, but these compounds are not necessarily protective to the cell. They may be mere participants in free radical chain reactions or may actually generate more toxic elements than the parent free radical (Freeman and Crapo, 1982).

Uric acid, a byproduct of purine catabolism, is a potent scavenger of $^1O_2$ and $OH^{\cdot}$. Circulating uric acid is thought to protect hemoglobin and red cell lipids from peroxidation. Like ascorbate, uric acid is present in extracellular fluids in much higher concentrations than SOD (Freeman and Crapo, 1982).

Reduced glutathione (GSH) acts as a low molecular weight antioxidant in the cytoplasm and mitochondrial matrix of most cells.

Glutathione is a tripeptide thiol that reduces lipid peroxides, H_2O_2, disulfides, $OH^{\bullet}$ and $^1O_2$. It reduces these species by undergoing an oxidation reaction to form oxidized glutathione, also called glutathione disulfide (GSSG). Several enzymes are thought to be inactivated by free radicals by the oxidation of essential thiol groups. Glutathione may repair this damage and restore function to these enzymes by this mechanism (Halliwell, 1974).

The removal of H_2O_2 and other peroxides by glutathione is catalyzed by glutathione peroxidase. The potentially harmful H_2O_2 is reduced to form water as GSH is oxidized to form GSSG. Lipid or protein peroxides are reduced by GSH forming a lipid or protein GSH adduct. Glutathione peroxidases are a group of enzymes, most containing selenium, which is essential for proper cell function (Halliwell, 1974).

All of the cytoplasmic GSH would soon be oxidized to GSSG, which is an oxidant for cell proteins itself, if it were not for the enzymatic system (glutathione reductase) keeping GSH in the reduced form. Glutathione reductase reduces GSSG to restore intracellular concentrations of GSH, using NADPH as a cofactor. NADPH is generated via a cytosolic enzyme acting in the early portion of the hexose monophosphate shunt, glucose-6- phosphate dehydrogenase (G6PD). Many humans are genetically deficient in G6PD, a condition which causes no problems in health or at times with low oxidative stress. These individuals are susceptible to hemolytic anemia due to the low concentration of erythrocyte GSH when stressed by a wide variety of

oxidative xenobiotics such as primaquin (antimalarial drug) isoniazid, adriamycin, and fava beans (Clark et al., 1985).

It is important to maintain a high ratio of GSH to GSSG because accumulation of GSSG may actually damage cellular proteins by disulfide interchange.

$$GSSG + 2R\text{-}SH \rightarrow 2GSH + R\text{-}SS\text{-}R$$

Erythrocytes, pulmonary and hepatic cells actively transport GSSG out of the cytoplasm to prevent these deleterious interchanges.

Catalase is another enzymatic scavenger for H_2O_2. This enzyme is found in high concentrations in peroxisomes (microbodies). Catalase is thought to catalyze the metabolism of H_2O_2 only when the substrate (H_2O_2) is present in very high concentrations (Km=1.1M). Glutathione peroxidase has a lower Km (higher affinity) for H_2O_2 and is thought to act at the lower H_2O_2 concentrations found in normal metabolism. The role of catalase may be secondary, used only to prevent the depletion of GSH at overwhelming H_2O_2 concentrations (Fridovich, 1976).

Superoxide dismutases (SOD) are enzymes responsible for the removal of $O_2^{\cdot-}$ radicals. Superoxide dismutase causes the oxidation of one molecule of $O_2^{\cdot-}$ to O_2 and the simultaneous reduction of another $O_2^{\cdot-}$ radical to form H_2O_2.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide dismutases are found ubiquitously inside aerobic and aerotolerant cells. These enzymes increase the rate of superoxide dismutation over 10,000 times the rate of the spontaneous reaction. The superoxide dismutases are a family of metalloenzymes, each

containing a different metal at its active site (Fridovich, 1979; Fridovich, 1978; Hassan, 1980; Fridovich, 1981). All catalyze the same reaction, using the same basic mechanism of reduction and reoxidation of the metal (Cu/Zn, Mn, or Fe) at the active site (Brawn and Fridovich, 1980). The participation of the metal avoids the electrostatic interactions between radicals.

The SOD that contains copper and zinc is the most thoroughly studied enzyme and is generally found in eukaryotic cytosol. Studies of this enzyme suggest a high degree of evolutionary conservatism because Cu/Zn SOD from widely differing eukaryotes show striking amino acids sequence homologies. The Cu/Zn SOD consists of two identical units, constituting a dimeric enzyme. Each unit contains a Cu++ and Zn++ in the active site. Cu++ is believed to be the catalytically active metal while Zn++ has a structural role.

The second type of SOD, which contains manganese at its active site, is found in prokaryotes and in the mitochondria of eukaryotes (Fridovich, 1979; Fridovich, 1978; Fridovich, 1981). The third type of SOD contains iron at its active site and is principally a prokaryotic enzyme (Hassan and Fridovich, 1977a; Gregory et al., 1973). The Fe SOD and Mn SOD have very similar primary amino acid sequences but differ greatly from the Cu/Zn SOD.

SOD is an intracellular scavenger because it is found in low to insignificant quantities in extracellular fluids (Fridovich, 1976).

Ceruloplasmin is thought to scavenge $O_2^{\cdot-}$ that is generated in circulation but is much less active (25-50,000 times) than SOD (Freeman

and Crapo, 1982).

Transferrin, lactoferrin and ferritin are also thought to be important in cellular defense because they sequester iron, making it less available to catalyze the Haber-Weiss reaction.

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THE EFFECTS OF GOSSYPOL ON THE ANTIOXIDANT DEFENSE SYSTEM OF THE LIVER

INTRODUCTION

The toxic effects of feeding large amounts of cottonseed meal as a protein supplement to livestock were first reported in 1859 (Adams et al., 1960). Nearly sixty years later, the toxic agent of cottonseed was identified as the yellow polyphenolic pigment, gossypol (Withers and Carruth, 1916). In 1957, gossypol was found to also have antifertility properties (Liu, 1985). Most of the studies since then concern its effects on the reproductive system of men and male animals (National Coordinating Group on Male Antifertility Agents, 1978).

Gossypol is reported as a potent hepatotoxin in swine. Pigs fed cottonseed rations containing high concentrations of gossypol showed clinical signs of dyspnea, panting, weakness and anorexia for several days prior to death. The livers of these pigs were congested and necrotic on postmortem examination. Microscopic examination of the livers revealed extensive centrilobular necrosis that was so severe in some cases that almost no viable parenchyma remained. The necrotic spaces around central veins were filled with pooled erythrocytes (Smith, 1957).

Adult ruminants are much less sensitive to the toxic effects of gossypol than monogastrics. Some investigators propose that gossypol binds to proteins in ruminal contents, rendering it relatively nontoxic. However, young calves are very susceptible to gossypol poisoning (Rogers and Henaghan, 1975; Orgad-Klopfer and Adler, 1986).

The differences in sensitivity to gossypol poisoning between age groups in cattle may be explained by differences in the gastrointestinal tract. The digestive system of young calves behaves much like that of a monogastric until the rumen developes. Gossypol associated lesions described in young calves are similar to the toxic hepatitis described in pigs.

The mechanism by which gossypol causes hepatic necrosis is unknown. A recent in vitro study examining human sperm and rat liver microsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals. The authors suggest that the common mechanism that underlies all of the toxic effects of gossypol may be related to free radical injury (DePeyster et al., 1984).

Reactive species of oxygen are implicated as a cause of a multitude of pathological changes. Free radical reactions with proteins and lipids often change the molecular configuration and may lead to cell death (Freeman and Crapo, 1982). The interaction of free radicals with DNA may cause damage to the molecular structure and induce strand breakage. Cells often die after this form of damage or those that survive may undergo mutations (Halliwell and Gutteridge, 1985). Cell death due to reactive species of oxygen is likely to be some combination of an overwhelming production of free radicals and a compromised antioxidant defense system.

The present study examines the effects of oral and injected gossypol acetic acid on the antioxidant defense mechanism of the rat liver.

MATERIALS AND METHODS

Animals - Gossypol was administered by two different routes in separate experiments. Thirty-one adult male Sprague-Dawley rats in a weight range of 431-477 g were dosed orally and 26 males in a range of 370-410 g were dosed by subcutaneous injection. The rats were obtained from Charles River Breeding Laboratory and housed according to NIH guidelines [DHEW Publication No. (NIH) 80-23] in a temperature (19-22°C) and humidity (50-60%) controlled vivarium. Each rat was assigned to a treatment group according to a random number table and grouped two to a cage. The animals had free access to water and commercial rat chow and were exposed to a photoperiod of 12 hours of light and 12 hours of darkness each day.

Gossypol Treatment, Oral - Gossypol acetic acid (Polyscience Inc., Warrington, PA) was homogenized each morning in a steroid suspending media (Saksena et al., 1981). Each rat was weighed daily and dosed according to body weight. The low dose rats received 40 mg/kg, the high dose groups received 60 mg/kg and control rats received the steroid suspending media. The gossypol stock solutions were prepared at different concentrations so that each animal received an equivalent volume of fluid per kg body weight. Control and low dose rats were dosed daily for thirty days; high dose rats were dosed daily for 10

days. The drug and/or vehicle was administered by gavage through a soft rubber feeding tube between 8:00 - 11:00 each morning.

Gossypol Treatment, Parenteral - Gossypol acetic acid was homogenized each morning in corn oil. The low dose group received 15 mg/kg, the high dose group received 30 mg/kg and control rats received an equivalent volume of corn oil per kg body weight. The treatments were administered by subcutaneous injection each day for seven days between 8:00-11:00 a.m. The rats were weighed daily and dosage was adjusted to individual body weight.

Tissue Collection - All rats were anesthetized with ether and a thoracotomy was performed to perfuse the entire body with cold 0.15 M phosphate buffered saline through the right ventricle on the day following the last day of treatment. The livers were removed, weighed and homogenized individually using a proportion of 1 g tissue to 4 ml of 0.15 M potassium phosphate buffer (pH 7.8) with a Polytron Homogenizer (Brinkman Instruments, Westbury NY). A 2 ml aliquot of homogenate was removed for the vitamin E assay and the remainder was sonicated for 15 seconds with a Sonicator Cell Disrupter (Heat Systems Ultrasonics, Inc., Plainview, NY) and centrifuged at 10,000xg for 30 minutes. The supernatant was used for all assays except for vitamin E.

Enzyme Assays -

Superoxide dismutase (SOD) - was assayed using the epinephrine autoxidation method of Misra (Misra, 1985). The total SOD activity (cytosolic plus mitochondrial) was determined spectrophotometrically at 480 nm. A unit of SOD is the amount of protein required to produce 50%

inhibition of the rate of accumulation of autoxidation products of epinephrine under specified conditions. The activity of Mn SOD was determined in a similar manner except that the reaction mixture contained 2mM CN^-. Thus, in the presence of 2mM CN^-, the decrease in total activity represents the CuZn SOD activity and the remaining activity in the presence of 2mM CN^- represents Mn SOD.

Catalase - was assayed according to a method adapted from Beers and Sizer (The Worthington Manual, 1972). The rate of disappearence of peroxide, followed spectrophotometrically at 240 nm, is proportional to the amount of enzyme present.

Glucose-6-Phosphate Dehydrogenase - was assayed by the procedure of Kornberg and Horecker (Kornberg and Horecker, 1955) as modified by Lohr and Waller (Lohr and Waller, 1974). The amount of enzyme determined spectrophotometrically at 340 nm, is proportional to the rate of reduction of $NADP^+$ in the presence of excess glucose-6-phosphate.

α-tocopheral - was extracted by the general SDS method of Burton et al (Burton et al. 1985). Vitamin E was extracted from the tissue homogenate using sodium dodecyl sulfate (SDS), ethanol and heptane. The organic phase was analyzed by high performance liquid chromatography. The method was adapted from the fluorometric procedure of Storer (Storer, 1974) using a reverse phase C18 column with 2% water in methanol.

Ascorbic Acid - was assayed by the method described by Carr et al. (Carr et al, 1983). The samples were deproteinized in trichloroacetic acid and perchloric acid. This colormetric assay uses 2,6

dichlorophenol-indophenol to oxidize ascorbic acid to dehydroascorbic acid and dinitrophenyl hydrazine to form a hydrazone derivative that is monitored spectrophotometrically at 524 nm.

Glutathione Peroxidase - was measured according to Tappel (Tappel, 1978). This assay uses cumene hydroperoxide and reduced glutathione (GSH) as substrates for glutathione peroxidase. The resulting oxidized glutathione (GSSG) is reduced in a coupled reaction with NADPH and glutathione reductase. Glutathione peroxidase is quantified by following the rate of NADPH oxidation spectrophotometrically at 340 nm.

Glutathione Reductase - was quantitated by the method of Racker (Racker, 1955). This procedure quantitates glutathione reductase by using oxidized glutathione and NADPH as substrates and measuring the rate of NADPH oxidation using a spectrophotometer at 340 nm.

Total Glutathione - was measured by the kinetic assay of Teitze (Tietze, 1969) as modified by Sies and Ackerboom (Sies and Ackerboom, 1984). This assay quantitates GSH plus GSSG by following the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) by NADPH and glutathione reductase. The reaction velocity is dependent on the concentration of glutathione and is monitored spectrophotometrically at 412 nm.

Protein - concentrations were determined according to the dye binding method of Bradford (Bradford, 1976) with recrystallized bovine serum albumen as the standard. Coomassie Brilliant Blue G250 binds to proteins and causes a shift in the absorption maximum of the dye from 465 to 595 nm. The protein is quantitated by monitoring the increase in absorption at 595 nm spectrophotometrically. All antioxidants were

expressed per mg protein except for ascorbic acid and α-tocopherol, which were expressed per g tissue.

Statistics - values were compared by analysis of variance and the Duncan's Multiple Range test (Kramer, 1956). P values $<$ .01 were considered significant. Data from gavaged and injected rats were analyzed separately.

RESULTS

Clinical Signs, Gavaged Rats - One control rat, 2 low dose (LD) rats and 2 high dose (HD) rats showed respiratory distress and died immediately following oral intubation on different days of the experiment. No gross lesions were found in any of these rats at necropsy. Most of the HD rats lost large amounts of body weight over the first 10 days; four of these rats had diarrhea and became extremely dehydrated. The HD rats were killed on day 11 of the experiment due to their extreme loss of physical condition. Five rats in the LD group exhibited diarrhea and became dehydrated during the first 15 days of dosing, but this resolved and all LD rats appeared clinically normal during the latter half of the experiment. None of the control rats exhibited diarrhea or dehydration.

Clinical Signs, Injected Rats - All of the gossypol treated rats developed subcutaneous swellings at the injection sites. None of the control rats had any evidence of a subcutaneous reaction to the corn oil. Five of the HD rats exhibited lethargy, weakness and diarrhea. The diarrhea was characterized by dark, pastey stools. Three of these

animals died and necropsies were performed. Histopathologic examination revealed subcutaneous necrosis with severe fibrin deposition and a heavy infiltrate of neutrophils that extended deeply among muscle bundles. Necrosis of muscle fibers was also noted.

Body and Organ Weights - No significant differences in body weights were found between control, low dose or high dose rats at the beginning of the experiment in either injected or gavaged rats. All control rats in both injected and gavaged groups gained weight during the course of the experiment. Seven of the 8 LD gavaged rats that survived the entire dosing interval and one of the nine LD injected rats gained weight by the end of the experiment. All high dose gavaged and injected rats lost weight during the experiment.

Body weights were statistically compared at day 11 and day 31 (Fig. 4) in the gavaged rats because the HD rats were killed on day 11 whereas LD and control rats were killed on day 31. On day 11, body weights of LD rats were significantly decreased compared to controls. Weights of HD rats were significantly decreased compared to both controls and LD rats. Body weights of LD rats were also significantly decreased compared to controls on day 31. Body weights of injected rats were all compared on day 8. The body weights of both injected LD and HD rats were decreased compared to injected controls (Fig. 4).

Adrenal weights were compared to assess stress in the injected and gavaged rats (Fig. 5). No statistically significant differences were found in adrenal weights between any groups. Adrenal weights were also compared in a ratio with body weights to account for differences in

body weights. Here, statistically significant increases in adrenal weight/body weight ratios were found in gavaged HD rats compared to gavaged controls and LD rats (Fig. 6). Adrenal weight/body weight ratios of injected LD and HD rats were increased compared to injected controls.

The effect of gossypol treatment on liver weights was also assessed between the treatment groups (Fig. 7). Liver weights were significantly decreased in HD gavaged rats compared to gavaged controls. The liver weights of injected HD and LD rats were also decreased compared to injected controls, but the differences were not statistically significant in all cases when liver weights were ratioed with body weights (Fig. 8).

The Hepatic Antioxidant Defense System

Catalase - Gavaged HD rats had significant decreases in liver catalase when compared to gavaged controls. Liver catalase was significantly decreased in both injected LD and HD rats compared to injected controls (Fig. 9).

Vitamin C - Gavaged LD and HD rats had decreased liver ascorbic acid when compared to gavaged control rats, but only differences in the HD rats reached statistical significance. Similar decreases in vitamin C were found in the injected rats, but both injected LD and HD rat liver vitamin C concentrations were significantly decreased (Fig. 10).

Vitamin E - The effect of gossypol administration on concentrations of hepatic α-tocopherol differed between the gavaged and

injected rats (Fig 11). As shown in this figure, the gavaged LD and HD rats demonstrated significant decreases in hepatic vitamin E compared to gavaged controls, but differences in injected LD and HD values were not significant compared to injected controls.

Glutathione Peroxidase - Hepatic glutathione peroxidase concentrations of gavaged HD and injected LD and HD rats decreased significantly compared to their respective controls (Fig.12).

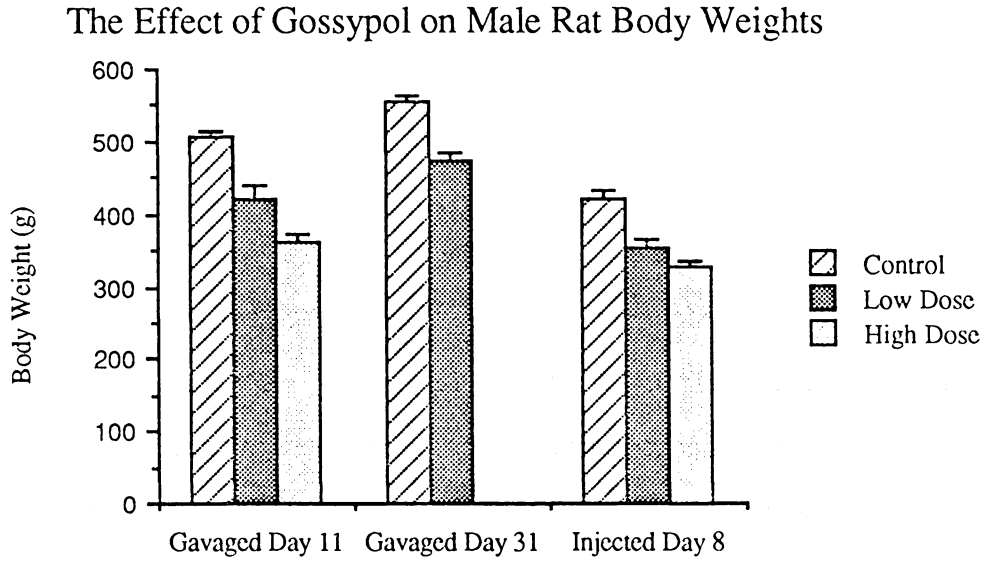
Total Glutathione - No significant differences in hepatic glutathione concentrations were found between groups (Fig. 13).

Glutathione Reductase - Hepatic glutathione reductase concentrations of gavaged LD rats were significantly increased compared to controls, but no other values reached statistical significance (Fig. 14) .

Copper-zinc Superoxide Dismutase - Enzyme concentrations of gavaged and injected LD rats decreased when compared to the respective controls. Enzyme concentrations of gavaged and injected HD rats also decreased compared to the respective LD and control rats (Fig. 15) .

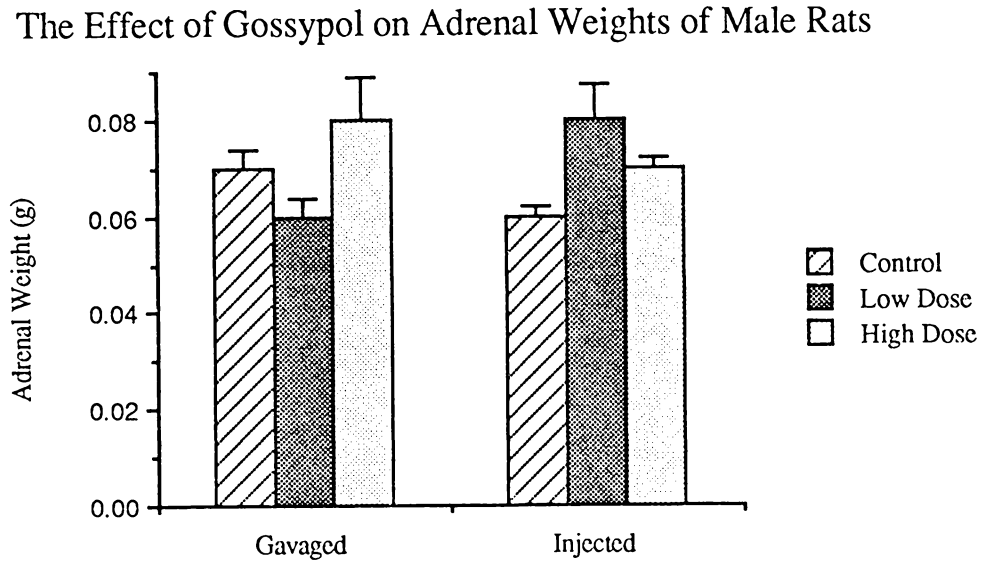
Manganese Superoxide Dismutase - The enzyme concentrations of injected LD rats were significantly decreased (Fig. 16)

Glucose-6-Phosphate Dehydrogenase - No significant differences were found in hepatic glucose-6-phosphate dehydrogenase values (Fig. 17).



DOSE ROUTE	DAY	CONTROL	LOW DOSE	HIGH DOSE
Gavage	11	508.4 ± 5.0 (9)a	422.5 ± 16.0 (8)b	360.4 ± 14.1 (9)c
Gavage	31	554.9 ± 8.8 (9)a	471.9 ± 11.8 (8)b	
Injection	8	421.3 ± 10.8 (8)a	355.1 ± 11.6 (9)b	329.2 ± 7.7 (6)b

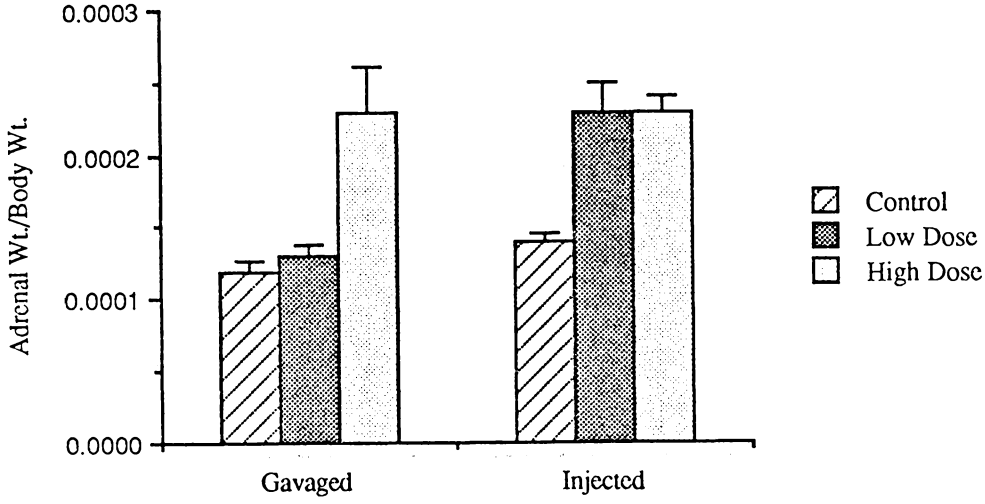
Figure 4. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript c are significantly different from both a and b.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	.07 ± .004 (9)a	.06 ± .004 (8)a	.08 ± .009 (9)a
Injection	.06 ± .002 (8)a	.08 ± .007 (9)a	.07 ± .002 (6)a

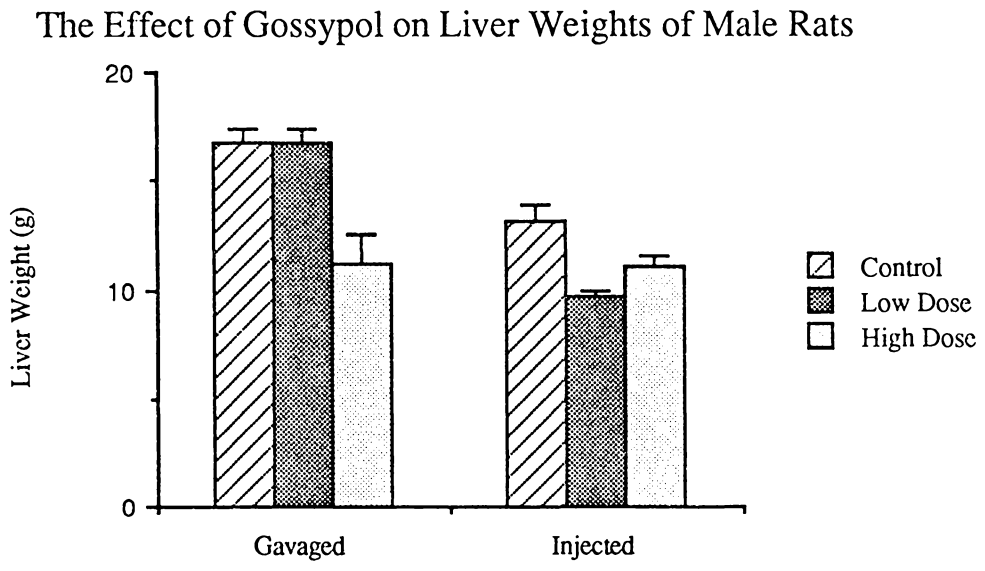
Figure 5. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

The Effect of Gossypol on Male Rat Adrenal/Body Weight Ratios



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	.00012 ± .000006(9)a	.00013 ± .000008(8)a	.00023 ± .00003(9)b
Injection	.00014 ± .000005(8)a	.00023 ± .00002(9)b	.00023 ± .00001(6)b

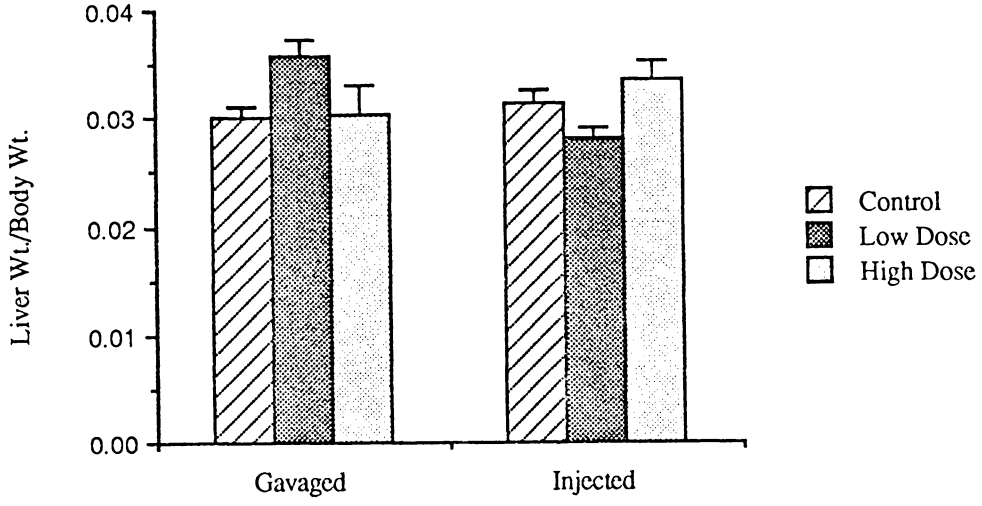
Figure 6. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data was analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	16.75 ± .65 (9)a	16.78 ± .65 (8)a	11.16 ± 1.36 (9)b
Injection	13.21 ± .70 (8)a	9.66 ± .33 (9)b	11.03 ± .53 (6)ab

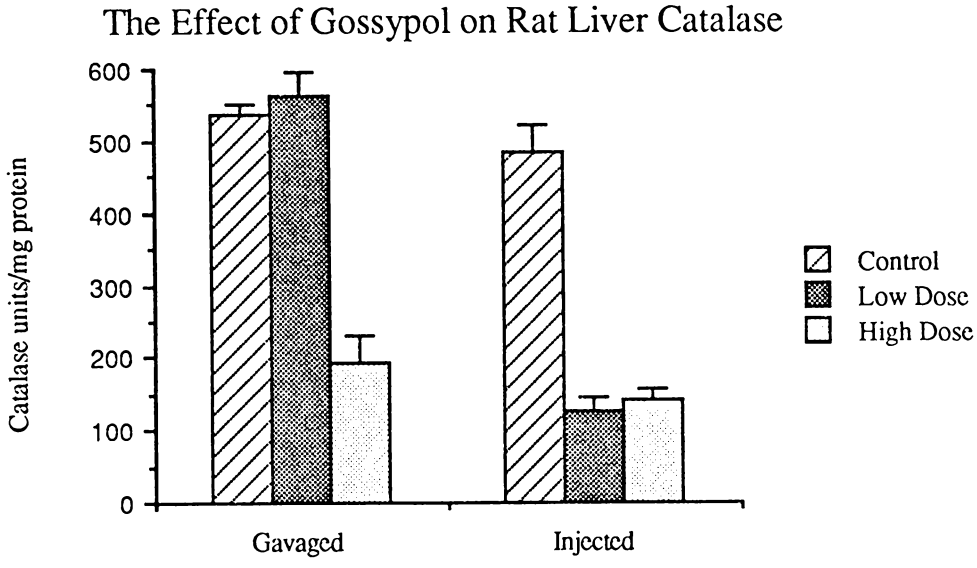
Figure 7. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.

The Effect of Gossypol on Male Rat Liver/Body Weight Ratios



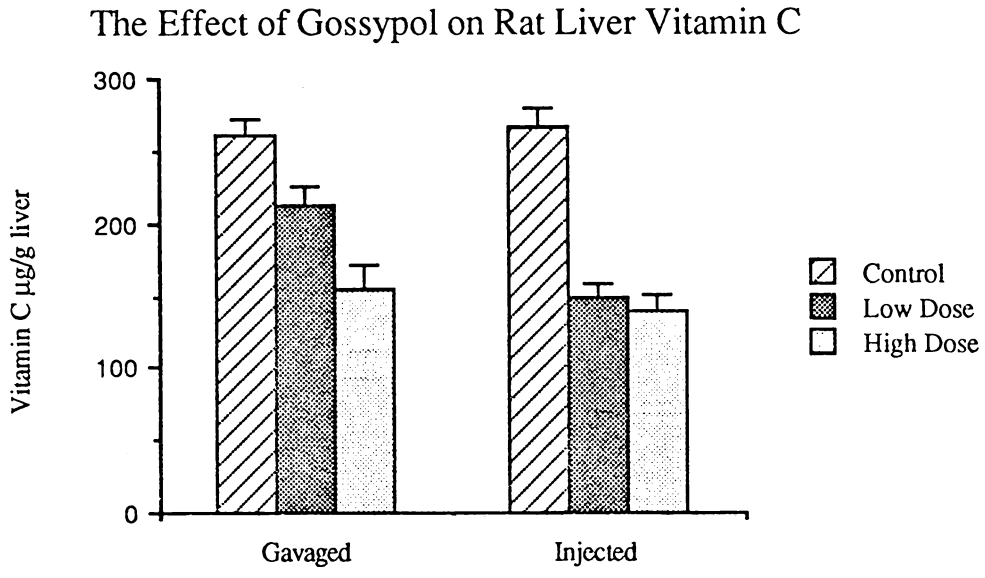
DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	.0301 ± .0010 (9)a	.0357 ± .0016 (8)a	.0303 ± .0027 (9)a
Injection	.0313 ± .0012 (8)a	.0280 ± .0011 (9)a	.0336 ± .0018 (6)a

Figure 8. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data was analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.



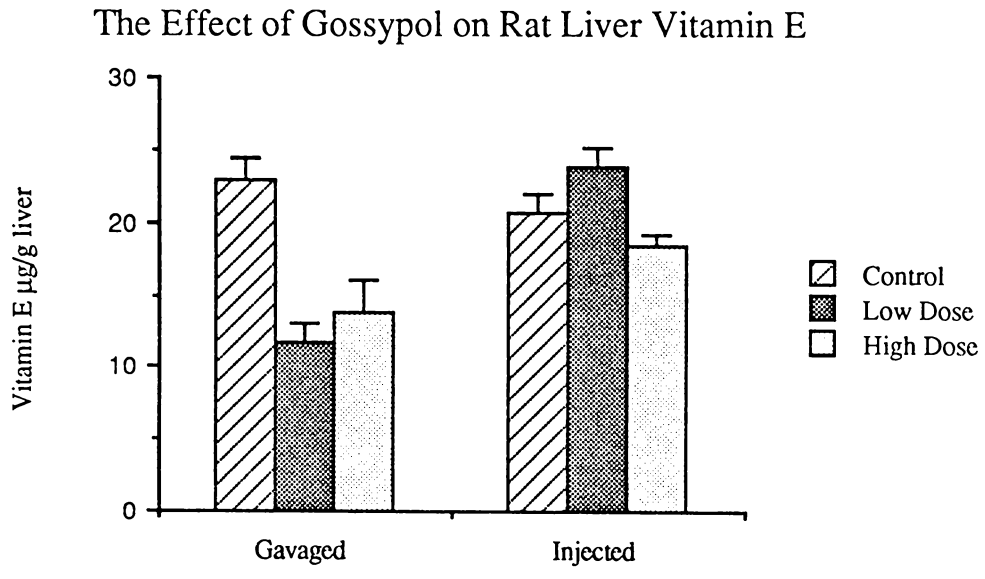
DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	534.88 ± 18.28 (9)a	564.31 ± 30.66 (8)a	193.97 ± 35.60 (9)b
Injection	486.10 ± 34.77 (8)a	127.74 ± 19.29 (9)b	143.31 ± 12.29 (6)b

Figure 9. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	260.37 ± 12.51 (9)a	213.01 ± 12.41 (8)ab	154.59 ± 17.15 (9)b
Injection	266.29 ± 12.76 (8)a	149.04 ± 10.14 (9)b	140.11 ± 10.69 (6)b

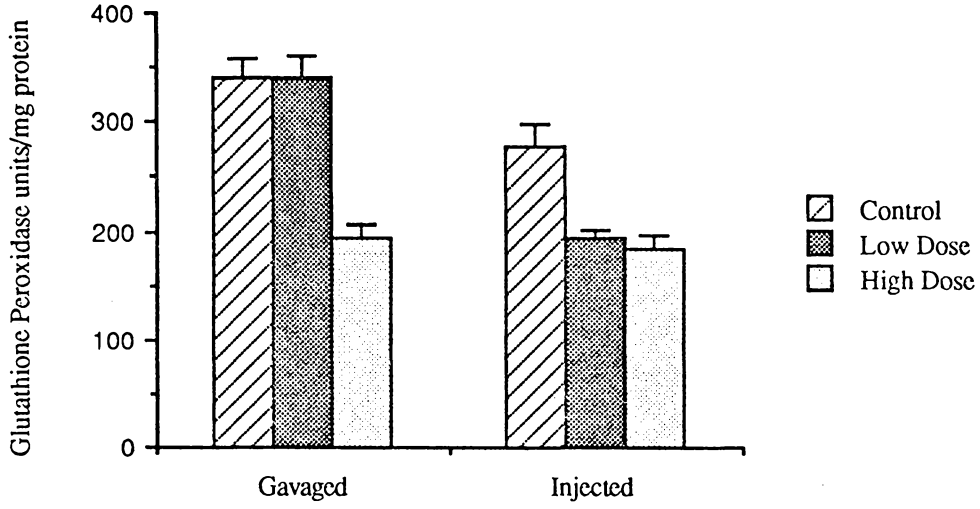
Figure 10. Values are means ± standard errors as determined in µg/g tissue. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	23.0 ± 1.5 (9)a	11.8 ± 1.2 (8)b	13.7 ± 2.3 (8)b
Injection	20.7 ± 1.3 (8)ab	23.9 ± 1.2 (9)a	18.5 ± 0.7 (6)b

Figure 11. Values are means ± standard errors as determined in µg/g tissue. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.

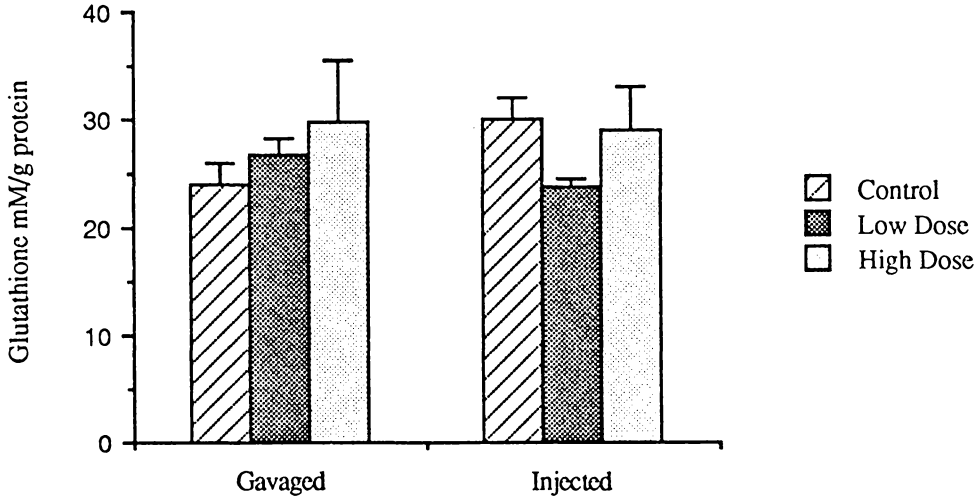
The Effect of Gossypol on Rat Liver Glutathione Peroxidase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	339.77 ± 16.94 (9)a	339.87 ± 19.16 (8)a	194.00 ± 11.17 (9)b
Injection	279.09 ± 18.91 (8)a	193.37 ± 8.12 (9)b	184.66 ± 11.86 (6)b

Figure 12. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

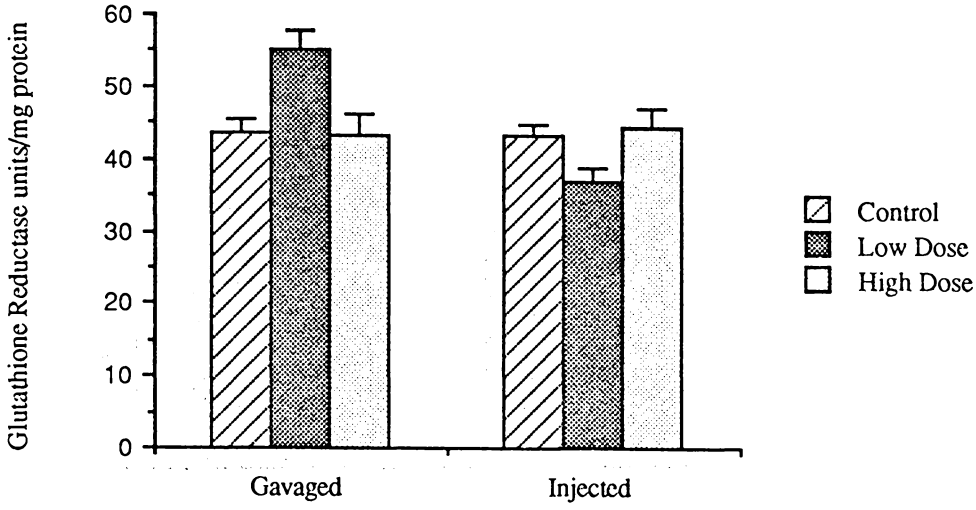
The Effect of Gossypol on Rat Liver Total Glutathione



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	24.01 ± 2.09 (9)a	26.73 ± 1.54 (8)a	29.64 ± 5.81 (9)a
Injection	29.98 ± 2.06 (8)a	23.63 ± 0.88 (9)a	29.02 ± 4.03 (6)a

Figure 13. Values are means ± standard errors as determined in mM/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

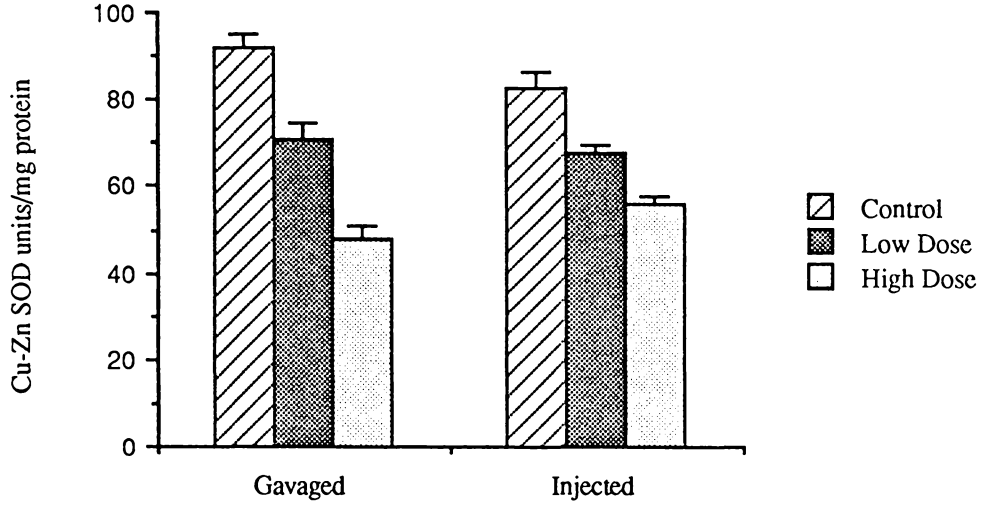
The Effect of Gossypol on Rat Liver Glutathione Reductase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	43.44 ± 2.16 (9)a	55.11 ± 2.68 (8) b	43.24 ± 2.80 (9)a
Injection	43.18 ± 1.47 (8)a	36.79 ± 1.91 (9)a	44.51 ± 2.62 (6)a

Figure 14. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

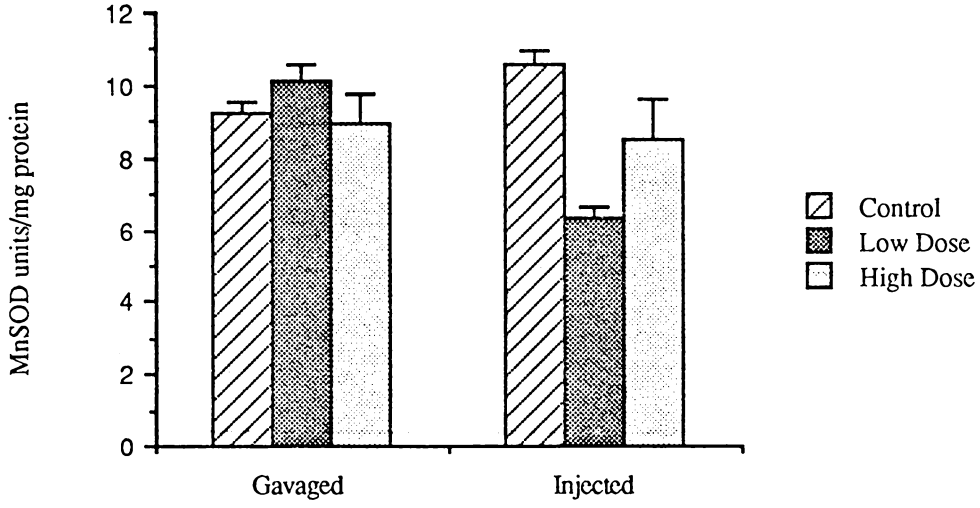
Effect of Gossypol on Rat Liver Copper-Zinc Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	91.76 ± 3.10 (9)a	70.68 ± 3.99 (8)b	48.01 ± 2.64 (9)c
Injection	82.70 ± 3.48 (8)a	67.49 ± 1.97 (9)b	55.81 ± 1.77 (6)c

Figure 15. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript c are significantly different from both a and b.

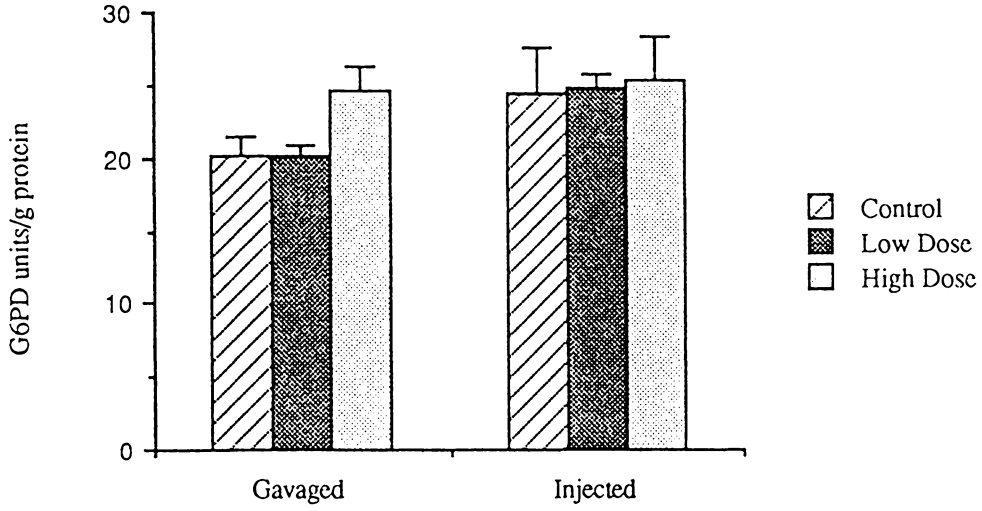
Effect of Gossypol on Rat Liver Manganese Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	9.21 ± .32 (9)a	10.14 ± .43 (8)a	8.91 ± .84 (9)a
Injection	10.60 ± .36 (8)a	6.35 ± .26 (9)b	8.50 ± 1.15 (6)ab

Figure 16. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.

The Effect of Gossypol on Rat Liver Glucose-6-Phosphate Dehydrogenase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	20.09 ± 1.33 (9)a	20.20 ± .59 (8)a	24.58 ± 1.67 (9)a
Injection	24.33 ± 3.30 (8)a	24.86 ± .88 (9)a	25.27 ± 2.96 (6)a

Figure 17. Values are means ± standard errors as determined in units/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

DISCUSSION

Body weight reductions, observed in both HD and LD rats, are a common consequence of gossypol treatment in the rat and are consistent with the findings of Zatuchni and Osborn, 1981; Heywood et al., 1986; Lagerlof and Tone, 1985. Two previous studies found that the growth rates of gossypol treated rats increased to control values after treatment was discontinued (Beaudin, 1985; Hadley et al., 1981). Some investigators attribute the reductions in weight to decreased food intake following anorexia (Zatuchni and Osborn, 1981; Beaudin, 1985). The diarrhea and dehydration observed in the present study were additional reasons for weight loss in the gossypol treated rats. Diarrhea associated with gossypol treatment was previously reported in rats (Zatuchni and Osborn, 1981; Lagerlof and Tone, 1985; Gafvels et al., 1984), monkeys (Shandilya et al., 1982) and calves (Rogers et al., 1975).

The statistical trends in organ weights between the various treatment groups changed when each organ weight was compared in a ratio with individual body weight. Liver weights generally decreased in gossypol treated rats. The liver to body weight ratios demonstrated that liver weight reductions were proportional to reductions in body weights in gossypol treated rats. In contrast, adrenal weights, which did not significantly differ between groups, were significantly increased in gavaged HD and injected LD and HD rats when ratioed with body weights. It is probable that the adrenal gland weight was not as affected as the liver and total body weight by mobilization of

glycogen, fat and protein reserves when the animals were experiencing anorexia and weight loss. Alternately, the stress of the gossypol treatment may have caused adrenocortical hyperplasia in the rats with increased adrenal to body weight ratios. The clinical impression of stress in the various treatment groups compared favorably with the statistically significant increases in adrenal to body weight ratios.

The subcutaneous lesions of multifocal necrotizing cellulitis and myositis were consistent with foreign body reactions. Other investigators reported similar inflammatory reactions at injection sites (Gafvels et al., 1984). It is not known whether this reaction was due to the gossypol itself or impurities in the gossypol preparation. The author prefers oral administration of gossypol to rats as it mimics the usual route of consumption of gossypol by livestock and people. Oral administration also avoids the additional stress of the inflammatory response associated with subcutaneous injection of gossypol.

Gossypol treated animals showed consistent and significant reductions in four of the nine quantitated components of the hepatic antioxidant defense system. Catalase, glutatione peroxidase, copper-zinc superoxide dismutase and vitamin C concentrations were significantly decreased in gavaged and injected rats that were treated with gossypol. Catalase and glutathione peroxidase are the two enzymatic scavengers of hydrogen peroxide (H_2O_2). The majority of hepatic catalase is located within peroxisomes with smaller amounts found in the cytosol (Masters et al., 1986). Glutathione peroxidase

is found in the cytosol and mitochondrial matrix of hepatocytes (Tappel et al., 1978). Catalase is thought to catalyze the conversion of H_2O_2 to water and oxygen, only when H_2O_2 is present in very high quantities (Km=1.1M). Glutathione peroxidase has a lower Km (higher affinity) for H_2O_2 than catalase, and is thought to scavenge H_2O_2 produced during normal metabolism. The role of catalase may be secondary, acting to protect cellular constituents during pathological conditions that generate H_2O_2 concentrations overwhelming to the glutathione peroxidase system (Fridovich, 1976).

Hydrogen peroxide is an oxidizing agent produced by the dismutation of two molecules of superoxide ($O_2^{\cdot-}$) or by reactions catalyzed by oxidases including D-amino acid oxidase and glucose oxidase. Hydrogen peroxide inactivates enzymes by oxidizing essential thiol groups. It readily crosses cell membranes and may react with $O_2^{\cdot-}$ in a reaction catalyzed by metal ions to form one of the most reactive species of oxygen, the hydroxyl radical (Halliwell and Gutteridge, 1985). Hydroxyl radical ($OH^{\cdot}$) reacts quickly and indiscriminately with all classes of molecules found in biological systems including sugars, amino acids, phospholipids, nucleotides and organic acids. Hydroxyl radical may inactivate enzymes, damage DNA, and cause lipid peroxidation, which may ultimately lead to cell death (Brawn and Fridovich, 1980).

Hepatic copper-zinc superoxide dismutase (Cu-Zn SOD) concentrations were also significantly decreased in gossypol treated animals in this experiment. Manganese superoxide dismutase (Mn SOD)

showed inconsistent reductions in the gossypol treated groups. Mammalian cells contain two different superoxide dismutases, each containing a different metal at the active site. The SOD that contains copper and zinc at its active site, Cu-Zn SOD, is found in the hepatic cytosol, nuclear matrix and lysosomes. The second type of SOD, which contains manganese at its active site, MnSOD, is found primarily in the mitochondrial matrix of hepatocytes, but also in small concentrations in the cytoplasmic matrix (Slot et al., 1986). Superoxide dismutases are enzymes responsible for the removal of $O_2^{\cdot-}$ radicals. These enzymes cause a dismutation reaction whereby one molecule of $O_2^{\cdot-}$ is oxidized to form molecular oxygen (O_2) and another $O_2^{\cdot-}$ is simutaneously reduced to form H_2O_2. Superoxide dismutases increase the rate of $O_2^{\cdot-}$ dismutation over 10,000 times the rate of spontaneous reaction (Fridovich, 1979).

Superoxide radicals are formed by the univalent reduction of molecular oxygen. Superoxide radical has been implicated in the inactivation of enzymes (Salin and McCord, 1977), peroxidation of lipids (Halliwell, 1978) and damage to DNA (Brown and Fridovich, 1980). Superoxide may not be the precise culprit causing all of this damage. Superoxide formed in physiological processes may dismute spontaneously or via a reaction catalyzed by SOD to form H_2O_2 (Fridovich, 1979). The H_2O_2 produced may subsequently react with an additional $O_2^{\cdot-}$ radical, as previously discussed, to form the highly destructive $OH^{\cdot}$ radical.

Hepatic vitamin C concentrations were significantly decreased in gossypol treated animals in this experiment. Vitamin C is a low molecular weight free radical scavenger and powerful reducing agent

found in the cytoplasm of hepatocytes and the surrounding plasma. Ascorbate is thought to protect cells from aqueous phase oxidative injury by scavenging reactive species of oxygen including $O_2^{\bullet}$ and $OH^{\bullet}$. Recent evidence suggests that the antioxidant properties of vitamin E are restored by interactions with vitamin C (Bendich et al., 1986; Halliwell, 1985). Concentrations of vitamin E, a lipid phase antioxidant were inconsistently decreased in gossypol treated rats in the present study. It is interesting that hepatic lesions associated with gossypol toxicity in the pig in a previous study were thought to resemble those of vitamin E deficiency (Smith, 1956).

The in vivo reductions in catalase, glutathione peroxidase, Cu-Zn SOD and vitamin C concentrations in the present experiment are supported by a recent study using gossypol treated rat liver microsomes. This study demonstrated that gossypol promoted the formation of H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet}$ in vitro (DePeyster et al., 1984). Without the protective effects of catalase, glutathione peroxidase, Cu-Zn SOD and vitamin C, hepatocyes would be vulnerable to oxidative injury by these reactive species of oxygen. Oxidative injury may be central to the pathogenesis of gossypol induced hepatic necrosis reported in rats (Qian and Wang, 1984) pigs (Smith, 1957) and calves (Rogers and Henaghan, 1975).

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THE EFFECTS OF GOSSYPOL ON THE ANTIOXIDANT DEFENSE SYSTEM OF THE TESTIS

INTRODUCTION

Gossypol is a yellow, polyphenolic pigment (MW518.54) found in cotton plants of the genus Gossypium. The cottonseed contains the highest concentrations of gossypol, but it is also found in the stem and roots (Adams et al., 1960; Merck Index, 1976).

The use of gossypol as a contraceptive was first suggested in the 1950's when people in several regions of China showed high rates of infertility. Epidemiologic studies linked the infertility to the use of cooking oil extracted from cottonseeds by a new method. Oil was traditionally pressed from cottonseeds after heating, but newer technology allowed extraction without heat. This new crude cottonseed oil caused infertility in men and interrupted menstrual cycles in women. On withdrawal of the oil, many of the women cycled normally and men gradually regained fertility (Zatuchni and Osborn, 1981). Recovery generally depended on the dose and length of time that the individual consumed gossypol (Liu, 1985). Animal experiments of the 1970's implicated gossypol as the antifertility agent in cottonseeds. Heat used in the original oil processing technique destroyed much of the gossypol, but the new method allowed high concentrations of gossypol to remain in the extracted oil (National Coordinating Group on Male Antifertility Agents, 1978). Extensive clinical trials involving 8806 Chinese men from 1972 through 1980, examined the use of purified

gossypol and its derivatives gossypol acetic acid and gossypol formic acid, as reversible male contraceptive agents. These trials established optimal dosage rates to achieve over 99% antifertility efficacy, while producing minimal side effects (Liu et al., 1981).

Administration of low oral doses of gossypol (20 mg/day) to men for several weeks causes decreased motility and numbers of spermatozoa. These effects are generally reversible within two to three months after withdrawal of gossypol, especially if treatment is confined to two years (Liu et al., 1981). Similar effects are found in rats (Hadley et al., 1981; Zhou and Lei, 1981), monkeys (Shandilya et al., 1982), hamsters (Waller et al., 1981; Chang et al, 1980), and mice (Coulson et al., 1980).

Studies in rats demonstrated damage to testis germinal epithelium that increased in severity with the length of time on daily gossypol treatment (Xue, 1981). Ultrastructural examination of epididymal spermatozoa and testicular spermatids in gossypol treated rats, demonstrated marked damage to the tail region, especially pronounced in the mitochondrial sheath of the midpiece. A high incidence of segmental aplasia of the mitochondrial sheath was found in both epididymal and testicular sperm (Oko and Hrundka, 1982). Decreased epididymal sperm counts were also found (Nadakavukaren et al., 1979). Sperm were immotile and sperm counts were decreased in the ejaculate of treated rats, but libido was not affected (Hadley et al., 1981). The rate of testicular fluid secretion was not affected by treatment with gossypol (Wong, et al., 1984).

Gossypol is also effective in decreasing sperm motility in vitro (Chongthammakun et al., 1986) and was investigated as a spermicide for use as a vaginal contraceptive in women (Ratsula, et al., 1983). These spermicidal actions were enhanced when gossypol was co-precipitated with polyvinyl pyrrolidine (Waller et al., 1980).

Some studies suggest that gossypol disturbs the normal reproductive endocrine balance in male rats. Gossypol treatment decreased serum LH and testosterone in male rats but serum FSH remained constant. Leydig cells from gossypol treated rats produced less testosterone than control cells when incubated in vitro with LH. LH stimulated control Leydig cells demonstrated decreased testosterone production when incubated with increasing amounts of gossypol (Hadley et al, 1981). These findings may indicate that gossypol causes a disruption of the hypothalamic pituitary axis, as well as a direct effect on Leydig cell testosterone production.

The antifertility actions of gossypol are attributed by some investigators to the inhibition of testicular enzymes. Experiments using ejaculated spermatozoa from monkeys demonstrated that glycolysis was inhibited by gossypol and that the site of inhibition was lactate dehydrogenase-X (Stephens et al., 1983). LDH-X is an isoenzyme with a unique electrophoretic mobility found only in mature testicular epithelium and spermatozoa. The role of this enzyme is unclear, but decreased LDH-X activity is found in some cases of infertility (Blanco, 1980; Maugh, 1981).

The mechanism by which gossypol causes infertility is unknown. A recent in vitro study examining human sperm and rat liver microsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals. The authors suggest that the common mechanism that underlies all of the toxic effects of gossypol may be related to free radical injury (DePeyster et al., 1984).

Reactive species of oxygen are implicated as a cause of a multitude of pathological changes. Free radical reactions with proteins and lipids often change the molecular configuration and may lead to the death of a cell (Freeman and Crapo, 1982). The interaction of free radicals with DNA may cause damage to the molecular structure and induce strand breakage. Cells often die after this form of damage or those that survive may undergo mutations (Halliwell and Gutteridge, 1985). Cell death due to reactive species of oxygen is likely to be some combination of an overwhelming production of free radicals and a compromised defense system.

The present study examines the effects of oral and injected gossypol acetic acid on the antioxidant defense mechanism of the rat testis.

MATERIALS AND METHODS

Animals, gossypol treatments, enzyme assays, statistics and tissue collection techniques are described in section 2, The Effects of Gossypol on the Antioxidant Defense System of the Liver, with the

exception that the testes were removed, weighed and homogenized for each individual animal.

RESULTS

Clinical signs, body weight and adrenal weight comparisons are as found in section 2. The effect of gossypol treatment on testis weights was assessed (Fig. 18) in both gavaged and injected rats. Testis weights of gavaged HD rats were significantly decreased compared to gavaged controls, but no other significant differences were found. When testis weights were compared in a ratio with body weights to account for differences in body weights between the groups, a similar pattern was found in both injected and gavaged rats. Thus, the testis weight/body weight ratios (Fig. 19) of both gavaged and injected LD rats were increased compared to controls, but these values did not reach statistical significance. Both HD groups, however, demonstrated significant increases in testis weight/body weight ratios compared to the respective controls.

The Antioxidant Defense System of the Testes -

Catalase - Significant decreases in catalase concentrations were found in injected LD and HD testes compared to injected controls. Decreases found in enzyme concentrations of gavaged LD and HD rats did not reach statistical significance (Fig. 20) .

Vitamin C - Ascorbic acid concentrations of gavaged HD rats decreased in significant quantities compared to gavaged controls. Both LD and HD injected groups had decreased ascorbic acid concentrations, but these values were not statistically significant (Fig. 21) .

Vitamin E - Injected LD and HD rats had significantly lower levels of testis α-tocopherol compared to controls. No significant differences were found in gavaged rats, although the LD rats had lower values than controls (Fig. 22) .

Glutathione peroxidase - Significant decreases were found in all groups compared to controls. The HD gavaged rats had significantly lower concentrations of glutathione peroxidase than either controls or LD rats (Fig. 23) .

Total glutathione - No significant differences were found in any group (Fig. 24) .

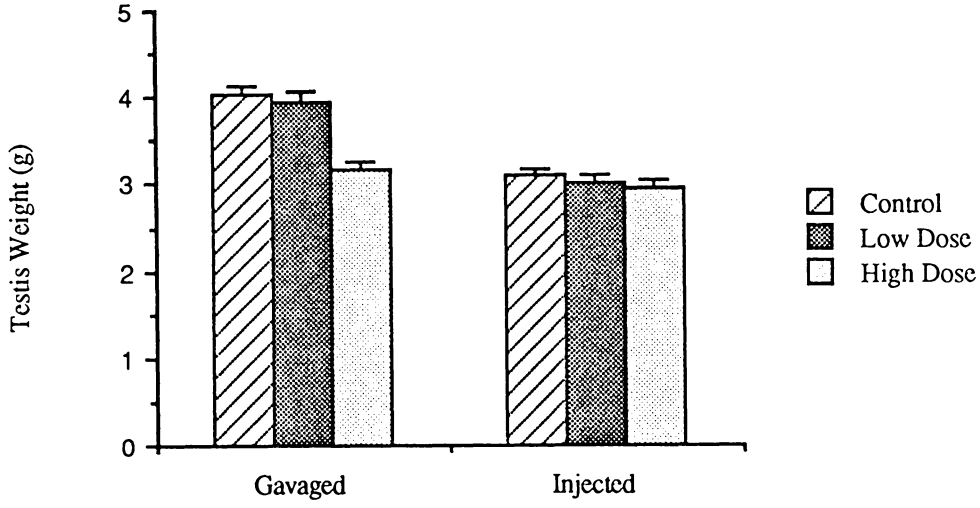
Glutathione Reductase - Glutathione reductase concentrations were significantly decreased in gavaged LD and HD rats, but no significant changes were found in the injected groups (Fig. 25) .

Copper-zinc superoxide dismutase - The only significant decrease in enzyme concentrations was found in LD gavaged rats (Fig. 26) .

Manganese superoxide dismutase - Inconsistant patterns were found in gavaged and injected rats. Enzyme concentrations significantly decreased in LD and HD gavaged rats, but increased in HD injected rats (Fig. 27) .

Glucose-6-phosphate dehydrogenase - Enzyme concentrations significantly decreased in gavaged HD rats and in both injected LD and HD rats compared to the respective controls (Fig. 28).

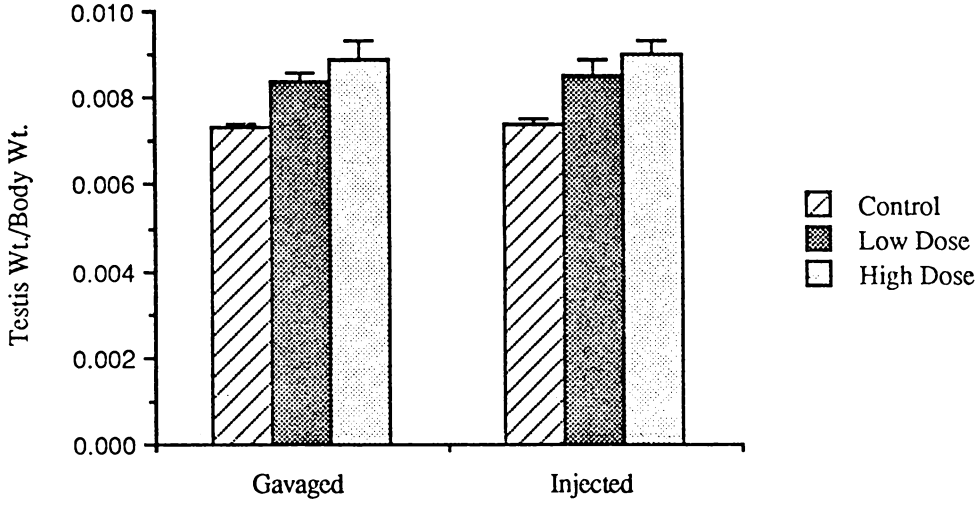
The Effect of Gossypol on Testis Weights of Male Rats



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	4.04 ± .08 (9)a	3.95 ± .12 (8)a	3.16 ± .09 (9)b
Injection	3.12 ± .05 (8)a	3.00 ± .12 (9)a	2.95 ± .10 (6)a

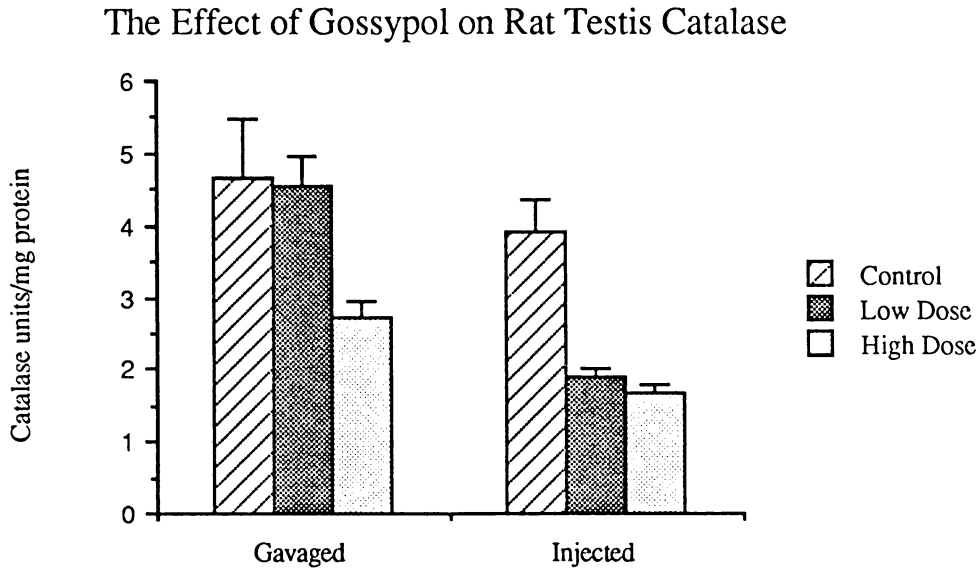
Figure 18. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

The Effect of Gossypol on Male Rat Testis/Body Weight Ratios



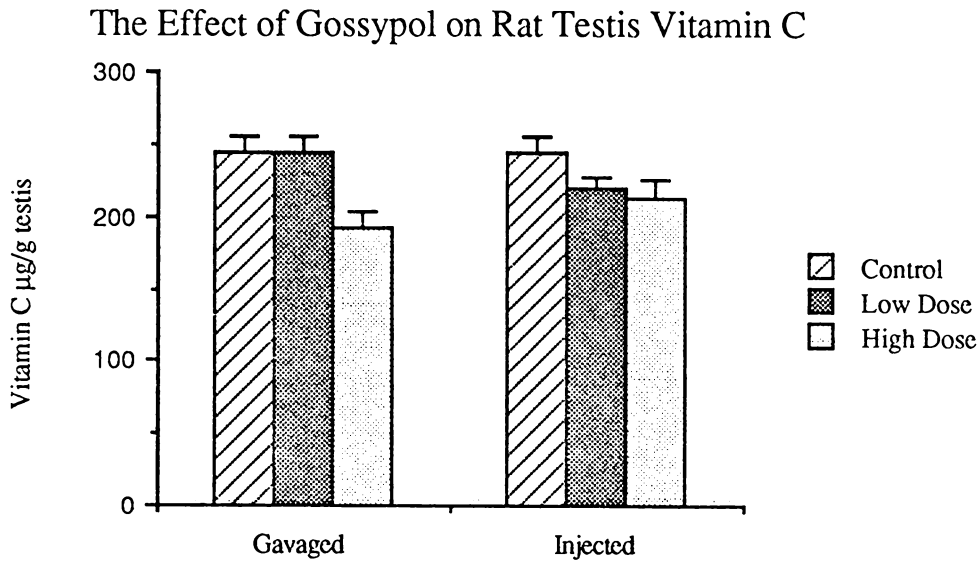
DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	.0073 ± .0001 (9)a	.0084 ± .0002 (8)ab	.0089 ± .0004 (9)b
Injection	.0074 ± .0001 (8)a	.0085 ± .0004 (9)ab	.0090 ± .0003 (6)b

Figure 19. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.



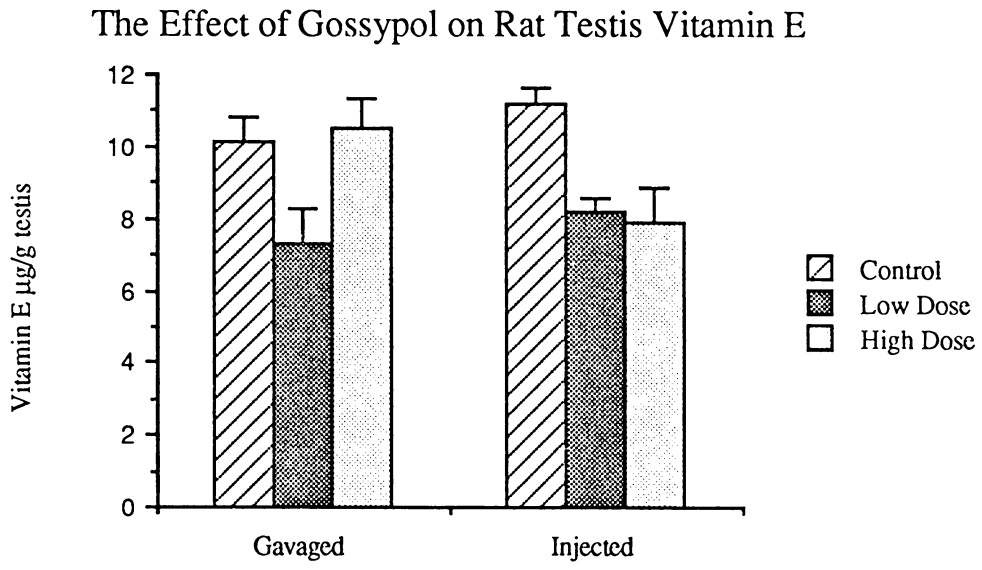
DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	4.67 ± .79 (9)a	4.55 ± .41 (8)a	2.72 ± .21 (9)a
Injection	3.90 ± .45 (8)a	1.90 ± .12 (9)b	1.66 ± .12 (6)b

Figure 20. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	243.68 ± 11.69 (9)a	244.05 ± 11.31 (8)a	191.67 ± 11.83 (9)b
Injection	244.01 ± 11.66 (8)a	220.02 ± 7.47 (9)a	211.57 ± 13.98 (6)a

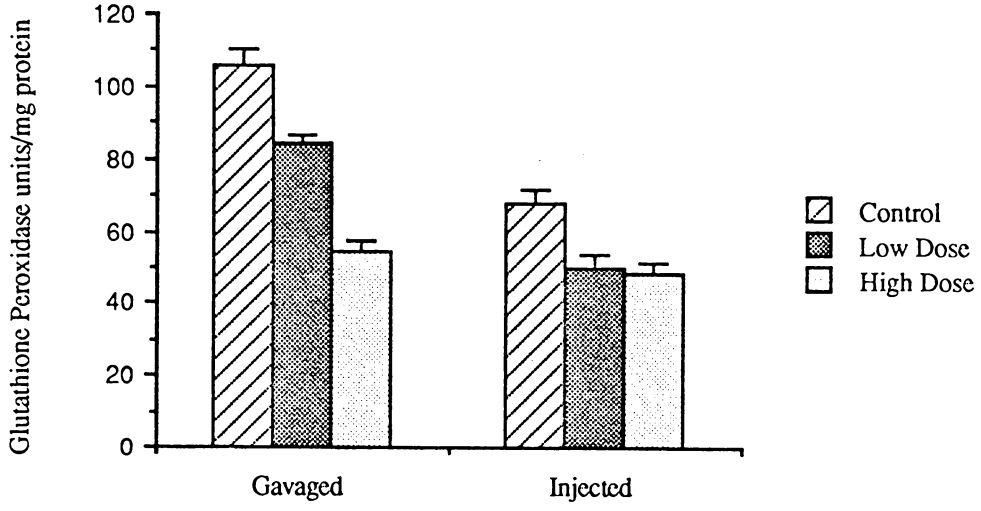
Figure 21. Values are means ± standard errors as determined in µg/g tissue. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	10.1 ± 0.7 (9)a	7.3 ± 1.0 (8)a	10.5 ± 0.8 (8)a
Injection	11.2 ± 0.4 (8)a	8.2 ± 0.4 (9)b	7.9 ± 1.0 (6)b

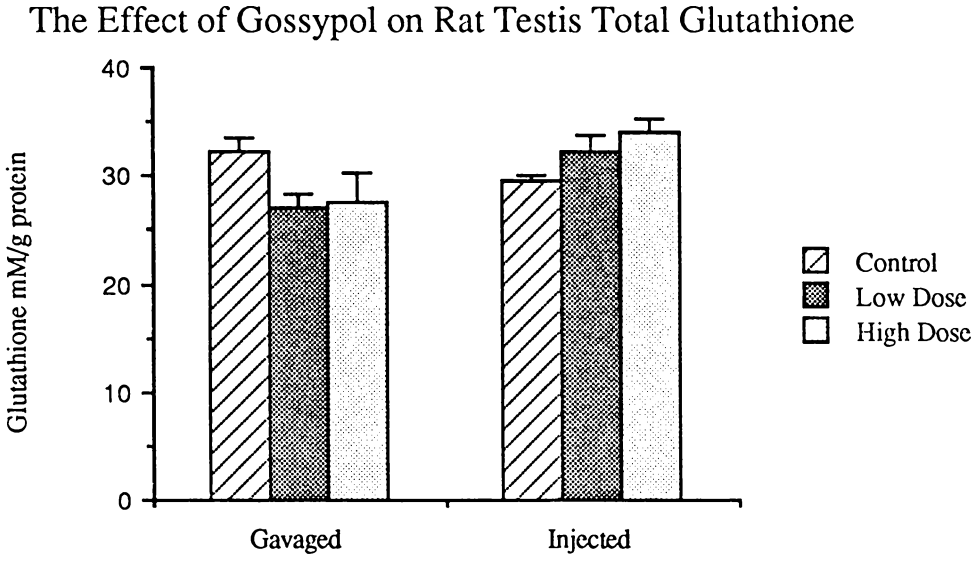
Figure 22. Values are means ± standard errors as determined in µg/g tissue. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

The Effect of Gossypol on Rat Testis Glutathione Peroxidase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	105.71 ± 4.36 (9)a	84.50 ± 1.62 (8)b	54.70 ± 2.53 (9)c
Injection	67.61 ± 3.78 (8)a	50.25 ± 3.33 (9)b	48.10 ± 3.27 (6)b

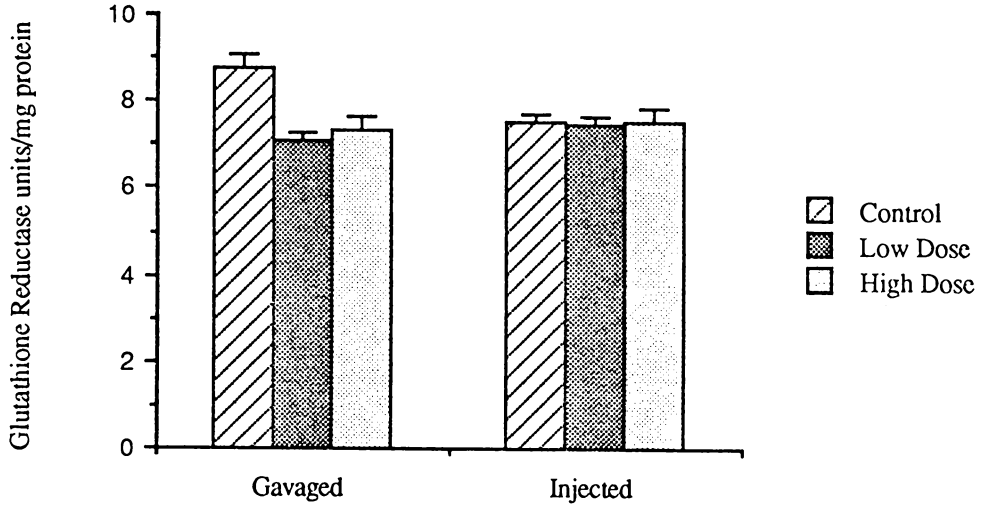
Figure 23. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript c are significantly different from both a and b.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	32.28 ± 1.24 (9)a	27.20 ± 1.13 (8)a	27.66 ± 2.75 (9)a
Injection	29.50 ± 0.66 (8)a	32.31 ± 1.49 (9)a	34.02 ± 1.31 (6)a

Figure 24. Values are means ± standard errors as determined in mM/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

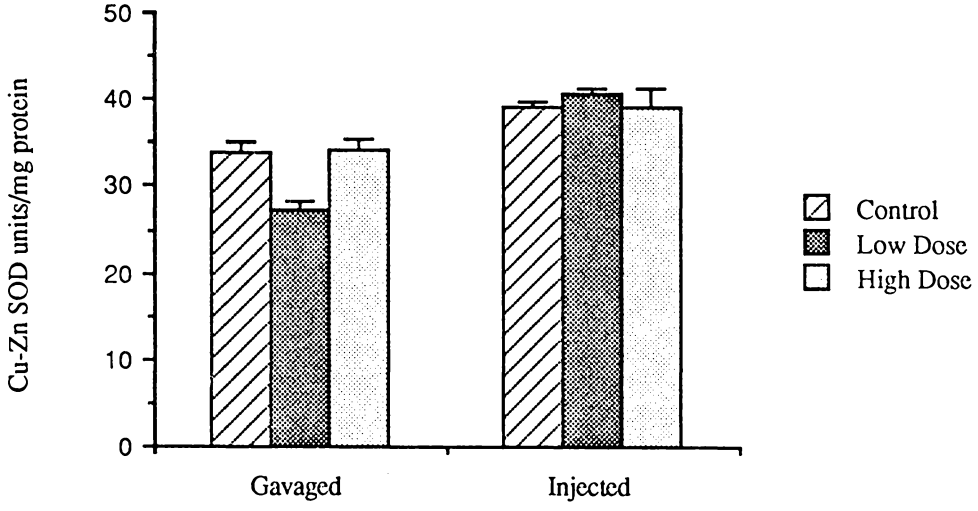
The Effect of Gossypol on Rat Testis Glutathione Reductase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	8.77 ± .31 (9)a	7.05 ± .24 (8)b	7.36 ± .28 (9)b
Injection	7.49 ± .20 (8)a	7.45 ± .19 (9)a	7.53 ± .30 (6)a

Figure 25. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

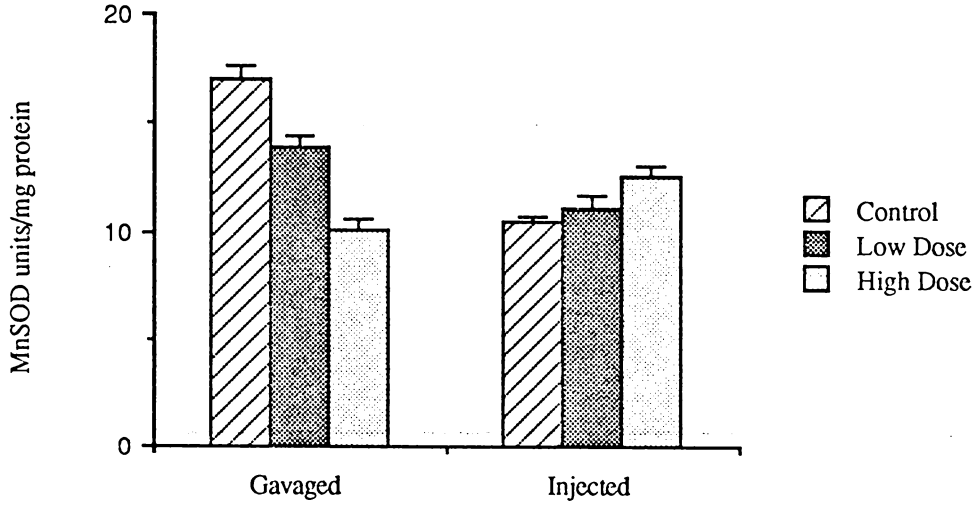
The Effect of Gossypol on Rat Testis Copper-Zinc Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	33.80 ± 1.19 (9)a	27.25 ± 1.05 (8)b	34.14 ± 1.40 (9)a
Injection	39.08 ± .75 (8)a	40.57 ± .75 (9)a	39.13 ± 2.26 (6)a

Figure 26. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

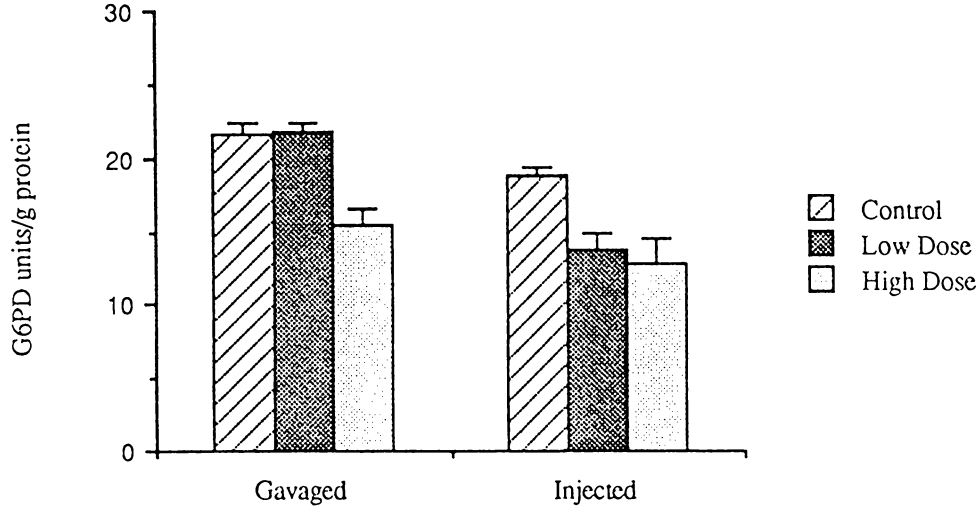
The Effect of Gossypol on Rat Testis Manganese Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	17.05 ± .56 (9)a	13.87 ± .56 (8)b	10.12 ± .45 (9)c
Injection	10.43 ± .30 (8)a	11.10 ± .52 (9)ab	12.50 ± .53 (6)b

Figure 27. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.

The Effect of Gossypol on Rat Testis Glucose-6-Phosphate Dehydrogenase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	21.63 ± .81 (9)a	21.77 ± .65 (8)a	15.44 ± 1.22 (9)b
Injection	18.89 ± .49 (8)a	13.86 ± 1.07 (9)b	12.90 ± 1.72 (6)b

Figure 28. Values are means ± standard errors as determined in units/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

DISCUSSION

Testis weights were reduced in gossypol treated rats, but these differences reached statistical significance only in the high dose (HD) gavaged group. A previous study of gossypol treated rats found similar reductions in testis weights (Oko and Hrundka, 1984). Testis weights were compared in a ratio with body weights because gossypol treated rats had lower body weights than controls at the end of the dosing interval. Testis to body weight ratios in all gossypol treated rats increased compared to controls, but these differences reached significance only in the HD groups. Some investigators attribute reductions in body weight in gossypol treated rats to decreased food intake following anorexia (Zatuchni and Osborn, 1981; Beaudin, 1985). It is reasonable to assume that the testis weight may not be affected proportionally to total body weight because the mobilization of glycogen, fat and protein reserves following anorexia would cause a greater percentage loss of body weight than testis weight. Other studies found increases in testis to body weight ratios in gossypol treated mice (Coulson et al., 1980). These investigators proposed that the proportional differences in testis and body weight were due to the antiandrogenic properties of gossypol. Other antiandrogenic agents are reported to cause testicular stimulation due to a lack of negative feedback by testosterone and a resultant increased release of luteinizing hormone (Newman and Steinbeck, 1974). Tissue edema from the destructive effects of gossypol on seminiferous epithelium (Xue, 1981) may also account for the increased testis to body weight ratios.

Gossypol treated animals showed significant reductions in the majority of antioxidants that were examined in this study. Catalase and glutathione peroxidase were decreased in all gossypol treated groups. Catalase and glutathione peroxidase are the two enzymes which scavenge hydrogen peroxide (H_2O_2). The majority of catalase in mammalian cells is located within peroxisomes (Masters et al., 1986) whereas glutathione peroxidase is found in the cytosol and mitochondrial matrix (Tappel et al., 1978). Catalase is thought to catalyze the conversion of H_2O_2 to water and oxygen, only when H_2O_2 is present in very high quantities (Km=1.1M). Glutathione peroxidase has a lower Km (higher affinity) for H_2O_2 than catalase, and is thought to scavenge the smaller quantities of H_2O_2 produced during normal metabolism. Hence, the role of catalase may be secondary, acting to protect cellular constituents, during pathological conditions that generate H_2O_2 concentrations overwhelming to the glutathione peroxidase system (Fridovich, 1976).

Hydrogen peroxide is an oxidizing agent produced by the dismutation of two molecules of superoxide ($O_2^{\cdot-}$) or by reactions catalyzed by oxidases including D-amino acid oxidase and urate oxidase. Hydrogen peroxide inactivates enzymes by oxidizing essential thiol groups. It readily crosses cell membranes and may react with $O_2^{\cdot-}$ in a reaction catalyzed by metal ions to form one of the most reactive species of oxygen, the hydroxyl radical (Halliwell and Gutteridge, 1985). Hydroxyl radical ($OH^{\cdot}$) reacts quickly and indiscriminately with all classes of molecules found in biological systems including sugars,

amino acids, phospholipids, nucleotides and organic acids. Hydroxyl radical may inactivate enzymes, damage DNA, and cause lipid peroxidation, which may ultimately lead to cell death (Brawn and Fridovich, 1980).

Decreases were found in concentrations of glutathione reductase in the gavaged rats, but these decreases in enzyme activity were not consistent with data from injected rats. It is reasonable to speculate that the route of gossypol administration, as well as differences in the length of the dosing interval may account for the inconsistent reduction in this enzyme. Glutathione reductase, an enzyme found in the cytosol and mitochondrial matrix of mammalian cells, keeps glutathione (a low molecular weight cellular antioxidant) in the reduced state. Glutathione (GSH) is a tripeptide thiol that reduces lipid peroxides, H_2O_2, disulfides, $OH^{\bullet}$ and singlet oxygen. It reduces these species by undergoing an oxidation reaction to form oxidized glutathione (GSSG). Several enzymes are thought to be inactivated via the oxidation of essential thiol groups (Halliwell, 1974). Glutathione may repair this damage and restore normal function to these enzymes. All cellular GSH would soon be oxidized to GSSG, which is an oxidant itself, if it were not for glutathione reductase. Glutathione reductase reduces GSSG to restore intracellular concentrations of GSH, using NADPH as a cofactor (Clark et al., 1985).

The NADPH used for reduction of GSSG is generated by the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase catalyzes the initial, committed, rate limiting step of the pentose phosphate

pathway. As presented in Fig. 28, glucose-6-phosphate dehydrogenase was significantly decreased in gavaged and injected gossypol treated rats. Inhibition of testicular glucose-6-phosphate dehydrogenase would decrease the number of NADPH molecules available for GSSG reduction and render this important component in the antioxidant defense system nonfunctional. NADPH is also required for steroidogenesis, therefore, decreases in testosterone levels reported by Hadley et al. could have been due to the decreased activity of glucose-6-phosphate dehydrogenase (Hadley et al., 1981).

Vitamin C concentrations were also decreased in gossypol treated rats in this experiment. Vitamin C is a low molecular weight free radical scavenger and powerful reducing agent found in the cytoplasm of mammalian cells and the surrounding plasma. Ascorbate is thought to protect cells from aqueous phase oxidative injury by scavenging rective species of oxygen, including $O_2^{\bullet-}$ and $OH^{\bullet}$. Recent evidence suggests that the antioxidant properties of vitamin E are restored by interactions with vitamin C (Bendich et al., 1986; Halliwell and Gutteridge, 1985). Since the concentrations of vitamin E, a lipid phase antioxidant, were also found to be decreased in the testes of all gossypol treated rats (with the exception of gavaged HD rats). It is reasonable to speculate that this organ is at greater risk from drugs that promote the formation of reactive oxygen species, such as gossypol.

This hypothesis is consistent with DePeyster et al. who demonstrated that gossypol promoted the formation of reactive species of oxygen including $O_2^{\bullet-}$ and H_2O_2 in human sperm <u>in vitro</u> (DePeyster et

al., 1984). Without the protective actions of catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, vitamin C and vitamin E, spermatogenic epithelium would be highly vulnerable to oxidative injury by these reactive species of oxygen. Oxidative injury may thus be central to the pathogenesis of the destruction of testis germinal epithelium and sperm abnormalties found in gossypol treated rats.

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THE EFFECTS OF GOSSYPOL ON THE ANTIOXIDANT DEFENSE SYSTEM OF THE HEART

INTRODUCTION

The toxic effects of feeding large amounts of cottonseed meal as a protein supplement to livestock were first reported in 1859 (Adams et al., 1960). Nearly sixty years later, the toxic agent of cottonseed was identified as the yellow polyphenolic pigment, gossypol (Withers and Carruth, 1916). In 1957, gossypol was found to also have antifertility properties (Liu, 1985). Most of the studies since then concern its effects on the reproductive system of men and male animals (National Coordinating Group on Male Antifertility Agents, 1978).

Gossypol is reported as a potent cardiotoxin in swine. Pigs fed cottonseed rations containing high concentrations of gossypol showed clinical signs of dyspnea, panting, weakness and anorexia for several days prior to death. Postmortem examination revealed congestive heart failure with dilatation of both ventricles. Microscopic examination revealed partial dissolution of some cardiac myocytes, and extreme atrophy of others (Smith, 1957).

Dogs on cottonseed meal rations are reported to die of a similar congestive heart failure with hypertrophic cardiomyopathy (West, 1940; Patton et al., 1985). Microscopic examination revealed many cardiac myofibers with perinuclear vacuoles and others with large hyperchromatic nuclei. Cardiac edema and atrophied myofibers were also reported.

Cottonseed meal rations caused similar lesions in the hearts of young calves (Rogers and Henaghan, 1975; Orgad-Klopfer and Adler, 1986). The rat heart is relatively insensitive to the toxic effects of gossypol (Qian and Wang, 1984).

The mechanism by which gossypol causes cardiomyopathy is unknown. A recent in vitro study examining human sperm and rat liver microsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals. The authors suggest that the common mechanism that underlies all of the toxic effects of gossypol may be related to free radical injury (DePeyster et al., 1984).

Reactive species of oxygen are implicated as a cause of a multitude of pathological changes. Free radical reactions with proteins and lipids often change the molecular configuration and may lead to cell death (Freeman and Crapo, 1982). The interaction of free radicals with DNA may cause damage to the molecular structure and induce strand breakage. Cells often die after this form of damage or those that survive may undergo mutations (Halliwell and Gutteridge, 1985). Cell death due to reactive species of oxygen is likely to be some combination of an overwhelming production of free radicals and a compromised antioxidant defense system.

Since the rat heart is known to be less sensitive to the toxic effects of gossypol than other organs, including the testis and liver (Qian and Wang, 1984), the present study was designed to examine the effects of oral and injected gossypol acetic acid on the antioxidant defense mechanism of the rat heart.

MATERIALS AND METHODS

Animals, gossypol treatment, enzyme assays, statistics and tissue collection techniques are described in section 2, The Effects of Gossypol on the Antioxidant Defense System of the Liver, with the exception that the perfused hearts were removed, weighed and individually homogenized. Vitamin E assays were not performed on the hearts and vitamin C assays were not performed on gavaged high dose (HD) rat hearts due to insufficient amounts of tissue homogenate.

RESULTS

Clinical signs, body weight and adrenal weight comparisons are presented in section 2. The effect of gossypol treatment on heart weights was assessed in both injected and gavaged rats. Heart weights decreased in all groups compared to controls (Fig. 29) . These values reached statistical significance in gavaged low dose (LD) and HD rats and injected LD rats. When heart weights were compared in a ratio with body weights (Fig. 30) to account for differences in body weights between the groups, no statistical differences were found.

The Antioxidant Defense System of the Heart -

Catalase - Increases in heart catalase were found in all gossypol treated groups compared to controls (Fig. 31). The changes were significant in injected LD and HD rats.

Vitamin C - No significant changes in cardiac vitamin C content were found between the groups (Fig. 32).

Glutathione peroxidase - No significant changes were found in any groups (Fig. 33).

Total glutathione - No significant changes were found between the groups (Fig. 34).

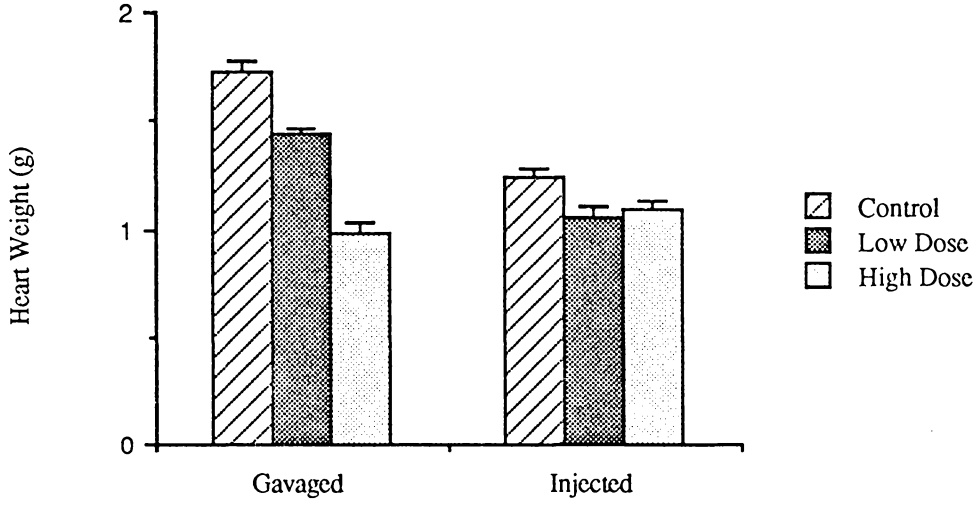
Glutathione reductase - Increases in glutathione reductase levels were found in all gossypol treated groups compared to controls (Fig. 35). These increases reached statistical significance in both HD groups compared to the controls.

Copper-zinc superoxide dismutase - No significant changes were found in any groups compared to control rats (Fig. 36).

Manganese superoxide dismutase - Statistically significant changes were found only in gavaged rats (Fig. 37). Gavaged LD rats had significant increases in enzyme concentrations compared to controls. The HD rats had significant increases compared to both controls and LD rats.

Glucose-6-phosphate dehydrogenase - Although the enzyme levels appear increased in all gossypol treated groups, the values did not reach statistical significance (Fig. 38).

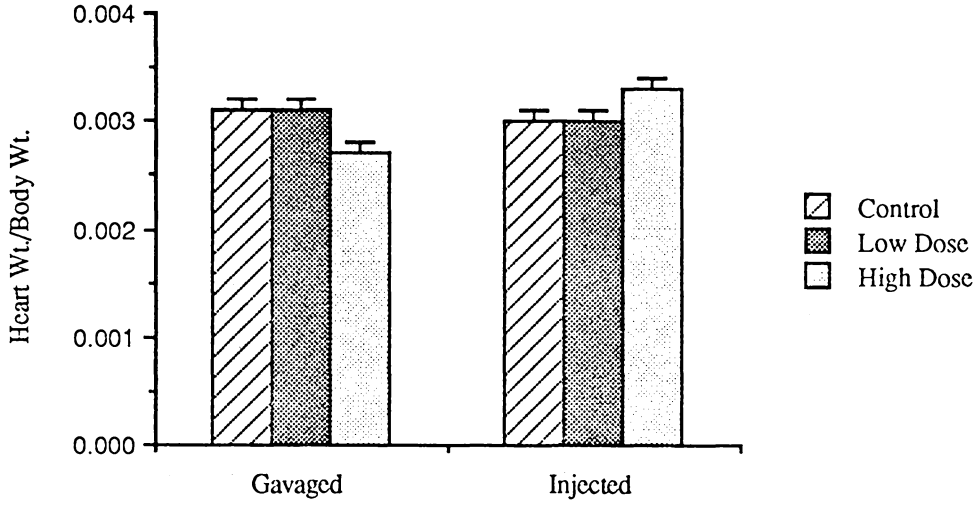
The Effect of Gossypol on Heart Weights of Male Rats



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	1.73 ± .05 (9)a	1.44 ± .02 (8)b	.98 ± .05 (9)c
Injection	1.24 ± .04 (8)a	1.06 ± .04 (9)b	1.09 ± .04 (6)ab

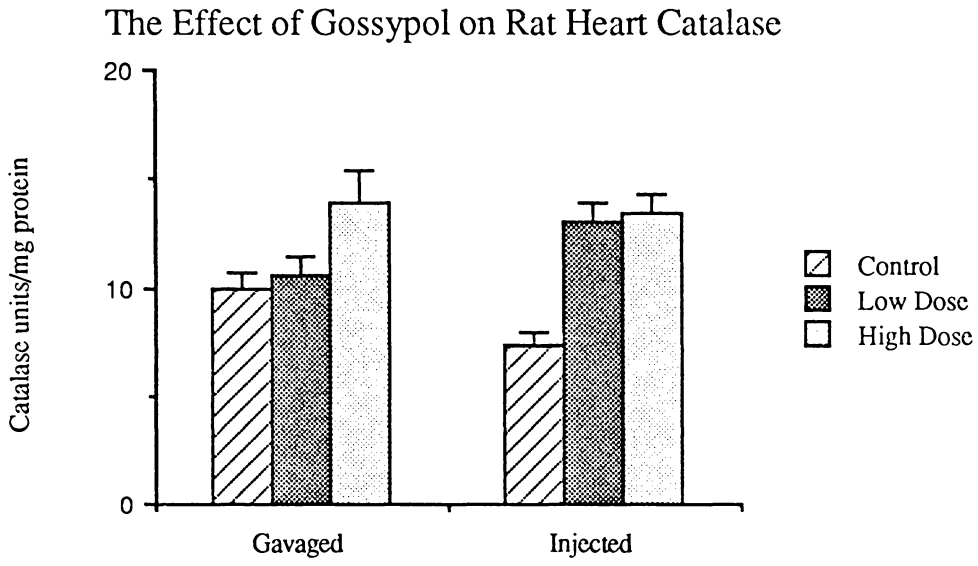
Figure 29. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript c are significantly different from both a and b. Values labeled ab are not significantly different from values labeled a or b.

The Effect of Gossypol on Male Rat Heart/Body Weight Ratios



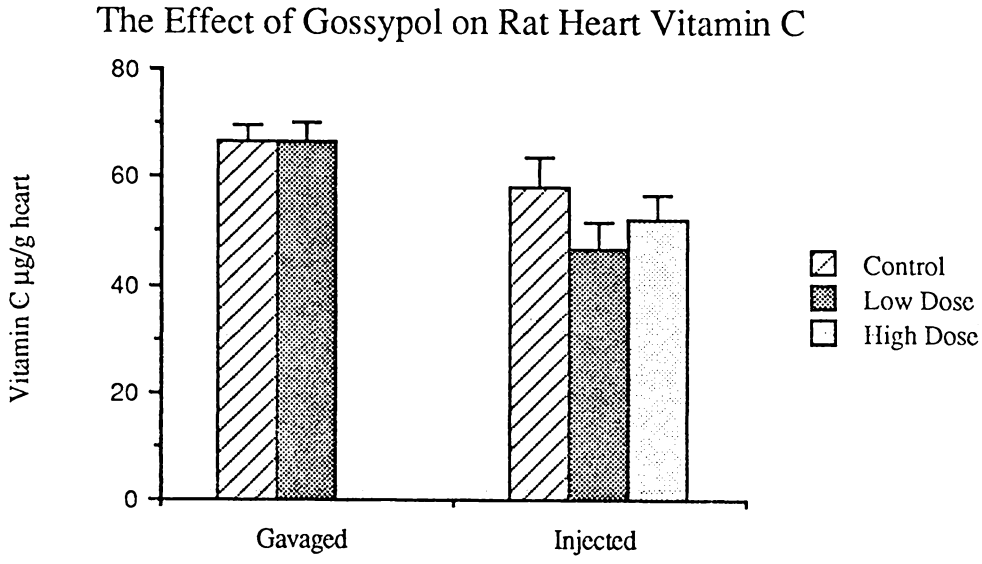
DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	.0031 ± .0001 (9)a	.0031 ± .0001 (8)a	.0027 ± .0001 (9)a
Injection	.0030 ± .0001 (8)a	.0030 ± .0001 (9)a	.0033 ± .0001 (6)a

Figure 30. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	9.95 ± .73 (9)a	10.61 ± .87 (8)a	13.88 ± 1.53 (9)a
Injection	7.28 ± .61 (8)a	13.04 ± .83 (9)b	13.41 ± .83 (6)b

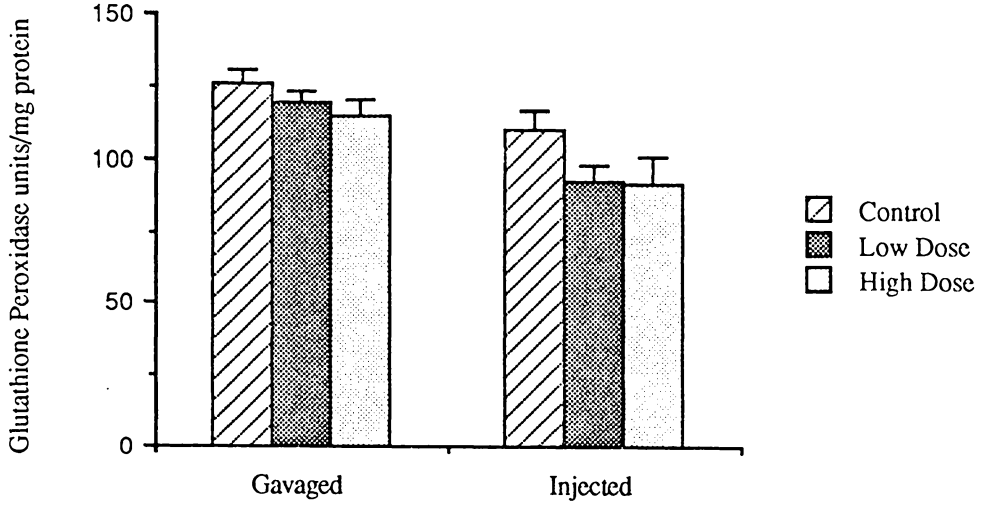
Figure 31. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	66.44 ± 2.97 (9)a	66.37 ± 3.77 (8)a	insufficient sample
Injection	58.25 ± 5.6 (8)a	46.56 ± 5.34 (9)a	52.25 ± 4.48 (6)a

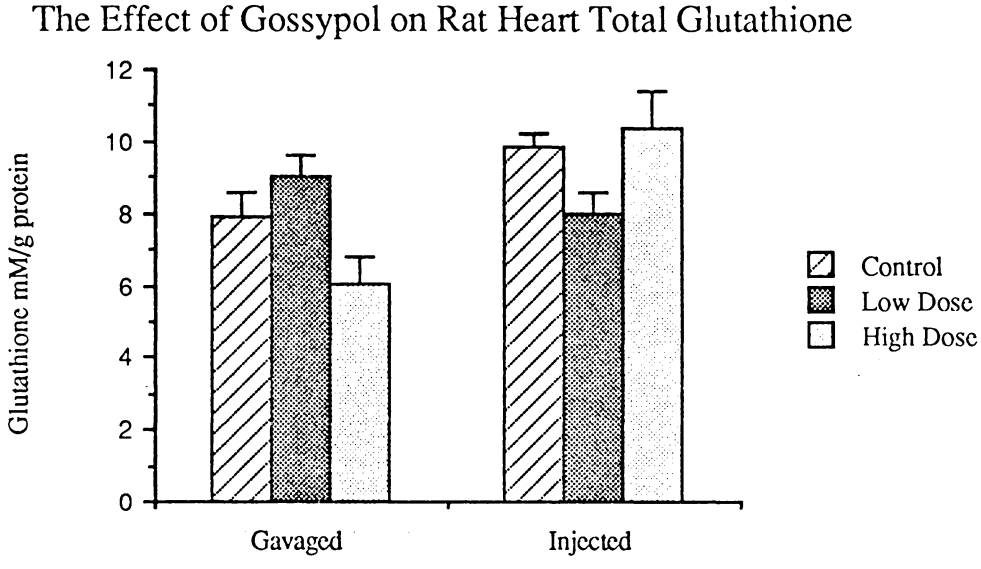
Figure 32. Values are means ± standard errors as determined in µg/g tissue. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

The Effect of Gossypol on Rat Heart Glutathione Peroxidase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	125.84 ± 5.06 (9)a	119.07 ± 3.56 (8)a	114.67 ± 5.27 (9)a
Injection	110.17 ± 6.52 (8)a	92.29 ± 5.61 (9)a	91.77 ± 8.57 (6)a

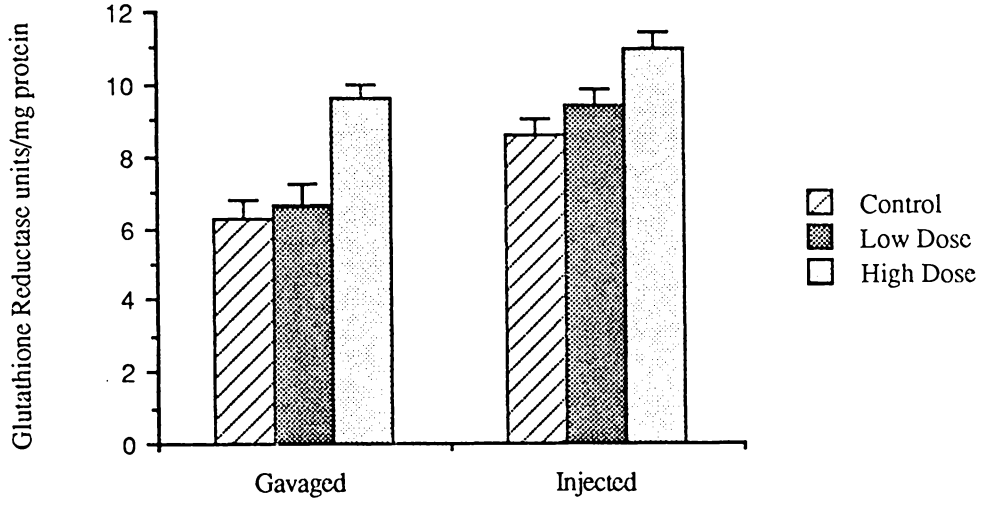
Figure 33. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	7.88 ± .68 (9)a	9.00 ± .60 (8)a	6.04 ± .77 (9)a
Injection	9.85 ± .36 (8)a	7.99 ± .58 (9)a	10.38 ± .99 (6)a

Figure 34. Values are means ± standard errors as determined in mM/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

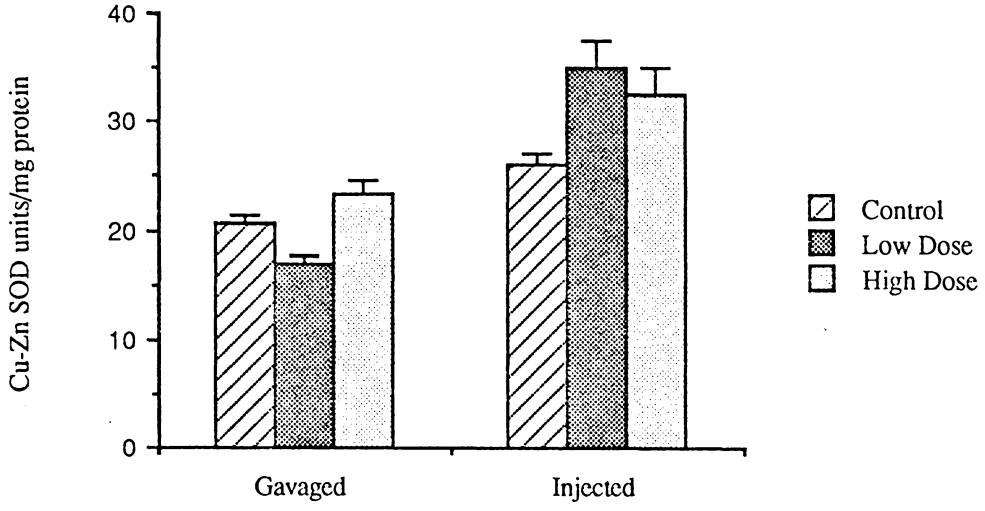
The Effect of Gossypol on Rat Heart Glutathione Reductase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	6.28 ± .47 (9)a	6.63 ± .62 (8)a	9.60 ± .41 (9)b
Injection	8.54 ± .48 (8)a	9.38 ± .42 (9)ab	10.93 ± .45 (6)b

Figure 35. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Values labeled subscript ab are not significantly different from values labeled subscript a or b.

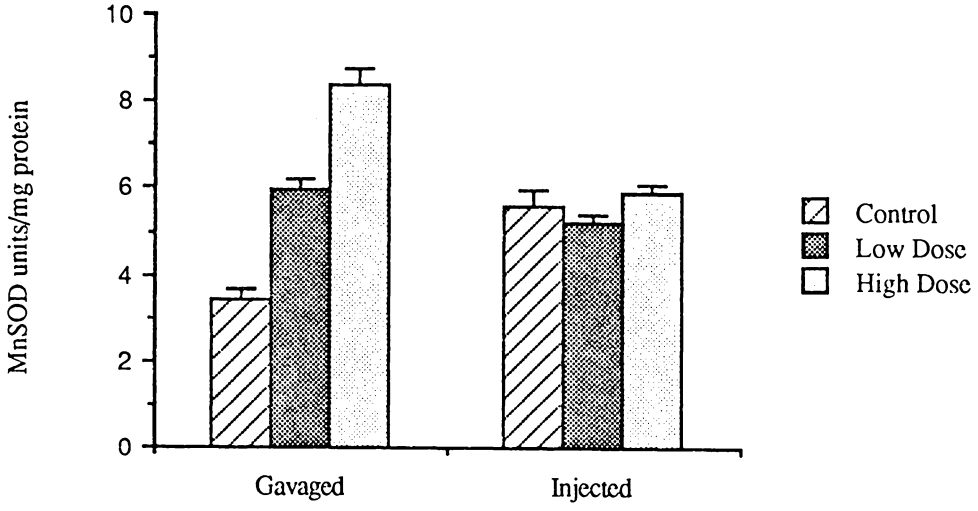
The Effect of Gossypol on Rat Heart Copper-Zinc Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	20.73 ± .65 (9) ab	16.98 ± .71 (8)a	23.28 ± 1.22 (9)b
Injection	26.05 ± 1.00 (8)a	35.05 ± 2.57 (9)b	32.50 ± 2.44 (6)ab

Figure 36. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled subscript a or b.

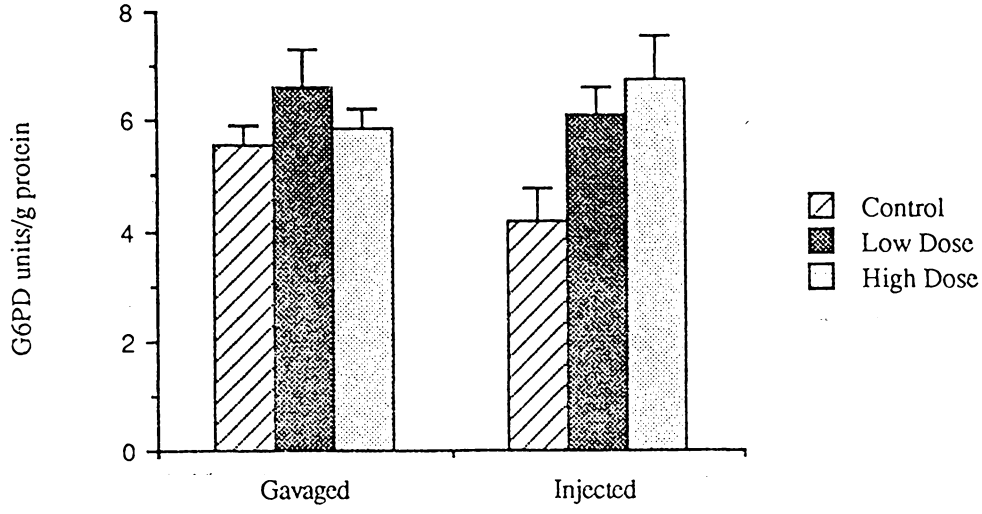
The Effect of Gossypol on Rat Heart Manganese Superoxide Dismutase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	3.42 ± .25 (9)a	5.95 ± .25 (8)b	8.36 ± .39 (9)c
Injection	5.61 ± .35 (8)a	5.19 ± .22 (9)a	5.90 ± .19 (6)a

Figure 37. Values are means ± standard errors as determined in units/mg protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript c are significantly different from both a and b.

The Effect of Gossypol on Rat Heart Glucose-6-Phosphate Dehydrogenase



DOSE ROUTE	CONTROL	LOW DOSE	HIGH DOSE
Gavage	5.57 ± .34 (9)a	6.60 ± .70 (8)a	5.88 ± .34 (9)a
Injection	4.15 ± .64 (8)a	6.11 ± .48 (9)a	6.74 ± .79 (6)a

Figure 38. Values are means ± standard errors as determined in units/g protein. The number of rats in each group is shown in parentheses. Data were analyzed separately for gavaged and injected rats by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

DISCUSSION

The gossypol treated rats had significantly reduced heart weights compared to controls. Statistical trends in heart weights changed when each heart weight was compared in a ratio with individual body weight. The heart to body weight ratios demonstrated that the reductions in heart weights were proportional to reductions in body weights in gossypol treated rats.

Gossypol treated animals showed consistant and significant increases in two of the eight quantitated components of the cardiac antioxidant defense system.

Catalase concentrations were significantly increased in gavaged and injected gossypol treated rats. Catalase, an enzymatic scavenger of hydrogen peroxide (H_2O_2), is located within peroxisomes of mammalian cells (Masters et al., 1986). Recent studies demonstrated that catalase catalyzes an important pathway for the detoxification of H_2O_2 in the rat myocardium (Thayer, 1986).

Hydrogen peroxide is an oxidizing agent, produced by the dismutation of two molecules of superoxide ($O_2^{\cdot-}$) or by reactions catalyzed by oxidases including D-amino acid oxidase and glucose oxidase. Hydrogen peroxide inactivates enzymes by oxidizing essential thiol groups. It readily crosses cell membranes and may react with superoxide in a reaction catalyzed by metal ions to form one of the most reactive species of oxygen, the hydroxyl radical (Halliwell and Gutteridge, 1985). Hydroxyl radical ($OH^{\cdot}$) reacts quickly and indiscriminately with all classes of molecules found in biological systems including sugars, amino acids, phospholipids, nucleotides and

organic acids. Hydroxyl radical may inactivate enzymes, damage DNA, and cause lipid peroxidation which may lead to the death of a cell (Brawn and Fridovich, 1980).

Glutathione reductase concentrations were also increased in gossypol treated animals. Glutathione reductase, an enzyme found in the cytosol and mitochondrial matrix of mammalian cells, keeps glutathione, a low molecular weight cellular antioxidant, in the reduced state. Glutathione (GSH) is a tripeptide thiol that reduces lipid peroxides, H_2O_2, disulfides, $OH^{\bullet}$ and singlet oxygen. It reduces these species by undergoing an oxidation reaction to form oxidized glutathione, also called glutathione disulfide (GSSG). Several enzymes are thought to be inactivated by free radicals via the oxidation of essential thiol groups (Halliwell, 1974). Glutathione may repair this damage and restore function to these enzymes. All cellular GSH would soon be oxidized to GSSG, which is an oxidant itself, if it were not for glutathione reductase. Glutathione reductase reduces GSSG to restore intracellular concentrations of GSH, using NADPH as a cofactor (Clark et al., 1985).

Cardiac manganese superoxide dismutase showed inconsistant increases in gossypol treated rats. Manganese superoxidase dismutase (MnSOD) was significantly increased in gavaged, but not in injected rats. Cardiac myocytes contain two different superoxide dismutases, each containing a different metal at the active site. The SOD that contains copper and zinc at its active site (CuZn SOD) is found mainly in the cytosol whereas the second type of SOD which contains manganese

at its active site (MnSOD) is found primarily in the mitochondrial matrix (Spanier et al., 1985). Superoxide dismutases are enzymes responsible for the removal of superoxide radicals ($O_2^{\cdot-}$). These enzymes cause a dismutation reaction whereby one molecule of $O_2^{\cdot-}$ is oxidized to form molecular oxygen (O_2) and another $O_2^{\cdot-}$ is simultaneously reduced to form H_2O_2. Superoxide dismutases increase the rate of $O_2^{\cdot-}$ dismutation over 10,000 times the rate of the spontaneous reaction (Fridovich, 1979).

Superoxide radicals are formed by the univalent reduction of molecular oxygen. Superoxide has been implicated in the inactivation of enzymes (Salin and McCord, 1977), peroxidation of lipids (Halliwell, 1978) and damage to DNA (Brawn and Fridovich, 1980). It is reasonable to speculate that the route of gossypol administration as well as differences in the length of the dosing interval may account for the inconsistent induction of MnSOD in the present study.

The *in vivo* increases in catalase, glutathione reductase and MnSOD reported here are consistent with recent *in vitro* findings. DePeyster et al., using gossypol treated rat liver microsomes and human sperm demonstrated that gossypol promoted the formation reactive species of oxygen including hydrogen peroxide, hydroxyl radical and superoxide radical (DePeyster et al., 1985). Antioxidant enzyme induction is a well documented consequence of chronic oxidative insult to tissues. Myocardial glutathione reductase concentrations were increased in rats that were chronically dosed with ethanol (Edes et al., 1986). Reactive species of oxygen are thought to be central to the pathogenesis of alcohol induced tissue injury. Erythrocytes from alcoholics contain

increased concentrations of the antioxidant, SOD (DelVillano et al., 1980). Administration of 100% oxygen to rats for 60-72 hours caused severe lung damage and death. However, rats exposed to gradual increases in oxygen tension survived in 100% oxygen. The ability to survive these conditions was correlated with induction of pulmonary SOD, catalase, glutathione reductase and glutathione peroxidase (Halliwell and Gutteridge, 1985). These antioxidants were thought to protect these animals from oxygen induced pulmonary tissue injury. Therefore, induction of some of the antioxidants by gossypol is not surprising, since gossypol is known to promote the formation of reactive species of oxygen, capable of causing oxidative stress in animals.

In studies of the effects of gossypol on the antioxidant system of two other tissues (The Effects of Gossypol on the Antioxidant Defense System of the Liver, section 2; The Effects of Gossypol on the Antioxidant Defense System of the Testis, section 3), the activites of many antioxidants were inhibited in gossypol treated rats. However, in the present study of myocardial tissue, antioxidant activities were generally unchanged or increased. Increased concentrations of antioxidants may have protected the myocardium from injury due to reactive species of oxygen and accounted for the apparent insensitivity of the rat heart to gossypol. Treatment of swine (Smith, 1957), dogs (West, 1940; Patton et al., 1985) and calves (Rogers and Henaghan, 1975; Orgad-Klopfer and Adler, 1986) is associated with myocardial injury, but similar lesions are found in gossypol treated rats only

after prolonged consumption of high doses of gossypol (Qian and Wang, 1984). Testis seminiferous epithelium is the most sensitive, followed by hepatocytes to the toxic effects of gossypol (Xue, 1981). The inhibition of antioxidant enzymes in the liver and testis of gossypol treated rats in this study may account for the greater sensitivity of these tissues to oxidative damage from gossypol treatment compared to the heart. A study of the heart antioxidant system of species sensitive to the cardiotoxic effects of gossypol is needed to further define this proposal.

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THE EFFECTS OF GOSSYPOL ON THE KINETICS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

INTRODUCTION

Recent studies of the tissue antioxidant defense system of rats demonstrated significant reductions in testis glucose-6-phosphate dehydrogenase concentrations in rats treated with gossypol acetic acid (The Effects of Gossypol on the Antioxidant Defense System of the Testis, section 3). Gossypol is a yellow polyphenolic compound with known antifertility properties in humans and many animal species (National Coordinating Group on Male Antifertility Agents, 1978). It is found in cottonseeds and cottonseed products that are used extensively in food for consumption by animals and people. Gossypol has undergone extensive clinical trials as a male antifertility agent in China to establish the optimal dosage rate to achieve over 99% antifertility efficacy. The mechanism by which infertility is achieved is not completely understood, but the germinal epithelium of the testis appears highly sensitive to the effects of gossypol. Pharmacokinetic studies of gossypol demonstrate that only small quantities accumulate in the testes; the majority is stored in the liver before it is secreted in the bile (Xue, 1981). It is interesting that glucose-6-phosphate dehydrogenase activity was decreased in the testis, but not in the liver or heart of gossypol treated rats.

Glucose-6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.49) catalyzes the initial, committed, rate

limiting step of the pentose phosphate pathway:

glucose-6-phosphate + $NADP^+$ → 6-phosphate gluconolactone + NADPH + H^+

This essential metabolic pathway is responsible for generating most of the cytosolic NADPH needed by mammalian cells. NADPH is used for reducing equivalents in many biosynthetic reactions including fatty acid and steroid synthesis (Schroeder et al., 1984; Lee et al., 1979; Schachet and Squire, 1976). Steroid producing tissues are thus generally rich in pentose phosphate enzymes (Criss and McKerns, 1968). NADPH also plays a major role in the antioxidant defense system of cells as a substrate for glutathione reductase. The reaction catalyzed by glutathione reductase helps to maintain adequate quantities of glutathione in the reduced form to protect cells from oxidative injury.

Two possible mechanisms may contribute to the sensitivity of the testis to the effects of gossypol. The inhibition of testis glucose-6-phosphate dehydrogenase would decrease the number of NADPH reducing equivalents available for steroidogenesis. This proposal is supported by studies that found reductions of serum testosterone in gossypol treated male rats (Hadely et al., 1981). An alternate mechanism for the action of gossypol on the testis is oxidative damage to the spermatogenic epithelium due to a decrease in available NADPH reducing equivalents and a compromised glutathione system. This proposal is supported by a recent <u>in vitro</u> study demonstrating production of reactive oxygen species when gossypol was incubated with human sperm and rat liver microsomes (DePeyster et al., 1984). The present study

examines the mechamism of inhibition (kinetics) of glucose-6-phosphate dehydrogenase purified from another steroid producing tissue, the bovine adrenal gland.

MATERIALS AND METHODS

Enzyme - Glucose-6-phosphate dehydrogenase purified from bovine adrenals was obtained from Sigma Chemical Company (St. Louis MO, lot 56F-8040) as a crystalline suspension in 2.8 M $(NH_4)_2SO_4$ solution at pH 7.6. The crystalline enzyme was diluted in 0.05 M glycine-buffer with 0.02 M 2-mercaptoethanol and 1% bovine serum albumen at pH 8.0. The concentration of the enzyme solution was adjusted to approximately 1.5 µg protein per ml to give a change of absorbance per minute of 0.04 when 0.1 ml enzyme solution was added to a 2.9 ml reaction mixture containing both substrates at a minimum of 10 times their respective Km values. The enzyme solution was used within three hours of dilution.

Substrates - Glucose-6-phosphate monosodium salt and $NADP^+$ sodium salt were obtained from Sigma Chemical Company. The substrates were diluted in 0.05 M Tris(base)-HCl buffer with 3.3 mM $MgCl_2$, pH 7.8, ensuring that the cuvette contained a final volume of 3.0 ml. The reaction was initiated by the addition of 0.1 ml of enzyme solution, and the initial velocity was determined by measuring the change of absorbance at 340 nm associated with $NADP^+$ reduction. Reaction velocities were determined using a Shimadzu-160 recording spectrophotometer at 30°C. Kinetic constants were determined by adding limiting concentrations of one substrate while the second substrate was

held at saturating concentrations, greater than 10 times the determined Km value. Each reported velocity is the mean of three to twelve determinations.

Inhibitor - Gossypol acetic acid, obtained from Polysciences, Inc., (Warrington PA), was dissolved in 95% ethanol. Four concentrations of gossypol solution were prepared fresh daily and a constant volume of 0.1 ml was added to the reaction mixture. The percent inhibition of glucose-6-phosphate dehydrogenase by 0.1 ml of 95% ethanol was determined at each substrate concentration. The reaction inhibition caused by gossypol acetic acid alone was determined by subtracting the percent inhibition due to ethanol from the values determined using gossypol plus ethanol. A constant enzyme activity was ensured by pairing each reaction mixture containing gossypol with a control mixture containing the same concentration of substrates for the enzyme tended to lose activity over the course of an experiment.

Statistics - Initial velocities of the kinetic experiments using bovine adrenal glucose-6-phosphate dehydrogenase with and without gossypol acetic acid were plotted as conventional reciprocal plots (Lineweaver and Burk, 1934). Kinetic constants were determined using a computer program employing the rules of Cleland (Cleland, 1979), modified for use on an IBM PC computer. Inhibition constants were analyzed by fitting the data to double reciprocal plots using Competitive, Noncompetitive and Uncompetitive programs. The type of inhibition was determined by choosing the plot with the lowest sigma values and standard errors of kinetic constants.

RESULTS

The initial velocity of each reaction mixture depended upon the concentration of the limiting substrate (Figs. 39A, 40A). The initial velocity data in each of Figures 39A and 40A approximates a rectangular hyperbola.

The effect of ethanol on the activity of glucose-6-phosphate dehydrogenase activity was investigated because ethanol was used to dissolve gossypol. The percent inhibition of glucose-6-phosphate dehydrogenase by ethanol (Fig. 41) generally increased with substrate concentration and remained constant after reaching the Km values of each substrate.

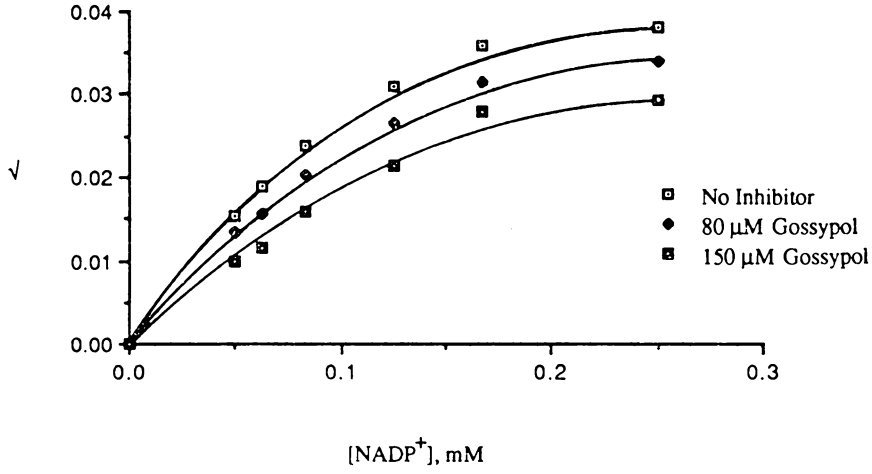
Double reciprocal graphs of the initial velocity data yield linear, intersecting plots (Figs. 39B and 40B). Analysis of initial velocity data by the Cleland computer program (Cleland, 1979) yields Km values of 135.59 ± 18.38 μM for $NADP^+$ and 30.76 ± 2.73 μM for G6P. The inhibition studies analyzed on the same program show that gossypol acetic acid inhibits bovine adrenal glucose-6-phosphate dehydrogenase in a competitive manner with respect to $NADP^+$ (Kis = 200.47 μM) and noncompetitively with respect G6P (Kis = 80.18 μM, Kii = 451.93 μM). Gossypol acetic acid was not used in concentrations higher than 150 μm in these experiments because of its insolubility in aqueous solutions. Table I summarizes and compares the Michealis constants, Vmax, Km and Ki values of glucose-6-phosphate dehydrogenase with respect to glucose-6-phosphate, $NADP^+$ and gossypol.

TABLE I

	Km(μM)	Vmax	Kis(μM)	Kii(μM)
$NADP^+$	135.59	.0635	200.47	--
G6P	30.76	.0374	80.18	451.93

Table I - Kinetic Constants for adrenal glucose-6-phosphate dehydrogenase. Kinetic constants were determined by Lineweaver-Burk plots of the data taken from assays determined at 30°C as described under Materials and Methods.

a Initial Velocity Data as [$NADP^+$] is Varied



b Double Reciprocal Plot as [$NADP^+$] is Varied

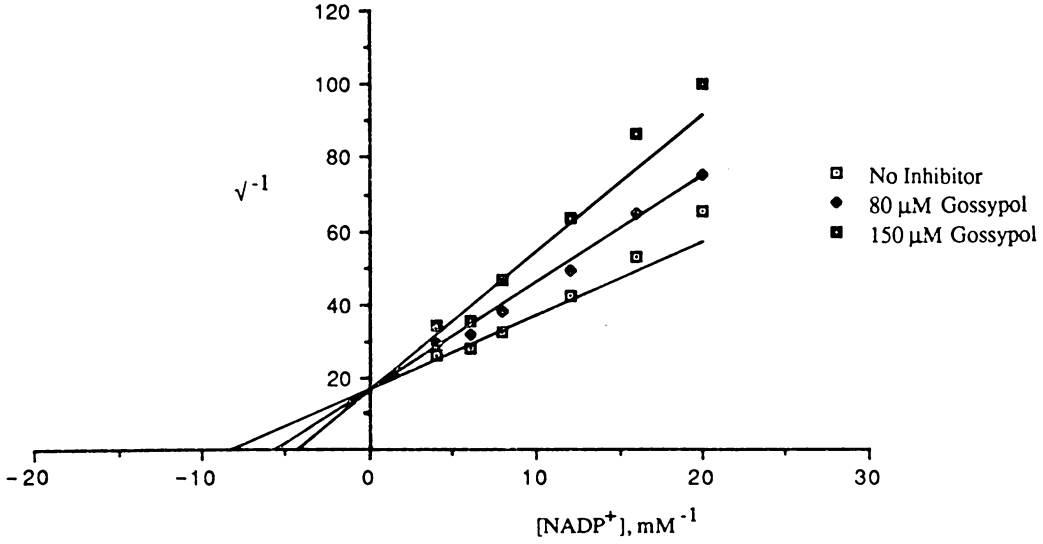
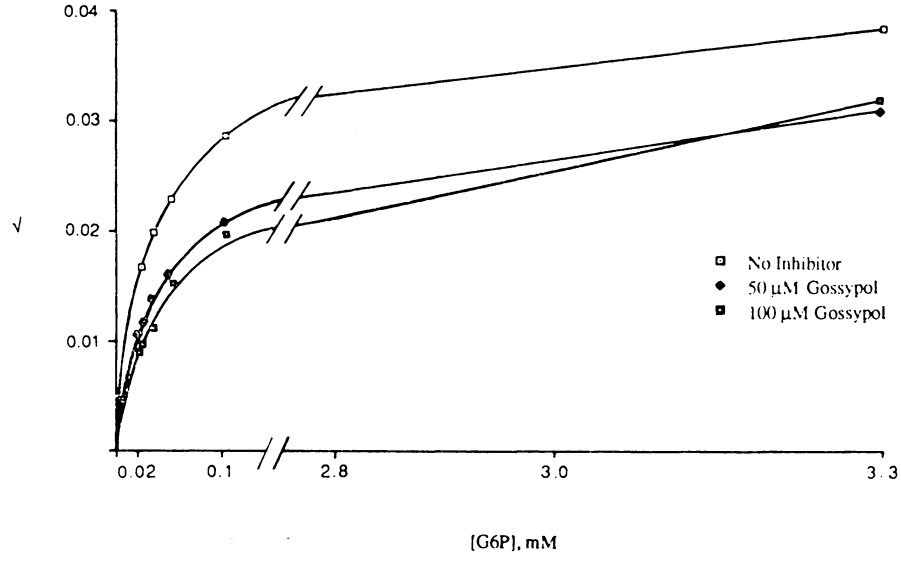


Figure 39-a. Initial velocity data and 39-b. Double-reciprocal plots of initial velocity data as $NADP^+$ was varied from .050 to .250 mM. Assays were performed with the concentrations of gossypol acetic acid indicated on the graph, keeping glucose-6-phosphate concentrations constant at 3.33 mM. The lines were fit according to the Competitive computer program (Cleland, 1979).

a

Initial Velocity Data as [G6P] is Varied



b

Double Reciprocal Plot as [G6P] is Varied

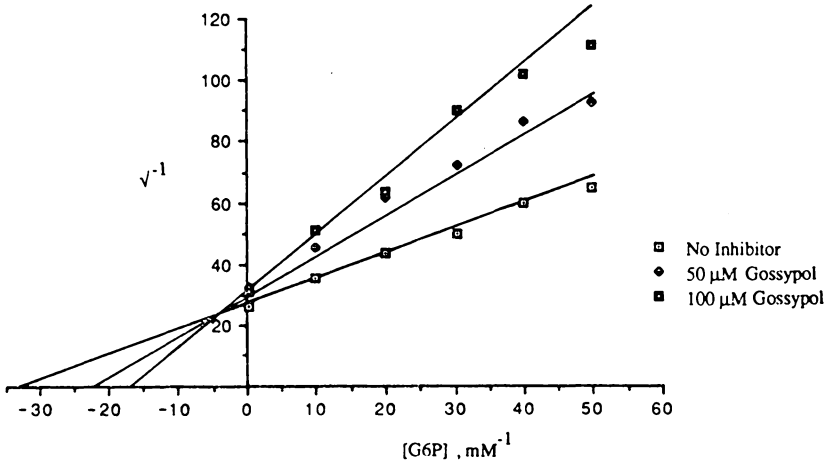
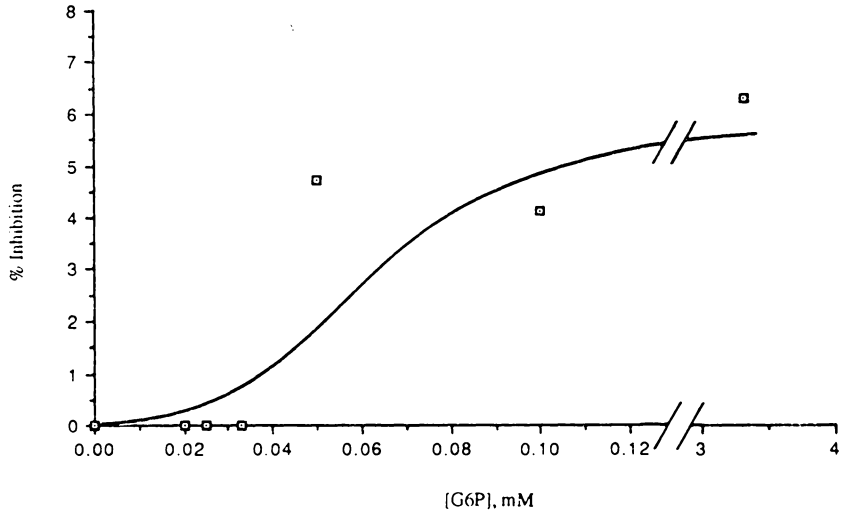


Figure 40-a. Initial velocity and 40-b. Double-reciprocal plots of initial velocity data as glucose-6-phosphate was varied from .020 to 3.33 mM. Assays were performed with the concentrations of gossypol acetic acid indicated on the graph, keeping $NADP^+$ concentrations constant at 1.33 mM. The lines were fit according to the Noncompetitive computer program (Cleland, 1979).

A % Inhibition of Glucose-6-Phosphate Dehydrogenase as [G6P] is Varied



B % Inhibition of Glucose-6-Phosphate Dehydrogenase as [$NADP^+$] is Varied

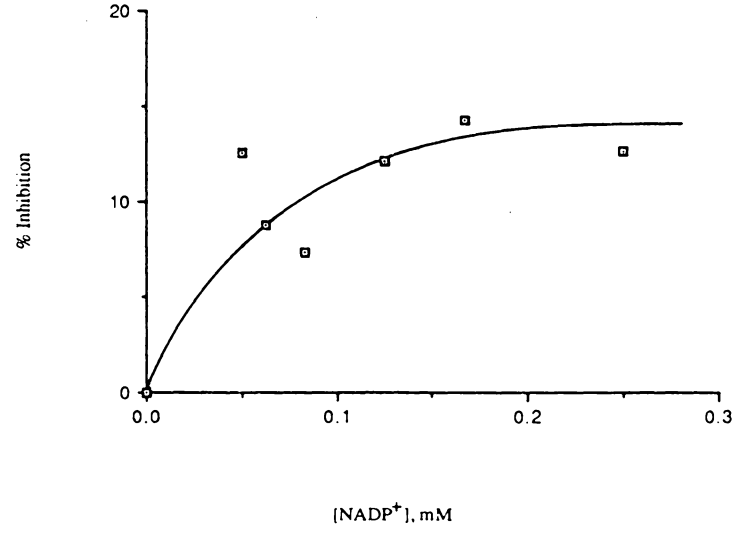


Figure 41. Percent inhibition of bovine adrenal glucose-6-phosphate dehydrogenase by 3.17% ethanol as A: Glucose-6-phosphate was varied from 0 to 3.33 mM, keeping $NADP^+$ concentrations constant at 1.33 mM and B: $NADP^+$ was varied from 0 to .250 mM, keeping glucose-6-phosphate concentrations constant at 3.33 mM.

DISCUSSION

The results of this study demonstrate that adrenal glucose-6-phosphate dehydrogenase is inhibited by micromolar quantities of gossypol acetic acid. This *in vitro* study may provide insight into the mechanism by which testis glucose-6-phosphate dehydrogenase activity was decreased *in vivo* in a previous study performed in our laboratory (The Effects of Gossypol on the Antioxidant Defense System of the Testis, section 3).

The reaction catalyzed by glucose-6-phosphate dehydrogenase generates NADPH reducing equivalents that are necessary as substrates for several metabolic reactions in the testis. The initial and rate limiting step in testosterone biosynthesis, the conversion of cholesterol to pregnenolone, requires three molecules of NADPH (McGilvery 1983). Additional molecules of NADPH are needed by hydroxylases and dehydrogenases in subsequent reactions in the synthesis of testosterone (Tepperman, 1981). Previous studies show decreased serum testosterone concentrations in gossypol treated rats (Gafvels et al., 1984; Hadley et al., 1981) hamsters (Saksena and Salmonsen, 1982) and rabbits (Saksena et al., 1981). Some investigators propose that gossypol causes inhibition of testosterone synthesis by a direct effect on testicular Leydig cells. Leydig cells isolated from gossypol treated rats produced less testosterone than controls when incubated with LH. LH stimulated control Leydig cells also demonstrated decreased testosterone production when incubated with increasing amounts of gossypol (Hadley et al., 1981). The site of the

testosterone biosynthetic pathway that is thought to be inhibited by gossypol is the conversion of cholesterol to pregnenolone (Lin et al., 1981). Gossypol could act by inhibiting glucose-6-phosphate dehydrogenase and thus reduce the number of NADPH molecules available for the conversion of cholesterol to pregnenolone. This mechanism could explain the decreased testosterone production in gossypol treated animals.

The direct effects of gossypol on sperm are not explained by decreased testicular steroid production. Gossypol was effective in decreasing sperm motility in vitro (Chongthammakum et al., 1986) and was investigated as a spermicide for vaginal contraceptive in women (Ratsula et al., 1983). Studies in rats demonstrated damaged to testis germinal epithelium that increased with the length of time on gossypol treatment (Xue, 1981). Ultrasound examination of epididymal spermatozoa and testicular spermatids demonstrated marked damage to the tail region. These effects could be explained by another NADPH requiring reaction, the reduction of glutathione. Glutathione is a tripeptide thiol found in the cytoplasm and mitochondrial matrix of most cells. Reduced glutathione (GSH) is an important part of the tissue antioxidant defense system. One of its most important roles is the removal of hydrogen peroxide and other peroxides. Reduced glutathione also scavenges other species of reactive oxygen such as singlet oxygen and hydroxyl radical. Reactive species of oxygen are known to injure tissues, causing a wide variety of pathological changes (Clark et al., 1985). Glutathione generally reduced these reactive

species of oxygen by undergoining an oxidation reaction to form oxidized glutathione (GSSG). All cellular GSH would soon be oxidized if it were not for the enzymatic system keeping it in the reduced form. Glutathione reductase reduces GSSG, using NADPH as a cofactor: GSSG + $NADPH+H^+ \rightarrow 2GSH + NADP^+$. Inhibition of testicular glucose-6-phosphate dehydrogenase would decrease the number of NADPH molecules available for glutathione reduction and this important component in the antioxidant defense system less effective.

These results are also consistent with the notion that gossypol promotes the formation of reactive oxygen species in human sperm (DePeyster et al., 1984). Thus, it would seem that gossypol intake would damage sperm and spermatogenic epithelium by not only generating excessive amounts of reactive oxygen species, but also by decreasing the antioxidant defense system.

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A MORPHOLOGIC STUDY OF THE EFFECTS OF GOSSYPOL ON THE FEMALE RAT

INTRODUCTION

Gossypol is a yellow polyphenolic pigment (MW 518.54) found in cotton plants of the genus Gossypium. The cottonseed contains the highest concentrations of gossypol, but it is also found in the stem and roots (Adams et al., 1960; Merck Index, 1976).

Investigators first implicated gossypol as an antifertility agent in the 1950's when men and women in several Chinese villages became infertile after the use of crude cooking oil contaminated by gossypol. Since these early reports, the antifertility properties of gossypol were extensively studied in men and male animals (National Coordinating Group on Male Antifertility Agents, 1978), but until recently few reports existed on the effects of gossypol in females.

In the 1950's, Chinese scientists reported a high incidence of infertility and amenorrhea in women from villages using crude cottonseed oil for over one year in the preparation of food (Zatuchni and Osborn, 1981). When cottonseed oil was replaced by soybean oil, these women gradually resumed regular menstrual cycles and many became pregnant (Liu, 1985). Women in the rural Southwestern United States use other gossypol containing parts of the cotton plant in traditional herbal medicines. New Mexican herbologists and curanderos (healers) claim that the extract of cotton root bark causes abortion and subsequently induces menstruation in women (Conway and Slocomb, 1979).

The estrous cycles of gossypol treated nonpregnant rats were investigated in recent studies. Estrous cycle irregularities, cycle duration and days in diestrus increased in rats treated with gossypol (Hahn et al., 1981; Gu and Anderson, 1985; Lagerlof and Tone, 1985; Lin et al., 1985). However, gossypol treatment did not inhibit ovulation in the rat (Hahn et al., 1981).

Gossypol may cause these changes by inhibiting reproductive hormone production because treated pregnant and nonpregnant rats had lower serum progesterone and estradiol than controls (Lin et al., 1985). Other studies show that gossypol treated rats had lower ovarian weights compared to controls (Gu and Anderson, 1985). The decidual reaction was also decreased in gossypol treated pseudopregnant rats compared to controls (Lin et al., 1984). Administration of gossypol to rats in early pregnancy reduced ovum implantation and the number of implanted embryos that were carried to term. When gossypol treated rats were supplemented with progesterone and estradiol in early pregnancy, implantation and litter sizes increased to control values (Lin et al., 1985). An ultrastructural study of rats in early pregnancy showed that gossypol treatment did not prevent implantation, but disrupted endometrial development on day 6 of pregnancy, resulting in fetal death (Rikihisa et al., 1985).

Gossypol is used clinically in China to treat menorrhagia, uterine leiomyoma and endometriosis in women. A histopathologic study of endometrial biopsies from women on chronic low doses of gossypol, revealed endometrial atrophy after 2 to 6 months of treatment. An

ultrastructural study of endometrial biopsies from women on a similar treatment regimen found irregular secretory activity, proliferative changes or atrophy of the endometrium. Some of the ultrastructural changes were consistent with reduction of steroid hormones, but others suggested direct toxic effects to the endometrium. No light microscopic or ultrastructural studies of the endometrium in nonpregnant rats are reported.

The effects of gossypol on the morphology of the testes and spermatozoa of the rat have been studied extensively, however few similar studies are reported on the reproductive organs of the female rat. This study was performed to examine histopathological and ultrastructural changes in the uterus and histopathological changes in other organs of gossypol treated nonpregnant rats and to find morphological evidence of the underlying mechanism of infertility.

MATERIALS AND METHODS

Experimental Animals: Thirty-one adult female Sprague Dawley rats in a weight range of 200 to 235 grams were obtained from Charles River Breeding Laboratory. The rats were housed according to NIH guidelines [DHEW Publication No. (NIH) 80-23] in a temperature (19-22°C) and humidity (50-60%) controlled vivarium. They were assigned to one of three treatments according to a random number table and grouped two or three to a cage. The animals were exposed to a photoperiod of 12 hours of light and 12 hours of darkness each day and had free access to water and commercial rat chow.

Gossypol Treatment: Gossypol acetic acid (Polyscience, Inc., Warrington, PA) was homogenized each morning in a steroid suspending media (Saksena et al., 1981). Each rat was weighed daily and dosed according to body weight for 30 days. The low dose rats received 40 mg/kg, the high dose rats received 60 mg/kg and control rats received the steroid suspending media. Gossypol stock solutions were prepared at different concentrations so that each animal received an equivalent volume of fluid per kg body weight. The gossypol and/or vehicle was administered by gavage through a soft rubber feeding tube each morning between 8:00-11:00.

Estrous Cycles: Daily vaginal smears were prepared from each animal between 8:00 and 10:00 a.m. to determine the stage of the estrous cycle. This procedure was performed each day of the dosing schedule plus the morning of tissue collection (31 days). A normal estrous cycle was defined as a four day sequence characterized by the predominance of nucleated epithelial cells in the cytological preparation (proestrus) on one day, followed the next day by a smear dominated by cornified epithelial cells (estrus) and then two days of smears containing largely leukocytes (metestrus and diestrus). A rat that was cycling normally throughout the experiment would have a maximum of 7.75 cycles in 31 days. Statistical comparison was performed only on rats that survived the entire dosing period.

Tissue Collection: All rats were weighed and then killed by methoxyfluorine anesthesia and cervical dislocation on the morning of day 31. Adrenals were removed and weighed prior to fixation. Tissues

collected for histopathological examination include heart, lung, liver, spleen, pancreas, urinary bladder, kidney, adrenal, stomach, duodenum, ileum, colon, skeletal muscle, salivary gland, mandibular lymph node, brain, pituitary, ovary, left uterine horn and vagina. These tissues were placed in 10% neutral buffered formalin. The right uterine horn was placed in fixative containing 3% glutaraldehyde, 5% formaldehyde and 25% picric acid in 0.1M cacodylate buffer immediately following collection for ultrastructural examination.

Tissue Processing: Tissues collected for histopathological examination were dehydrated in a series of graded alcohols and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined through a light microscope. Tissues fixed for ultrastructural examination were washed twice in 0.1M cacodylate buffer (pH 7.4) for fifteen minutes and post-fixed for 1 hour in 1% osmium tetroxide and 1.5% potassium ferrocyanide. Tissue blocks were stained in 1% uranyl acetate in maleate buffer (pH 5.2), dehydrated in increasing concentrations of ethanol and propylene oxide and embedded in Poly/Bed 812 (Polyscience, Inc., Warrington, PA). Thin 60-90 nm sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined using a JEOL 100 CX-II electron microscope.

Statistics: Adrenal and body weights were compared by analysis of variance and the Duncan's Multiple Range test (Kramer, 1956). P values $<.01$ were considered significant.

RESULTS

Estrous Cycles: The number of regular four day estrous cycles decreased significantly in HD rats compared to control rats (Fig. 42). This difference was even greater when the statistical comparison included estrous cycle data from the rats that died during the dosing period. These rats were eliminated from the comparison to avoid attributing the lack of cycling from a shortened lifespan, to the specific effect of gossypol on the reproductive tract. Six of the HD group rats had periods of diestrus lasting longer than one week. Other abnormalities included cycles lasting longer than four days and irregular sequences in the days of the cycles. The number of estrous cycles in the LD group was also decreased compared to controls, but these differences did not reach statistical significance. One control rat cycled normally for 19 days and remained in diestrus for the remainder of the study.

Clinical Signs: Diarrhea, dehydration and distended abdomens were observed in eight rats after the first week of dosing. Six of these rats were in the HD group, and two in the LD group. Four of the rats in the HD group died prior to the end of the study. One of these rats was extremely dehydrated and had diarrhea prior to death and the other three had no abnormal clinical signs prior to death. Diarrhea in the other affected rats resolved prior to the end of the study. No rats in the control or LD groups died before day 31.

Necropsy and Histopathologic Findings: The control rat that was observed to remain in diestrus for the latter portion of the study had

bilateral multifocal renal abscesses. Suppurative foci were found microscopically in the cortex and medulla of both kidneys, with destruction of tubules and glomeruli. The urinary bladder contained purulent material and the mucosa was hyperplastic. Papillary hyperplasia was found in the mucosa of the urinary bladder and a dense severe purulent inflammation was present in the submucosa. The spleens of most of the treated rats were 1.5 to 2 times larger than control rat spleens. Two LD rats and one HD rat had distended small and large intestines due to excessive content. Histopathological examination of the intestines of one of the affected LD rats revealed a thickened duodenal inner muscular layer which contained small numbers of lymphocytes and neutrophils, but no microscopic lesions were found in the intestines of the other two affected rats. No other significant microscopic lesions were observed, although a few incidental lesions were noted. Small focal collections of macrophages were present in the pulmonary alveoli of the two control group rats. One HD rat had several macrophages and neutrophils in most alveoli throughout the lung. Each of two HD rats had a focus of lymphocytes in one small area of the interstitium of the heart. One LD rat had a small focus of lymphocytes in the pituitary and another focus in the adrenal gland. The thyroid of one HD rat had several follicles containing cellular debris.

Body and Adrenal Weights: Body weights were significantly decreased in the LD and HD groups compared to controls (fig. 43). No statistical differences were found in adrenal weights when compared either by

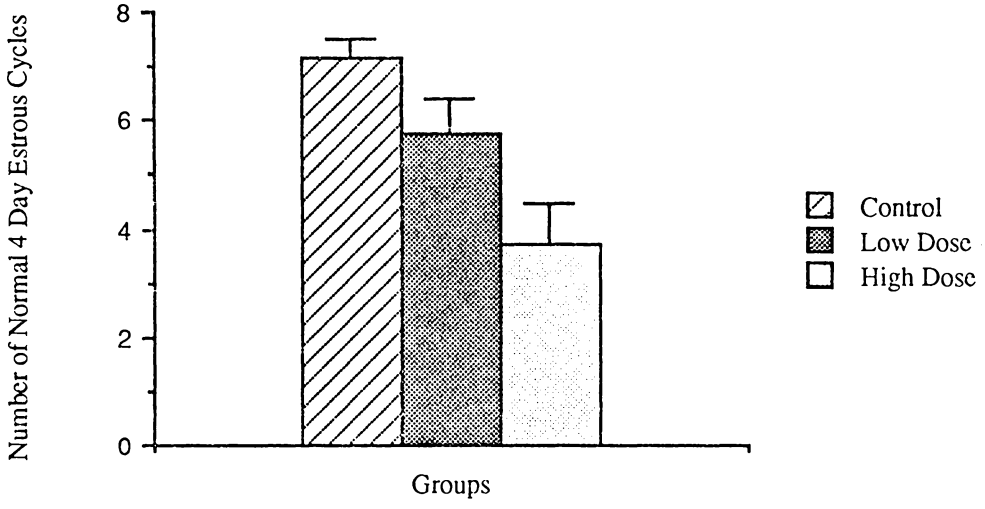
direct means or when ratioed over body weight to correct for differences in total body weight (Figs. 44, 45).

Histopathologic and Ultrastructural Examination of the Reproductive Tract: No morphologic differences were found in the vagina, uterus or ovaries of gossypol treated rats compared to controls at the light microscopic level. Ultrastructural examination of uterine endometrial cells from control rats revealed that the epithelium was composed of tall cuboidal to columnar cells with apical microvilli, moderate numbers of mitochondria and lateral infoldings that were especially prominent in the basal region (Fig. 46). Golgi complexes were prominent and generally present within endometrial cells. The cytoplasm was moderately rich in glycogen and contained moderate amounts of rough endoplasmic reticulum (Fig. 47). The nuclei were characteristically convoluted, filled with euchromatin and contained a single nucleolus (Fig. 48). Large membrane bound vesicles containing amorphous debris and degenerating organelles were found in the supranuclear region of many epithelial cells (Fig. 49). Smaller membrane bound, electron lucent vesicles were found in the apical region of epithelial cells (Fig. 50) and in the subnuclear area (Fig. 51). Junctional complexes were found at the lateral apical regions of cells and moderate numbers of spot desmosomes (macula adherentes) were present along the lateral borders (Fig. 52).

Examination of the endometrium of LD gossypol treated rats revealed no apparent ultrastructural changes. Ultrastructural changes were found in the macula adherentes of the endometrium in six of seven rats in the HD group. Desmosomes showed a wide array of irregularities

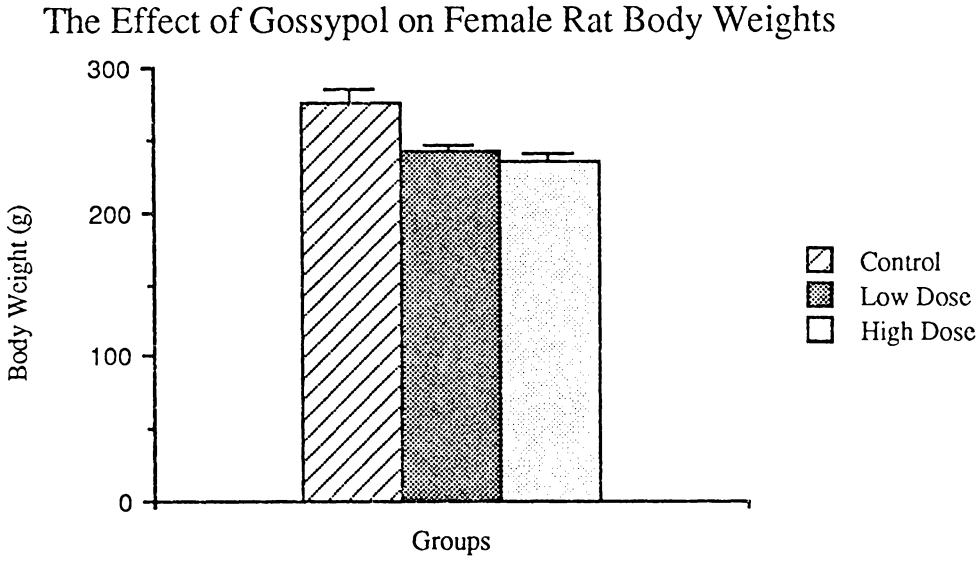
including an increase in number and prominent filamentous interconnections between desmosomes (Fig. 53). Other desmosomes were asymetrical and appeared to be decreased in number (Fig. 54). Some cell junctions had disorganized tonofilaments and desmosomal plaques did not appear to line up with the adjacent cell (Fig. 55). Other desmosomal plaques appeared to be separated (Fig. 56).

The Effect of Gossypol on the Estrous Cycles of Female Rats



CONTROL	LOW DOSE	HIGH DOSE
7.18 ± .31 (10)a	5.75 ± .64 (10)ab	3.72 ± .74 (7)b

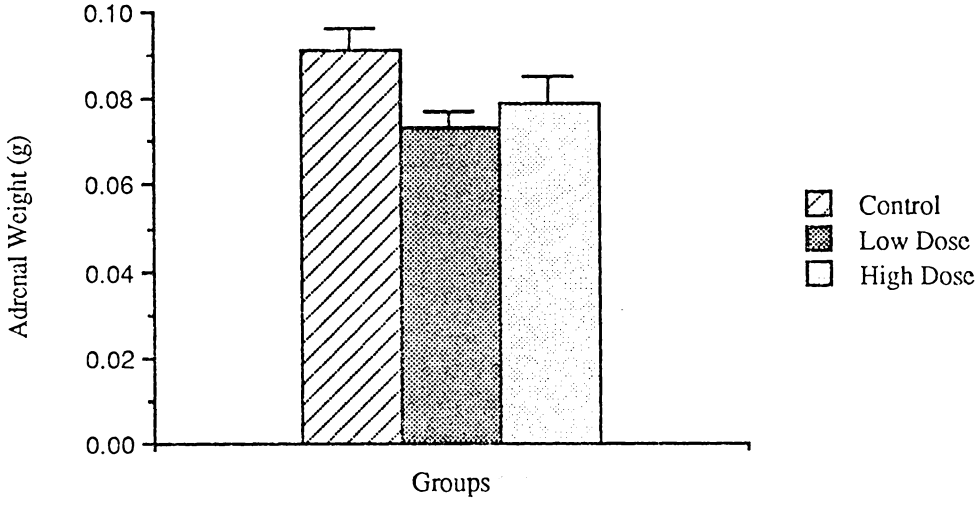
Figure 42. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data were analyzed by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a. Values labeled subscript ab are not significantly different from values labeled a or b.



CONTROL	LOW DOSE	HIGH DOSE
276.3 ± 9.3 (10)a	243.1 ± 3.3 (10)b	235.0 ± 5.5 (7)b

Figure 43. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed by the Duncan's Multiple Range Test ($p < .01$). Subscript b indicates values significantly different from values labeled subscript a.

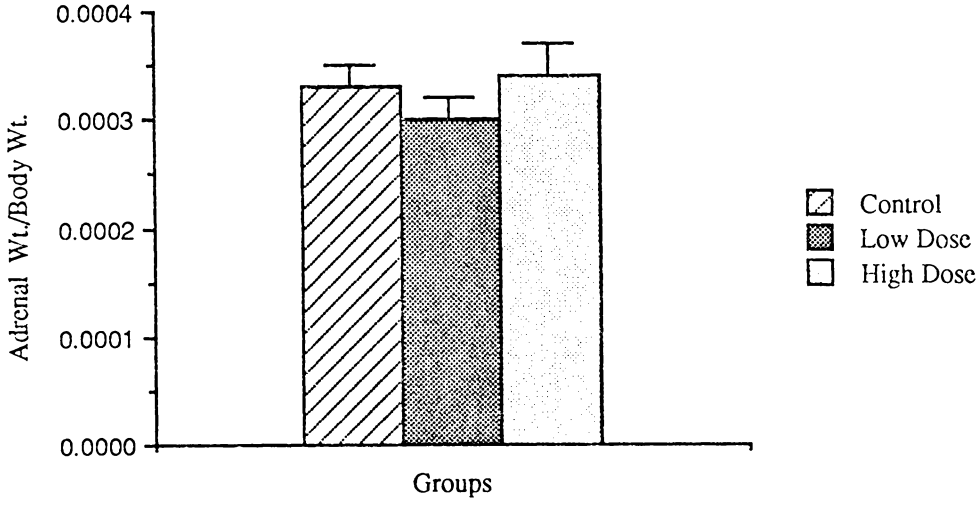
The Effect of Gossypol on Adrenal Weights of Female Rats



CONTROL	LOW DOSE	HIGH DOSE
.091 ± .005 (10)a	.073 ± .004 (10)a	.079 ± .006 (7)a

Figure 44. Values are means ± standard errors as determined in grams. The number of rats in each group is shown in parentheses. Data were analyzed by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

The Effect of Gossypol on Female Rat Adrenal/Body Weight Ratios



CONTROL	LOW DOSE	HIGH DOSE
.00033 ± .00002 (10)a	.00030 ± .00002 (10)a	.00034 ± .00003 (7)a

Figure 45. Values are means ± standard errors. The number of rats in each group is shown in parentheses. Data were analyzed by the Duncan's Multiple Range Test ($p < .01$). No significant differences were found between values labeled subscript a.

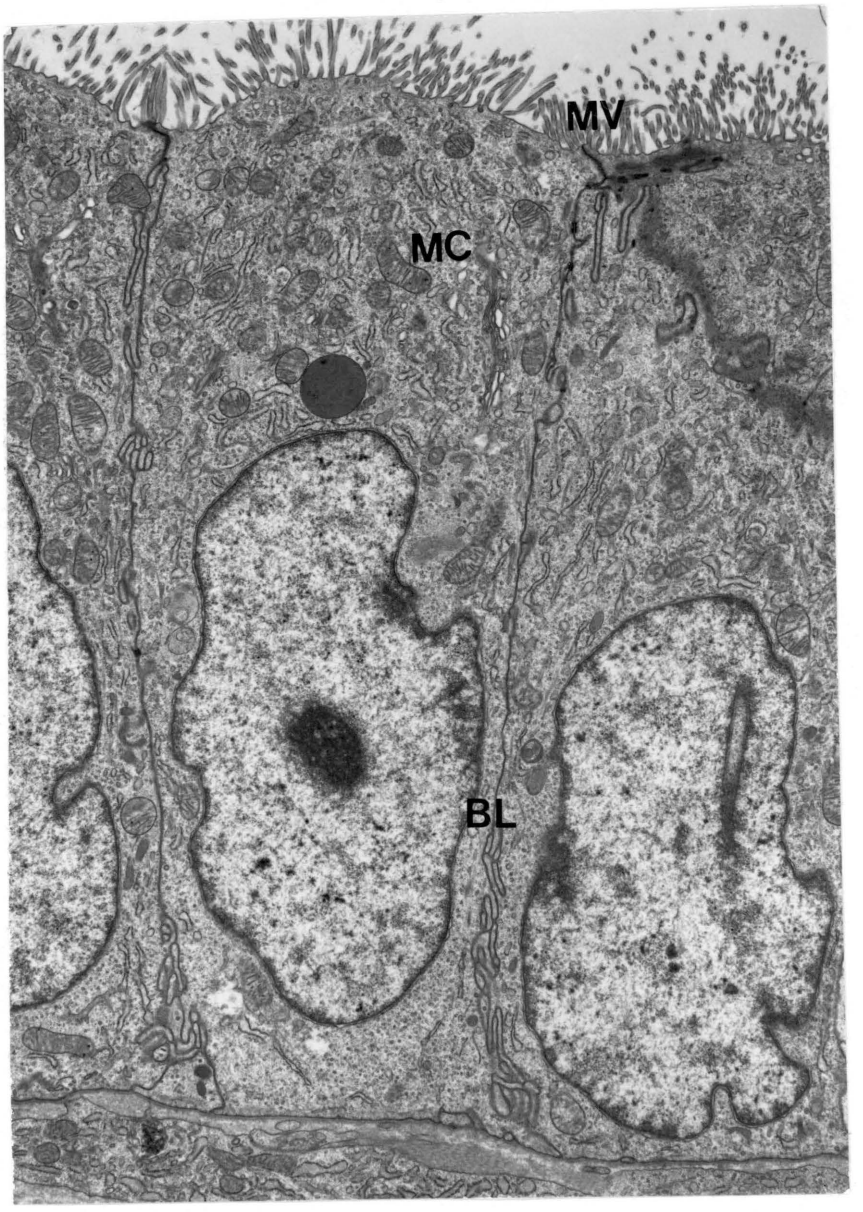


Figure 46. Endometrial cells from a control rat composed of columnar cells with microvilli (MV), mitochondria (MC) and prominent basolateral infoldings (BL) (total magnification = 6300X).

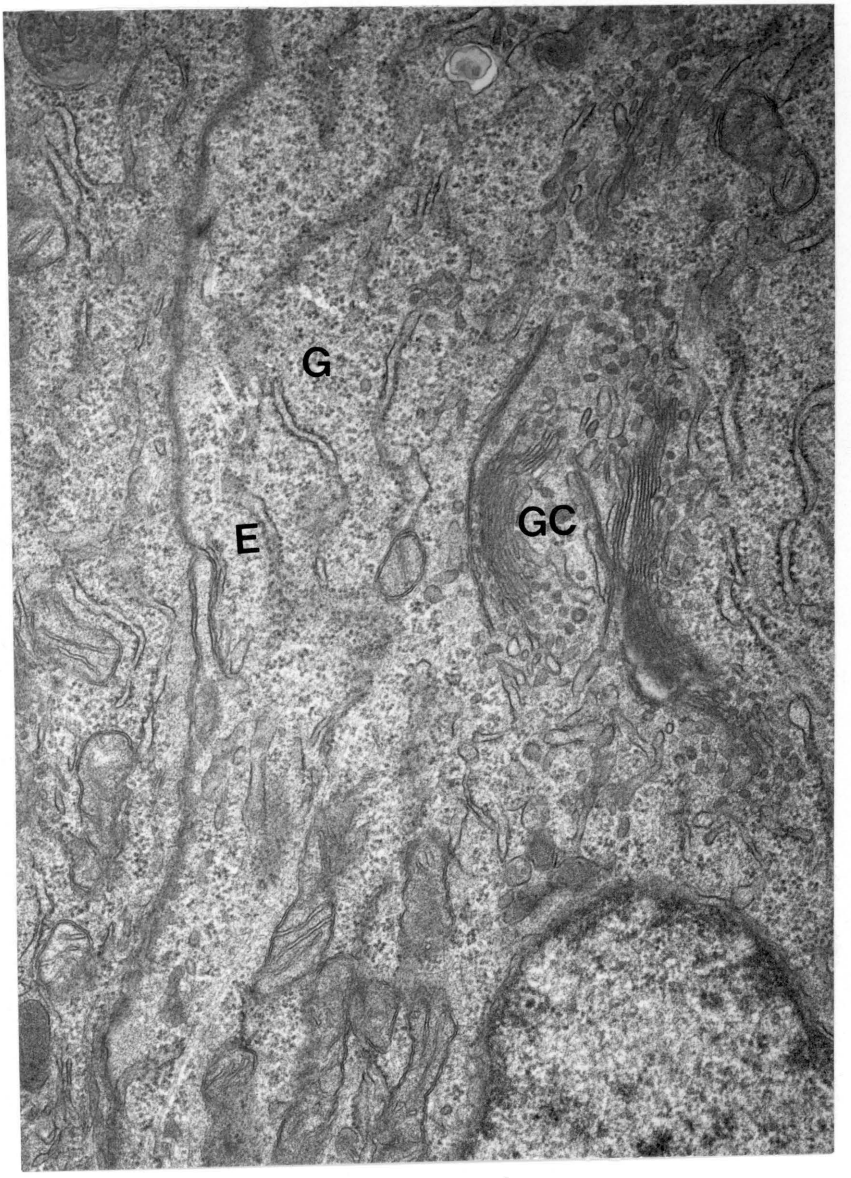


Figure 47. Endometrial cells from a control rat demonstrating golgi complexes (GC), glycogen (G) and rough endoplasmic reticulum (E) (total magnification = 24,500X).

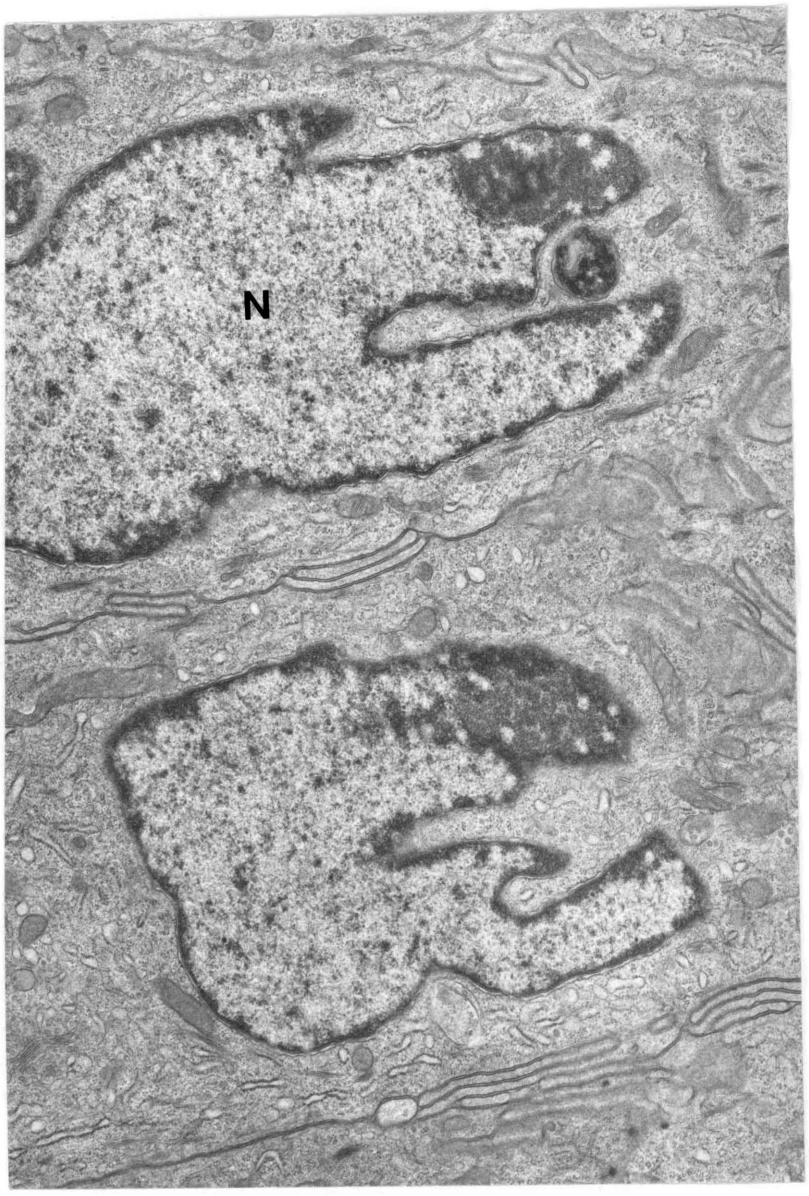


Figure 48. Control endometrial cell nuclei (N) with characteristic convolutions, euchromatin and nucleoli (total magnification = 12,600X).

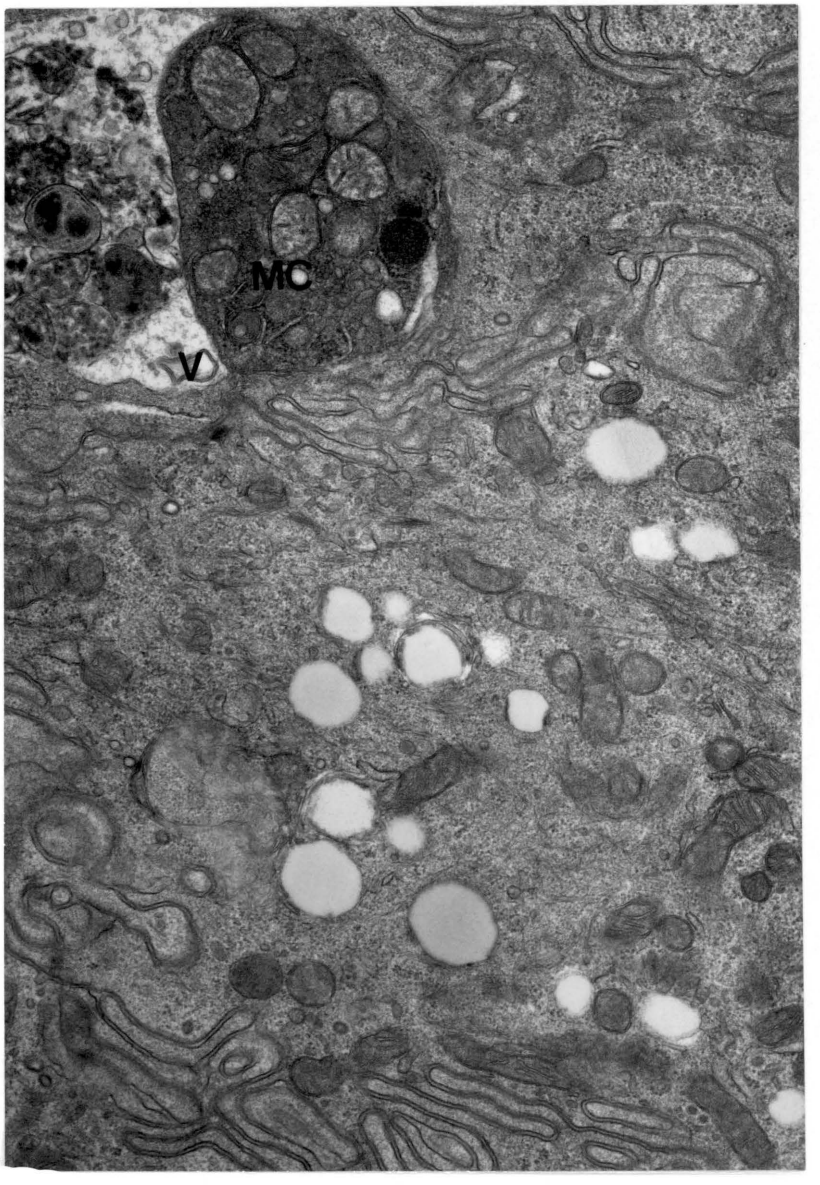


Figure 49. Control endometrial cells with a large membrane bound vesicle (V) containing amorphous debris and degenerating mitochondria (MC) (total magnification = 17,500X).

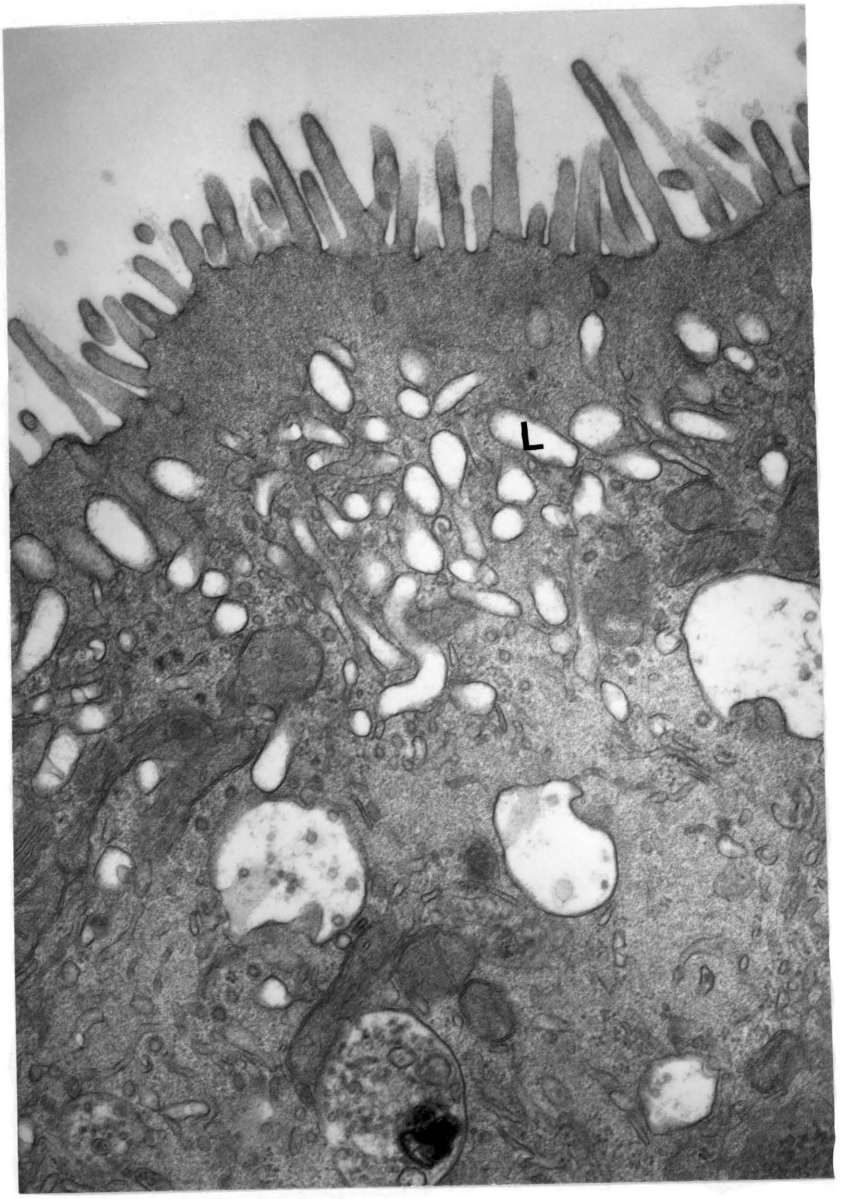


Figure 50. Control endometrial cell with apical electron lucent vesicles (V) (total magnification = 24,500X).

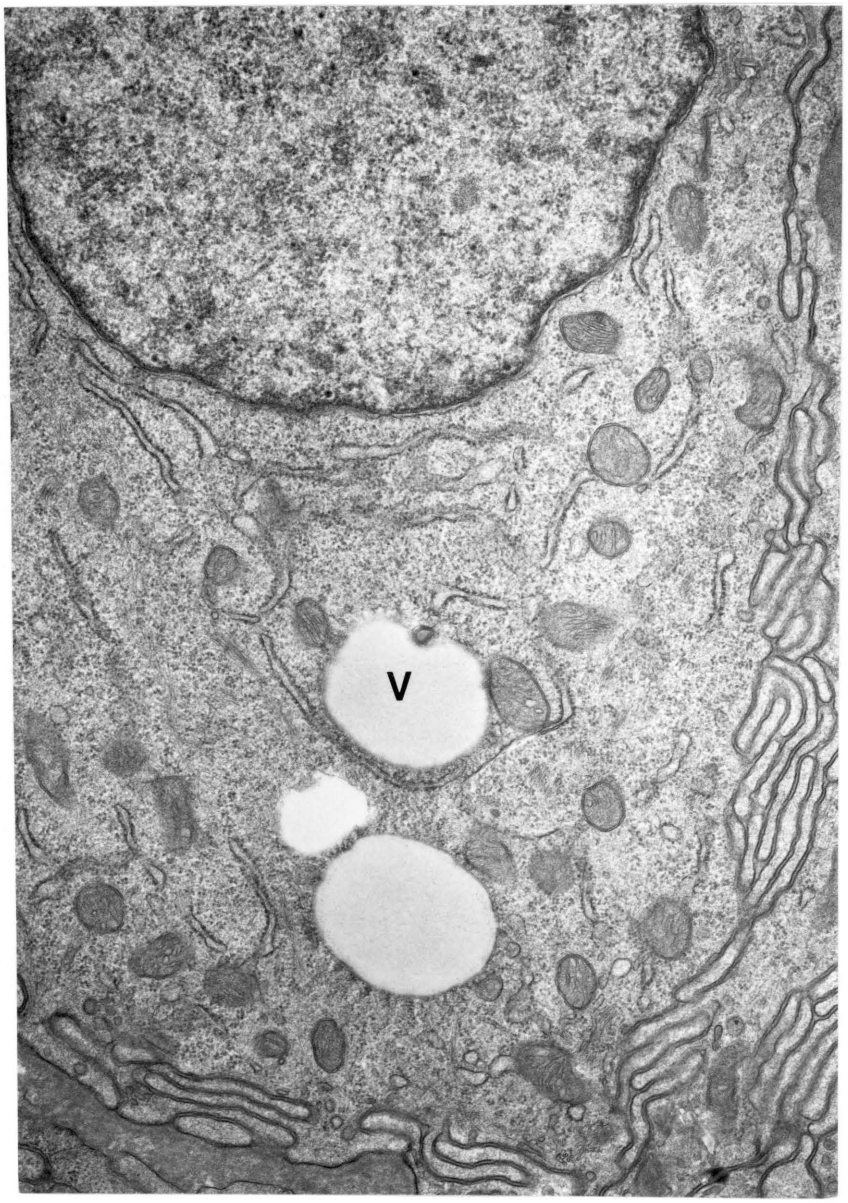


Figure 51. Control endometrial cell with subnuclear vacuoles (V) (total magnification = 17,500X).

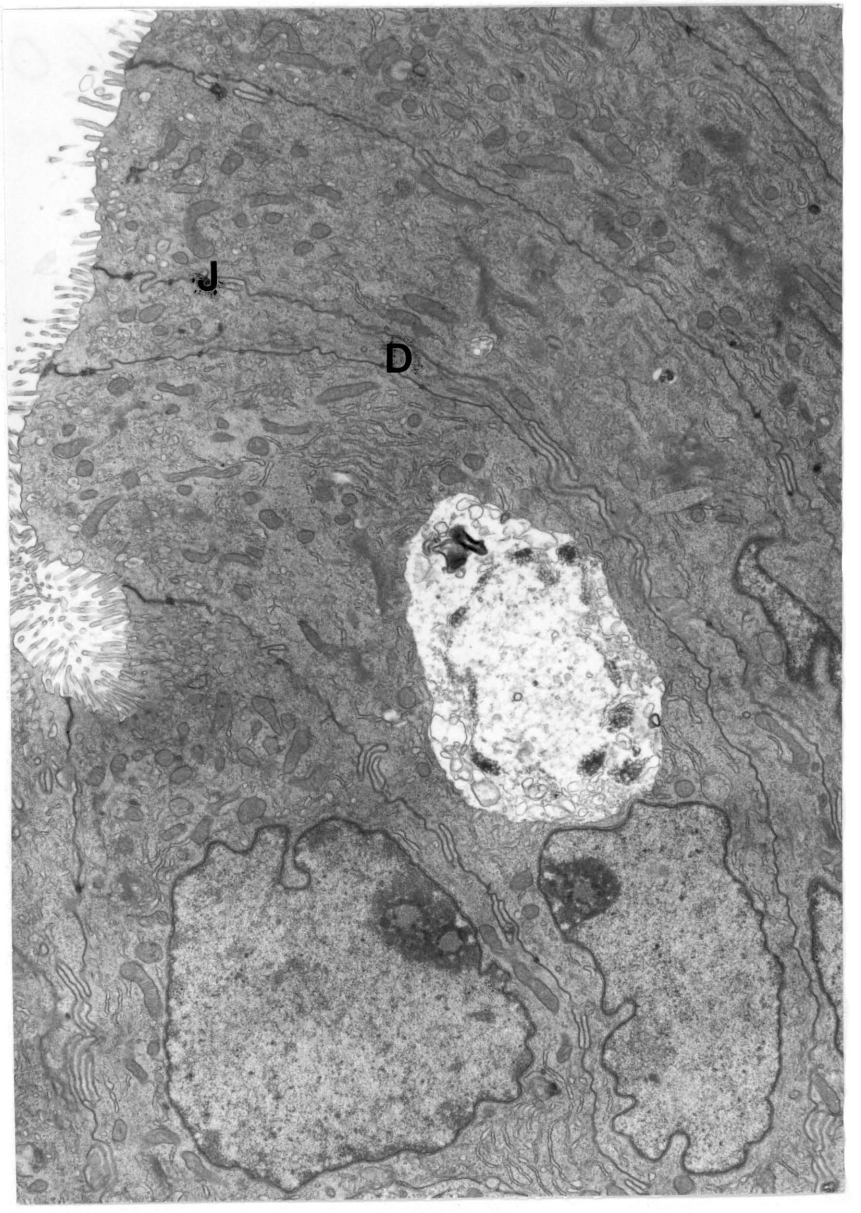


Figure 52. Control endometrial cells with junctional complexes (J) and spot desmosomes (D) (total magnification = 6,300X).

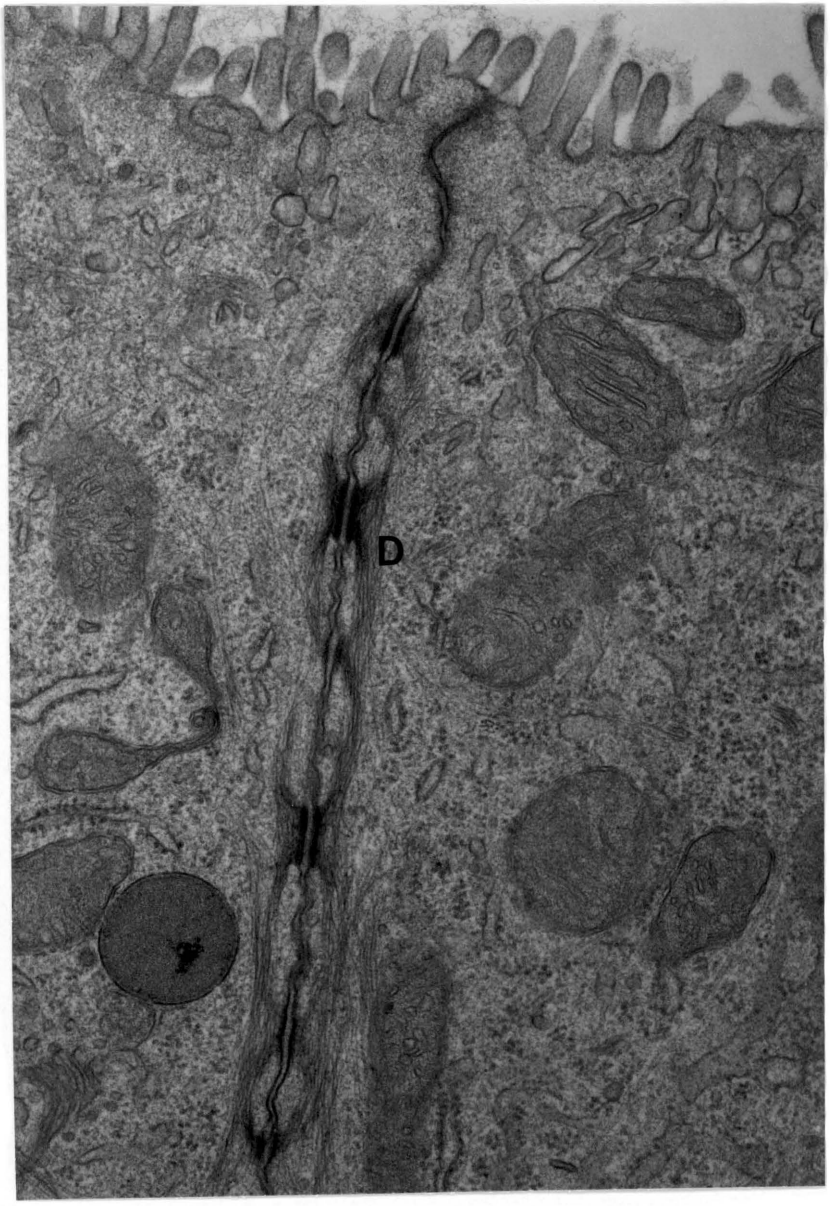


Figure 53. Endometrial cells from a HD gossypol treated rat showing desmosomes (D) with prominent filamentous interconnections (total magnification = 33,250X).

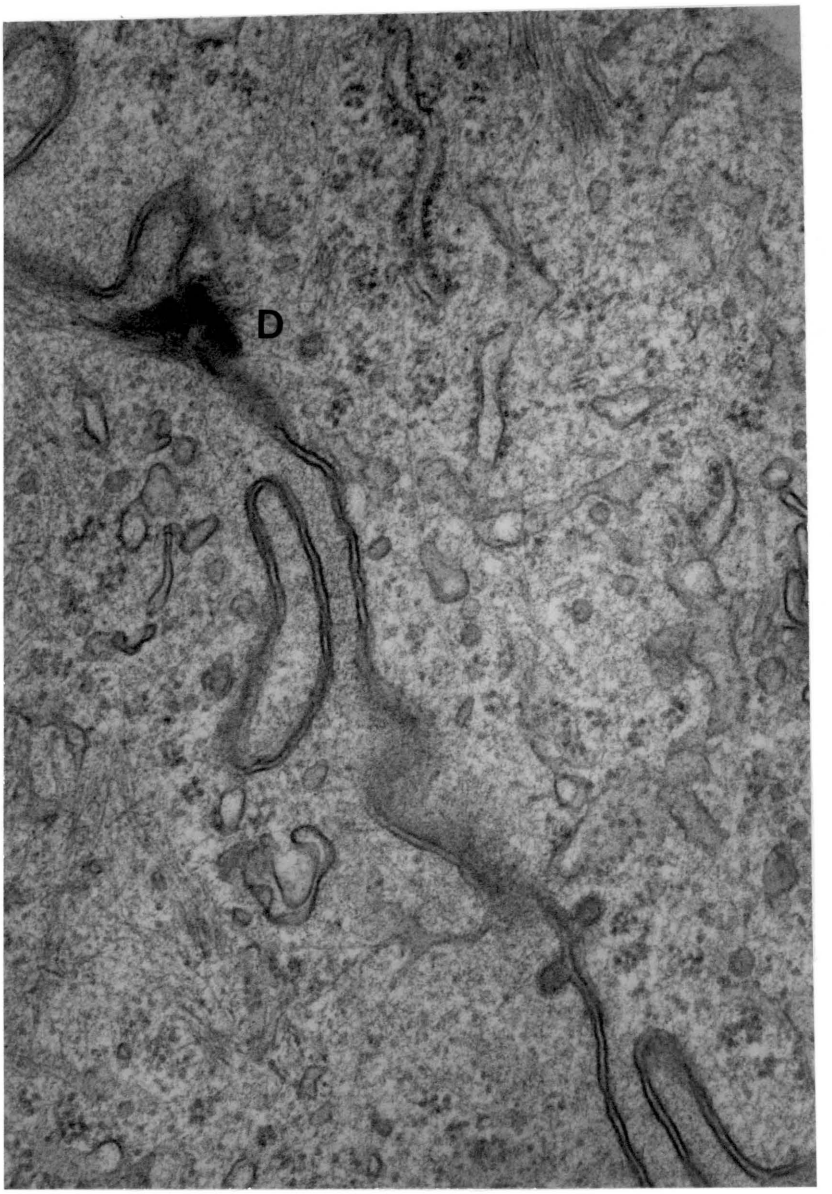


Figure 54. Asymmetrical desmosome (D) from a HD gossypol treated rat (total magnification = 50,750X).

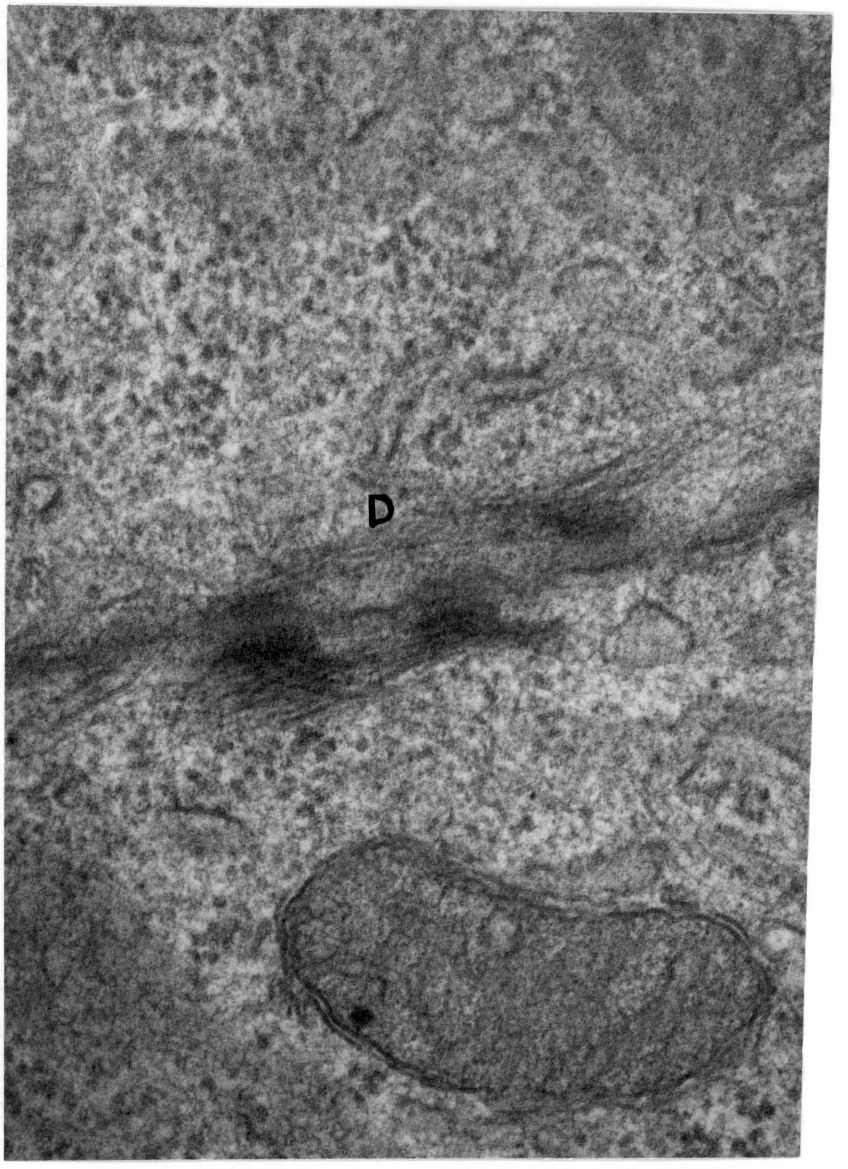


Figure 55. Desmosomal plaques (D) that did not appear to line up from a HD gossypol treated rat (total magnification = 84,000X).

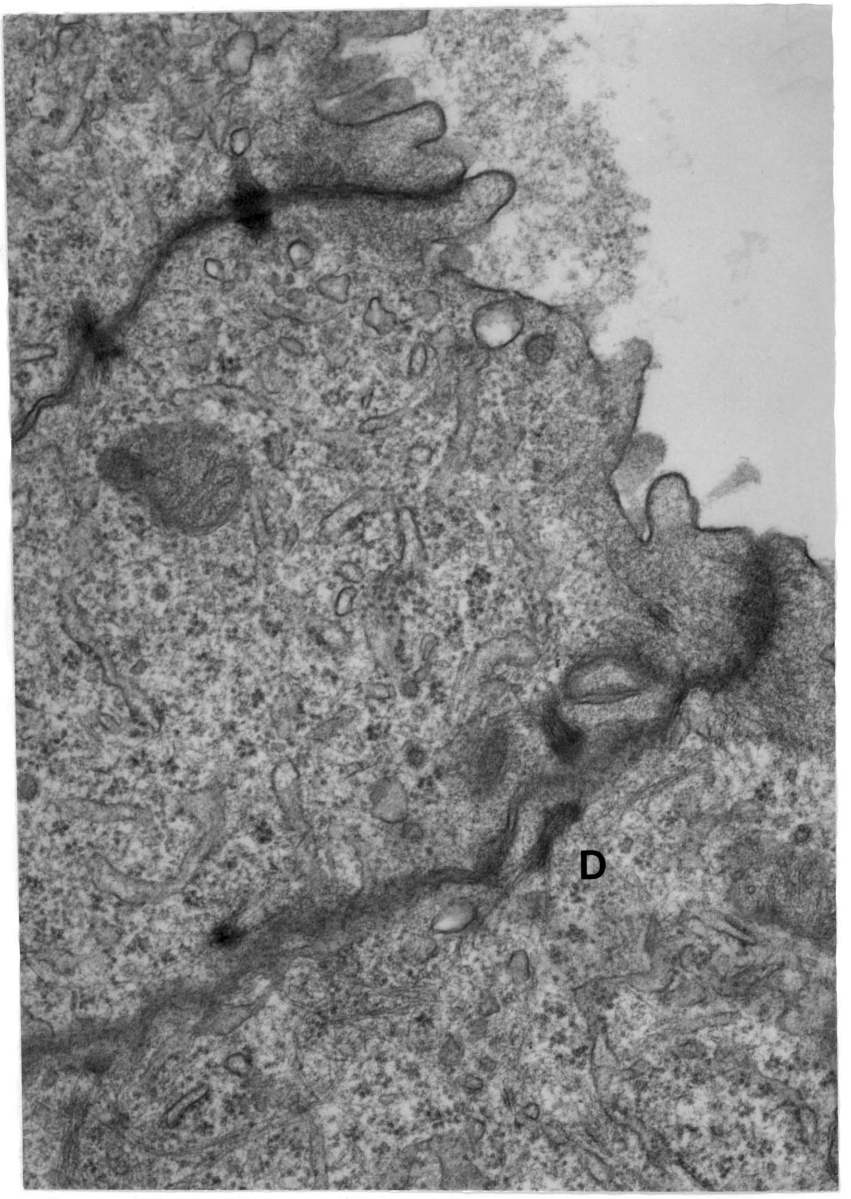


Figure 56. Desmosome (D) from a HD gossypol treated rat that appears to be separated (total magnification = 33,250X).

DISCUSSION

The reduction in the number of normal estrous cycles in gossypol treated rats in this study is consistent with evidence from other recent studies (Hahn et al., 1985; Gu and Anderson, 1985; Lagerlof and Tone, 1985; Lin et al., 1985a). These irregularities may be due to the inhibition of reproductive hormone production by gossypol as previously found (Lin et al., 1985a). Inhibition of estrogen and progesterone production does not explain the results of a recent study using rats treated with micromolar concentrations of gossypol solution, injected directly into one horn of the uterus on the day of breeding (Lin et al., 1985b). The opposite (control) uterine horn of each rat was injected with an equivalent amount of the suspending vehicle. Embryos implanted and developed in the control horn, but failed to implant in the gossypol treated horn. An ultrastructural study of endometrial cells from women on chronic gossypol treatment demonstrated swollen rough endoplasmic reticulum, golgi, and mitochondria with increased numbers of lysosomes (Zhu et al., 1984). These results imply that gossypol has a local effect on the endometrium.

Body weight reductions, consistent with the results of this study, are a common consequence of gossypol treatment in the rat (Zatuchni and Osborn, 1981; Heywood et al., 1986; Lagerlof and Tone, 1985). Two previous studies found that growth rates increased to control values after treatment was discontinued (Beaudin, 1985; Hadley et al., 1981). Some investigators attribute these reductions in weight to decreased food intake following anorexia (Zatuchni and Osborn, 1981; Beaudin,

1985). The diarrhea and dehydration observed in the present study were additional reasons for weight loss in the gossypol treated rats. Diarrhea associated with gossypol treatment was previously reported in rats (Zatuchni and Osborn, 1981; Lagerlof and Tone, 1985; Gafvels et al., 1984), monkeys (Shandilya et al., 1982) and calves (Rogers et al., 1975). The diarrhea in most cases resolved prior to the end of this study. Three gossypol treated rats had evidence of diarrhea and intestinal dilatation on postmortem examination. Intestinal dilatation is a well documented, but poorly understood sequellae to gossypol treatment in the rat (Gafvels et al., 1984; Zatuchni and Osborn, 1981) and mouse (Hahn et al., 1981).

Adrenals were weighed at necropsy and examined microscopically for a crude assessment of stress. No evidence of adrenal hyperplasia was found in any group as determined by comparing adrenal histology, weight, or adrenal to body weight ratios.

Ultrastructural examination of the endometrium revealed changes in macula adherentes in the HD group. Macula adherentes are electron dense plaques found on the lateral borders of epithelial cells, cardiac myocytes and meninges. These desmosomal plaques are thought to provide attachment sites for adjacent cells (Steinberg et al., 1986). They also anchor intermediate sized (10 nm) filaments, often called tonofilaments, to the plasma membrane. These filaments are generally composed of cytokeratin in epithelial cells and are thought to suspend cellular organelles within the cytoplasm to create specialized cytoplasmic domains (Franke et al., 1986). The molecular composition

of the proteins and glycoproteins of desmosomal plaques are only partly characterized and are not well understood (Mattey et al., 1986). It is possible that gossypol causes a derrangement of desmosome formation which may account for its local effects on the endometrium. Cell junctions were less distinguishable and intercellular spaces increased in the endometrium of women on chronic doses of gossypol (Zhu et al., 1984). No previous studies have examined the specific effects of gossypol on desmosomes, but in vitro studies show that gossypol binds to amino groups of many proteins including tubulin (Medrano et al., 1986). This binding inhibits the assembly of tubulin into microtubules (Kanje et al, 1986). If gossypol causes a similar inhibition of desmosome assembly, cell adhesion could be altered as well as the microenvironment within cytoplasmic domains. These changes may make the endometrial surface unfavorable for the implantation of embryos. Further investigation is needed to test this hypothesis.

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