The Role of TNF-α in Aflatoxin B-1 Induced Hepatic Toxicity in Isolated Perfused Rat Liver Model

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Abstract- Aflatoxin B-1 (AFB1) is one of the major mycotoxins causing food contamination. Previous studies have shown that AFB1 can induce carcinogenicity and toxic effects in the isolated perfused rat liver and these effects are associated with its metabolites and peroxidation activity. Here we surveyed whether these pathogenic effects of AFB1 are associated with TNF-α as an inflammatory cytokine in general liver damages. In this study, we used twenty male Wistar rats (250-300 g). Rats were divided into four groups. Control group was pre-treated with LPS and then perfused with KHBB. The second group was pretreated with PTX and LPS and then perfused with KHB. The third group was pre-treated with LPS and then perfused with AFB-1 and KHB. The last group was pretreated with LPS and PTX and then perfused with AFB1 and KHB. Results revealed that aflatoxin B1 significantly increased the enzyme activity of aminotransferase and levels of lipid peroxidation. Also, the levels of Glutathione decreased in the aflatoxin group significantly. TNF-α released in perfusate and increased in aflatoxin B1 group significantly and decreased in AFB-1+PTX. Exposure to Aflatoxin B1 may induce reactive oxygen species, so these species may induce overproduction of proinflammatory cytokines such as TNF-α and may cause more damage to hepatic cells.

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Introduction

Aflatoxin B-1 (AFB1) is one of the secondary toxic fungal metabolites produced by Aspergillus flavus and A. parasiticus. This aflatoxin is found in edible tissues, milk and eggs after consumption of contaminated food via farm animals and consumption of such foods cause hepatic injuries, including hemorrhage, parenchymal cell necrosis and injury to intrahepatic bile ducts (1-3). AFB1 plays an important role in the etiology of human liver cancer. The crucial step in the onset of hepatocarcinogenicity of AFB1 in hepatic tissue is the formation of AFB1-8,9-epoxide by the hepatic cytochrome P450 enzyme system that produces DNA and protein adducts (3,4). On the other hand, numerous studies have shown hepatotoxicity and initial carcinogenicity activity of AFB1 in the liver by induction of oxidative damage in this organ. One of the main mechanisms of toxicity is through the generation of intracellular reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) during the metabolic processing of AFB1 by cytochrome P450 (3,5,6).

With regard to the production and effects of inflammatory mediators, the liver is one of the most important organs, and these mediators may incorporate in pathological events (necrosis and fibrosis), protective and repair process in case of exposure to different

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chemicals. Because Kupffer cells of the liver are one of the major sources of these mediators such as TNF-α, so this mediator is produced and released in response to inflammatory stimulants (1,7-9).

The initial toxic injury makes focal tissue damage and necrosis in a target organ. In response to necrosis, tissue-fixed macrophages, along with adjacent endothelial cells and epithelial cells are activated and secrete inflammatory products, subsequently. The proinflammatory cytokine tumor necrosis factor (TNF) is one of these products, which is a central regulator that aids in tissue repair by arousing apoptosis and cell proliferation as well as amplify cell damage by initiating an overly aggressive inflammatory process. The latter ultimately results in the recruitment and activation of neutrophils and monocytes into the damaged site and the release of reactive oxygen species and the nitrogen-centered radical, nitric oxide, producing cell damage. Over production of these cytokines leads to more activation of macrophage and neutrophils in the site of damage and cause generalized liver damage (8,10-12). The purpose of this study is to investigate the role of liver TNF-α in AFB1-induced liver toxicity in perfused rat liver and in order to confirm the involvement of TNF-α in the toxic effects of AFB1 in this model. Also in our study, PTX was used as an inhibitor of TNF-α release.

Materials and Methods

Chemical

AFB1, LPS, PTX, MDA and GSH stocks were purchased from Sigma (St. Louis, MO, USA). ELISA kit for TNF-α detection in rats was provided by the Elabscience company. ALT and AST were provided from Teb Gostaran Hayan (Tehran, Iran). The perfusion fluid was prepared with Krebs-Henseleit bicarbonate buffer. The perfusion medium was consisted of 118mmol/l NaCl, 6 mmol/l KCl, 1.1 mmol/l MgSo4, 24 mmol/l $NaHCo_3$ and 1.25 mmol/l $CaCl_2$.

Animals

Male Wistar rats (250-300 g) were obtained from the vivarium section of the department of pharmacology, Tehran University of Medical Sciences, Tehran, Iran. Animals were housed in cages in room temperature (22±2° C). They had free access to murine standard diet supplied by Behparvar Company, Iran, and tap water until 2 h before surgery. An experimental study was carried out based on the standard guidelines for animal welfare in the faculty of veterinary medicine, Semnan University.

Perfusion apparatus

The liver perfusion apparatus used in our laboratory is based on the description of Bessems *et al*., (13) and Cheung *et al*., (14) which was originally developed by Wolkoff *et al*., and *et al*., (15).

Preparation of isolated perfused rat liver (IPRL)

The rats were anesthetized by an intraperitoneal injection of Ketamine/Xylazine (60:8 mg/ml) (15,16). The anterior abdomen was shaved and cleaned with alcohol and a ventricle longitudinal midline incision made extending from pubis to upper chest. The animals heparinized by injecting the solution into the inferior vena cava anterior to the renal vein and immediately vena cava was ligated. Then, hepatic portal vein (inlet) and the thoracic inferior vena cava (outlet) were cannulated. The liver was perfused with buffer KHBB (*p*H=7.4±2) saturated by 95% O_2/5% CO_2 through the catheter cannulated into the portal vein. The perfusate was collected from a catheter placed into the superior vena cava via the right atrium. Perfusion flow rate and pressure were 20 ml/min and 15-20 cm H_2O, respectively. The flow rate was measured by fractionating the effluent.

Experimental design

Rats were divided randomly into four groups, each including five animals. The control group was injected intraperitoneally with LPS (30 μg/kg) 30 min before the start of perfusion with Krebs-Henselit buffer. The second group was injected intraperitoneally with LPS (30 μg/kg) 30 min and pentoxifylline (100 mg/kg) 60 min before the start of perfusion. The third group was injected intraperitoneally with LPS (30 μg/kg) 30 min before the start of perfusion and perfused with 100 ppm of aflatoxin B1, 15 min after start of perfusion. In the fourth group, rat livers were pretreated with LPS and Pentoxifylline and then perfused with 100 ppm of AFB1, 15 min after start of perfusion, as with the third group.

Sample collection

Samples (1.5 ml) were collected from the outlet at 0, 15, 30, 45, 60, 75, 90, 105 and 120-minute post perfusion operation and stored at -20° C. The perfused livers were used for measurement of MDA, GSH, and total proteins.

Sample analysis

ALT and AST levels were quantitated based on colorimetric methods using a commercial kit guideline. MDA level was determined in homogenized liver tissue, according to the thiobarbituric acid (TBA) method (17). GSH was estimated by Kuo and Hook standard method (18), total proteins were evaluated by the Bradford method (19) and TNF- α determined by ELISA method.

Statistical analysis

Values are presented as mean±SE. Data were analyzed by one-way ANOVA, followed by Tukey test for multiple comparisons. Differences were considered to be statistically significant when <0.05.

Results

Enzyme release

Damage to perfused rat liver was detected by measuring the outlet AST and ALT, as indexes of hepatic injury (Figures 1,2). In control group and second group, levels of enzymes were below 60 IU/L. In third and fourth groups, increase in AST and ALT levels were observed from 15 min after perfusion. Significant differences were observed in the third group in comparison with the control group, statistically, but in spite of the decrease of AST levels in the fourth group, significant differences were not observed in comparison with the third group. Also, we didn't find significant differences in levels of AST and ALT between fourth and control groups. In companion of third and fourth groups in ALT levels, we observed statistically significant differences, that was shown decrease of ALT levels in the fourth group.

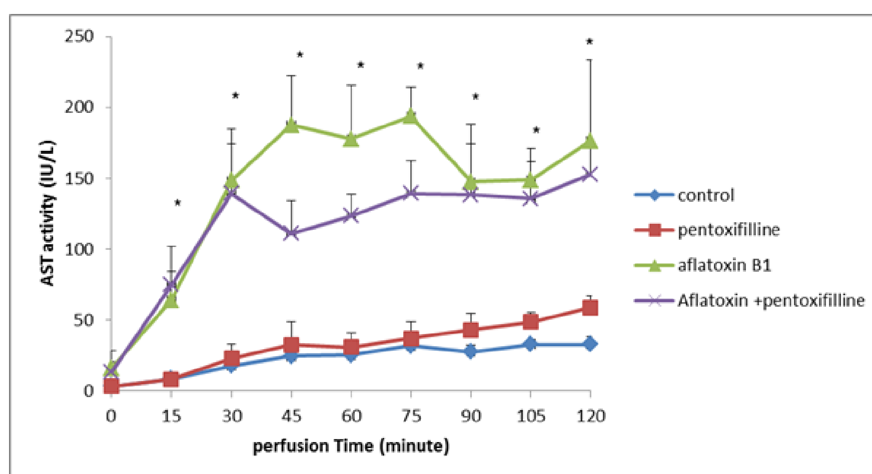

Figure 1. Time courses of alanine aminotransferase (AST) release from the perfused liver rat for 2 h. Values are presented as mean±S.E. of group four. * Statistically significant difference (P<0.05) compared with control group

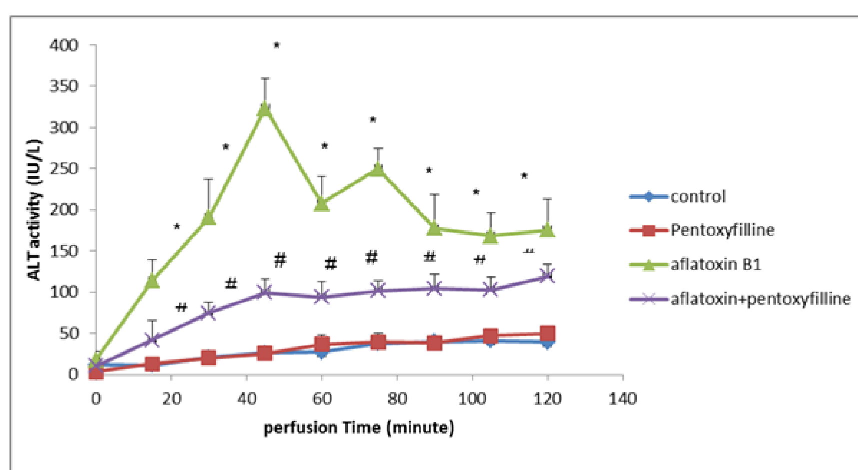

Figure 2. Time courses of alanine aminotransferase (ALT) release from the perfused liver rat for 2 h. Values are presented as mean±S.E. of group four. * Statistically significant difference (P<0.05) compared with control group. # statistically significant difference (P<0.05) from AFB1 group

Effects of aflatoxin B1 on lipid peroxidation, GSH, and total protein

The MDA level significantly increased in the third group, GSH level significantly decreased in the third group, and protein values significantly increased in the third group, compared to those in the control group. In comparison to the third and fourth group, in GSH level significant increase statistically was shown in the third group and in MDA level and total value, significant decrease statistically were observed (Table 1).

Table 1. Concentrations of MDA, glutathione and total proteins in different groups

Groups	Concentrations	MDA (nmol/µg pro.)	Proteins (µg/ml)	Glutathion (nmol/µg pro.)
Control (LPS)	30 µg/kg	0.405±0.13	148.15±16.5	569±85.1
PTX+LPS	100 (mg/kg) +30 (µg/kg)	0.6±0.12	193.7±39.4	427.9±11.2
AFB1+LPS	100(ppm)+30(µg/kg)	2.18±0.17*	727.5±37.1*	91.7±8.2*
AFB1+PTX+LPS	100 (ppm)+ 100 (mg/kg) + 30(µg/kg)	$0.945±0.1^{\#}$	$523.2±78.2^{\#}$	$239.6±66^{\#}$

Values are presented as mean±S.E. of group four. * Statistically significant difference (*P*<0.05) compared with control group. # Statistically significant difference (*P*<0.05) between third and fourth group

Concentrations of tumor necrosis factor-α in perfusate

Concentrations of TNF-α detected in treatment groups from 5 different time-point in the outlet. The increase of release of TNF-α in perfusate was from 60 min onwards that significant difference significantly (*P*<0.05) was observed from 90 min until 120 min. In comparison to the third and fourth group, in TNF-α level, significant decrease statistically (*P*<0.05) was shown in the fourth group at 120 min.

Discussion

Several studies conducted about hepatotoxicity and carcinogenicity mechanisms of AFB1 in vitro, in situ and in vivo models. Documented evidence indicates that AFB1-induced liver toxicity and initial carcinogenesis are through oxidative stress. They have shown that AFB1 metabolism via the cytochrome system leads to the production of reactive oxygen species that damage cell membranes and components. These reactive species change intracellular antioxidant, such as GSH and will begin the process of lipid peroxidation of cell membranes, which ultimately leads to cell lysis and cell necrosis (4,6,20-27).

Recent studies suggested that initial inflammation plays a role in many toxicities including hepatotoxicity, neurotoxicity, and pulmonary toxicity. Initial damage causes focal tissue necrosis. As result of damage and necrosis, proinflammatory cytokines such as TNF-α, IL-6, IL-1 and chemokines are produced and released by Kuppfer cells along with adjacent nonparenchymal cells (12,28-30). Keeping in mind that 80% of body resident macrophages are localized in liver, and these cells are source of inflammatory cytokines including TNF-α that is produced in response to immunity stimuli such as lipopolysaccharides (LPS), their overproduction influences liver function such as metabolism and may result massive hepatic failure (7,8,29).

A lot of studies showed the role of TNF-α has been in liver toxicity and liver damage by aflatoxin, carbon tetrachloride, fumonisin and monocrotaline in vivo models (9,31-33).

Despite numerous reports on TNF-α levels in animal models and isolated Kupffer cells, the data about TNF-α levels in perfusion model is scarce (29,34,35). Mehvar and Zhang 2002 designed a model for the study of stimulation and inhibition of TNF-α used in isolated perfused rat liver model (16).

In this study, we have evaluated the role of TNF-α as an inflammation mediator in AFB1-induced liver damage in perfused rat liver by PTX inhibitory effects in the levels of TNF-α release in the liver. For this study, we used the isolated perfused rat liver since this model is useful system for studying hepatotoxicity induced by chemicals (36,37). Further, a few studies are found in Medline database about effects of Aflatoxin-B1 on the isolated perfused rat liver.

Our results showed that markers of liver damage (ALT and AST activity) and oxidative injury of lipid are group-depended. The increase of ALT and AST activities in perfusate, increase of TBARS and decrease of GSH in liver homogeneous suggested that AFB1 play a role in the oxidative damages, as other authors have pointed to it (37). In the fourth group, decrease of ALT and AST activities in perfusate, decrease of TBARs and increase of GSH in liver homogenous was observed in comparison with the third group that infers PTX may have antioxidative properties and may protect the liver from AFB1-induced oxidative damages. In the other

word, this indicates not only that AFB1 exposure elicited the liver damage in this model, but also that toxic effect by exposure of AFB1 in this model was inhibited by treatment of PTX.

Another assessment in our study was determination of TNF-α levels in the perfusate. A significant increase of TNF-α levels observed in the third group in comparison with control group from 90 min onwards, and significant decrease statistically ($P<0.05$) was shown in the fourth group at 120 min in comparison with the third group.

Taken together, it seems that antioxidative properties of PTX in a part is through inhibition of release of TNF-α in Kupfer, polymorphonuclears (PMNs) (38), and it may be concluded that Kupffer cell blockade protects the liver by inhibition of hepatic TNF release (29).

PTX, a derivative of the methylxanthine theobromine has been used for many years in the treatment of peripheral vascular diseases. A number of studies have shown PTX's effects on the cytokine network and antifibrogenic actions (38).

It is generally accepted that PTX exerts its pharmacological effects by inhibiting intracellular cAMP phosphodiesterases, thus leading to the intracellular concentration of cAMP. A lot of studies indicated that the effects of PTX on the cytokine network are due to an intracellular elevation of cAMP, increased intracellular cAMP reduced phagocytic activity, superoxide anion production, and lysosomal enzyme release by PMNs. High levels of intracellular cAMP suppress the TNF-α gene transcription and ultimately inhibit TNF-α release (29,38,39).

Initial aflatoxin toxic damage causes focal necrosis in the target organ. As a consequence of this damage, resident macrophages and other cells are activated and secrete inflammatory exudate. The overproduction of proinflammatory cytokines such as TNF-α stimulates cell tissue damage by the onset of the systemic severe inflammatory process. Finally, mobilization and activation of neutrophils and monocytes happen to the sites of injury lead to the release of reactive oxygen species and radicals with central nitrogen, nitric oxide, and the cell damage. Pentoxifylline as an inhibitor of TNF-α release, may protect hepatocytes from AFB-1 induced liver damage and lead to reduce liver cell damage caused by free radical production.

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