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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).