AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 34 NUMBER 1 MARCH 2005

ISSN 1116—4077

Afr. J. Med. med. Sci. (2005) 34, 37-43

The effect of modulation of glutathione levels on markers for Aflatoxin B_1–induced cell damage

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Summary

The modulatory effect of glutathione levels on markers for aflatoxin B_1 (AFB_1)–induced cell damage has been investigated in the rat (susceptible specie) and the (mouse resistant specie). The concentration of GSH was depleted and increased by administering paracetamol (PAM) and cysteine respectively and activities of glutathione *S*-transferase (GST) and γ–glutamyl transpeptidase (γ-GT) were determined. The effect of AFB_1 on hepatic lipid peroxidation in both species was also investigated. Treatment of rats with 2 mg/kg.bwt AFB_1 intraperitoneally caused a depletion of GSH in the liver to a minimum at 6 h (80% of the control value) and the level returned to normal after 24 h. GST was similarly increased to a maximum at 6 h and the level also returned to normal after 24 h. GSH and GST activities were not significantly affected in AFB_1-treated mice. Orally administered PAM (400 mg/kg.bwt) caused a depletion of GSH with a minimum at 6 h (59% and 36% of the control rats and mice respectively). Pretreatment of AFB_1 with PAM produced a serious depletion at 6 h (34% and 35% of the control rats and mice respectively). GST activities were also marginally increased in both animals. AFB_1 pretreatment mediated ($P<0.001$) hepatic lipid peroxidation in rats but not in mice as assessed by the formation of thiobarbituric acid reactive substances (TBARS). Treatment of rats and mice with oral cysteine (50mg/kg bwt) elicited a significant elevation of GSH. Administration of cysteine with AFB_1 to rats attenuated the toxic effects of AFB_1 on GSH and inhibited the formation of TBARS. γ-GT activity was significantly increased when AFB_1 alone was administered to rats but was not increased ($P>0.05$) when cysteine was pretreated alone with AFB_1. Combined treatment of AFB_1 and PAM induced 177% increase in γ–GT activity. Overall, our results suggest that the metabolism of aflatoxin B_1 by GSH does not lead to the formation of toxic products but rather GSH plays a protective role in AFB_1-induced cell damage and GSH pathway is less utilised in mice.

Keywords: *Aflatoxin B_1, Glutathione S-transferase, γ-Glutamyl transpeptidase, Hepatotoxicity, Glutathione, lipid peroxidation.*

Résumé

Les effets modulateurs des niveaux du glutathione sur les marqueurs d' aflatoxin B1 (AFB1) la destruction cellulaire -provoqué a été etudie sur le souris (specie susceptible) et le (souris specie résistant). La concentration de GSH etait réduite et augmentée en administrant le paracetamol (PAM) et la cysteine respectivement et activités de glutathione S-transferase (GST) et transpeptidase du g-glutamyl (g-GT) ont été déterminés. L'effet d'AFB1 sur la peroxidation lipide hépatique dans les deux l'espèce a aussi été etudié. Le Traitement du sourris avec 2 mg/kg.bwt AFB1 avec l'intraperitoneal a causé un reduction de GSH dans le foie au minimum à 6 h (80% de la valeur du contrôle) et le niveau est revenu à la normale après 24 h. Le GST a aussi été augmenté au maximum à 6 h et le niveau est revenu à la normale après 24 h de la même façon. Les activités de GSH et de GST n'ont pas été affectées considérablement dans les souris traitees de l'AFB1. Le PAM oralement administré (400 mg/kg.bwt) a causé une reduction de GSH avec un minimum à 6 h (59% et 36% des rats et des souris etudiés respectivement). Le prétraitement d'AFB1 avec le PAM a produit une reduction sérieuse à 6 h (34% et 35% des rats et de souris respectivement), les activités GST ont aussi été augmentées d'une manière marginale dans les deux animaux. Le pretraitement de l'' AFB1 a servi de médiateur ($P<0.001$) peroxidation lipide hépatique dans les rats mais pas dans les souris comme évalué par la formation de substances thiobarbituriques acides réactives (TBARS). Le traitement de rats et souris avec le cysteine oral (50mg/kg bwt) a montré une élévation considérable de GSH. L'administration de cysteine avec AFB1 aux rats ont atténué les effets toxiques d'AFB1 sur GSH et ont inhibé la formation de TBARS. L'activité du g-GT avait été considérablement augmentée quand l'AFB1 seul a été administré aux rats mais n'a pas été augmenté ($P>0.05$) quand le cysteine a été prétraité avec AFB1 seul. Le traitement combiné d'AFB1 et le PAM a induit 177% d'augmentation dans l'activité du g-GT. En général, nos résultats suggèrent que le métabolisme d'aflatoxin B1 par GSH ne mène pas à la formation de produits toxiques mais plutôt le GSH joue un rôle protecteur dans dans la sestruction cellulaire induite de l'AFRBI et la voie GSH est moins utilisé dans les souris.

Introduction

Aflatoxin B_1 (AFB_1), the most hepatotoxic, mutagenic and carcinogenic of the difuranocoumarins, is a mycotoxin produced by *Aspergillus flavus* and related fungi, which is of considerable concern as health risk in several parts of the world [1]. Susceptibility to the toxic and carcinogenic effects of AFB_1 varies markedly between species [2]. The duck, trout and rat are highly susceptible to the carcinogenic effects of AFB_1, whereas monkey, mouse and hamster are relatively resistant [3].

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AFB_1 is biotransformed by the cytochrome P450 (CYP450) mixed function oxygenase system to the highly electrophilic reactive intermediate, AFB_1-8, 9 – epoxide, the isomer of which avidly binds to cellular macromolecules including DNA [4,5]. AFB_1 metabolites have been shown to be detoxified by various mechanisms including glucuronic acid conjugation [6] and glutathione conjugation, which serves as a crucial pathway for AFB_1 detoxification [7]. Also, the 8,9 epoxide of AFB_1 may alternatively hydrolyze spontaneously or via the action of epoxide hydrolase yielding the AFB_1-8, 9-dihydrodiol [5]. The reduction of AFB_1 to aflatoxicol and CYP450-mediated hydroxylation to aflatoxin M_1 and aflatoxin Q_1 or demethylation to aflatoxin P_1 have been reported [8,9].

Glutathione has long been known to be of value in the maintenance of tissue integrity by conjugating electrophilic compounds, which results in the formation of mercapturic acid. In many mammalian species, the primary pathway for AFB_1 detoxification is through glutathione *S*-transferase (GST)-mediated conjugation of AFB_1-8, 9 – epoxide with reduced glutathione [7,10]. Studies however, have demonstrated that enzymatically formed glutathione *S*-conjugates are toxic [11,12]. Glutathione conjugates which are synthesised in the liver by hepatic cytosolic and microsomal glutathione transferases are converted to cysteine-*S*-conjugates by the activity of γ-glutamyltranspeptidase (γ-GT) and dipeptidases present in the biliary tree, the gut or the kidney [13]. Uptake of cysteine-*S* conjugates and subsequent metabolism by β-lyase may lead to the formation of unstable thiol or to stable but toxic product. Both products may be involved in the expression of toxicity [14]. Glutathione metabolism by γ-GT has also been demonstrated to cause oxidative damage as measured by lipid peroxidation [15].

Studies have shown that the levels of GSH can be modulated by administration of chemicals. Thus, compounds such as diamide, diethylmaleate, paracetamol and t-butylhydroperoxide can be used to reduce GSH levels [16]. At the same time administration of cysteine may raise cellular GSH levels because it is usually the limiting amino acid in GSH biosynthesis [17]. GSH modulation models are useful for understanding the role of GSH in health and disease and for assessing potential therapies for raising cellular GSH levels.

In the present study, we have examined the role that glutathione plays in AFB_1 –induced cell damage by depleting and increasing its concentration using paracetamol and cysteine respectively and the activities of GST and γ-GT determined in the two animal species- rat a sensitive and mouse a resistant specie to the hepatocarcinogenesis of AFB_1. Since it has been recently reported that reactive oxygen specie is implicated as a probable mechanism of AFB1-induced hepatocarcinogenesis, the effect of AFB_1 on hepatic lipid peroxidation in both species was also investigated.

Materials and methods

Chemicals

AFB_1, 1-chloro 2, 4 dinitrobenzene (CDNB), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA) and other chemicals were purchased from Sigma Chemical Co. Poole, Dorset. Paracetamol, obtained from a local chemist in Ibadan, Nigeria, was extracted and recrystalized in hot boiling ethanol to obtain pure crystals. The melting point was determined and compared to literature value. All other reagents and solvents were of analytical grade.

Animals and drug pretreatment

Two separate experiments were carried out in the present investigation. In the first experiment, AFB_1 (2 mg/kg) was administered intraperitoneally to male albino rats (5) (Wistar strain) weighing between 180-220 g and 5 male mice (20-30 g) in corn oil as vehicle. Control rats (5) and mice (5) were treated with distilled water. Similarly, Paracetamol (PAM) (400 mg/kg) was administered to rats and mice orally by intubation.

In the second experiment, 36 male rats and 36 male mice were randomly divided into 6 groups. Group 1 serves as control, animals in group 2 were treated intraperitoneally with single dose of AFB_1 (2mg/kg). Animals in group 3 were administered cysteine (50mg/kg) and group 4 animals were co administered AFB_1 and cysteine. Group 5 animals received 400mg/kg PAM, while animals in group 6 received both AFB_1 and PAM. Animals were maintained on a 12h/light cycle and had access to standard laboratory chow and water *ad libitum*. Rats and mice were sacrificed by cervical dislocation at different time intervals ranging from 0-24 h.

Analytical procedures

Livers were removed, washed, dried with filter paper and weighed livers were homogenised in 4 vol 0. 1 M phosphate buffer pH 7.4.

Reduced GSH was determined in 10,000g supernatant fraction according to Jollow *et al.*, [18]. Briefly, an aliquot of liver homogenate was deproteinised by the addition of equal vol of 4% sulfosalicylic acid and the resulting solution was centrifuged at 10,000 g for 15 min at 4°C. 0.5 ml of the supernatant was then added to 4.5 ml (Ellman reagent) 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB). Reduced glutathione was proportional to the absorbance at 412 nm.

GST was assayed according to Habig *et al.* [19] using 1-chloro, 2,4 dinitrobenzene as substrate. The incubation mixture contained 50 µl of CDNB, 10 µl 0.1M reduced glutathione, 0.93 ml 0.1 M phosphate buffer (pH 6.5) and 10µl cytosolic fraction in a total volume of 1 ml. The change in absorbance at 340 nm was followed for 60 seconds in a Perkin Elmer spectrophotometer 548 at 30°C. γ-GT activity was assayed by following the *p*-nitroaniline released from gamma-glutamyl-*p*-nitroanilide in the presence of glycyl-glycine as glutamyl acceptor at 405nm

Table 1: Effect of aflatoxin B_1 (2mg/kg, i.p) on rats and mice hepatic glutathione and glutathione S-transferase activity.

Time (hr) After Treatment	Concentration of glutathione (mg/100g liver weight)				Enzyme activity (μmol/mg prot/min)			
	Rat		Mouse		Rat		House	
	Control	Test	Control	Test	Control	Test	Control	Test
0	150 ± 0.8	150 ± 0.7	145 ± 0.3	145 ± 10.5	2.7 ± 0.7	2.7 ± 0.7	2.2 ± 0.6	2.2 ± 0.7
1	150 ± 0.9	146 ± 0.3	145 ± 0.6	142 ± 0.4	2.6 ± 0.4	2.6 ± 0.3	2.2 ± 0.4	2.2 ± 0.6
2	149 ± 0.7	140 ± 0.3	146 ± 0.2	140 ± 0.8	2.7 ± 0.5	3.1 ± 0.4	2.2 ± 0.2	2.2 ± 0.5
4	150 ± 0.8	138 ± 0.2	144 ± 0.5	140 ± 0.4	2.7 ± 0.4	3.3 ± 0.6	2.1 ± 0.3	2.2 ± 0.5
6	150 ± 0.7	120 ± 0.7*	145 ± 10.3	139 ± 0.2	2.6 ± 0.3	3.7 ± 0.4*	2.2 ± 0.6	2.4 ± 0.4
8	151 ± 0.3	130 ± 0.4	145 ± 0.4	142 ± 0.3	2.6 ± 0.4	3.4 ± 0.4	2.2 ± 0.7	2.2 ± 0.8
10	150 ± 0.3	137 ± 0.7	145 ± 0.4	144 ± 0.3	2.7 ± 0.4	3.0 ± 0.5	2.2 ± 0.9	2.2 ± 0.7
24	149 ± 0.2	149 ± 0.6	145 ± 0.4	145 ± 0.2	2.7 ± 0.3	2.7 ± 0.2	2.2 ± 0.1	2.2 ± 0.1

*Values are means ± SD for four animals * Significantly different from control ($P < 0.001$)*

Table 2: Effect of orally administered paracetamol (400 mg/kg) on rats and mice hepatic glutathione.

Time (hr) After Treatment	Concentration of glutathione (mg/100g liver weight)				Enzyme activity (μmol/mg prot/min)			
	Rat		Mouse		Rat		House	
	Control	Test	Control	Test	Control	Test	Control	Test
0	150 ± 0.8	150 ± 0.7	145 ± 0.5	145 ± 0.8	2.7 ± 0.7	2.7 ± 0.5	2.2 ± 0.7	2.2 ± 0.6
2	150 ± 0.6	148 ± 0.5	146 ± 0.7	132 ± 0.6	2.7 ± 0.5	2.8 ± 0.4	2.2 ± 0.6	2.3 ± 0.5
4	149 ± 0.7	91 ± 0.6*	145 ± 0.4	61 ± 0.5*	2.7 ± 0.6	3.4 ± 0.5*	2.1 ± 0.5	3.4 ± 0.4*
6	150 ± 0.5	88 ± 0.7*	145 ± 0.6	52 ± 0.4*	2.8 ± 0.4	4.1 ± 0.3*	2.2 ± 0.4	3.6 ± 0.3*
8	150 ± 0.6	115 ± 0.9	145 ± 0.5	70 ± 0.3	2.7 ± 0.4	3.8 ± 0.4	2.2 ± 0.5	3.2 ± 0.2

Values are means ± SD for five animals
**Significantly different from control ($P< 0.001$)*

Table 3: Effects of Aflatoxin B_1 (2mg/kg, i.p) and paracetamol (400 mg/kg) on rats and mice hepatic glutathione and the activity of glutathione S-transferase.

Time (hr) After Treatment	Concentration of glutathione mg/100g) liver weight)				Enzyme activity (μmol/mg prot/min)			
	Rat		Mouse		Rat		House	
	Control	Test	Control	Test	Control	Test	Control	Test
0	150 ± 0.7	150 ± 0.6	145 ± 0.6	145 ± 0.5	2.7 ± 0.6	2.7 ± 0.5	2.1 ± 0.9	2.2 + 0.7
6	150 ± 0.7	51 ± 0.4*	144 ± 0.5	50 ± 0.6*	2.7 ± 0.7	5.5 ± 0.2*	2.2 + 0.4	3.9 + 0.4*

Values are means ± SD for five animals
**Significantly different from control ($P< 0.001$)*

[20]. Protein content of various fractions was determined by the method of Lowry *et al.* [21].

Lipid peroxidation was assessed in rats and mice by measuring the thiobarbituric acid reacting substances (TBARS) formation as described by Farombi *et al.*, [22]. Briefly, 1 mg/ml of the 10,000 g supernatant fraction in isotonic phosphate buffer, pH 7.4 was incubated for 6 hr at 37ºC in a shaking water bath. 0.5 ml of 0.75% thiobarbituric acid in 0.1 M HCl was added to 0.5 ml of incubation mixture already quenched with 0.5 ml of 10% TCA. The mixture was heated at 90-95ºC for 20 min and after cooling centrifuged for 10 min at 780 g. The supernatant was transferred into acid resistant tubes and centrifuged at 32,000 g for 10 min. The absorbance of the resulting clear solution was

determined at 532 nm using a Perkin Elmer spectrophotometer 548..

Statistics

The data were analyzed by a two tailed Students t-test. P values less than 0.05 were considered statistically significant.

Results

Effects of AF B_1 and PAM on GSH and GST in rats and mice.

Rats pretreated with AFB_1 (2mg/kg) showed a time-dependent decrease in the concentration of hepatic GSH, which reached a minimum at 6 h (80% of control) and increased thereafter to the initial level at 24 h (Table 1). There was a corresponding increase in the activity of GST at 6 h (137%) returning to the initial level at 24h (Table 1). The results showed that hepatic GSH was much less depleted in mice than in rats following AFB_1 treatment. In addition, mice hepatic GST was not significantly affected (Table 1). Table 2 shows the effect of orally administered PAM (400 mg/kg) on rat and mouse hepatic GSH. In rat, at 4 and 6h there were significant depletions in hepatic GSH level (61% and 59% at 4 and 6h). Similarly, GST activity was increased at 4 and 6h maximally (Table 2). Mice hepatic GSH was depleted to 36% of the control at 6h following treatment with 400mg/kg PAM. (Table 2).

Since GSH level was maximally depleted at 6 hr when the animals were administered with AFB1, and PAM singly, in the subsequent experiment, the animals were co-administered with AFB1 and PAM and GSH level determined at 0 and 6 hr post treatment. At 6 h after PAM and AFB_1 co-administration, rat and mouse hepatic GSH levels were seriously depleted to 34% and 35% respectively of the control. GST activities in both species were similarly increased ($P<0.001$) (Table 3).

Table 4: Effects of Aflatoxin B1 (2mg/kg, i.p) and cysteine (50mg/kg, p.o) on rats and mice hepatic glutathione concentra-tion.

Treatment	Concentration of glutathione mg/100g liver weight Rat	Mouse
Control	150 ± 0.8	145 ± 10.3
AFB_1	120 ± 0.7*	139 ± 0.2
Cysteine	164 ± 0.3*	176 ± 0.3*
AFB_1 + Cysteine	141 ± 0.4**	172 ± 0.4

Values are mean± SD for 5 animals.
**Significantly different from control (P<0.001)*
***Significantly different from AFB1 group (P<0.001)*

Modulatory effect of cysteine and PAM on AFB_1–induced lipid peroxidation and alteration of GSH and γ-GT activity in rats and mice.

Cysteine administered at a dose of 50mg/kg bwt elicited 9% and 21% increases in rats and mice hepatic GSH levels respectively 6h after pretreatment. Following cysteine pretreatment with AFB_1 a slight decrease in hepatic GSH (6%) level was observed in rat while 18% increase in GSH level was observed in mice compared to control (Table 4).

AFB_1 pretreatment significantly increased the formation of thiobarbituric acid reacting substances (TBARS) in rats but not in mice assessed at 24 h after treatment (Table 5). Cysteine pretreated with AFB_1 inhibited the formation of TBARS in rats 24 h after treatment.

Table 5: Effects of Aflatoxin B1 (2mg/kg, i.p), paracetamol (400mg/kg,p.o) and cysteine (50mg/kg, p.o) on rats and mice hepatic lipid peroxidation.

Treatment	Thiobarbituric acid reacting substances (TBARS) A 532/mg protein Rat	Mouse
Control	0.34 ± 0.3	0.21 ± 0.2
AFB_1	0.62 ± 0.1*	0.31 ± 0.3
Cysteine	0.23 ± 0.1*	0.26 ± 0.1
AFB_1+Cysteine	0.33 ± 0.1**	0.29 ± 0.2
PAM	0.68 ± 0.2*	0.64 ± 0.1*
AFB_1+PAM	0.85 ± 0.4*	0.68 ± 0.2

Values are means SD for five animals. Determination of TBARS was performed 24 hour after treatment.
**Significantly different from control (P<0.001;*
***Significantly different from AFB1 group (P<0.01)*

Table 6: Alterations of g–glutamyl transpeptidase activity in rats and mice following pretreatments with Aflatoxin B_1 (2mg/kg, i.p), paracetamol (400mg/kg, p.o) and cysteine (50mg/kg, p.o).

Treatment	Enzyme Activity (U/l) Rat	Mouse
Control	16.9 ± 0.2	14.8 ± 0.3
AFB1	21.2 ± 0.4*	16.9 ± 0.1
Cysteine	16.3 ± 0.1	15.1 ± 0.2
AFB1+Cysteine	19.0 ± 0.7	16.9 ± 0.3
PAM	23.2 ± 0.2*	18.1 ± 0.3*
AFB1 + PAM	29.9 ± 0.2*	23.3 ± 0.1*

Values are means ± SD for five animals. Assays were performed 24 h after treatment.
** Significantly different from control (P<0.001)*

The effects of AFB_1, PAM, and cysteine given alone or in combination on the activity of γ-GT in rats and mice are presented in (Table 6). γ-GT activity was significantly increased (125 % of control) when AFB_1 alone was administered to rats whilst cysteine was observed to prevent this increase when given before AFB_1 treatment. Combined treatment with AFB_1 and PAM lead to a 177 % increase in γ-GT activity. Mice were observed to be more resistant to changes in γ-GT with no statistically significant effect seen

for AFB_1 treatment. Furthermore, the percentage increases in the enzyme activity were less for PAM (122 %) and PAM + AFB_1 (157 %)

Discussion

In this study, AFB_1 was observed to deplete rat glutathione levels to a minimum at six hours and increased the activity of GST. This corresponds to the time of maximum binding of AFB_1 to hepatic macromolecules and suggests a competitive process between the binding of AFB_1 to liver macromolecules with attendant cell damage [23] and the binding to GSH, which is believed to be protective.

This pattern however was not obtained in mice treated with the same dose of AFB_1. The difference observed in this study may be due to differences in susceptibility of rats and mice to AFB_1 toxicity. In addition, the metabolites produced in these two species are somehow different [3]. Species comparison of *in vitro* metabolism by 9,000 x g supernatant fraction of livers of many animal species showed that AFQ_1 was the principal chloroform soluble metabolite produced by monkey, human, and rat liver whereas AFP_1 was produced by monkey, human and mouse liver but not by rat and duck [3,7]. Aflatoxicol was also a major metabolite in rat plasma whereas this metabolite was not detected in the plasma of similarly dosed mice and monkeys which are resistant to AFB_1 carcinogenicity [24]. AFP_1, the *O*-demethylated product of AFB_1 has been detected in the urine of several animal species chiefly as the glucuronide, sulphate and as free phenol [8]. This may account for why mice hepatic GSH was less affected in this study.

Our data indicate that GSH is of value in protection of liver cells against AFB_1-induced damage. However, other studies have implicated GSH *S*-conjugates in the toxicity of several compounds such as the halo alkanes [25, 26] through the activities of GST which catalyze the formation of S-halo alkenyl GSH-conjugates and subsequent metabolism by γ-GT to form cysteine *S*-conjugates which may be converted by β-lyase to an unstable thiol that gives rise to reactive electrophilic products or to stable, but toxic products, of which both may be involved in the expression of toxicity [14,27,28].

The significant depletion of mice hepatic GSH observed when AFB_1 and PAM were fed in combination compared with GSH level observed in AFB_1 alone treated mice suggests that mice are not resistant to PAM toxicity [29]. The result is also indicative of the effect of PAM primarily and very little contribution if at all from AFB_1 since mice are resistant to its toxicity.

γ-GT is an enzyme used as marker of liver toxicity and induction of high levels is a frequent early event in experimental toxicity and hepatocarcinogenesis [13,15]. Induction of γ-GT in preneoplastic lesions has been studied most extensively in the rat liver model [13]. Our data showed a significant increase in the activity of γ-GT when AFB_1 was administered singly and in combination with PAM indicating toxicity to liver cell. The results further revealed that γ-GT activity was not increased when cysteine and AFB_1 were fed together. As expected the enzyme activity would have increased if GSH was biotransformed to toxic cysteine S-conjugates [26]. This result confirms the protective effect of GSH in AFB1-induced toxicity. The AFB_1- induced lipid peroxidation assessed by TBARS formation confirms earlier investigation [30,31]. Reactive oxygen species have been implicated in the mechanism of AFB_1 –induced cell damage [32,33]. The ability of cysteine, one of the components of the tripeptide glutathione and its precursor, to attenuate the AFB_1 mediated lipid peroxidation suggests a protective effect of GSH in AFB_1–induced lipid peroxidation unlike the S-halo alkanes whose metabolism by GSH results in the formation of toxic metabolites [11, 12, 28].

Taken together, our results suggest that the metabolism of aflatoxin by GSH does not lead to the formation of toxic products but rather GSH plays a protective role in AFB_1-induced cell damage as assessed by the levels of γ-GT, GST and TBARS formation. The data further indicate that GSH pathway is less utilised in mice presumably due to a major metabolite, AFP_1, in this specie that utilizes sulphate and glucuronide for detoxification [7]. This probably explains why the rat is susceptible to AFB_1-induced cell damage while the mouse is not.

Acknowledgements

We appreciate the gift of Aflatoxin B_1 from Mr. Opeoluwa Odusan of Abbot Pharmaceuticals in Chicago, USA.

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Received: 30/12/03
Accepted: 10/11/04