

The pretreatment effects of pentoxifylline on aflatoxin B1-induced oxidative damage in perfused rat liver

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Abstract

This study was conducted to investigate the hepatoprotective and antioxidant effects of pentoxifylline (PTX) against aflatoxin B1 (AFB1) exposure in perfused rat livers by evaluating damage marker enzymes, antioxidant defense systems (glutathione, GSH) and lipid peroxidation (malondialdehyde, MDA). Sixteen rats were divided randomly into four experimental groups: control, PTX, AFB1 and AFB1 + PTX. Rats in the control group were infused with Krebs–Henseleit bicarbonate buffer. Rats in the AFB1-treated group received approximately 1 ppm and the PTX-treated group received 100 mg/kg intraperitoneally 24 h before surgery. Alanine aminotransferase and lactate dehydrogenase levels were increased by AFB1 and decreased by PTX. PTX also ameliorated the increased concentration of MDA caused by AFB1. PTX did not compensate for the decrease in GSH caused by AFB1. These results imply that PTX has an antioxidant effect by inhibiting free radicals, and prior treatment with PTX ameliorates the effects of AFB1-induced lipid peroxidation but does not compensate GSH depts.

Introduction

Aflatoxins are toxic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* (Choi *et al.*; Preetha, Kannappan *et al.* 2006). There are four naturally occurring aflatoxins: B1, B2, G1 and G2. Aflatoxins induce many forms of toxic damage, including hepatotoxicity, hepatocarcinogenesis and mutagenic effects. Aflatoxin B1 (AFB1) is the most highly toxic form. Humans and animals can be exposed to aflatoxins both directly and indirectly, but predominantly by forms present in various foods and feedstuffs (Choi *et al.*, 2010). AFB1 plays a major role in the etiology of human liver cancer (Towner *et al.*, 2002). The crucial step in the hepatocarcinogenicity of AFB1 is the formation of AFB1-8, 9-epoxide by the hepatic cytochrome P450 enzyme system. This epoxide produces both DNA and protein adducts *in vivo* and *in situ* (Essigmann *et al.*, 1980; Shen *et al.*, 1994; Awney *et al.*, 2002; Towner *et al.*, 2002; Towner *et al.*, 2003; Preetha *et al.*, 2006). Numerous studies have shown AFB1-induced oxidative damage and its role in cytotoxicity and carcinogenicity in the liver (Shen *et al.*, 1995; Rastogi *et al.*, 2001; Towner *et al.*, 2002; Lee *et al.*, 2005; Gesing and Karbownik-Lewinska, 2008; Yener *et al.*, 2009). Studies also suggest that oxidation of

AFB1 can produce reactive oxygen species (ROS) including the superoxide anion, hydrogen peroxide (H₂O₂), and the hydroxyl radical via cytochrome P450 metabolism and an iron-mediated redox mechanism (Choi *et al.*, 2010; Shen *et al.*, 1995; Preetha *et al.*, 2006). ROS cause oxidative stress by damaging cellular membranes and components. Evidence has been accumulated that AFB1-induced hepatotoxicity and hepatocarcinogenesis via oxidative stress are inhibited by non-natural and natural antioxidants such as silymarin (Rastogi *et al.*, 2001), crocetin (a natural carotenoid) (Wang *et al.*, 1991), green tea (Qin *et al.*, 1997) and butylated hydroxyanisole (Choi *et al.*, 1991).

Pentoxifylline (PTX) is a derivative of methylxanthine that has hematologic and immunomodulating properties. It also has anti-inflammatory and antioxidant effects (Abdin *et al.*, 2010; Abdel-Salam *et al.*, 2003; Radfar *et al.*, 2005). PTX has an inhibitory effect on xanthine oxidase, which is involved in the formation of oxygen free radical, and down-regulates tumor necrosis factor alpha (TNF- α) production. This cytokine causes increased H₂O₂ production from mitochondria. PTX is a nonselective inhibitor of phosphodiesterase (PDE), which plays a role in nitric oxide production, and decreases lipid peroxidation via inhibition of PDE (Abdin *et al.*, 2010;

Maiti *et al.*, 2007; Radfar *et al.*, 2005).

The present study was designed to investigate the protective effect of against AFB1-induced oxidative damage in perfused rat liver.

Material and Methods

Chemicals

AFB1 and glutathione (GSH) were obtained from Sigma (St. Louis, MO, USA). Perfusion fluids comprised Krebs–Henseleit bicarbonate buffer (KHBB; 118 mmol/l NaCl, 6 mmol/l KCl, 1.1 mmol/l MgSO₄, 24 mmol/l NaHCO₃ and 1.25 mmol/l CaCl₂). These materials were purchased from Merck.

Animals

Male Wistar rats (200–300 g) were obtained from the vivarium section of the Department of Pharmacology, Tehran University of Medical Sciences, Tehran, Iran. The animals were housed in cages at room temperature. They had free access to standard diet and tap water until surgery, when they were deprived of food. All procedures in this study were performed in accordance with the guidelines for the Care and Use of Laboratory Animals adopted by the Ethics Committee of the School of Medicine of Tehran University of Medical Sciences (130/8970, March, 2002).

Experimental design

Sixteen rats were divided into four groups of four rats each: control (A), PTX (B), AFB1 (C) and AFB1 + PTX (D). Group B and D rats were injected with PTX (100 mg/kg intraperitoneally) (Leist *et al.*, 1996; Barton *et al.*, 2001) 24 h before being anesthetized. All rats were anesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine (60 mg/ml and 8 mg/ml, respectively) (Wolkoff *et al.*, 1987; Mehvar and Zhang, 2002). The anterior abdomen was cleaned with alcohol and a ventral longitudinal midline incision was made extending from the pubis to the upper chest. The animals were heparinized by injecting into the inferior vena cava anterior to the renal vein with immediate ligation of the vena cava. The hepatic portal vein (inlet) and the thoracic inferior vena cava (outlet) were then cannulated. The liver was perfused with KHBB (pH 7.4 ± 2) saturated by 95%O₂/5%CO₂, through a catheter cannulated into the portal vein. The perfusate was collected from a catheter in placed in the superior vena cava via the right atrium. The flow rate of perfusion was 20 ml/min at 15–20 cm H₂O. The flow rate was measured by fractionating the effluent. In the rats of groups C and D, 1 ppm AFB1 was infused (about 3 ml/g liver/min) into the portal vein. This concentration was selected based on our preliminary study of different doses of AFB1 (0.01, 0.1 and 1 ppm). Group A rats were infused with KHBB.

Sample collection

Samples (1.5 ml) were taken from the outlet at 0, 15, 30, 45, 60, 75, 90, 105 and 120 min and stored at –20°C for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels. The middle lobe of the perfused livers was used for measurements of malondialdehyde (MDA), GSH and total protein.

Sample analysis

Enzymes levels were quantified based on ultraviolet light methods using a commercial kit from Teb Gostaran Hayan (Tehran, Iran). Lipid peroxidation was determined in liver tissue homogenate according to the thiobarbituric acid method (Esterbauer and Cheeseman, 1990). GSH was estimated by the Kuo and Hook standard method (Kuo and Hook, 1982) and total protein was estimated by the Bradford method (Bradford, 1976).

Statistical analysis

Values are presented as mean ± SE. Data were analyzed by one-way analysis of variance, followed by Tukey's post hoc test for multiple comparisons. Differences were considered to be statistically significant at $p < 0.05$.

Results

Liver viability and damage parameters

Flow rate showed a time dependent decrease but there were no significant differences among the groups (Figure 1). ALT is an enzyme in the hepatocyte cytoplasm that is released following minor liver damage (Bessems *et al.*, 2006). The increase in ALT release in the AFB1 was significant after 60 min in comparison with the control group (Figure 2). In the others groups, we also observed a statistically significant difference, particularly in the AFB1 + PTX group. AST is an enzyme in both hepatocyte cytoplasm and mitochondria and is released in response to major liver damage (Bessems *et al.*, 2006). A statistically significant difference was observed between the AFB1 group and the controls, but not between AFB1 and AFB1 + PTX (Figure 3). LDH is a general tissue damage marker; it present in hepatocytes and non-parenchymal cells but is not a liver-specific enzyme (Bessems *et al.*, 2006). An increase in LDH release manifested from 30 min to 120 min with fluctuations in the AFB1 group. There were significant differences between the AFB1 and control groups and between AFB1 and AFB1 + PTX (Figure 4).

MDA and GSH concentration in liver

AFB1 reduced GSH concentrations in comparison with the control group. In the PTX + AFB1 group, the increase of GSH concentration was not significant

Figure 1: Flow rate in perfused rat liver over 120 min. Values are means \pm S.E. of four experiments.

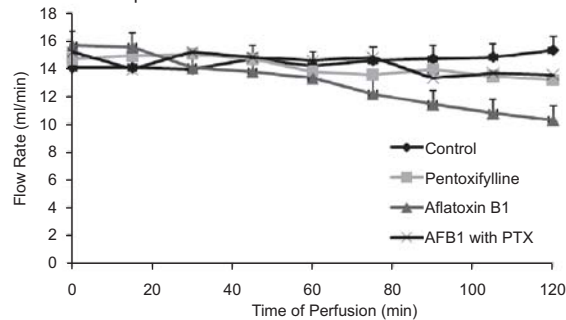


Figure 2: Time course of ALT release from perfused rat liver over 120 min. Values are means \pm S.E. of four experiments.

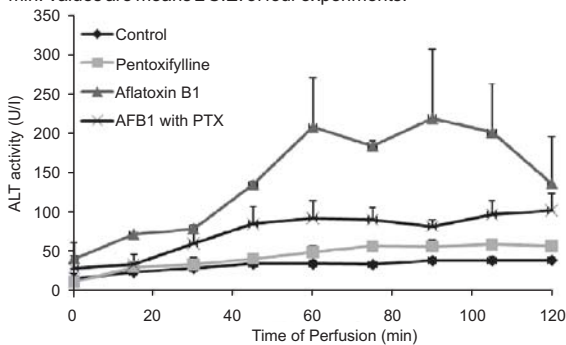


Figure 3: Time course of AST release from perfused rat liver over 120 min. Values are means \pm S.E. of four experiments.

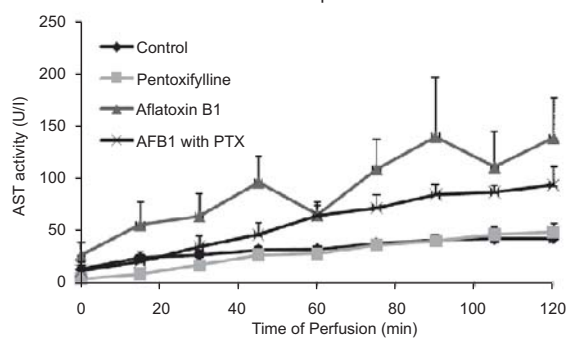


Figure 4: Time course of LDH release from perfused rat liver over 120 min. Values are means \pm S.E. of four experiments.

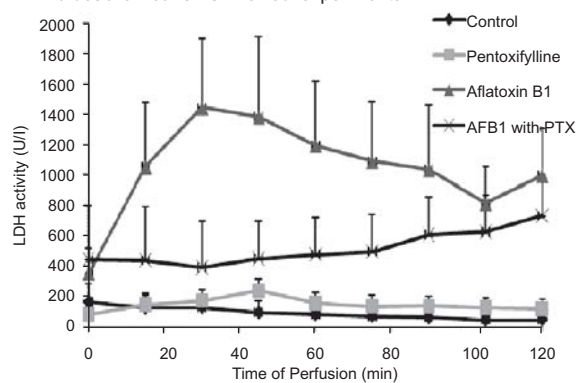


Table 1: Concentrations of GSH, protein and MDA in perfused rat liver at the end of the experiment.

Group	Concentration	MDA (nmol)	Protein (μ g/ml)	GSH (nmol)
Control	KHBB	0.405 \pm 0.13	148.15 \pm 16.5	569 \pm 85.1
PTX	100 mg/kg	0.6 \pm 0.12	193.7 \pm 39.4	428 \pm 112.3
AFB1	1 ppm	2.18 \pm 0.17*	727.5 \pm 37.1*	91.7 \pm 8.2**
AFB1 + PTX	1ppm + 100 mg/kg	0.945 \pm 0.1†	523.2 \pm 78.2††	239.6 \pm 66

Values are means \pm S.E. of four experiments.

*Statistically significant difference at $p < 0.001$ compared with control group. **Statistically significant difference at $p < 0.01$ compared with control group. †Statistically significant difference at $p < 0.001$ compared with AFB1 group. ††Statistically significant difference at $p < 0.05$ compared with AFB1 group

compared to the AFB1 group (Table 1). The MDA concentration in liver was significantly increased by AFB1 and decreased by PTX. Protein levels in the AFB1 and AFB1 + PTX groups were significantly greater than in the control group (Table 1).

Discussion

The aim of this study was to investigate the effect of pretreatment with PTX on AFB1-induced hepatotoxicity in perfused rat liver. We used the perfused rat liver model because it is considered a suitable system for chemically induced hepatotoxicity (Lupo *et al.*, 1986). Most studies on the effects of AFB1 in isolated perfused rat liver have focused on hepatic uptake and disposition, net synthesis of albumin, fibrinogen and α_1 -glycoprotein, and carcinogenicity (John and Miller, 1969; Unger *et al.*, 1977; Essigmann *et al.*, 1980). Furthermore, the first site of AFB1 damage is the liver. In our study, AFB1 at 1 ppm concentration affected ALT, AST and LDH activity in rat liver. The increase of enzyme levels was partially time dependent. Elevations of AST levels were significant in comparison with the control group, showing that AFB1 caused major liver damage. Changes in ALT and LDH levels are a manifestation of minor liver damage. Rat liver pretreated with PTX before administration of AFB1 showed a decrease of ALT, AST and LDH activity, but this was not statistically significant. The perfusion flow rate was approximately constant in all four groups. Only in the AFB1 group after 60 min of perfusion was a decrease of flow rate observed. Numerous studies have reported increased activity of ALT, AST and LDH in liver during aflatoxin treatment *in vivo*. Most studies of perfused rat liver have demonstrated an increase of ALT, AST and LDH levels (Deters *et al.*, 1998; Yokoyama *et al.*, 2006; Alexandrova *et al.*, 2007). PTX slowed the release of AST, ALT and LDH induced by aflatoxin B1 but there were no significant differences among the groups. MDA and GSH concentrations are measured as signs of oxidative stress and lipid peroxidation in liver tissue. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids (Yener *et al.*, 2009).

Increased MDA concentrations and decreased hepatic GSH levels were evident after infusion of 1 ppm concentration of AFB1. In the group pretreated with PTX before administration of AFB1, the decrease of MDA concentration was significant in comparison with the group given AFB1 only, but the increase of GSH levels was not significant. Protein aggregates were produced in cell membranes, which underwent lipid peroxidation induced by AFB1. These proteins impair the physiological function of tissues and are metabolized in the liver. It seems that PTX protects against AFB1-induced oxygen free radicals but could not compensate for reduced GSH. PTX is a nonselective inhibitor of phosphodiesterase capable of increasing cAMP and cGMP levels (Radfar *et al.*, 2005). These nucleotides prevent oxidative stress by reducing lipid peroxidation and thus PTX reduces hepatic cellular damage due to free radicals (Radfar *et al.*, 2005). PTX also has anti-inflammatory effects and prevents transcription of TNF- α . This cytokine plays a role in the augmentation of AFB1-induced liver damage, and its inhibition by PTX and anti-TNF- α antibodies prevents further damage (Abdin *et al.*, 2010; Maiti *et al.*, 2007; Abdel-Salam *et al.*, 2003).

Conclusion

One of the underlying mechanisms of AFB1-induced hepatic cell injury is oxidative damage that can be prevented by various natural antioxidants. In the present study, exposure to AFB1 caused an increase in MDA concentration as an indicator of cellular lipid peroxidation, and a decrease in the GSH content of perfused rat liver, which is in agreement with findings reported previously. Pretreatment with PTX significantly decreased MDA levels in comparison with the group that received AFB1 only, and GSH levels increased significantly in comparison with the AFB1 group. These results imply that prior treatment with PTX ameliorates the effects of AFB1-induced lipid peroxidation.

References

1. Abdel-Salam, O. M. E.; Baiuomy, A. R. *et al.* (2003) The anti-inflammatory effects of the phosphodiesterase inhibitor pentoxifylline in the rat. *Pharmacological Research* 47(4): 331-340.
2. Abdin, A. A.; El-Hamid, M. A. A. *et al.* (2010) Effect of pentoxifylline and/or alpha lipoic Acid on experimentally induced acute pancreatitis. *European Journal of Pharmacology* 643(2-3): 289-296.
3. Alexandrova, A.; Kebis, A. *et al.* (2007) Copper impairs biliary epithelial cells and induces protein oxidation and oxidative DNA damage in the isolated perfused rat liver. *Exp Toxicol Pathol* 58(4): 255-61.
4. Awney, H. A.; Attih, A. M. *et al.* (2002) Effect of melatonin on the production of microsomal hydrogen peroxide and cytochrome P-450 content in rat treated with aflatoxin B1. *Toxicology* 172(2): 143-148.
5. Barton, C. C.; Eva, X. B. *et al.* (2001) Bacterial lipopolysaccharide enhances aflatoxin B hepatotoxicity in rats by a mechanism that depends on tumor necrosis factor alpha. *Hepatology* 33(1): 66-73.
6. Bessems, M.; 't Hart, N. A. *et al.* (2006) The isolated perfused rat liver: standardization of a time-honoured model. *Lab Anim* 40(3): 236-46.
7. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-54.
8. Choi, C. Y.; Park, S. H. *et al.* (1991) Alteration of aflatoxin B1 metabolic profiles and reduction of aflatoxin B1 mutagenicity by hepatic microsomes of rats fed butylated hydroxyanisole. *The Journal of toxicological sciences* 16: 119.
9. Choi, K. C.; Chung, W. T. *et al.* (2010) Inhibitory effects of quercetin on aflatoxin B1 induced hepatic damage in mice. *Food and Chemical Toxicology* In Press.
10. Deters, M.; Siegers, C. P. *et al.* (1998) Influence of glycine on the damage induced in isolated perfused rat liver by five hepatotoxic agents. *Toxicology* 128(1): 63-72.
11. Essigmann, J. M.; Donahue, P. R. *et al.* (1980) Use of the isolated perfused rat liver to study carcinogen-DNA adduct formation from aflatoxin B1 and sterigmatocystin. *Cancer Res* 40(11): 4085-91.
12. Esterbauer, H.; Cheeseman, K. H. (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* 186: 407-21.
13. Gesing, A.; Karbownik-Lewinska, M. (2008) Protective effects of melatonin and N acetylserotonin on aflatoxin B1-induced lipid peroxidation in rats. *Cell Biochem Funct* 26(3): 314-9.
14. John, D. W.; Miller, L. L. (1969) Effect of aflatoxin B1 on net synthesis of albumin, fibrinogen, and alpha-1-acid glycoprotein by the isolated perfused rat liver. *Biochem Pharmacol* 18(5): 1135-46.
15. Kuo, C. H.; Hook, J. B. (1982) Depletion of renal glutathione content and nephrotoxicity of cephaloridine in rabbits, rats, and mice. *Toxicol Appl Pharmacol* 63(2): 292-302.
16. Lee, J.-K.; Choi, E. H. *et al.* (2005) Alleviation of aflatoxin B1-induced oxidative stress in HepG2 cells by volatile extract from *Allii Fistulosi Bulbus*. *Life Sciences* 77(23): 2896-2910.
17. Leist, M.; Auer-Barth, S. *et al.* (1996) Tumor necrosis factor production in the perfused mouse liver and its pharmacological modulation by methylxanthines. *Journal of Pharmacology and Experimental Therapeutics* 276(3): 968.
18. Lupo, S.; Hewitt, W. R. *et al.* (1986) Cadmium toxicity in the isolated perfused rat liver. *Toxicol Lett* 34(1): 5-11.
19. Maiti, R.; Agrawal, N. K. *et al.* (2007) Effect of Pentoxifylline

- on inflammatory burden, oxidative stress and platelet aggregability in hypertensive type 2 diabetes mellitus patients. *Vascular Pharmacology* 47(2-3): 118-124.
20. Mehvar, R.; Zhang, X. (2002) Development and application of an isolated perfused rat liver model to study the stimulation and inhibition of tumor necrosis factor- α production ex vivo. *Pharm Res* 19(1): 47-53.
 21. Preetha, S. P.; Kanniappan, M. *et al.* (2006) Lupeol ameliorates aflatoxin B1-induced peroxidative hepatic damage in rats. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 143(3): 333-339.
 22. Qin, G.; Gopalan-Kriczky, P. *et al.* (1997) Inhibition of aflatoxin B1-induced initiation of hepatocarcinogenesis in the rat by green tea. *Cancer Letters* 112(2): 149-154.
 23. Radfar, M.; Larijani, B. *et al.* (2005) Effects of pentoxifylline on oxidative stress and levels of EGF and NO in blood of diabetic type-2 patients; a randomized, double-blind placebo-controlled clinical trial. *Biomedicine & Pharmacotherapy* 59(6): 302-306.
 24. Rastogi, R.; Srivastava, A. K. *et al.* (2001) Long term effect of aflatoxin B(1) on lipid peroxidation in rat liver and kidney: effect of picroliv and silymarin. *Phytother Res* 15(4):307-10.
 25. Shen, H.-M.; Ong, C.-N. *et al.* (1995) Involvement of reactive oxygen species in aflatoxin B1-induced cell injury in cultured rat hepatocytes. *Toxicology* 99(1-2): 115-123.
 26. Shen, H. M.; Shi, C. Y. *et al.* (1994) Aflatoxin B1-induced lipid peroxidation in rat liver. *Toxicol Appl Pharmacol* 127(1): 145-50.
 27. Towner, R. A.; Mason, R. P. *et al.* (2002) In vivo detection of aflatoxin-induced lipid free radicals in rat bile. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1573(1): 55-62.
 28. Towner, R. A.; Qian, S. Y. *et al.* (2003) In vivo identification of aflatoxin-induced free radicals in rat bile. *Free Radical Biology and Medicine* 35(10): 1330-1340.
 29. Unger, P. D.; Mehendale, H. M. *et al.* (1977) Hepatic uptake and disposition of aflatoxin B1 in isolated perfused rat liver. *Toxicol Appl Pharmacol* 41(3): 523-34.
 30. Wang, C.-J.; Hsu, J.-D. *et al.* (1991) "Suppression of aflatoxin B1-induced hepatotoxic lesions by crocetin (a natural carotenoid)." *Carcinogenesis* 12(10): 1807-1810.
 31. Wolkoff, A. W.; Johansen, K. L. *et al.* (1987) The isolated perfused rat liver: preparation and application. *Anal Biochem* 167(1): 1-14.
 32. Yener, Z.; Celik, I. *et al.* (2009) Effects of *Urtica dioica* L. seed on lipid peroxidation, antioxidants and liver pathology in aflatoxin-induced tissue injury in rats. *Food Chem Toxicol* 47(2): 418-24.
 33. Yokoyama, H.; Horie, T. *et al.* (2006) Naproxen-induced oxidative stress in the isolated perfused rat liver. *Chem Biol Interact* 160(2): 150-8.