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GLUTATHIONE S TRANSFERASE PROTEIN, NUCLEIC ACID, CHROMATIN, CELL NUCLEI AND STRUCTURAL VARIATION ANALYSIS OF ERYTHROCYTE, BONE MARROW CELL AND HEPATOCYTES OF RATS UNDER THE INFLUENCE OF ACRYLAMIDE

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ABSTRACT
Glutathione s transferases (GSTs; EC, 2.5.1.18), a family of multifunctional and versatile proteins, are found almost in all organisms that are living in aerobic environment. The present study was conducted on rat GSTs using dot blot, ELISA, genomic DNA analysis, micronuclei comet assay and histopathological studies in control and acrylamide treated rat liver tissues. In dot blot analysis studies rat serum GSTs showed high immunoprecipitation and ELISA analysis results showed higher O.D values at 12mg and 36mg of acrylamide treatment than control values. In isolated genomic DNA, degradation was observed in all treated tissues indicating damage to hepatocytes of rats. The positive genotoxic effects were also observed by the formation of micronuclei. In these studies more of micronuclei were observed at 36mg AC. In comet assay the diffusion of nuclear head formation was started from 24mg of dosage and a full comet characteristic of necrosis was appeared at 36mg of dosage. To confirm the production of more of GST proteins, damage to DNA, chromatin and nuclei the histological changes were conducted. These studies on rat liver tissues have revealed a pathological change in hepatocyte on exposure to Acrylamide with different doses at different time intervals. Therefore our experimental analysis has revealed that the GSTs can be used as biomarkers for the detection of cancers and chemical toxicity which are caused by acrylamide and other drugs/chemicals.

INTRODUCTION

Glutathiones transferases (GSTs; EC, 2.5.1.18) are a family of structurally related multifunctional enzymes found in all organisms that are living in aerobic environment (Mannervick *et al.,* 1985). These enzymes can catalyze reactions between a tripeptide, glutathione (γ-Glu-Cys-Gly; GSH) and a wide variety of endogenous and exogenous electrophilic compounds. In this enzymatic reaction glutathione conjugation is the first step to lead the mercapturic acid pathway for the elimination of toxic compounds. GSTs are abundant in most of life forms and exists about 10% of proteins in liver of various organisms.

Acrylamide is a hemotoxicant and also causes damage to liver and neurons. During the process of liver damage hepatocytes can produce and regulate GSTs as defence enzymes. In this process new GST proteins may be produced and are used as markers of the tissue damage by the analysis of several tests.

The micronuclei (MN) assay is one of the most widely used test for the determination of mutagenesis and potential carcinogenesis. The micronuclei assay system automatically finds and categorises micronucleated cells in bone marrow or peripheral blood preparations. The micronuclei (MN) is a small, membrane bound DNA fragments contained tiny organelle structure in the cytoplasm formed by the condensation of chromosome fragments or by whole chromosome, lagging behind the cell division. Fenech, (1993) also briefly demonstrated that the MN are acentric chromosome fragments or whole chromosomes left behind during mitosis and appear in the cytoplasm of interphase cells as small additional nuclei. So, this is only biomarker, which allows the simultaneous evaluation of both clastogenic and aneugenic in a wide range of cells.

The dot blot analysis, DNA comet analysis and histopathological studies are also used for the identification of liver damage at the level of protein, chromosome and cell, respectively, under the influence of various chemical toxicants. Therefore to analyse the protein variation in GST, DNA fragmentation in hepatocyte and damage at membrane level experiments were conducted in control and AC treated rat liver and the results are presented based on the performed experiments as studied below.

MATERIALS AND METHODS

The male wistar rats weighing about 150-200 g with the age of 3 month old were purchased from Sri Venkateswara Enterprises, Bangalore and were allowed to adjust to the laboratory conditions.

In this study rats were divided into seven groups and each group consisting of six rats (n = 6). The rats were treated with various concentrations of acrylamide in water and control were given water. The six groups of rats were treated with 1, 2, 3, 4, 5 and 6mg of acrylamide, respectively, per 100g of body weight for 48 hr interval times, for a total of six doses and the total doses of acrylamide given to rats were of 6, 12, 18, 24,30 and 36mg, respectively. After the treatment the animals were sacrificed using decapitation after 48 hr interval of the last injection.

Production of antibodies: The antibodies to GSTs were raised in Newzeland male rabbits and were isolated as serum using centrifugation of collected blood.

The dot blot, ELISA, Isolation of genomic DNA, Micronuclei, Comet assay, histopathological studies, were carried out using standard protocols of Towbin *et al.,* (1979); Engvall (1980); Herrmann *et al.,* (1994); De Diddle and Verhaegen, (1994); Ahuja and Sarang (1999); Harris (1900) respectively.

RESULTS

Immunoanalysis

In dot blot studies rat serum GSTs upon immunoprecipitation with anti GST proteins of rat liver showed high intensity of precipitation at 12mg and followed by slow decrease from 18, 24 and 30mg (Fig. 1). However at the 36mg of AC treatment the intensity was found to be identical to that of 12mg of AC treatment.

In the experiment conducted with Acrylamide by the above treatment on ELISA showed similar results *i.e.* higher O.D values at 12mg AC and 36mg AC than control values (Table 1).

Isolation of Genomic DNA

In the present study DNA was isolated for all the treated, control samples and its electrophoretic pattern was observed on agarose gel electrophoresis to identify apoptosis or necrosis. 25μgm of DNA was loaded into each well and in each well concentration of the DNA bands were varied.

The DNA degradation observed was more in lane 4, 6, 7 and 8 damage was also found in lanes 3 and 5. However no damage was seen in control liver DNA (lane 2) (Fig. 2).

Micronuclei test in bone marrow cells

The positive genotoxic effects may be observed by the formation of a micronucleus or micronuclei. In these studies more of micronuclei were observed at 36mg of AC treatment.

Table 1: ELISA for serum GST with liver antibodies

Dose	Indirect ELISA O.D at 405nm	Direct ELISA O.D at 405nm
Control	0.06	0.145
6mg	0.046	0.209
12mg	0.084	0.335
18mg	0.051	0.186
24mg	0.048	0.147
30mg	0.026	0.122
36mg	0.057	0.283

The 36mg of Acrylamide treated bone marrow cells showed 5 micronuclei (Fig. 3).

Comet assay in peripheral blood lymphocytes for the DNA fragmentation analysis

In this treatment the diffusion of nuclear head formation was started from 24mg (Fig. 4) of dosage and a full comet characteristic of necrosis was appeared at 36mg of dosage (Fig. 4).

Histopathology

The control rat liver showed normal architecture, normal hepatic central vein, hepatocytes with nuclei (Fig. 5). Histological analysis of liver tissues of rats in the present investigation revealed a pathological condition on exposure to Acrylamide with different doses at different times.

In this treatment the rats treated with (1mg/100gm body weight/ 48hrs interval for 6 doses) multiple dose administration of 6mg of AC showed central vein congestion and degenerative changes (Fig. 5b). The 12mg AC treatment liver showed sinusoidal haemarrahages and mitotic hepatocytes changes (Fig. 5c). On exposure of rats to 18mg AC observed to have more number of mitotic, binucleated hepatocytes and hypertrophy of nuclei (Fig. 5d). On 24mg of AC exposure the liver sections contained granularity of sinusoidal, binucleated and vascular congestion (Fig. 5e). The 30mg AC exposed rat liver exhibited prominant nuclear, mononuclear round cell collection, hyperplastic hepatocytes and binucleated cells (Fig. 5f). Finally on 36mg AC treatment the rat liver showed proliferation of sinusoidal, hyperplastic sinusoidal, sinusoidal haemorrhages and binucleated hepatocytes on microscopic analysis (Fig. 5g).

DISCUSSION

The influence of acrylamide was tested on various systems of rat. The systems of rat include are serum, bonemarrow cells

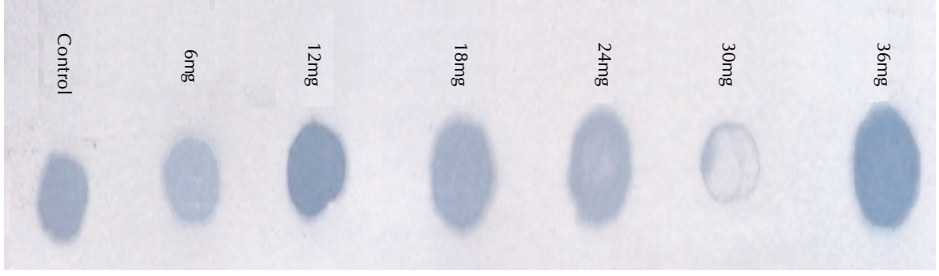

Figure 1: Dot - blot analysis of acrylamide induced serum GST with liver antisera
Lane 1: Control; Lane 2-7: acrylamide treated samples (6mg, 12mg, 18mg, 24mg, 30mg, 36mg)

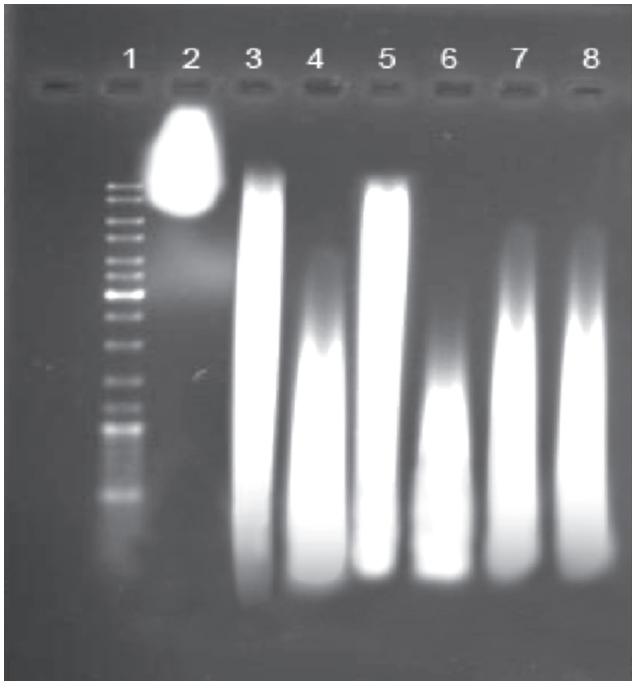

Figure 2: Analysis of DNA damage on Agarose gel electrophoresis
Lane 1: Marker DNA; Lane 2: Control DNA; Lanes 3-8: Acrylamide treated liver with 6mg, 12mg, 18mg, 24mg, 30mg and 36mg

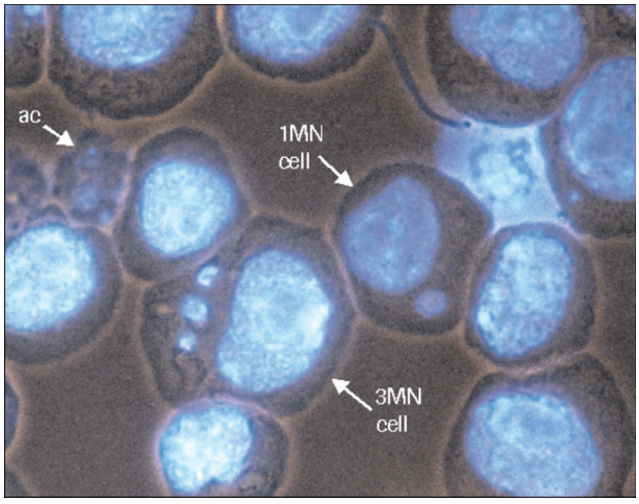

Figure 3: Micronuclei test with acrylamide treated sample
MN: micronuclei; Ac: apoptotic cells

and hepatocytes. The serum was used for the analysis of GST proteins, bonemarrow cells were used for the formation of DNA fragmentation using comet assays, the hepatic tissue was used for DNA analysis and cell modification under the influence of acrylamide.

The immunological analysis of GSTs were performed using dot blot and ELISA tests for acrylamide influenced GST proteins of serum by cross reactivity of anti GSTs of rat liver. These two tests confirmed the elevation of GST proteins at the acrylamide dose of 12mg and 36mg and having slight decrease in cross reactivity from 18 to 30 mg of acrylamide.

Hence the dot blot analysis of serum samples of this treatment

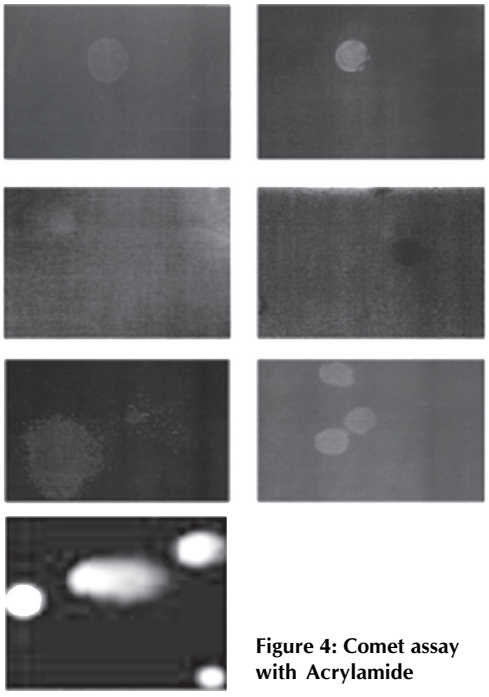

Figure 4: Comet assay with Acrylamide

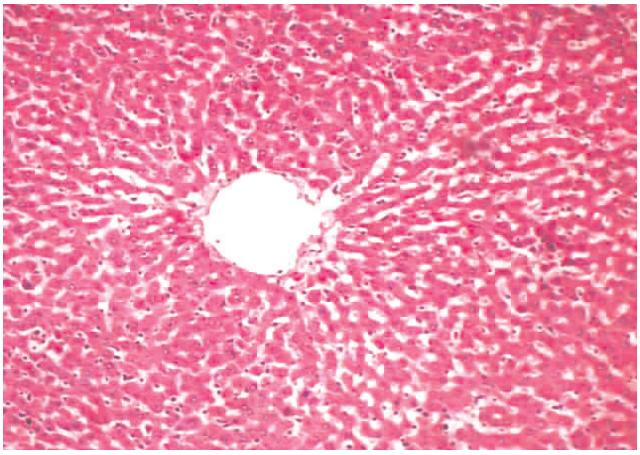

Figure 5a: Control liver

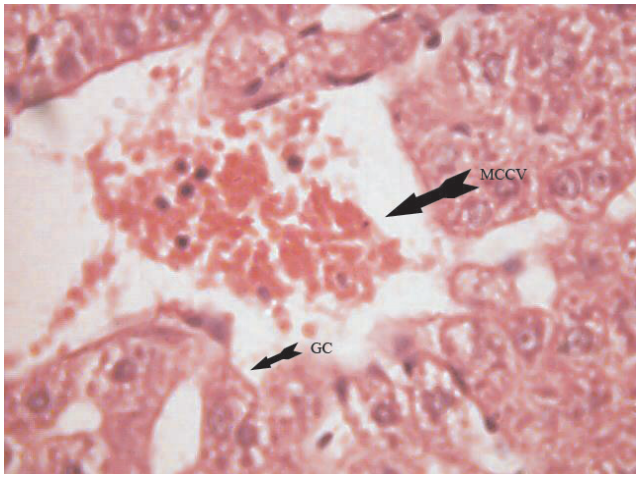

Figure 5b: Acrylamide treated with 6mg
MCCV - Mild Congestion of the central vein GC - Granularity of the cytoplasm

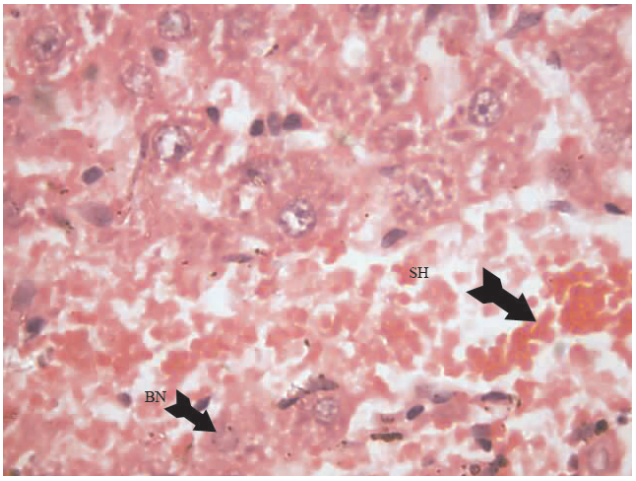

Figure 5c: Acrylamide treated with 12mg
SH - Sinusoidal Haemorrahages; BN - Binucleated cells

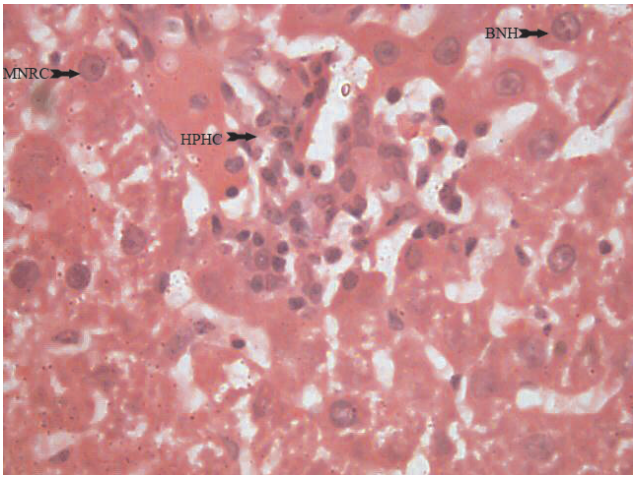

Figure 5f: Acrylamide treated with 30mg
NRC - Mononuclear round cell collection; HPHC - Hyperplastic Hepatocytes

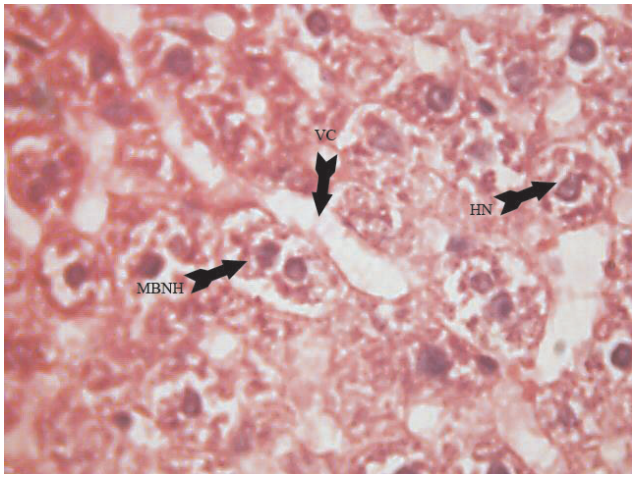

Figure 5d: Acrylamide treated with 18mg
HN - Hypertrophy of nuclei; MBNH - Mitotic and binucleated hepatocytes; VC - Vascular Congestion

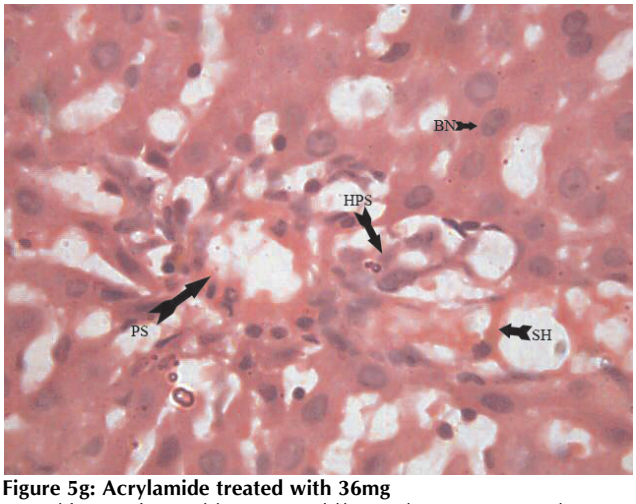

Figure 5g: Acrylamide treated with 36mg
PS - Proliferation of sinusoidal; SH - Sinusoidal haemorrhages; HPS - Hyperplastic sinusoidal

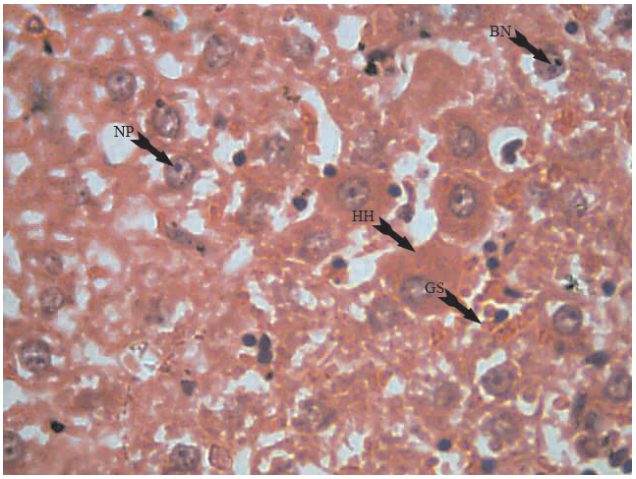

Figure 5e: Acrylamide treated with 24mg
NP - Nulcear Prominance; GS - Granularity of sinusoidal; HH - Hyperplastic hepatocytes

showed high immunoprecipitation at 12mg and 36mg AC. In ELISA the identical results were also observed with higher O.D values at 12mg and 36mg AC than control values in this treatment. These studies have indicated the damage to blood cells and hepatocytes, since these two are major sites to produce the GST and defend the body systems.

Further to analyse the DNA fragmentation at nucleus level, micronucleus test was performed to the bone marrow cells, since bone marrow is generally used as source to provide new cells to the system. The micronucleus test is based on the following principle that during cell mitosis, chromosomes get fragmented or whole chromosomes which have not undergone segregation will not be located in the main nucleus during telophase and may form a micronuclei and gets separated from the main nucleus. The DNA fragments, which give rise to the micronuclei (Fig. 3) may be caused by either lesion to DNA (clastogenic or aneugenic effects of genotoxic compounds) or cleavage subsequent to apoptosis (Savage). In these studies the micronuclei were observed at only 36mg of AC in rat bone marrow cells. Therefore protection of the

cells may be regulated by the bone marrow upto the high toxic concentrations.

In addition to above the treatment AC lead to the diffusion of nuclear head 24mg of dosage and a full comet characteristic to necrosis was appeared at 36mg of dosage (Fig. 4). This comet assay confirm that the hepatocytes are able to get damage from 24mg onwards and the bonemarrow cells are damaged only at higher doses of toxic compounds.

The present results have made a crucial role on the primary and secondary GSTs induction in rats due to the increased acrylamide toxicity and the AC administration to rats have pronounced pathological changes to liver when exposed to multiple doses. Our results are in accordance of the reports of production of cancer as revealed by the phenobarbitol (Pb) (Thyagaraju *et al*., 2003), butylated hydroxy tolune, poly chlorinated biphenyls (Pitot and Sirica, 1980); prolactin (Welsch and Nagasawa, 1997) and acids (Narisawa *et al*., 1974; Reddy *et al*., 1977) has been found to influence carcinogenesis in several systems.

Liver is the major metabolizing organ which detoxifies a number of drugs and xenobiotics. Liver was affected with PB and Methylcholanthrene with certain doses with hypertrophy of hepatocytes and also the nuclei was binucleated and pyknotic in hepatocytes (Devi *et al*., 2002). Centrilobular necrosis, hypertrophoid nuclei, adenomas, hepatocellular carcinoma after time periods in benzene hexa chloride (Kandarkar *et al*., 1983), treatment to rats. In our study, with doubling dosage of AC (6-36mg for 12days), liver was affected more with proliferation of sinusoidal, hyperplastic sinusoidal, sinusoidal haemorrhages and binucleated hepatocytes. These studies on histology support that the acrylamide influences more on hepatocytes to release GST to protect themselves from toxic molecules.

CONCLUSION

In conclusion the acrylamide, a neurotoxicant and a compound which formed during cooking, can cause not only damage to serum GSTs at various levels of acrylamide treatments but also degrades nucleus, chromosomes and DNA in bone marrow cells and hepatocytes. Therefore to protect rats from these damages induction of GST proteins are necessary in hepatocyte and also bone marrow cells.

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