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Original Article

T2-Toxin Hepatotoxicity in the *in situ* Rat Liver Model

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Abstract

T-2 toxin, a trichothecene mycotoxin, is considered to be one of the most toxic compounds that are produced by molds, particularly the Fusarium species. Fusarium species have been recognized as a great agricultural problem. They occur worldwide on a variety of plant hosts and cereal grains. The aim of this study was to investigate T-2 toxin-induced liver injury using in situ perfused rat liver. The in situ perfused rat liver (IPRL) was chosen because it permits studies of liver function in a system that resembles normal physiology. Elevation of aminotransferase activities have shown to be a good indicator of hepatocellular damage. In addition, glutathione levels have also shown to be an indicator of liver damage through lipid peroxidation. Male Sprague-Dawley rats (6-8 weeks) weighing 250-300 g were used in this study. They were randomly divided into 5 groups of 3-4 rats per cage. In group 1, liver was perfused by Krebs-Henseleit buffer alone (Control). Groups 2-5 received different concentration of T-2 toxin (4, 9, 21, 43 ρmol/L) in Krebs-Henseleit buffer and biochemical changes in the liver were examined within 2 h. There was a significant increase in both ALT and AST activity in all dose levels compared with the control group ($p<0.01$ and $p<0.05$). T-2 toxin treatment enhanced lipid peroxidation in the liver, as indicated by the increased MDA content in liver homogenates. The MDA level was maximal 2 h after the T-2 toxin challenge ($p<0.01$ and $p<0.05$). The results also show that T-2 toxin causes an increase in lipid peroxidation while causing a decrease in glutathione (GSH) content in bile secretion ($p<0.01$). This result suggests that both lipid peroxidation and glutathione (GSH) depletion play a role in T-2 toxin liver induced damages.

Keywords: T-2 Toxin, Glutathione, Lipid Peroxidation, MDA, Aminotransferase activity, IPRL

Introduction

T-2 toxin, a *trichothecene mycotoxin*, is considered as one of the most toxic compounds that are produced by molds, particularly the Fusarium species (1). Structurally, they are sesquiterpenes and have an epoxy ring at C-12, 13 (1). Contamination of cereals such as barley, wheat, rice and maize with Fusarium mycotoxins has been reported worldwide (2-7). Because of their widespread natural occurrence and diverse toxic effects, the presence of these mycotoxins in food and feed is considered potentially hazardous to humans and animals (4-7). Different mechanisms of action have been proposed for T-2 toxin. They react with the thiol groups of sulfhydryl enzymes and as a result are potent protein and DNA synthesis inhibitors (8, 9, 10, and 25). Further, morphological and functional changes in biological membranes have been observed in the heart (11), red blood cells (12) and liver (13). Previous studies have shown that there is a similarity between the hemolysis of rat erythrocytes caused by T-2 toxin and that caused by the free radicals (8). Furthermore, T-2 toxin administration leads to a pronounced increase in the thiobarbituric acid reactive compounds in liver homogenate of T-2 toxin-treated rats. This was interpreted as an indicator of the presence of lipid peroxidation

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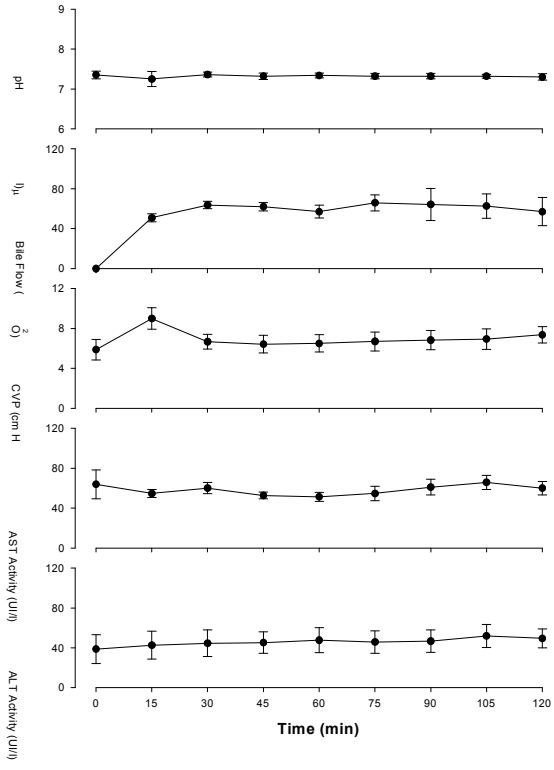

Figure 1. Results of monitoring in situ perfused rat liver preparation in the control group. Over the 120 min time period of this study, there is little change in pH, bile flow, central vein pressure, or perfusate aminotransferases (ALT and AST) activities. This indicates stability and consistency of perfusion method. Data are mean ± SEM of 3-4 animal per group. The data is in conformation with those reported by Wolkoff et al. (14).

substances such as 2-alkenals, 4-hydroxy-alkenals and malondialdehyde (MDA), which were reported to be present in the liver after acute exposure to T-2 toxin (8, 25). Hoehler et al. used a specific species of yeast (*Kluveromyces marixianus*) to study oxidative damage induced by T-2 toxin (26). They demonstrated that MDA content increased when the concentration of T-2 toxin was raised. These studies further suggest that T-2 toxin stimulates lipid peroxidation by promoting free radical production. Despite reports on the induction of lipid peroxidation by T-2 toxin, others reported no effect on lipid peroxidation property of trichothecenes (1). Overall, the evidence suggests that one of the modes of action of T-2 toxin is to enhance the peroxidation of lipids, with free radicals involved in the process. Studies on liver tissue were undertaken since this organ has been shown in previous studies (8, 9, 13, and 27) to be a target for T-2 toxin.

Experimental

Materials

T-2 toxin and all other chemicals were of analytical grade obtained from Sigma-Aldrich (St Louis, MO, USA).

Methods

Animal

Male Sprague-Dawley rats (6-8 weeks) weighing 250-300 g were obtained from Razi Vaccine Institute, Tehran, Iran. They were housed in standard stainless-steel cages at a 12 h cycle of light and dark. Room temperature was kept at 22°C and humidity maintained at 50%. Rats were allowed to become acclimatized to standard laboratory condition for at least 7 days and standard food and water was provided *ad libitum*. Food was withdrawn 12 h before starting the experiment.

Administration of T-2 toxin

One mg crystalline T-2 toxin was dissolved in 1 ml ethanol (96%) and stored at 4°C until use. The stock solution was appropriately diluted to the concentration needed (25, 28).

Experimental design

Animals were divided into 5 groups. Each group contained 3-4 male rats. In group 1, liver was perfused by Krebs-Henseleit buffer alone (Control). Groups 2-5 contained different concentrations of T2 toxin, with the same buffer. Before performing the experiment, perfusate was circulated for 30 min to permit stabilization. Then, the perfusion was recirculated with Krebs-Henseleit buffer.

Liver perfusion

Livers from male Sprague-Dawley rats were perfused at 37°C with Krebs-Henseleit solution. D-glucose (0.1% w/v) was added to provide energy source. The perfusion medium was gassed continuously with carbogen (95% O_2, 5% CO_2) essentially as described by Wolkoff et al. (14). Briefly, an incision was made along the length of the abdomen to expose the liver under ketamine and xylazine (70 mg/kg and 15 mg/kg, i.p., respectively) anesthesia. Heparin, 500 units, may also be injected i.p. to prevent blood

clotting, although this is not obligatory for successful perfusion. An incision was made along the length of the abdomen to expose the liver. Sutures were placed loosely around the common bile duct, which were canulated with PE10 tubing, secured and bile was collected for a period of 120 min. Sutures were then placed loosely around the inferior *vena cava*, above and below the renal veins. The distal suture around the *vena cava* was tightened and then an 18G polyethylene catheter was inserted, placed above the renal vein and secured with the proximal suture. The portal vein was immediately cannulated. The diaphragm was incised and the inferior *vena cava* was legated super-hepatically. Following attachment of the perfusion tubing to the cannulae, the liver was perfused in situ through the portal vein. Temperature, perfusion fluid pressure, flow rate and perfusion fluid pH were closely monitored during the perfusion (14) and the experiment was not begun until they all had reached to constant and acceptable values. Perfusion pressure was not raised above 10-15 cm of water with a flow rate of approximately 3 ml/min/g liver weight to provide an adequate oxygenation. The pH of perfusion fluid was always set between 7.2 and 7.4 by adjusting the Carbogen gas. As soon as perfusion was begun, the liver should blanch to a uniform, light brown color. Blotches or discoloration means that liver is not well-perfused. Serum aminotransferase activities (ALT and AST), central vein pressure (CVP), bile flow and pH, serve as indicators of liver viability during perfusion, which were determined in samples of perfusion medium every 15 min (Figure 1).

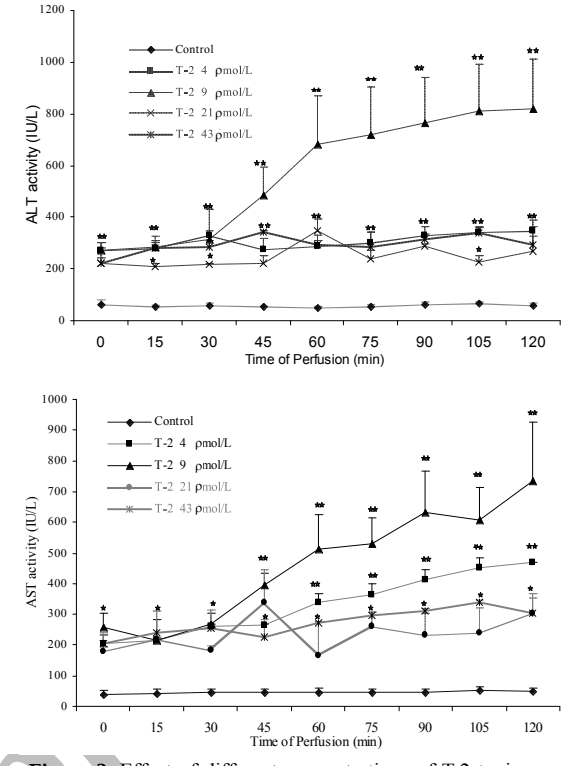

Figure 2. Effect of different concentrations of T-2 toxin on aminotransferases activities (ALT and AST) in perfusate of the isolate perfused rat liver. There was a significant increase in both the ALT and AST activity in all dose levels compared with the control group ($p<0.01$ and $p<0.05$). However, this increase was more significant ($p<0.01$) at the T-2 toxin dose of 4 and 9 ρmol/L. All data are given as mean±SEM, 3-4 animals per group.*$p<0.05$, ** $p<0.01$ statistically different from the control group.

Determination of the activities of aminotransferases

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the perfusion fluid and homogenate sample were assayed using the commercial kit of Darman Kave (Tehran, Iran).

Determination of the total protein and glutathione (GSH) contents

Glutathione contents in tissue homogenates and bile secretion were determined as described by Kuo and Hook (15). Briefly, the tissue was homogenized in 20% (w/v) trichloroacetic acid and centrifuged at 6000 rpm for 20 min. To determine glutathione (GSH) concentration in the tissue, an aliquot of the deproteinized supernatant fraction was added to 2 ml of 0.3 M Na2HPO4 solution followed by the addition of 0.5 ml of 0.04 %, 5,5-dithiobis-[2-nitrobenzoic acid] dissolved in 10% sodium citrate. The absorbance at 412 nm was measured immediately after mixing and the glutathione (GSH) values were determined by extrapolation from standard curve. The amount of total protein was determined in liver homogenates after 120 min of perfusion, colorimetrically according to the Bradford method, using the coomassi brilliant blue G-250 reagent (16).

Lipid peroxidation

Lipid peroxidation was determined in liver tissue homogenates according to the thiobarbituric acid (TBA) method (17).

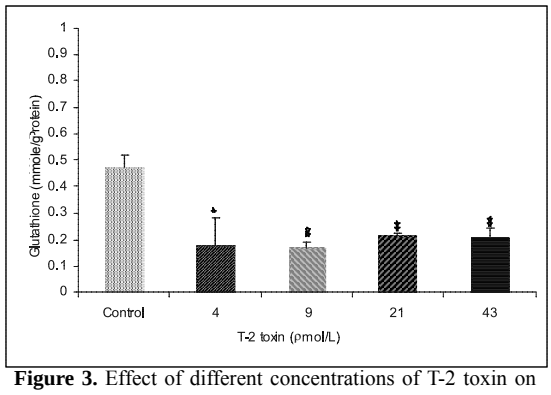

Figure 3. Effect of different concentrations of T-2 toxin on the glutathione (GSH) liver tissue content in the rat liver perfusion. T-2 toxin caused a significant ($p<0.01$ and $p<0.05$) reduction in the glutathion content in liver tissue. All the data are given as mean±SE, 3-4 animals per group.*$p<0.05$, ** $p<0.01$ statistically different from the control group.

Statistical analysis

Statistical analysis was performed using the one way analysis of variance (ANOVA) followed by the Student Newman Keuls post test. Level of significance was set at $p<0.05$ and $p<0.01$.

Results and Discussion

T-2 toxin has the highest toxicity among the 16 trichothecene mycotoxins studied (18). In this study we showed that T-2 toxin causes an alteration in the biochemical parameters in the in situ perfused rat liver. Evaluation of viability of the perfused liver by different parameters in control groups showed no significant change, which indicated stability and consistency of the method. The data is in agreement with those reported by Wolkoff et al. (Figure1) (14). Perfusion of rat liver with the Krebs-Henseleit buffer containing different concentrations of T-2 toxin (4, 9, 21, 43 ρmol/L) showed a significant increase in both AST and ALT activities in all concentrations, compared to the control group at $p<0.01$ and $p<0.05$ (Figure 2). However, this increase was more significant at $p<0.01$ with doses of 4 and 9 ρmol/L of T-2 toxin. T-2 toxin doses of 4, 9, 21, 43 ρmol/L caused a significant decrease in glutathione (GSH) level in liver homogenates, compared to the control group, with $p<0.01$ and $p<0.05$ (Figure 3). It was also shown that the addition of toxin to perfusion buffer resulted in a significant decrease ($p<0.01$) in GSH concentration in biliary excretion at doses of 21 and 43 ρmol/L, compared with their respective control groups (Figure 4). T-2 toxin is known to bind to SH-proteins (22). The epoxy group of T-2 toxin competes with the substrates of glutathione-s-transferase (19). This could result in a decrease in the content of GSH. Prevention of lipid peroxidation is primarily connected with the glutathione (GSH) metabolism and also the ability of hepatic glutathione peroxidase to remove H2O2 and organic peroxides in the compartments where catalase is absent (23). It is possible that T-2 toxin reduces the level of glutathione (GSH), when it is required for the elimination of peroxide radicals.

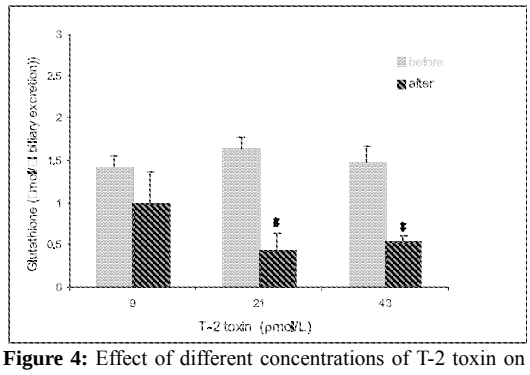

Figure 4: Effect of different concentrations of T-2 toxin on the glutathione (GSH) biliary excretion content in the rat liver perfusion. It was shown that the addition of toxin to perfusion buffer resulted in a significant ($p<0.01$) decrease in GSH concentration at doses of 21 and 43 ρmol/L as compared with their respective control griups. All data are given as mean±SE, 3-4 animals per group.** $p<0.01$ statistically different from the control group.

The TBA values increased in the perfused liver, 2 h after administration of T-2 toxin, reaching values of approximately six (21 ρmol/L) and seven (9, 43 ρmol/L) times higher than control (Figure 5). This study showed that T-2 toxin causes an increase in lipid peroxidation, while causing a decrease in glutathione (GSH) in the in situ perfused rat liver. Glutathione (GSH) and lipid peroxidation are both sensitive indicators of oxidative stress. Some investigators have suggested that T-2 toxin produces its toxic effects by inducing membrane lipid peroxidation mediated by superoxide anion radicals (19). Since lipid peroxidation is one of the causes of cell membrane disruption and necrosis, T-2 toxin may induce modifications in membrane permeability, releasing significant quantities of

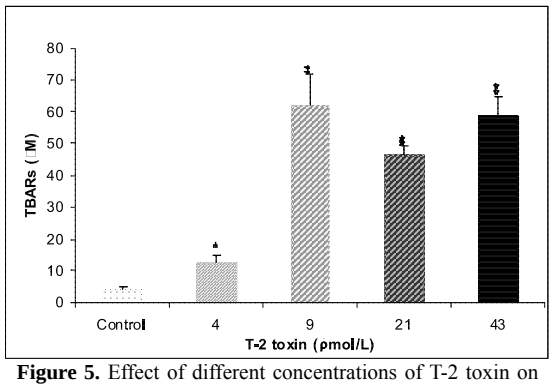

Figure 5. Effect of different concentrations of T-2 toxin on the liver tissue lipid peroxidation in the rat liver. There was a significant increase in lipid peroxidation of liver tissue exposed to various concentrations of T-2 toxin. All the data are given as mean±SE, 3-4 animals per group. *p<0.05, ** p<0.01 statistically different from the control group.

aminotransferase enzymes into the perfusion fluid. Moreover, glutathione cycle imbalance induced by T-2 toxin produced an increase in lipid peroxidation which affected membrane integrity, in turn causing enzymes leakage. Increasing both lipid peroxidation and aminotransferase enzymes release could result either from a primary increase in membrane vulnerability to oxidant damage, or a possible increase in free radical production. Liver lipid peroxidation caused by certain drugs (20) and ethanol (21) has been shown to be due to the depletion of hepatic reduced glutathione (GSH). Moreover, T-2 toxin is a lipophilic substance and is readily absorbed into the membrane, resulting in structural alteration within the membranes that triggers the stimulation of membranous lipid peroxidation. Stimulation of lipid peroxidation by T-2 toxin in liver cells rich in membranes appears to be carried out through the electron transport system and polyunsaturated fatty acids. T-2 toxin stimulates lipid peroxidation by promoting free radical production. The reactive oxygen molecules interact with phosopholipids of cellular membranes, resulting in an increased level of lipid peroxidation especially in the liver (25). In this study, toxin treatment also enhanced lipid peroxidation in the liver, as indicated by the increased MDA content in liver homogenate. The MDA level was maximal 2 h after the T-2 toxin challenge. The formation of lipid peroxides following T-2 toxin treatment in mammals has been reported in previous studies (24-26). Schuster et al., in contrast to the results obtained by several different groups, concluded that lipid peroxidation is not involved in T-2 toxicity (1). The reason for these differences was not established. Nevertheless, the collective results of several different research groups together with the results discussed in this study, suggests that T-2 toxin induces lipid peroxidation in the biological systems. T-2 toxin and some other mycotoxins are believed to enhance the production of oxygen radicals to such extent that the normal body radical scavengers are overwhelmed, resulting in cell injury (25).

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