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Brief communication

Effectiveness of albumin-conjugated gossypol as an immunogen to prevent gossypol-associated acute hepatotoxicity in rats

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ABSTRACT

Gossypol is a highly reactive compound present in cotton (*Gossypium* spp.). The aim of this work was to determine whether the administration of gossypol conjugated to albumin can immunize rats and thereby prevent the acute hepatotoxicity associated with gossypol. The first experiment consisted of administering the immunogen gossypol–BSA, with or without the Freund's incomplete adjuvant, to rats. The production of antibodies against gossypol was subsequently verified. The second experiment comprised three groups of Wistar rats: VG, CG and CO. The rats from the VG cohort were injected with gossypol–BSA associated with Freund's incomplete adjuvant, and the animals from the CG and CO groups were injected with saline solution. After 21 days, the rats from the VG and CG cohorts were treated with 30 mg/kg of gossypol by intraperitoneal injection, whereas the rats from the CO group received corn oil. After 24 h, the rats were evaluated for clinical signs of pathology, and their serum was biochemically analyzed. It was found that gossypol promoted hepatotoxic effects that were not prevented by the administration of gossypol–BSA. In conclusion, the administration of gossypol–BSA associated with Freund's incomplete adjuvant may be lightly to prevent the acute hepatotoxicity associated with gossypol.

1. Introduction

Cotton (*Gossypium* spp.) is a plant grown primarily for use in the textile industry for its fiber content. Cottonseed oil is also harvested as a by-product of cotton and is often used as a high-protein supplement for animal feed; however, it contains gossypol (2,2-bi(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene), a polyphenolic yellow pigment produced by cotton pigment glands present in the roots, branches, leaves, and seeds (Randel et al., 1992; Soto-Blanco, 2008; Gadelha et al., 2011). The function of this compound is to deter many insects from feeding on the plant (Abdurakhimov et al., 2009; Kong et al., 2010).

Gossypol is a highly reactive compound that rapidly binds to a range of substances, including minerals and amino acids. Iron is the most important mineral capable of binding to gossypol, and in so doing, it produces a gossypol–iron complex that causes iron deficiency, thereby affecting the synthesis of hemoglobin (Abou-Donia, 1976; Lindsey et al., 1980). However, this complex is found recently a hepatoprotective and less toxic than gossypol (El-Sharaky et al., 2009). Experimentally, gossypol has been shown to induce apoptosis and inhibit proliferation in a variety of cells (Gilbert et al., 1995; Jiang et al., 2004; Huang et al., 2006; Balakrishnan et al., 2008; Xu et al., 2009).

Signs of gossypol toxicosis include decreased growth rate, anorexia, labored breathing, and dyspnea (Randel et al., 1992). In males, gossypol causes a reduction in sperm motility and concentration (El-Sharaky et al., 2010; Guedes and Soto-Blanco, 2010; Nunes et al., 2010; Gadelha et al., 2011) and also affects the expression of the androgen receptors in Leydig and Sertoli cells from rat testes (Timurkaan et al., 2011). In females, the estrous cycle, folliculogenesis and early embryonic development may also be affected by gossypol (Randel et al., 1992; Gadelha et al., 2011). Moreover, gossypol is hepatotoxic (Wang and Lei, 1987; Haschek et al., 1989; Manabe et al., 1991; Deoras et al., 1997) and causes cytotoxic effects in lymphocytes, leading to immunodeficiency (Quintana et al., 2000; Xu et al., 2009; Braga et al., 2012).

Some preventive measures are carried out to minimize or eliminate the toxic effects of gossypol; these methods include heat treatment of grains (Arieli, 1998), pelleting of the diet, dietary supplementation with ferric sulfate (Soto-Blanco, 2008), dietary supplementation with selenium (sodium selenite) (El-Mokadem

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et al., 2012) and dietary supplementation with vitamin E (Velasquez-Pereira et al., 1998). Another preventative measure could be the use of vaccines to stimulate the production of neutralizing antibodies against the toxin. This type of vaccine has been developed to overcome the toxic effects of various plants (Stewart et al., 1988; Edgar et al., 1998; Filipov et al., 1998; Rice et al., 1998; Than et al., 1998; Lee et al., 2003; Tong et al., 2007; Tong et al., 2008). The objective of this study was to determine whether the administration of gossypol-conjugated albumin can immunize rats against the hepatotoxic effects of gossypol.

2. Materials and methods

2.1. Preparation of the immunogen

Bovine serum albumin (BSA – Sigma–Aldrich, St. Louis, MO, USA) and (±)-gossypol–acetic acid (Fluka, Steinheim, Germany) were used to prepare the gossypol–BSA conjugates as follows. Gossypol (4.9 mg) was dissolved in 1.2 ml of methanol, and BSA (32 mg) was dissolved in 9.0 ml of phosphate buffered saline (PBS); these two solutions were then combined by continuous stirring at 5 °C for 48 h in the dark. The final product was lyophilized.

2.2. Measurement of antibodies by ELISA

ELISA technology was employed for determination of antibody titers against gossypol using two polystyrene flat-bottom plates from MaxSorp (Nunc, Roskilde, Denmark) and Immunolon (Thermo Scientific, Waltham, MA, USA). The assay was performed as described below. Gossypol–BSA was diluted in PBS at 50 μg/ml for the Nunc plate and 100 μg/ml for the Immunolon plate; this conjugate was added to wells in 100 μl aliquots. Both plates were incubated overnight (16 h) at 4 °C and then washed with 3 × 300 μl/well of 0.05% Tween in PBS. Wells were filled with 200 μl/well of 2% BSA in PBS (blocking solution) and incubated for 1 h at 37 °C. After incubation, the solution was removed, and the wells were washed with 3 × 300 μl/well of 0.05% Tween in PBS.

Sera samples were diluted at a 1:5 ratio in PBS, and 100 μl aliquots of diluted serum were added to the wells in triplicate. Following incubation for 3 h at 37 °C, the solution was removed, and the wells were washed with 3 × 300 μl 0.05% Tween in PBS. After washing, 100 μl/well of 1/10,000 diluted rabbit anti-rat peroxidase-conjugated IgG (Rheabiotech, Campinas, SP, Brazil) was added to the Immunolon plate. In parallel, 100 μl/well of 1/10,000 diluted rabbit anti-rat phosphatase-conjugated IgG (Rheabiotech, Campinas, SP, Brazil) was added to the Nunc plate. Both plates were incubated for 1 h at 37 °C and then washed with 3 × 100 μl/well of 0.05% Tween in PBS.

O-phenylenediamine (OPD) substrate (1 mg/ml in citrate buffer and 3% H_2O_2) was added to the Immunolon (peroxidase-conjugated) plate at 100 μl/well. After incubation for 1 h at 25 °C in the dark, the peroxidase–OPD reaction was blocked by adding 50 μl/well of 2 N sulfuric acid. The absorbance was measured at 492 nm (ASYS Expert Plus microplate reader, Biochrom, Cambridge, UK). For the Nunc plate (alkaline phosphatase conjugated), para-nitrophenyl phosphate (pNPP) substrate (1 mg/ml in diethanolamine buffer pH 9.6) was added at 100 μl/well. The alkaline phosphatase–pNPP reaction was measured at 405 nm (ASYS Expert Plus microplate reader, Biochrom, Cambridge, UK).

2.3. Animals

The Wistar rats used in the present study were obtained from the Animal Sciences Department, UFERSA, Mossoró, RN, Brazil. The average weight of the rats was approximately 100 ± 15 g, and they were provided regular rodent chow (Labina, Purina, São Lourenço da Mata, PE, Brazil) *ad libitum* and given free access to tap water. During the entire study period, the animals were housed in cages under hygienic conditions in a controlled environment with a 12-h light/dark cycle that was maintained at 24 ± 3 °C.

The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal Rural do Semi-Árido.

2.4. Trial 1: evaluation of antibody production in mice immunized against gossypol

Three cohorts of five female and five male Wistar rats (mean weight 224 g) each were enrolled in the study. The rats from the first group were immunized with 0.2 mg of gossypol–BSA in 0.2 ml of PBS. The second group was injected with 0.2 mg of gossypol–BSA in 0.2 ml of PBS/Freund's incomplete adjuvant (Sigma Chem. Co., St. Louis, MO, USA – 1:1, v/v). The rats from the third group served as a control and were injected with 0.2 ml of PBS. All injections were subcutaneous. After 30 days, all rats were deeply anesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (60 mg/kg) to collect blood samples from the vena cava. The sera were frozen for storage until antibody detection.

2.5. Trial 2: determination of the efficacy of the immunization

Three cohorts of Wistar rats (mean weight 90 g) were formed: VG (seven female and eight male rats), CG (six female and six male rats) and CO (five female and five male rats). Rats from the VG group were injected subcutaneously with 0.2 mg of gossypol–BSA in 0.2 ml of PBS/Freund's incomplete adjuvant (1:1, v/v). Animals from the CG and CO groups were injected subcutaneously with 0.2 ml of PBS. After 21 days, rats from the VG and CG groups were dosed with 30 mg/kg of gossypol dissolved in Mazola corn oil (1.0 ml/kg) by intraperitoneal injection. Rats from the CO group received 1.0 ml/kg of Mazola corn oil by intraperitoneal injection. The rats were monitored closely for 24 h. After this period, all surviving rats were deeply anesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (60 mg/kg) to collect blood samples from the vena cava. The sera were frozen for storage. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined by using specific commercial kits (InVitro®, Itabira, MG, Brazil) and a semiautomatic analyzer (HumaStar 300®, Wiesbaden, Germany). Fragments of liver, kidneys and lungs were collected and fixed in 10% formalin. The paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological analysis.

2.6. Statistical analysis

The data were statistically analyzed using BioEstat software (version 5.0). The Kruskal–Wallis' test was employed and then followed by Student–Newman–Keuls' test to compare groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Trial 1

No rats died following gossypol–BSA administration. The antibody titers against gossypol were determined by indirect ELISA (Table 1). The optical densities obtained using the peroxidase–OPD reaction and the alkaline phosphatase–pNPP reaction were very similar. The mean optical density observed in rats treated with gossypol–BSA associated to Freund's incomplete adjuvant was statistically higher ($p < 0.05$) than in the other two groups.

3.2. Trial 2

No rats presented any sign of poisoning following gossypol–BSA administration. Within 24 h of the gossypol administration, three male and four female rats from the VG group died. Depression was the only clinical sign observed in the seven females and five males from this group. At necropsy, the observed gross pathologies included ascites, fibrin deposition in the abdominal cavity, adhesions between the liver and diaphragm, firm viscera, and yellow nasal discharge.

In the CG group, only one female rat died within 24 h of gossypol administration. Depression and severely labored breathing was observed in a female rat from the CG group. Necropsy revealed ascites, fibrin deposition in the abdominal cavity, adhesions between the liver and diaphragm, and firm viscera. The animals of the CO group demonstrated no clinical signs or gross pathology at necropsy.

The assessment of serum biochemistry (Table 2) revealed that the activity of AST and ALT was significantly higher in rats from the CG and VG groups than in those from the CO group. Micro-

Table 1
Optical density determined by ELISA as a measurement of antibody titers against gossypol in mice treated with 0.2 mg of gossypol–BSA (G1), 0.2 mg of gossypol–BSA associated to Freund's incomplete adjuvant (1:1, v/v) (G2), or 0.2 ml saline (G3).

Chromogen	G1	G2	G3
Peroxidase–OPD	0.686 ± 0.153[a]	1.460 ± 0.110[b]	0.561 ± 0.016[a]
Alkaline phosphatase–pNPP	0.680 ± 0.172[a]	1.580 ± 0.122[b]	0.576 ± 0.017[a]

[a,b] Different letters indicate a significant difference ($p < 0.05$, Kruskal–Wallis test followed by Student–Newman–Keuls).

Table 2
Serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in rats injected with 0.2 mg of gossypol–BSA associated to Freund's incomplete adjuvant (1:1, v/v) (VG) or saline (CG and CO) and dosed 21 days thereafter with 30 mg/kg of either gossypol dissolved in corn oil (VG and GC) or only corn oil (CO).

	CO (n = 10)	CG (n = 11)	VG (n = 8)
AST (in U/l)	84.8 ± 3.75[a]	383.3 ± 20.9[b]	286.5 ± 64.1[b]
ALT (in U/l)	77.0 ± 4.56[a]	291.9 ± 22.9[b]	261.0 ± 77.1[b]
ALP (in U/l)	755.6 ± 53.2	725.9 ± 120.4	824.7 ± 154.8

[a,b] Different letters indicate a significant difference ($p < 0.05$, Kruskal–Wallis test followed by Student–Newman–Keuls).

scopic evaluation of liver fragments from CG and VG cohorts revealed the occasional presence of hepatocytes with strong cytoplasmic eosinophilia and nuclear pyknosis (Fig. 1), observed in five rats from CG group and six rats from VG group. No microscopic lesion was found in CO group.

4. Discussions

The effects of toxic plants can be reduced by vaccination, which induces the production of antibodies that neutralize the toxic compounds. Some vaccines have been developed to prevent poisoning of livestock by several plant species, such as *Lolium rigidum* (annual ryegrass) infected by the bacterium *Corynebacterium toxicum* (Than et al., 1998), *Lupinus* spp infected by *Diaporthe toxic* (Edgar et al., 1998), *Festuca arundinacea* (''tall fescue'') infected with endophytes (Filipov et al., 1998; Rice et al., 1998), *Lantana camara* (Stewart et al., 1988), *Delphium* spp (larkspur) (Lee et al., 2003), and *Oxytropis kansuensis* (locoweed) (Tong et al., 2007; Tong et al., 2008). Vaccination to protect sheep against prolonged exposure to cotyledoside, a neurotoxic bufadienolide present in some plants such as *Tylecodon wallichii* and *Tylecodon ventricosus*, has also shown some promise (Botha et al., 2008).

The immunogenic compound assessed in this study was gossypol-conjugated bovine serum albumin; compounds of low molecular weight are generally unable to induce the formation of antibodies by lymphocytes unless conjugated to other compounds (Janeway et al., 2004). In agreement with previous studies, the administration of gossypol conjugated to albumin produced no observable toxic effect (Manabe et al., 1991).

This study determined that animals injected with the conjugated gossypol–BSA produced antibodies against gossypol when Freund's incomplete adjuvant was included in the vaccine; however, the administration of the conjugate without the adjuvant was unable to induce significant antibody production. Thus, the combination with Freund's incomplete adjuvant was essential for the production of antibodies to gossypol. Adjuvants are compounds associated with an immunogen, which can promote the immune response by acting on antigen-presenting cells; these cells may (1) have greater recruitment to the inoculation site, (2) have extended contact with antigen, or (3) induce cytokine production by activating T cells (McKee et al., 2010). Antibodies against gossypol have also been induced in rabbits using Freund's incomplete adjuvant to measure gossypol levels by ELISA in various cotton products (Wang and Plhak, 2000).

Gossypol is a hepatotoxic substance (Wang and Lei, 1987; Haschek et al., 1989; Manabe et al., 1991; Deoras et al., 1997), as verified in the present study by the occurrence of ascites and the degeneration of hepatocytes following gossypol exposure (30 mg/kg BW). The hepatocyte degeneration seen here strongly resembled the degeneration observed previously in rats that received a single intraperitoneal dose of 25 mg/kg BW (Deoras et al., 1997). Rats dosed with lower gossypol dosage (15 mg/kg/day for four weeks or 30 mg/kg/day for 2 weeks) presented morphological changes in liver observed at electron microscopy, characterized as mitochondrial vacuolation, enlarged endoplasmatic reticulum, expanded perinuclear space, and proliferation of collagen fibers in perisinusoidal space (Wang and Lei, 1987). In the present study, the occurrence of fibrin deposition in the abdominal cavity, the adhesions between the liver and diaphragm, and the presence of hemorrhagic ascites are indicative of local inflammation. In other studies in rats, the oral administration of gossypol was associated with diarrhea (Bender et al., 1988; Silva et al., 2002), which may have resulted from irritation of the gastrointestinal mucosa.

ALT, AST and ALP are conventional serum biomarkers of hepatobiliary injury. ALT and AST enzymes are found mainly in the liver, kidneys, heart, and skeletal muscle. ALT is an exclusively cytoplasmatic enzyme whereas AST has both mitochondrial and cytoplasmic forms (Dufour et al., 2000; Ennulat et al., 2010). Usually ALT activity after liver injury is higher than that of AST (Dufour et al.,

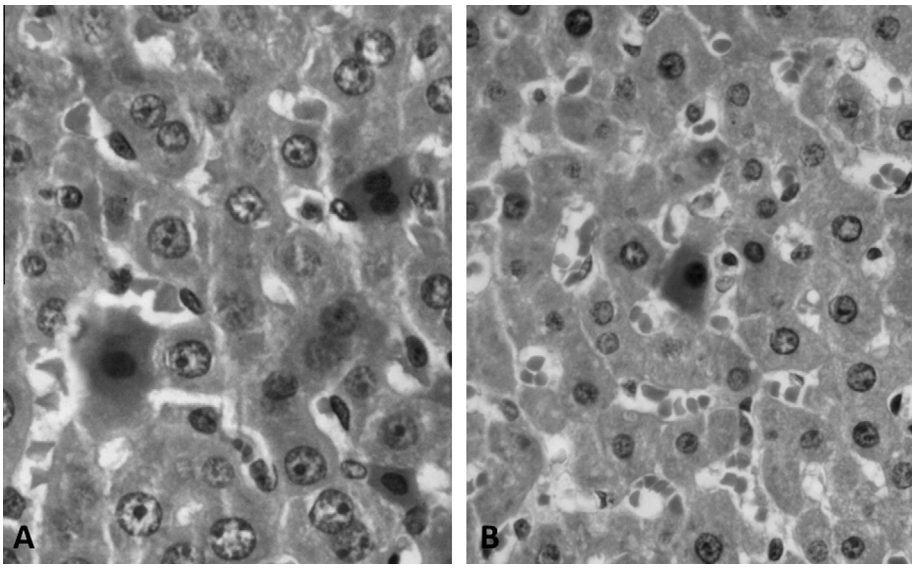

Fig. 1. Liver fragments of rats from CG (A) and VG (B) cohorts showing the occasional presence of hepatocytes with strong cytoplasmic eosinophilia and nuclear pyknosis (HE, obj.100x).

2000), which was the opposite of the observed in the present study. It is feasible to suppose that gossypol might induce release of mitochondrial AST.

The production of antibodies against a toxin does not guarantee neutralization of its toxicity. In fact, the administration of ergot-alkaloid conjugates to rabbits has been shown to induce antibody production that confers only temporary protection against intoxication (Filipov et al., 1998). In the present study using rats, the application of gossypol–BSA associated with Freund's incomplete adjuvant was insufficient to prevent intoxication despite successful antibody production against gossypol at the present dose. In fact, the immunized rats showed increased susceptibility to gossypol toxicity. Increased susceptibility to toxicity has also been observed with pyrrolizidine alkaloid senecionine conjugates in rats (Culvenor, 1978), the mycotoxin sporidesmin in sheep (Fairclough et al., 1984) and the mycotoxin zearalenone in pigs (MacDougald et al., 1990).

It has been hypothesized that the protective function of vaccines against toxins is due to the breaking of bridges and peptide bonds between carbohydrates by antibodies, resulting in the destruction of the toxin; however, compounds not degraded by antibodies might alter the systemic distribution of the toxin (Edgar, 1994). Previous reports of increased susceptibility to poisons following vaccination (Culvenor, 1978; Fairclough et al., 1984; MacDougald et al., 1990) support our findings in the present study and suggest that the temporary binding of antibodies to the toxin interferes with its biotransformation and/or disposal (Lee et al., 2003).

In conclusion, the administration of gossypol–BSA associated with Freund's incomplete adjuvant was able to promote the production of antibodies against gossypol. However, the antibodies produced were unable to prevent the acute hepatotoxicity associated with gossypol exposure.

Conflict of Interest

The authors declare that there is no conflict of interest.

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