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The Effects of Gossypol (a phytochemical) on the Development of *Trypanosoma musculi* in Two Strains of Mice.

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ABSTRACT

The effect of gossypol on *Trypanosoma musculi* infection in Fawn (FN) male mice indicated that the gossypol administered prior to and simultaneously with inoculation of trypanosomes affected the parasitemic level during the course of infection in favor of the host. In one experiment, gossypol administered to NIH Beige (BG) male mice simultaneously with and one day after an inoculation of a standard dose of 5×10^4 trypanosomes increased the host's resistance to the infection. In the other experiment the parasitemia of the experimental BG mice was generally lower than the infected but untreated controls, however the difference was not statistically significant. The infection was measured by the numbers of trypanosomes at the peak and throughout the course of the infection. The results suggest that in all the experiments, gossypol treated experimental mice developed lower levels of parasitemias when compared with the untreated but infected controls.

Key Words: Gossypol; FN and BG Mice; *Trypanosoma musculi.*

INTRODUCTION

Gossypol is a compound isolated from cottonseed and derives its name from the genus of cotton plants, *Gossypium.* It is a polyphenolic compound that contains two free aldehyde groups and has been found to be toxic to a variety of nonruminant animals (Zatuchni and Osborn, 1981). The compound first gained international recognition for its antifertility activity in males (Briggs and Briggs, 1974; Steinberger and Smith, 1977; Dai et al., 1978; Xue, 1980; Quian and Wang, 1984; Zatuchni and Osborn, 1981). The number of spermatozoa in rats given gossypol orally, was reduced and the spermatozoa underwent ultrastructural changes which resulted in sterility (Nadakavukaren and Sorensen, 1979). There is also evidence that gossypol can induce sterility in hamsters, and rabbits (Chang and Gu, 1980).

Further research has been conducted to examine the pharmaceutical properties of gossypol beyond its antifertility activity. Gossypol inhibited the infection of human amniotic epithelial by herpes simplex virus 2 (HSV-2) (Wichmann et al., 1982). The authors added gossypol and HSV-2 sequentially to cultures of human amniotic epithelial cells. A concentration of 100 μM inhibited the infection totally. When the gossypol was added to the culture after the virus, doses as low as 3 μM were effective. The antiviral activity of gossypol also extends to the human immunodeficiency virus 1 (HIV-1) (Prusoff and Lin, 1993; Polasky and co-workers,1987). Others reported that various types of cancer cell lines have also been shown to be susceptible to the

plant extract gossypol *in vitro* and *in vivo* studies (Wu et al., 1989; Tuszynski and Cossu, 1984; Tso, 1984). According to Gonzalez-Garza and Martin (1993) gossypol has a potent *in vitro* anti-amoebic effect against *Entamoeba histolytica*. The gossypol enantiomer (-), which has a lower affinity for proteins, had a stronger effect on the *E. histolytica* cultures.

Gossypol has also been shown to be a potent trypanocidal agent *in vitro* (Montamat and Burgos, 1982; Blanco and Aoki, 1983). The epimastigote stage of *Trypanosoma cruzi* was found to be immobilized and structurally altered when exposed to gossypol (Blanco and Aoki, 1983). Gossypol had a similar effect on the trypomastigote stage of *T. cruzi* . Trypomastigotes exhibited a reduction in motility and structural change when exposed to varying concentrations of gossypol (Rovai and Aoki, 1990).

Pharmaceutical treatments for trypanosomiasis with gossypol have a wide range of effectiveness and the reported side effects of gossypol are relatively mild and seem to be well tolerated (National Coordinating Group on Male Fertility, 1978). Current trypanocidal drugs are few and varying degrees of effectiveness are possible (Gill and Sen,. 1964; Hawking and Sen, 1960). The present investigation has been designed to investigate the effects of gossypol as an antiprotozoan agent using a nonpathogenic strain of stercorarian hemoflagellate, *Trypanosoma musculi* and Fawn (FN) and NIH (BG) strain of male mice.

Our results indicate that gossypol treated animals developed a certain degree of resistance against *T. musculi* infection in all the experiments.

MATERIALS AND METHODS

Three experiments were conducted using FN and BG male mice. Both strains were selected from breeding colonies maintained in the Parasitology Laboratory at Virginia State University. The selected animals weighed in the range of 23g-28g. *Trypanosoma musculi* strain used for the study was obtained from Howard University, Washington, DC. The trypanosomes were maintained in Swiss Webster (SW) mice population by intraperitoneal (i/p) injection of infected blood washed in 1% sodium oxalate solution (1g/100mL physiological saline) as described by Lincicome and Watkins (1963).

In the first experiment, a total of 15 BG male mice were used to determine the effects of gossypol as antiprotozoan agent against *T. musculi* development *in vivo*. Mice in the prior treated group died within two days in the first trial (Table 1, Experiment I). In the second experiment, 19 BG male were employed (Table 2, Experiment II). In the third experiment, 27 mice were selected from the FN strain (Table 3, Experiment III). In these experiments, the control groups received trypanosome infection, but did not receive any drug. Mice were housed individually in polypropylene cages with stainless steel wire lids. The animals were given Purina Laboratory Chow® and water ad libitum. The animal room temperature was maintained between 23° and 25° C.

Lethal dose 50 (LD_{50})

Gossypol was tested by i/p injection in concentrations of 150, 200, 250 and 300 μg to determine the LD_{50} in mice. Four mice were used at each dosage level. A 250 μg inoculum caused paralysis and shortness of breath and death in 50% of the treated mice. Some of the older mice weighing over 28g could tolerate the 300 μg dosage. A dose of 250 μg of gossypol was determined to be the LD_{50} for mice weighing 23-28g.

TABLE 1. *Trypanosoma musculi* parasitemia in BG male mice with and without gossypol treatment . (Experiment I).

Days	Group Mean (Trypanosomes in thousands/mm^3 of blood)* ± SD		
	Simultaneous (5)**	After (5)	Control 5)
7	2.00 ± 0.0a	4.00 ± 0.7b	7.60± 0.8c
9	4.8 ± 1.3b	3.60 ± 1.3b	16.8 ± 5.2a
11	27.2 ± 7.5$^+$ab	16.8 ± 5.3b	35.6 ± 4.7a
13	22.8 ± 5.7ab	16.4 ± 5.4b	38.4 ± 9.8$^+$a
15	14.8 ± 3.2b	19.6 ± 5.7$^+$ab	36.8 ± 8.4a
17	7.20 ± 2.3a	12.8 ± 2.5a	15.2 ± 6.9a
19	0.80 ± 0.9a	0.80 ± 0.9a	5.60± 2.9a

* Group means within any sampling period followed by different letters (a, b, c) are significantly different $P<0.05$. Mean separation by Duncan's multiple range test. The a, b, c, symbols used to interpret Duncan's multiple range test.
** Number of animals used.
+ Peak Parasitemia

TABLE 2. *Trypanosoma musculi* parasitemia in BG male mice with and without gossypol treatment. (Experiment II).

Days	Group Mean (Trypanosomes in thousands/mm^3 of blood)* ± SD			
	Prior (4)**	Simultaneous (6)	After (3)	Control (6)
7	2.0 ± 1.2b	2.40 ± 1.2b	37.0 ± 26.3a	2.60 ± 0.8b
9	2.6 ± 1.9a	4.00 ± 0.8a	30.5 ± 23.5a	5.40 ± 1.6a
11	8.6 ± 3.9a	3.60 ± 1.0a	38.0 ± 30.8$^+$a	3.4 ± 4.4a
13	6.6 ± 3.0a	2.60 ± 1.0a	24.0 ± 18.1a	11.6 ± 2.0a
15	9.6 ± 3.3$^+$a	15.6 ± 5.3$^+$a	31.0 ± 20.4a	29.6 ± 8.5$^+$a
17	5.0 ± 3.1a	5.00 ± 2.3a	8.00 ± 3.6a	7.60 ± 1.3a
19	1.0 ± 1.0a	4.00 ± 2.0	4.60 ± 1.0a	4.00 ± 1.5a

* Group means within any sampling period followed by different letters (a, b) are significantly different $P<0.05$. Mean separation by Duncan's multiple range test. The a, b symbols used to interpret Duncan's multiple range test.
** Number of animals used.
+ Peak Parasitemia

TABLE 3. *Trypanosoma musculi* parasitemia in FN male mice with and without gossypol treatment. (Experiment III).

Days	Group Mean (Trypanosomes in thousands/mm^3 of blood)* ± SD Prior (5)**	Simultaneous (6)	After (6)	Control I (5)	Control II (5)
7	4.40± 1.0a	1.40± 1.1a	1.60 ± 0.7a	4.40 ± 1.7a	2.80 ± 1.0a
9	12.0 ± 4.2$^+$a	4.60± 1.6a	7.00 ± 2.9a	10.0 ± 2.7a	11.2 ± 2.4a
11	5.20± 0.8b	11.0 ± 3.4$^+$b	13.0 ± 2.4ab	20.0 ± 4.3a	7.20 ± 0.8b
13	8.80± 1.5b	4.00± 0.8b	25.6 ± 6.8$^+$a	25.3 ± 7.3$^+$a	12.8 ± 3.5$^+$ab
15	4.80± 1.9a	2.60± 0.9a	6.40 ± 2.9a	7.20 ± 2.1a	6.40 ± 1.7a
17	4.00± 1.7a	1.60± 0.7a	1.60 ± 1.4a	2.40 ± 1.2a	2.4 ± 1.2a

* Group means within any sampling period followed by different letters (a, b) are significantly different $P<0.05$. Mean separation by Duncan's multiple range test. The a, b symbols used to interpret Duncan's multiple range test.
** Number of animals used.
+ Peak Parasitemia

Standard dose (gossypol)

Gossypol - (1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl[2,2'-binaphthalene]-8,8'-dicarboxaldehyde), molecular weight of 518.54, empirical formula $C_{30}H_{30}O_8$ (Edwards, 1958) is one of fifteen pigments that have been isolated from certain species of cotton plants. Gossypol is a yellowish pigment which is concentrated in the resin glands of the cotton seed (Marchlewski, 1989). It occurs in three tautomeric forms: the aldehyde (the major tautomeric form), the hemiacetal and the phenolic quinoid . The compound is markedly reactive and exhibits acidic properties. Early methods of extracting gossypol used petroleum and diethyl ethers, this ether extracted gossypol was termed free gossypol. Gossypol that reacted with proteins in the seed was termed bound gossypol because it could not be extracted using solvents (Zatuchni and Osborn, 1981). For this investigation the compound was obtained from Sigma Chemical Company and stored at 0°C. A stock solution was prepared by dissolving 2000 μg of the test compound (gossypol) to 2 ml of ethanol. An aliquot of 0.20 mL of the solution was injected by i/p injection as a standard dose of inoculum to ensure administration of 200 μg of the drug in the experimental animals.

The timing of the administration of the standard dose of gossypol was designed after the procedure of Sen et al. (1995/1996). The dose of gossypol consisted of (a) a single dose of 200 μg administered intraperitoneally (i/p) to 5 mice in Experiment I, 4 animals in Experiment II and 5 other in Experiment III, a day before i/p inoculum of 50,000 trypanosomes per mouse, (b) 200 μg administered by i/p injection of gossypol to 5 mice in Experiment I, 6 mice in Experiment II and 6 other mice in Experiment III, infected simultaneously with 50,000 trypanosomes per mouse, (c) and 200 μg administered by i/p injection of gossypol to 5 mice in Experiment I, 3 mice in Experiment II, and 6 other in Experiment III, a day after trypanosome inoculation. Limited number

of FN mice were available at the time of this study. The control counterparts in all experiments (5 in Experiment I, 6 in Experiment II, and 5 in Experiment III) were inoculated with a standard dose of 50,000 trypanosomes but did not receive gossypol. In the third experiment, a second control group was added which was administered with the standard inoculation of trypanosomes and injected intra- peritoneally with 0.20 ml of solvent (ethanol).

Parasitemias in all animals were determined by estimations of trypanosome cell populations in blood taken from the tail of mice during a 7-19 day post inoculation period. Numbers of trypanosomes were estimated by standard hemocytometer counts at 2 day intervals starting at day 7 of infection (Hartsell and Sen, 1997). One way ANOVA test was used to analyze the data.

RESULTS

The parasitemic levels in two experimental groups, after (post treated) and simultaneously treated, in one experiment (Experiment I) showed no difference in parasite numbers on most days of experimentation. These differences were not significant at five percent level of significance. BG male mice treated simultaneously with and after inoculation with a standard dose of 5×10^4 *Trypanosoma musculi* developed resistance against the trypanosomiasis. The parasitemic levels in those groups were significantly lower when compared to the untreated but infected controls on certain days of observation (Table 1, Fig. 1). The parasitemia in the simultaneously treated group was significantly lower than the controls on days 7 and 15 PI (post infection). On day 7 PI, the simultaneously treated group developed an average of 2,000 trypanosomes/mm^3 of blood which was significantly lower when compared to an average of 7,600 trypanosomes/mm^3 of blood in the controls on the same day of observation. On day 9 PI, the simultaneously treated group had a significantly lower level of parasitemia of 4,800 trypanosomes/mm^3 of blood compared to an average of 16,800 trypanosomes/mm^3 of blood in the control counterpart on the same observation period. The mice receiving the treatment of gossypol simultaneously with a standard dose of trypanosome inoculation had an average cell population of 14,800 trypanosomes/mm^3 of blood on day 15 PI compared to an average of 36,800 trypanosomes/mm^3 of blood in the control on the same day of observation. These differences in parasitemic levels were significant at the 5% level of significance (Table 1).

During days 7-19 PI, the BG male mice receiving gossypol after a trypanosome infection developed a lower level of parasitemia as compared to the controls. The parasitemic levels in the after treated group were significantly lower than the level observed in the untreated controls during days 9 through 13 PI. On day 9 PI, the average parasitemia of the after group was 3,600 trypanosomes/mm^3 of blood which was significantly lower as compared to an average of 16,800 trypanosomes/mm^3 of blood in the untreated control group. On day 11 PI , the mice receiving the treatment after the trypanosome inoculation developed an average of 16,800 trypanosomes/mm^3 of blood which was significantly lower as compared to an average of 35,600 trypanosomes/mm^3 of blood in the controls. On day 13 PI, the mice receiving treatment after the trypanosome inoculation developed a parasitemic level of 16,400 trypanosomes/mm^3 of blood compared to an average of 38,400 trypanosomes/mm^3 of blood in the control counterpart. This difference in parasitemic level was significant at the 5% level of significance (Table 1).

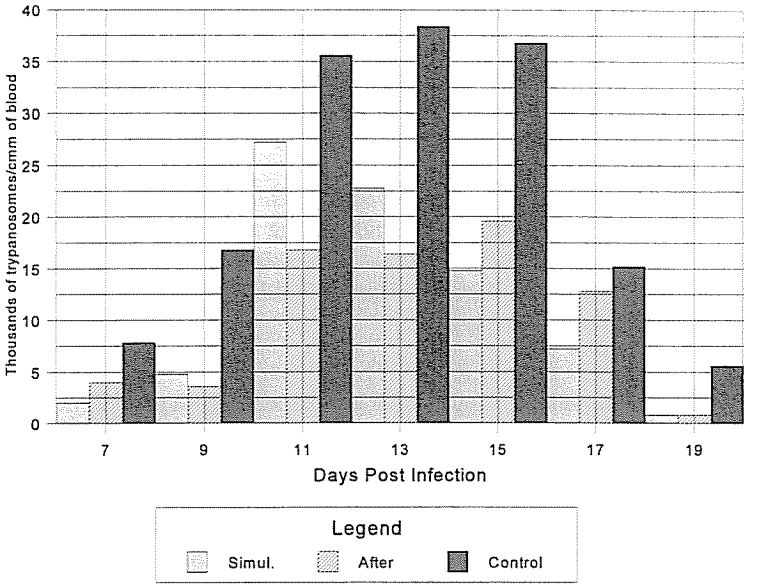

Fig. 1. *Trypanosoma musculi* parasitemia in BG male mice with and without gossypol treatment. Experiment I.

In a second trial (Experiment II), BG male mice treated with gossypol prior to and simultaneously with an inoculation of a standard dose of 5×10^4 trypanosomes also developed resistance against infection. Although these differences in parasitemic levels were not different from the level observed in the control counterpart at the 5% level of significance. The after treated group showed an elevated level of parasitemia when compared to the other experimental groups and controls on days 7 through 19 PI. These differences were not significant at the 5% level of significance. On most of the observation period, the parasitemic levels of the experimental groups were not significantly different from each other (Table 2, Fig. 2).

In another trial (Experiment III), a new FN strain of mice were employed. FN male mice treated with gossypol prior to and simultaneously with a standard dose of inoculum of *T. musculi* developed lower parasite population when compared to the untreated but infected controls (I) on days 11 and 13 PI (Table 3, Fig. 3). On day 11, the mice receiving gossypol prior to the inoculation of trypanosomes, developed an average of 5,200 trypanosomes/mm^3 of blood compared to an average of 20,000 trypanosomes/mm^3 of blood in the controls (I). This difference was found to be significant at the 5% level of significance. On day 11, the group of mice receiving the treatment simultaneously with an inoculation of trypanosomes developed an average of 11,000 trypanosomes/mm^3 of blood compared to 20,000 trypanosomes/mm^3 of blood in the controls (I). This difference was also significant at the 5% level of

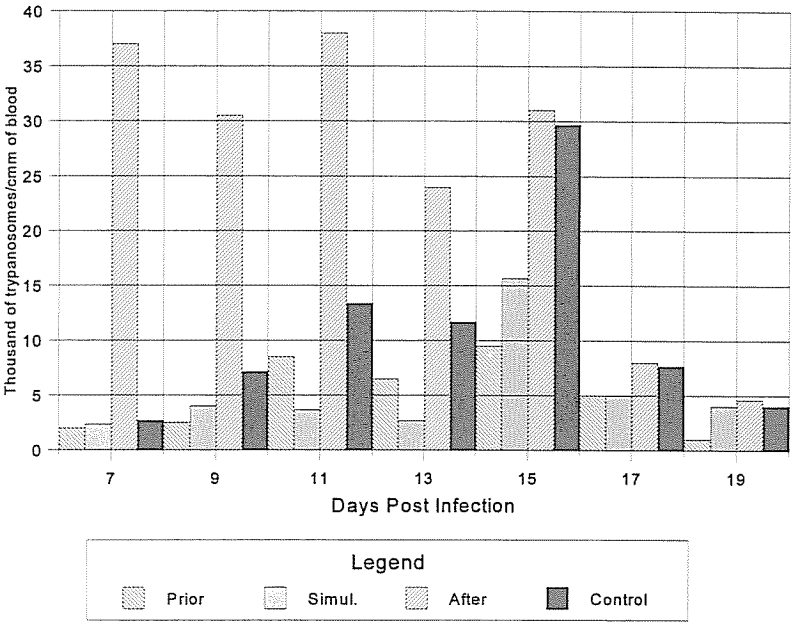

Fig. 2. *Trypanosoma musculi* parasitemia in BG male mice with and without gossypol treatment. Experiment II.

significance. On day 13 PI, the simultaneously treated group developed an average of 4,000 trypanosomes/mm^3 of blood which was significantly lower when compared to an average of 25,300 trypanosomes/mm^3 of blood in the control I counterpart on the same day of observation. On day 13 PI, the prior treated group had a significantly lower level of parasitemia of 8,800 trypanosomes/mm^3 of blood compared to 25,300 trypanosomes/mm^3 of blood in the control I on the same observation day. The after treated group showed no difference in parasitemic level when compared to the level in the controls on most of the observation period. Also, the parasitemic levels between the experimental groups were not significantly different from each other on most of the observation days. The parasite numbers of the control II group (a new incorporation in this experiment) failed to show any difference in parasitemic levels compared to the experimental and control I groups on most days of experimentation.

DISCUSSION

The results of the present investigation show that BG male mice administered gossypol simultaneously with and after inoculation with *Trypanosoma musculi* generally increased their resistance to the infection. The parasitemic levels in all the BG experimental male mice in one experiment (Experiment I) were significantly lower when compared to the untreated but infected controls on certain observation days. In Experiment II, BG male mice treated with gossypol prior to and simultaneously with

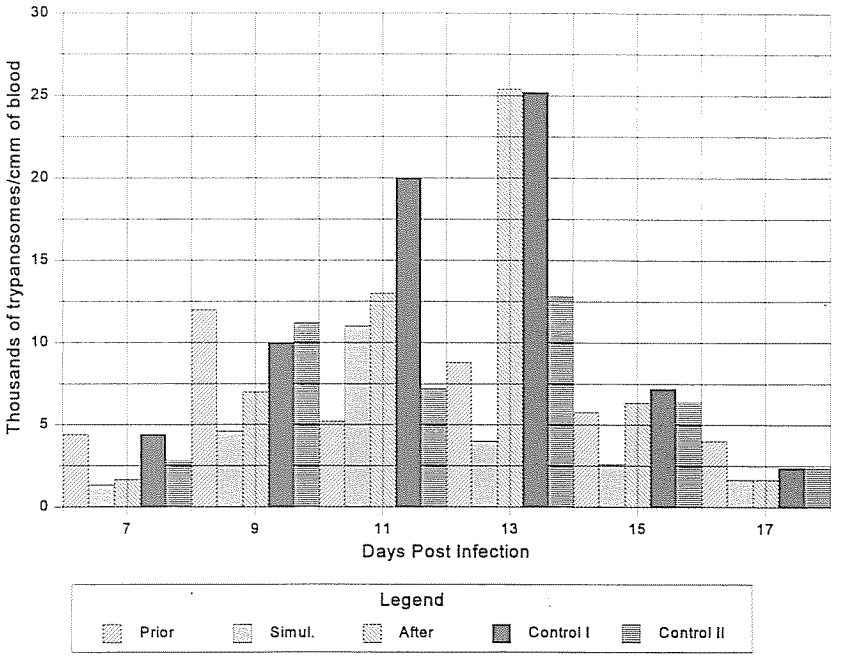

Fig. 3. *Trypanosoma musculi* parasitemia in FN male mice with and without gossypol treatment. Experiment III.

an inoculation of trypanosomes developed resistance to the infection. Although these differences in parasitemic levels were not significantly different than the level observed in the untreated control. The investigation also showed FN male mice receiving gossypol prior to and simultaneously with an inoculation of trypanosomes also developed resistance to trypanosomiasis. In Experiment III, FN male mice treated with gossypol one day prior to and simultaneously with an inoculation of *Trypanosoma musculi* developed a lower parasite population when compared to the control counterpart. Physiological differences in the two strains used in this investigation may account for the variance in the effectiveness of the gossypol treatment.

Preliminary studies indicate the action of gossypol is inhibition of lactate dehydrogenase (Maugh, 1981; Segal, 1985). Further studies indicate that it may have a broader scope and inhibit other NAD-linked enzymes. *Trypanosoma cruzi* containd the enzyme α-hydroxyacid dehydrogenase which is involved in energy generation for flagellar movement. When the parasites were incubated in 50 and 100 μM of gossypol only a few were observed as motile. The inhibitive effect of gossypol on spermatozoa is related to its ability to interfere with the enzyme lactate dehydrogenase isozyme X (LDI-X), which is specific to spermatozoa (Maugh, 1981; Burgos et al., 1986). In *T. cruzi* α-hydroxyacid dehydrogenase is believed to have a similar function to LDI-X. Gossypol can also inhibit oxidoreductase enzymes from *T. cruzi* (Gerez de Burgos et al., 1984). The same result was reported after 30 min in a 25 μM concentration. It was

also noted that the epimastigotes were rounded and contained various membranous structures that could not be related to any known structure (Blanco and Akoi, 1983). In another study examining gossypol and its effects on α-hydroxyacid, parasites incubated for 5 minutes in 100 μM gossypol were reported as completely immobilized. It was also found that concentrations of gossypol as low as 0.01 μM markedly reduced the growth rate of *T. cruzi* in culture (Montamat and Burgos, 1982).

Rovai and Aoki (1990) examined the effects of gossypol on the trypomastigotes and amastigotes of *Trypanosoma cruzi*. Ultrastructural damage was noted in the kinetoplast-mitochondrion complex in the epimastigotes. Motility of the trypomastigotes was decreased greatly by the presence of gossypol in the media. The introduction of serum albumin was found to decrease the effectiveness of the gossypol and higher concentrations were needed to achieve the same effect.

Other studies have indicated gossypol is able to inhibit enzymes and interfere with energy metabolism. In tumor cell lines treated with gossypol the destruction of mitochondria was observed accompanied by a decrease in intracellular ATP. This may suggest that gossypol's cytotoxic effects may result from the uncoupling of oxidative phosphorylation (Tuszynski and Cossu, 1984). A study by Lee and Moon (1982) suggested that LDH isozymes and MDH may be inactivated by gossypol. It was also found that gossypol can be partially blocked by NADH which would indicate that the process of inactivation may be coenzyme-binding-site-directed. It has been well documented that gossypol is capable of enzyme inhibition and in particular the enzyme LDH-X which is found in sperm (Lee and Moon, 1982; Montamat and Burgos, 1982; Tuszynski and Cossu, 1984). Gossypol particularly inhibits enzymes involved in redox reactions and that involve NAD and NADP cofactors (Coyle and Levante, 1994).

From the present investigation it is impossible to determine the mode of action of gossypol on *T. musculi* development in BG or FN strains of mice. The mechanism by which gossypol causes damage to the protozoan organism is still not clearly understood. Rodent trypanosome infections have been shown to vary depending on strain of the isolate, and the age and strain of the host (Dusanic, 1975; Lee et al., 1978). Toxicity remains an obstacle to the pharmaceutical use of gossypol and further information in this area may contribute to the development of new trypanocidal agents. Further research is also needed to fully ascertain the potential of gossypol as a trypanocidal agent.

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Andrew Colley submitted this piece of work to the Faculty of the Graduate School, Virginia State University, in partial fulfillment for the Degree of Master of Science, September 1996. Dr. Dilip Sen served as Thesis Advisor for this project.

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