DIFFERENTIAL EFFECTS OF ACETAMINOPHEN AND AFLATOXIN B1 ON EXPRESSION OF LIVER CLASS-P GLUTATHIONE S-TRANSFERASE IN GROWING RATS[*]

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Abstract - Glutathione S-transferase (GST) is a superfamily enzyme which plays a major role in detoxification of xenobiotic compounds by catalyzing the conjugation of xenobiotic to cellular glutathione (GSH). GST-P is an important class of GSTs which is expressed during the early stage of life and during developmental stages. Its activity is relatively high during embryogenesis and immediately after birth and diminished in normal adult rat liver. To investigate the effects of hepatotoxic agents such as acetaminophen (APAP) and aflatoxin B1 (AFB1) on liver GST-P in rats during postnatal age, suckling rats age (14±2 days old) were divided into groups (n=5) and treated with both APAP (250 or 450 mg/kg B.W) and AFB1 (3 mg/kg B.W). Livers were removed at different time intervals (2, 6, 12, 18 and 24 h) and processed for GST and GST-P activity at protein and mRNA levels (RT-PCR). Administration of a single high dose of AFB1 (3 mg/kg BW) and APAP (450 mg/kg BW) to weanling rats caused a significant ($P < 0.05$) induction in total GST activity in developing rats. Based on the Western blotting technique and GST-P specific mRNA amplification by RT-PCR, the GST-pi protein level and its expression were not affected by APAP or AFB1. Despite the inducible effects of AFB1 and APAP on liver total GST activity, GST-P remained unaffected in response to the drugs at protein and mRNA levels.

Keywords - Acetaminophen, aflatoxin B1, glutathione S-transferases, expression

1. INTRODUCTION

Glutathione S-transferases; GSTs (EC: 2. 5. 1. 18) are a family of enzymes involved in the detoxification of foreign compounds. GSTs exist as homo-or hetero-dimers, and on the basis of sequence similarities, mammalian cytosolic GSTs have been grouped into at least seven classes *viz*. Alpha, Mu, Pi, Theta, Sigma, Omega and Zeta. GSTs catalyze the conjugation reaction of GSH to xenobiotics. The conjugate complex undergoes a further enzymatic reaction and mercupturic acid is produced. This metabolite is exerted in urine [1, 2]. A developmentally regulated expression of GSTs, especially GST-P is important in the ability of the liver to detoxify drugs and chemicals, and is a critical determinant of developmental toxicology. GST-P is the main isoform of GST in normal placental tissue**,** and comprises 36% of the total GST activity [3] and 67% of the total GST concentration [4]. GST-P expression is relatively higher in fetal liver than in normal adults, and its concentration slowly decreases after birth and is normally absent in adult rat liver. GSTs are involved in the detoxification processes of several xenobiotic compounds, particularly chemical carcinogens. The mechanism of GST-mediated inactivation of xenobiotics by conjugation to cellular glutathione (GSH) is well established in vitro and in vivo [5]. Aflatoxin B1 (AFB1) is a procarcinogen which is converted to potent hepatocarcinogen after undergoing activation by

microsomal cytochrome P-450. The metabolic activation products are good substrates for phase II detoxification enzymes. AFB1-8, 9-epoxide is a reactive metabolite which is inactivated by conjugation to cellular GSH, a reaction mediated by GST. AFB1 shares common properties with toxic doses of APAP in hepatotoxicity and metabolic pathways [6, 7]. APAP is metabolized primarily by conjugation with glucuronate or sulfate in the liver of many species. A smaller fraction of APAP is metabolized by hepatic cytochrome P-450 to N-acetyl-p-benzoquinone imine (NAPQI), a chemically reactive metabolite. This metabolite binds irreversibly to cellular macromolecules, an event often associated with hepatocellular damage [8, 9], or it can be conjugated to reduced GSH by GST. The latter step is considered a detoxification pathway [6]. Our previous reports on growing rats revealed that the GSH conjugation pathway is significantly induced in response to high doses of AFB1 or APAP [10-12]. The aim of the present study was to examine if placental GST, which has a high activity in the pre- and post- natal period, is altered in response to these chemicals**.**

2. MATERIALS AND METHODS

a) Chemicals

Acetaminophen (APAP), aflatoxin B1 (AFB1), acrylamid and bis-acrylamid, anti-glutathione S-transferase-P (anti-GST-pi), 1-chloro 2,4 dinitro benzene (CDNB), bovine serum albumin (BSA), glutathione S-transferase-P (GST-P), dimethyl sulfoxide (DMSO), anti-IgG conjugated to horseradish peroxidase (anti-IgG-HRP), RNase, diethyl pyrocarbonate (DEPC) and GST-pi standard were purchased from Sigma chemical Co., (St. Louis, USA). ECL-plus reagent, Hyperfilm-ECL and Hybond-C extra nitrocellulose membrane were bought from Amersham Life Science, UK. RNasin, Reverse transcriptase (Moloney Murine Leukemia Virus (M-MuLV)), oligo-dt mixture and Taq DNA polymerase were prepared from Sinagene, Tehran, Iran. ALT (Alanin amino Transferase) and AST (Aspartate amino Transferase) Kits were purchased from Pars Azmon Company, Iran, and Primers for β-actin and GST-P genes were prepared by Farayand-Danesh Co., Tehran, Iran. All other chemicals and reagents were analytical grade and locally available.

b) Animals and treatments

In this experiment, 20-28 g suckling rats (14 ± 2 days old) were selected. Each set of experiments comprised of case (n=5) and control groups (n=5). The treated groups were treated either with APAP or with AFB1 and sacrificed at different time intervals. APAP was administrated IP at doses of 250 or 450 mg/kg B. W, dissolved in 0.5 ml of PBS; pH 7.3 and controls received an equal volume of the vehicle. Likewise, AFB1 (3.0 mg/kg B. W, dissolved in DMSO) were injected (IP). Rats in the control groups received DMSO only. The treated and their matching control groups were sacrificed at different time intervals after treatment (2, 6, 12, 18, and 24 h).

c) Tissue preparation and enzyme assay

A 20% w/v tissue homogenate was prepared after the removal of livers, washing in ice-cold phosphate buffer (100 mM, pH 7.0), and homogenization [13]. Cytosolic fraction was prepared by ultracentrifugation (100000 g), divided into aliquots and stored at -70°C for further use. Liver cytosolic GST activity was measured spectrophotometrically according to the method described by Habig et al., [14]. GST activity was measured by using CDNB as the substrate.
The levels of ALT and AST were measured in cytosolic fraction on a autoanalyser (Technicon RA 1000) using commercially available Kits (Pars Azmun Co). The enzymes were measured based on the kit' method.

d) Western blot analysis of GST-pi

Western blot technique was performed based on the procedure of Towbin et al. [15]. For this purpose, cytosolic proteins were resolved by SDS-PAGE on a 12% acrylamide gel. Proteins were electrophoretically separated at a constant voltage of 100 V in 25 mM Tris-HCl, pH 8.3, containing SDS and glycine [15]. Proteins were blotted onto a nitrocellulose membrane (45 μm pore size) at 100 V for 1 h by the Protein Blotter System (Bio-Rad) and using 25 mM Tris, pH 8.3, 192 mM glycine and 15% v/v methanol. After transfer, the membranes were rinsed by a Tris-buffered saline containing Tween 20 (TBS-T), blocked in 10% skimmed milk/TBS-T, rinsed three times with TBS-T, and 60 min incubation in the primary antibody (anti-GST-pi). Following three rinsings with TBS-T, the membranes were incubated for 60 min by horseradish peroxidase-conjugated secondary antibody. The blots were rinsed with TBS-T and visualized using the Amersham ECL-Plus chemiluminescence system. Quantitation of the Western blots was accomplished using densitometric scanning (LabImage Software, version 2.6; Kapelan, GmbH Co., Germany). Relative density of immunoreactivity was measured by using Adobe Photoshop, version 7.0 for Windows (Adobe Systems Incorporation, San Jose, CA).

e) Isolation of RNA & cDNA synthesis

Total RNA was isolated using a phenol extraction mixture e.g., RNX (Sinagene, Tehran, Iran). Briefly, 50-100 mg fresh liver was homogenized in 1ml of the extraction mixture and incubated 5min at room temperature. 200 μl of chloroform was then added and gently mixed. The mixture was centrifuged at 12000×g for 15 min at 4°C, and 1ml of its supernatant, containing nucleic acids, separated and transferred into a micro tube. An equal volume of iso-propanol (prepared in DEPC-treated water) was added and centrifuged for 10 min at 12000×g. RNA was suspended in 1ml ethanol (75%) and centrifuged again at 7500×g for 5 min at 4°C. Finally RNA was dissolved in 50 μl DEPC-treated water, checked spectrophotometrically (OD=260 nm), and loaded on 3% agarose gel electrophoresis.

The first strand cDNA was synthesized from the isolated RNA by using oligo-dT primers. The reaction mixture contained: 5 μg RNA; 5 μl (10 pmol) oligo-dT; 3 μl deionized water and 1 μl (10 mM) dNTP mixture which was finally mixed and incubated for 5 min at 70 °C. 4 μl of PCR buffer (5X), 0.5μl of $MgCl_2$ and 0.5μl RNasin; one μl of AMV reverse transcriptase were then added to the reaction mixture and incubated at 42° C for 60 min.

f) PCR amplification of GST-P

PCR reaction: Specific primers for GST-P and β-actin, as an internal control, were used. The Rat GST-P primers sequences were as follows:
Forward: 5′-CCT CAC CCT TTA CCA ATC TA-3′. (NCBI Accession number Xo.2904).
Reverse 5'-TTC GTC CAC TAC TGT TTA CC-3'.
Molecular weight=5994 g/mol, Tm=58 C̊, GC=45%. The above mentioned primers amplified a segment of rat cDNA from nucleotide number 219 to number 680.
Corresponding primes for β-actin were as listed below:
Forward 5′-TAC GTA GCC ATC CAG GCT GTG-3′
Reverse: 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′.
These primers amplify a 590 bp segment corresponding to nucleotide number 400 - number 990 of β-actin.
(NCBI Accession number NM-031144).
Five microliters of the cDNA (reverse transcription mixture) and oligonucleotides at a final concentration of 20 μM of primers were analyzed in a 50-μL volume. PCR amplification for the target sequence (GST-

P) and internal control (β-actin) for each sample were performed. The first strand cDNA derived from the tissue was directly used to amplify the target sequence (GST-P) and internal control (GST-P) genes for each sample. For this, 5 μL of the first strand cDNA at a final concentration of 20 μM of primers were used in a 50 μL total volume. The amplification protocol was: 1 cycle at 90°C for 150s for the initial denaturation and 1 cycle at 95°C for 10 min., followed by 35 cycles at 95°C for 30s (denaturation), 55°C for annealing and at 72° C for 1 min for extension. The reaction continued for 10 min at 72° C for the final extension.

Other assays: Protein concentration of plasma samples were measured by Bradford's method [16] using bovine serum albumin as the standard.

g) Statistical analysis

All of the samples and standards were run in duplicate; the results are presented as mean ± SEM. Differences between control and treated animals were analyzed by using Student's t-test, considering $P<0.05$ as significant.

3. RESULTS

a) Effects of APAP and AFB1 on liver total GST activity

As shown in Fig. 1a, a sub-lethal dose of APAP i.e. 450 mg/kg B.W, caused a significant increase ($P<0.05$) in liver total GST activity of suckling rats sacrificed 18 h after drug administration and returned to the control level in 24 h. Likewise, AFB1 treatments (3 mg/kg B.W), caused a significant ($P<0.05$) increase in total hepatic cytosolic GST activity during 12-24 h after the toxin administration (Fig. 1b).
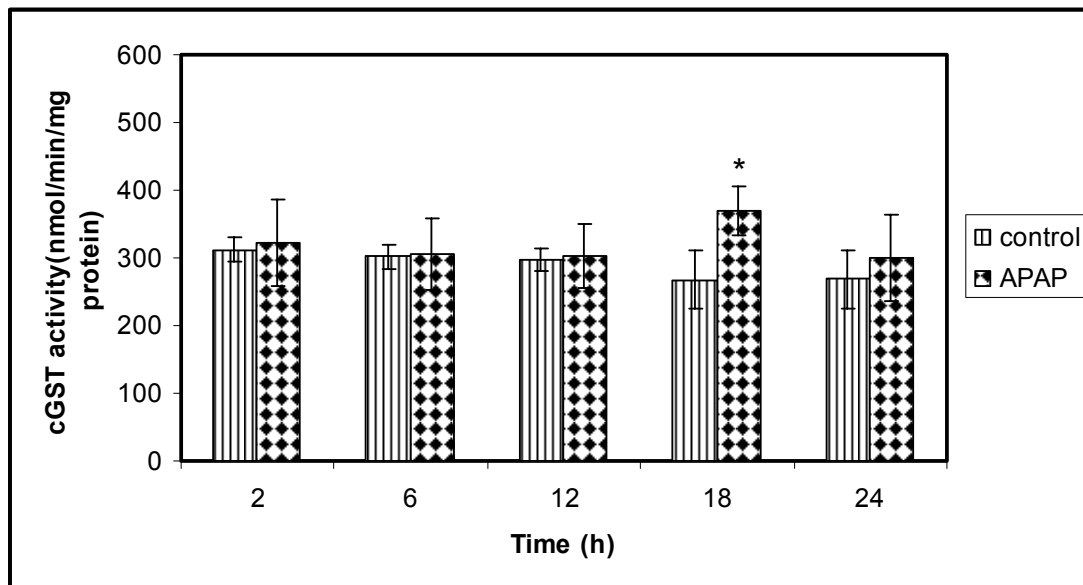

Fig. 1a. Effects of 450 mg/kg B.W APAP on liver cytosolic GST in weanling rats at different time intervals. Results are mean ± SEM of four analyses on four individual liver samples. CDNB was used as the substrate and enzyme activity is expressed as nmol/min/mg protein.
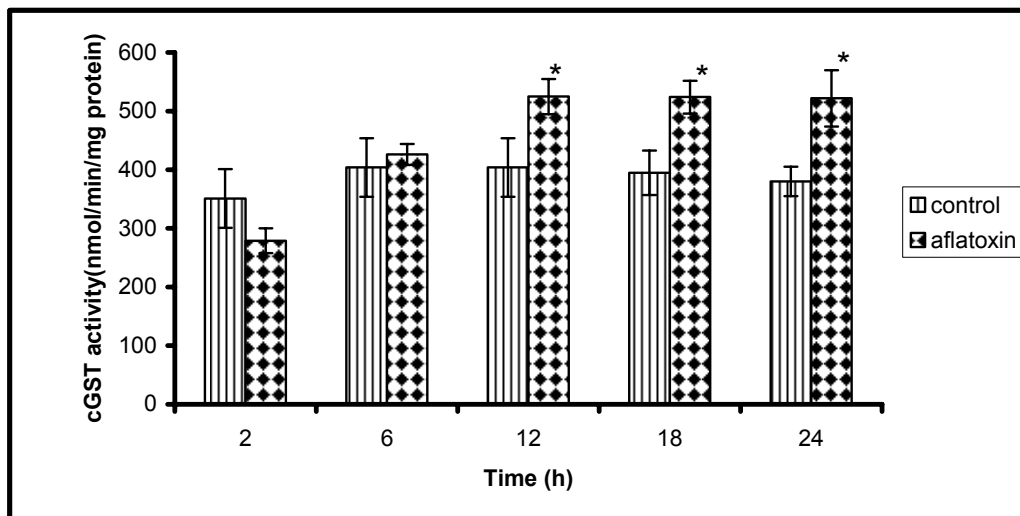$P<0.05$ is considered significant

Fig. 1b. Effects of 3 mg/kg B.W AFB1 on liver cytosolic GST in weanling rats at different time intervals. Results are mean ± SEM of four analyses on four individual liver samples. CDNB was used as the substrate and enzyme activity is expressed as nmol/min/mg protein. *P<0.05 considered significant

b) Effect of APAP and AFB1 on GST-pi

A possible effect of APAP on the GST-pi level was first examined by western blotting technique using specific antibodies against GST-pi. The data showed that a single high dose APAP (450 mg/kg B.W) caused no changes in the enzyme content in animals sacrificed during 18 and 24 h of treatments (p>0.05) (Fig. 2).

Similarly, there were no obvious changes in liver GST-pi levels in weanling rats treated with AFB1 (3 mg/kg B.W) that were sacrificed at 6 or 12 and 24h post-treatment (p>0.05) (Fig. 3a and 3b).
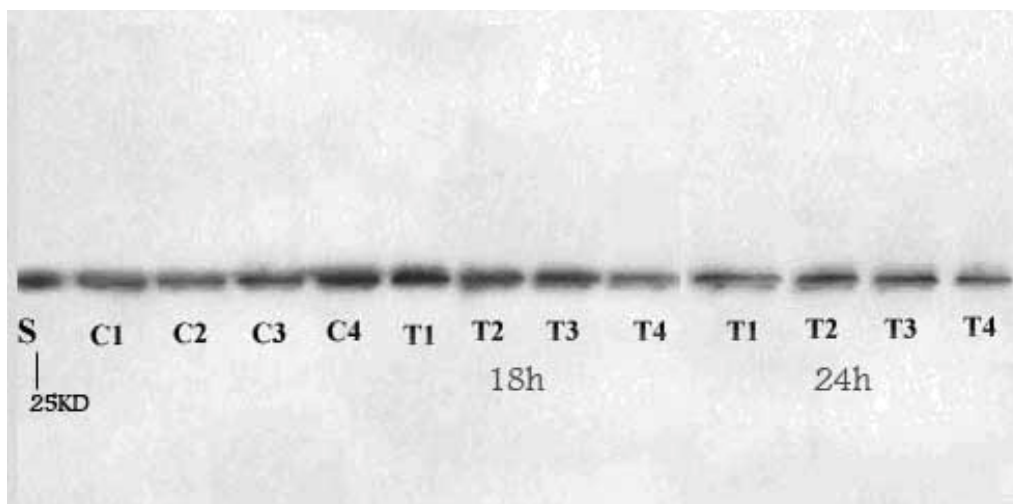

Fig. 2. Western blot analysis of GST-pi in weanling livers exposed to APAP (IP). *T*; Treated group, weanling liver cytosolic samples obtained from weanling rats exposed to 450 mg APAP /kg B.W. *C*; Control samples from weanling rat liver exposed to vehicle alone i.e., PBS. *S;* GST-pi standard sample purchased from Sigma Chemical Co.
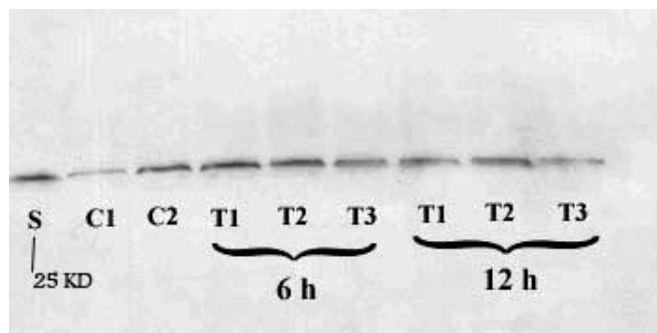
Fig. 3a. Western blot analysis of GST-pi in weanling livers exposed to AFB1 (IP). *T;* Treated group,
weanling liver cytosolic samples obtained from weanling rats exposed to 3 mg AFB1/kg B.W.
C; Control samples from weanling rat liver exposed to vehicle alone i.e., DMSO.
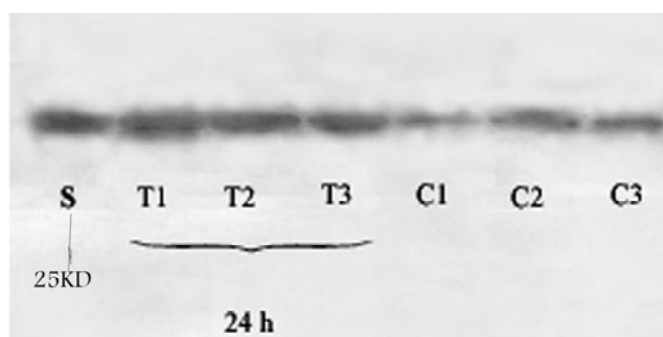*S;* GST-pi standard sample purchased from Sigma Chemical Co.

Fig. 3b. Western blot analysis of GST-pi in weanling livers exposed to AFB1 (IP). *T*; Treated group,
weanling liver cytosolic samples obtained from weanling rats exposed to 3 mg AFB1/kg B.W.
C; Control samples from weanling rat liver exposed to vehicle alone i.e., DMSO.
S; GST-pi standard sample purchased from Sigma Chemical Co.

c) Effects of APAP and AFB1 on GST-P at mRNA levels

There were no differential effects of APAP and AFB1 on GST-P at mRNA levels. AFB1 (3 mg/kg
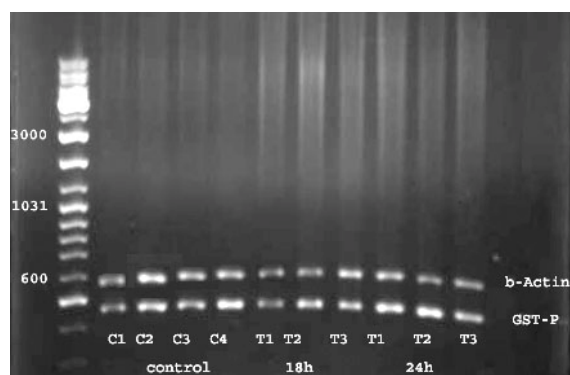B.W) and APAP (450 mg/kg B.W) failed to alter GST-P at mRNA levels (Fig. 4a and 4b).

Fig. 4a. RT-PCR analysis of GST-P RNA in weanling livers exposed to APAP (IP). The size of the GST-P
and β-actin PCR products were 461 and 590 bp, respectively. GST-P was subjected to PCR
using primers specific for GST-P. Rats in treated group (*T*) were exposed to APAP
and control group were exposed to vehicle alone i.e., PBS. The expression of
GST was normalized against the β-actin as the housekeeping
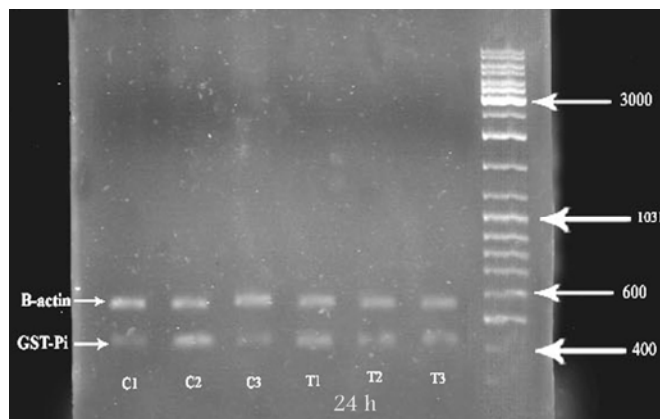gene. The first Lane from left is 100bp DNA ladder.

Fig. 4b. RT-PCR analysis of GST-P mRNA in weanling livers exposed to AFB1 (IP). The size of the
GST-P and β-actin PCR products were 461 and 590 bp, respectively. GST-P was subjected to
PCR using primers specific for GST-P Rats in treated group (T) were exposed to APAP
and control group were exposed to vehicle alone i.e., DMSO. The expression of
GST was normalized against the β-actin as the housekeeping gene.
The first Lane from right is 100bp DNA ladder.

d) Effects of APAP and AFB1 on serum transaminase activity

The activity of ALT was found to be significantly increased by 3 mg/kg BW AFB1 ($P<0.05$) (Fig. 5).
APAP (450 mg/kg BW) and AFB1 (3 mg/kg BW) also cause a significant increase ($p<0.05$) in AST
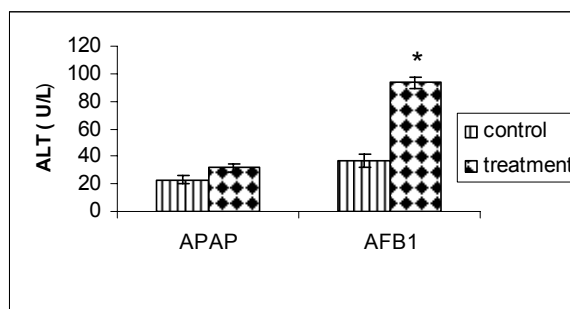activity after 2h (Fig. 6).

Fig. 5. Effects of 3 mg/kg B.W AFB1 and 450 mg/kg B.W APAP on liver ALT activity in weanling rats
Results are mean ± SEM of four analyses on four individual liver samples. CDNB was used as the
substrate and enzyme activity is expressed as (U/L). *$P<0.05$ considered significant

Fig. 6. Effects of 3 mg/kg B.W AFB1 and 450 mg/kg B.W APAP on liver AST activity in weanling rats
Results are mean ± SEM of four analyses on four individual liver samples. CDNB was used as
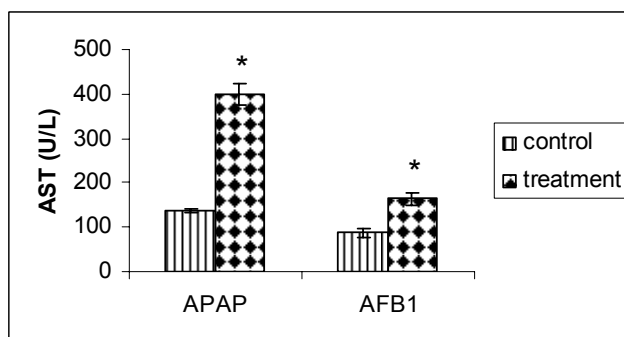the substrate and enzyme activity is expressed as (U/L). *$P<0.05$ considered significant

4. DISCUSSION

The molecular basis of resistance and susceptibility of animals to the adverse effects of chemical carcinogens is the subject of several scientific papers. Only limited information is available on the efficacy of the drug metabolizing system during growth and development and the fate of the chemical carcinogens in the tissues of immature animals. It is generally believed that developing organs during the maturation process exhibit differential sensitivity to several xenobiotics, due to poorly developed biotransformation and excretory mechanisms. Despite having an underdeveloped drug metabolizing system [17, 18, 19, 10, 11], a growing rat is capable of efficiently metabolizing hepatotoxic drugs and preventing cell injury. Comparison of AFB1 and APAP conjugation by GSH in newborns and adults also indicated that the conjugation rate of AFB1 and APAP by liver GSH is higher in the liver of suckling rats in comparison to adults [18, 10, 20]. AFB1 and APAP are two important hepatotoxins which differ in their metabolic pathways and mechanism of toxicity [6, 7]. It was therefore important to verify the differences in GST-P induction at protein and mRNA levels in neonatal rats. Basically, liver total GST activity is lower in growing rats, but following drug administration, the GST induction occurs more readily in young rats as compared to adults [12]. The elevation of GST activity 18h after APAP administration (Fig. 1a) and 12-24h after AFB1 treatment (Fig. 1b) attest to these findings. Conjugation of GSH with the toxic metabolite of APAP and AFB1 namely, NAPQI and AFB1-8, 9-epoxide is an important reaction carried out by GST and plays an important role in determining the resistance of different animal species to the hepatotoxic effects of these compounds [21, 22, 10, and 12]. Therefore differential effects of xenobiotics (APAP, AFB1) on liver GST activity could be beneficial to young rats having relatively more inducible GSTs. Nevertheless, the lack of influence of hepatotoxic compounds on liver GST-P may suggest that other classes of GST are responsible for handling the biotransformation of these compounds. In this connection, Hayes et al reported that GST-A5 is the dominant GST isoenzyme involved in AFB1 detoxification [2]. In this study, both APAP and AFB1 failed to change GST-P mRNA expression and it is possible that other factors in post-transcription or post-translational modification are involved in GST-P regulation.

It seems that there are other factors that affect GST-P expression in response to xenobiotic. It has been shown that, in animal models with hepatocellular carcinoma (HCC), after exposure to an initiating carcinogen, hyperplasic nodules containing liver cells displayed an increased expression of the GST-P [23- 25]. In human studies, it was found that there was an overall reduction in the expression of several different GST isoenzymes in HCC tissues [26]. The protective role of GSTs in liver damage is well established [27, 6, and 8]. Elevation of hepatotoxic markers such as ALT and AST by either AFB1 or APAP (Fig. 5, 6), together with histopatological observation, (results not shown) confirms these findings.

Overall, results show that hepatic GST activity using CDNB as the substrate is induced in response to APAP and AFB1. However lack of influence of the toxins on GST-P specific protein and mRNA (RT-PCR) may suggest the contribution of other classes of GST in detoxification of AFB1 and APAP.

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