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Pathological and biochemical evaluation of coumarin and chlorophyllin against aflatoxicosis in rat

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ABSTRACT

Aflatoxin contamination of animal diet has adverse effects on animal health and productivity. This study was performed to investigate the effect of using coumarin and/or chlorophyllin in rat diet against aflatoxicosis. Fifty-four rats were assigned into 7 groups (6 rats each). G1 was a negative control. G2 received water with coumarin 0.5%. G3 received water with chlorophyllin 0.5%. G4 received water with coumarin 0.5% and chlorophyllin 0.5%. G5-8 fed aflatoxin B_1 1000 ppb in diet. Group 6–8 were administered similar treatments as G2-4. The experiment ended after 8 weeks. Random glucose, total lipid, total cholesterol, total triglycerides, total protein, serum ALT, AST, creatinine, and urea were measured. Histopathology of liver, kidney and pancreas and immunohistochemical staining of placental glutathione-S-transferase (GST-P) in liver were performed. The glucose serum level, cholesterol, AST, and ALT were elevated in G5 compared to G6-8. The liver and kidney lesions in G5 included vacuolation and necrosis which subsided in G6-8. The necrosis and inflammatory cells infiltration in the pancreas of G5 were absent in G6-8. GST-P positive hepatocytes were abundant in G5, few in G6 and absent in G7 and G8. In conclusion, the chlorophyllin and coumarin possessed protective and anti-carcinogenic effect against aflatoxicosis in rats.

1. Introduction

Acute aflatoxicosis causes hepatitis, hemorrhage, immune suppression, genetic damage (carcinogenicity, teratogenicity, and mutagenicity) and death. Growth impairment and lowering of reproductive performance are the most sensitive clinical signs of chronic aflatoxicosis (Shehata, 2010; Shouman et al., 2012). Scientific efforts were directed towards using physical, chemical and biological techniques for detoxification or inactivation of aflatoxins (Park, 1993). These techniques have not been used on a commercial scale due to high costs, the need for special facilities, losses of important nutrients and the questionable safety of chemical degradation products of aflatoxins. One of the effective methods to overcome the toxic and carcinogenic effects of aflatoxins is to enhance aflatoxin metabolism towards its detoxification in humans or animals (Tulayakul et al., 2007).

More than 1300 coumarins have been identified from natural sources especially green plants (Hoult and Paya, 1996). Coumarins are antioxidants, contain the nucleus of benzo-α-pyrone and occur in plants like Tonka beans, Sweet clover, Woodruff and Cassia leaf (Lake et al., 1989). It is present also in a variety of plants families like Loganiaceae (Bhattacharyya et al., 2008), Orchidaceae, Leguminaceae, Rutaceae, Umbelliferae and Labiatae (Vyas et al., 2009). The synthetic coumarin (4-methyl-7 hydroxy coumarin) derived from resorcinol and ethyl aceto-acetate in presence of concentrated sulphuric acid is structurally close to scopoletin, being a coumarin derivative. Naturally derived and synthetic coumarins have been used in treatment of edemas (Casley-Smith et al., 1993), and possessed anti- tumorigenic (Prince et al., 2009; Bhattacharyya et al., 2009), anti- mutagenic (Pillai et al., 1999), antibacterial (Devienne et al., 2005), anticoagulants, anti- thrombotic and vasodilatory effects (Hoult and Paya, 1996).

Chlorophyllin (CHL), a water-soluble form of chlorophyll, was recently evaluated as chemopreventive agent in a population at high risk for exposure to aflatoxin and subsequent development of hepatocellular carcinoma (Kensler et al., 2002; Kumar et al., 2012). CHL, which is used extensively as a food colorant, has numerous

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medicinal applications. CHL is a safe and effective agent suitable for use in individuals unavoidably exposed to aflatoxins (Enger et al., 2003). Naturally, chlorophyll and CHL strongly inhibits aflatoxin B_1 preneoplasia biomarkers, stomach and liver tumor in rats and rainbow trout (Sudakin, 2003; Simonich et al., 2007, 2008). Moreover, the CHL was more effective than chlorophyll-A in reducing aflatoxin B_1–DNA adduct and liver tumor (Dashwood et al., 1998).

GST-placental form (GST-P) is an isoenzyme of glutathione S-transferases (GST) family which is metabolic enzymes (Ito et al., 1992). This isoenzyme plays a crucial role in detoxifying carcinogens, which made it the molecular target for the development of chemoprevention strategies (Chow et al., 2007; Gross-Steinmeyer and Eaton, 2012). The liver GST-P positive cells and foci are considered sensitive biomarkers for AFB_1 toxic effect and are correlated with the biochemical alterations in F344 rats (Qian et al., 2013).

2. Materials and methods

2.1. Animals

Fifty-four Sprague–Dawley male albino rats with average body weight 90 ± 10 g was used. The animals were housed individually in stainless steel cases in a controlled environment 25 ± 2 °C, 50–60% relative humidity and 12 h light- dark cycle, all over the experimental period (8 weeks). Free access to water and maintenance ration twice daily were available. This study was in accordance with Institutional Animal Use and Care Committee (IACUC) guidelines, Cairo University.

2.2. Coumarin preparation

Coumarin (4-Methyl-7-hydroxycoumarin) was prepared according to a method of Furniss et al. (1989) which is summarized as follow: 1 Liter of concentrated sulphuric acid was placed in a 3-l necked flask. The flask was immersed in an ice bath; a solution of 100 g (0.91 mol) of resorcinol in 134 g (130.5 mL, 1.03 mL) of ethyl aceto-acetate was added dropwise and with stirring for 2 h. The reaction mixture was kept at room temperature for about 18 h., then it was poured with vigorous stirring into a mixture of crushed ice and water, the crude yield (yield = 155 g, 97% concentration) was collected, recrystallization in ethanol 95% and air dried. The dried coumarin was added to water 0.5%.

2.3. Chlorophyllin preparation

All chlorophyll extractions were performed under dim green light to minimize photo – oxidative reaction (Shioi, 2006). Fresh spinach leaves were purchased from local market. Spinach leaves were separated from the stem and then about 100 g were weighed and put into a blender. Cold acetone was then added as much as 500 mL. Then the spinach leaves were ground in a blender for about 3 min. The filtrate of crude chlorophyll was separated using a Buchner funnel with a Whatman filter paper No.1. The residual solids that are not filtered were washed with 100 mL of acetone. Then the filtrate was added to dioxane approximately one-seventh of its volume. After that, the mixture was added with deionized water as much as one-seventh of the volume of the mixture by dropwise and then stirred using a magnetic stirrer. The mixture was precipitated by storing in the freezer at −20 °C 861 for 1 h until a dark green precipitate was obtained at the bottom of the solution and a yellow liquid on top. The precipitate of crude chlorophyll was separated by filtration using two layers of Whatman filter paper No. 1 with a Buchner funnel. The solution then was added with dioxane as much as one-seventh of the volume of the solution and also deionized water as much as one-seventh of the volume of the solution while slowly stirred. The precipitation was then performed for the second time in the same way at −20 °C for 1 h. After that, the precipitate was filtered using a Buchner funnel and two layers of Whatman paper No.1. The crude chlorophyll solids were filtered and diluted with acetone until colorless paper return. Then a solution of crude chlorophyll is evaporated from the solvent so that only the remaining solid green crude chlorophyll was left. Thus, the chlorophyll was dissolved using the small volume of diethyl ether in order to obtain the chlorophyll solution prior to any further separation using column chromatography according to Sandiningtyas and Suendo (2010).

2.4. Aflatoxin preparation

Aflatoxin B_1 production was carried out according to Davis et al. (1966) using liquid media (2% yeast extract and 20% sucrose) and *Aspergillus flavus* stain (NRRL 3145). The media which contained detectable amount aflatoxin was mixed well with the basal diet to get the aflatoxin- contamination diet. The total aflatoxin content in liquid medium was determined according to Roos et al. (1997) and AOAC (2006) method using monoclonal antibody columns for total aflatoxins (Vicam Science Technology, Water Town, MA, USA). Aflatoxin identification was performed by a modification of the HPLC – AFLATEST procedure Agillent 1200 series USA. HPLC equipment with two pumps, column C18, Lichrospher 100 RP-18, (5 Nm × 25 cm) was used. The mobile phase consisted of water: methanol: acetonitrile (54: 29:17, v/v/v) at a flow rate of 1 mL/min. The excitation and emission wavelengths for all aflatoxin were 362 and 460 nm (Flourcenses detector) respectively.

2.5. Experimental design

Fifty-four rats were randomly assigned into 7 groups (6 rats each). Group 1 received basal diet (negative control) (G1). Group 2 received basal diet and water with coumarin 0.5% (G2). Group 3 received basal diet and water with chlorophyll 0.5% (G3). Group 4 received basal diet, water with coumarin 0.5% and chlorophyll 0.5% (G4). Group 5 received a basal diet with aflatoxin B_1 1000 ppb (positive control) (G5). Group 6 received a basal diet with aflatoxin and water with coumarin 0.5% (G6). Group 7 received a basal diet with aflatoxin and water with chlorophyll 0.5% (G7). Group 8 received a basal diet with aflatoxin and water with coumarin 0.5% and chlorophyll 0.5% (G8). The ingredients of basal diet are shown in Table 1. Daily fresh water was available the whole time. At the last day of the experiment, the rats fasted overnight. The experiment was terminated after 8 weeks and animals were euthanized by cervical decapitation.

2.6. Serum analysis

Blood samples were collected from each rat from the retro-orbital vein and were received into clean dry centrifuge tubes. Serum was separated by centrifugation at 3000 rpm for 15 min and kept in deep-freeze at −20 °C until used for estimation of random glucose level, total lipid, total cholesterol, total triglyceride, total protein, serum ALT, AST, creatinine, and urea.

2.7. Histopathological examination

Liver, kidneys, and pancreas samples were collected at the end of the experiment and were fixed in 10% neutral buffered formalin solution for at least 48 h. Tissue specimens were processed using routine paraffin embedded technique in which tissues was dehydrated in ascending concentration of alcohol and cleared in xylene. Sections 3–5 μ thick were made using microtome (Leica