CHAPTER-II

EFFECT OF ACRYLAMIDE ON MICE KIDNEY AND TESTIS GLUTATHIONE S-TRANSFERASES
Virtually all aspects of our lives involve daily exposures to xenobiotics that are either present naturally in the environment or have been placed there by humans. Over 53,000 chemicals are utilized as drugs, pesticides, food and feed additives to enhance nutritional value, as well as additives to improve the stability and safety of chemical formulations. In spite of this heavy dependence on chemicals for our existence, little or no toxicity data are available for 80% of the agents that are inventoried under the Toxic Substances Control Act (ember, 1985).

There are over 75,000 toxic chemicals classified by the Environmental Protection Agency (EPA) as potentially or definitely hazardous to human health. And new chemicals are being tested in the U.S. at the rate of 6,000 or more per week. More than 35,000 pesticides are registered with the US EPA, and 3600 food additives are approved by the US Food and Drug Administration for routine use. The vast increase of chemicals in our environment, foods and medicines has greatly altered the body's ability to rid itself of toxins. Pollutants of all types are abundant in the aquatic environment. Pharmaceuticals are newly recognized as aquatic pollutants.

Biomarkers were originally defined as markers exists due to any biochemical, histological, or physiological alterations or manifestations of environmental stress (NRC, 1987). They have been classified as biomarkers of exposure to a toxicant, biomarkers of effects of exposure, and / or biomarkers of susceptibility to the effects of exposure (Peakall and Shugart, 1993). More recently, this definition has been challenged by several authors and the term biomarker is now more commonly used in a more restrictive sense, namely biochemical sublethal changes resulting from individual exposure to xenobiotics. Some of the xenobiotics carbon monoxide, of course, comes from auto exhaust, cigarette smoke and smog. Lead is found in dyes, gasoline, paint, plumbing, pottery, insecticides, tobacco smoke, textiles and scrap metal. Many of these chemicals are also present in water, food, and air we breathe. All of them elicit existence of specific markers depending upon their metabolism.
The effects of neuroactive compounds, which have been used as insecticides for the last 50 years, and the exposure to neurotoxicants can be assessed through the measurement of biomarkers related to their target activity or detoxification processes (Casida and Quistad, 1998). Several environmental carcinogens have been reported to be transferred to small sucklings via mother’s milk. Such as cycasin, pyrrolidine alkaloids, petridium aquilinum toxin, aflatoxin B-1 and G-5 are examples of the carcinogens which have been found in the milk of lactating mothers. In the embryo, transplacental transmission is the main route of environmental carcinogenesis and at least 30 chemicals have been found to be carcinogenic transplacentally. After delivery, mother’s milk plays a main role in the transfer of carcinogens to their sucklings. The mammary glands and placenta require protection from these molecules. Therefore specific biomarker GST proteins are expressed and are analyzed using a specific substrate reaction.

Most people have no idea that their bodies are contaminated with synthetic or natural chemicals found in every-day activities. During their manufacture and use, chemicals are readily released into the environment, traveling vast distances by air or water to be absorbed by humans and wildlife alike through the skin or ingested in food and water. Decline of sperm counts, loss of functional competence of spermatozoa, defects in several acrosomal enzymes and disintegration of sperm nucleus were observed due to chronic exposure to different chemicals. Therefore testis also contain a defence mechanism to protect the sperm and spermatozoa. This protection can avoid future malformations in the new born babies by the removal of mutations of germ plasm.

At present a study was made on the mice kidney and testis tissue for specific GST expression after 24 hours exposure of mice to acrylamide and also determine the expression of new GST proteins which are involved in the detoxification process. The separation and identification of various subunits due to the effect of β-MC on testicular GSTs was reported and several GST isoforms are expressed in the rat and rabbit brain (Li et al., 1996).
In order to determine the variation of the expression of GSTs in the mice kidney and testis the effect of acrylamide was proposed. Acrylamide, a highly water-soluble α, β-unsaturated amide, reacts with nucleophilic sites in macromolecules in Michael-type additions (Calleman, 1996; Segerbäck et al., 1995). Monomeric AC readily participates in radical-initiated polymerization reactions, whose products form the basis of most of its industrial applications (Calleman, 1996).

To study the effect above chemicals the following objects were selected.

Objectives

1. To study the effect of acrylamide on the expression of mice kidney and testis GSTs using battery of substrates and

2. To analyze the relatedness of kidney and testis GSTs using immunological experiments.

3. The mice treatments with acrylamide, the enzyme assays, protein determinations and western blot analysis were performed as discussed in “Materials and Methods” Chapter.
RESULTS

The mice were treated with various concentrations of acrylamide with an interval of 24 hours and GST and Gpx activities were tabulated for kidney in Table-12 & for testis in Table-13.

Effect of Acrylamide on mice kidney GSTs in 24 hours interval of treatment:

Substrate specificity studies using seven classical substrates CDNB, EPNP, pNPA, pNBC, BSP, CHP and H$_2$O$_2$ were performed and GST activities were determined.

The mice kidney tissue GST activities were increased from control to acrylamide treatment of 1 mg, 2 mg, 3 mg and 4 mg in the fold of 1.4x, 1.6x, 3.2x and 3.8x, respectively, using the substrate CDNB (Fig-19A), in the fold of 0.8x, using the substrate EPNP (Fig-19B), in the fold of 0.83x, using the substrate pNPA (Fig-19C), in the fold of 0.76x, using the substrate pNBC (Fig-19D), in the fold of 0.73x, respectively, using the substrate BSP (Fig-19E).

The Gpx II levels of mice kidney were increased to acrylamide treatment of 1 mg, 2 mg, 3 mg and 4 mg with CHP, in the fold of 0.75x (Fig-20A), and the Gpx 1 (Se-independent) levels were also increased with H$_2$O$_2$ in the fold of 0.78 (Fig-20B), when compared to control mice kidney for 24 hours interval.
Table-12: Effect of Acrylamide (24 hours interval) on the levels of GSTs and GPx of mice kidney with different substrates

<table>
<thead>
<tr>
<th></th>
<th>CDNB</th>
<th>EPNP</th>
<th>pNPA</th>
<th>pNBC</th>
<th>BSP</th>
<th>CHP</th>
<th>H_2O_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>18.22±2.0</td>
<td>0.32±0.4</td>
<td>0.040±0.006</td>
<td>0.268±0.03</td>
<td>0.062±0.004</td>
<td>0.362±0.025</td>
<td>0.323±0.032</td>
</tr>
<tr>
<td>1mgAC</td>
<td>26.12±3.8</td>
<td>0.40±0.62</td>
<td>0.048±0.008</td>
<td>0.342±0.04</td>
<td>0.084±0.006</td>
<td>0.482±0.030</td>
<td>0.414±0.038</td>
</tr>
<tr>
<td>2mgAC</td>
<td>32.18±4.4</td>
<td>0.49±0.64</td>
<td>0.072±0.14</td>
<td>0.398±0.82</td>
<td>0.124±0.031</td>
<td>0.578±0.384</td>
<td>0.438±0.040</td>
</tr>
<tr>
<td>3mgAC</td>
<td>58.25±5.2</td>
<td>0.66±0.72</td>
<td>0.098±0.016</td>
<td>0.422±0.93</td>
<td>0.168±0.026</td>
<td>0.698±0.046</td>
<td>0.481±0.046</td>
</tr>
<tr>
<td>4mgAC</td>
<td>69.27±5.0</td>
<td>0.82±0.82</td>
<td>0.128±0.18</td>
<td>0.452±0.10</td>
<td>0.189±0.17</td>
<td>0.728±0.40</td>
<td>0.523±0.048</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as micromoles of GSH conjugate formed/min/mg protein (CDNB, EPNP, pNPA, pNBC and BSP).

One unit is defined as micromoles of NADPH oxidized/min/mg protein (CHP, H_2O_2).

Values are average of three separate experiments of three samples. Mean ±SD significant (t-test).
FIGURE-19: Effect of acrylamide (24 hours interval) on levels of GSTs of mice kidney with battery of substrates

(A) CDNB

(B) EPNP

(C) pNPA
Con 2mgPvC 3mgAC 4mg~C

(D) pNBC

(E) BSP
FIGURE-20: Effect of acrylamide (24 hours interval) on levels of Gpx of mice kidney with CHP and H$_2$O$_2$

(A) CHP

(B) H$_2$O$_2$
Effect of Acrylamide on mice testis GSTs in 24 hours interval of treatment:

Substrate specificity studies using seven classical substrates CDNB, EPNP, pNPA, pNBC BSP. CHP and H2O2 were performed and GST activities were determined.

The mice testis tissue GST activities were increased from control to acrylamide treatment of 1mg, 2mg, 3mg and 4mg in the fold of 0.38x using the substrate CDNB (Fig-21A), in the fold of 0.63x, using the substrate EPNP (Fig-21B), in the fold of 0.52x, using the substrate pNPA (Fig-21C), in the fold of 0.60x, using the substrate pNBC (Fig-21D), in the fold of 0.39x, using the substrate BSP (Fig-21E).

The Gpx II levels of mice testis were increased to acrylamide treatment of 1mg, 2mg, 3mg and 4mg with CHP, in the fold of 0.78x (Fig-22A), and the Gpx I (Se-independent) levels were also increased with H2O2 in the fold of 0.58 (Fig-22B), when compared to control mice testis for 24 hours interval.
Table-13: Effect of Acrylamide (24-hours interval) on the levels of GSTs and GPx of mice testis with different substrates

<table>
<thead>
<tr>
<th></th>
<th>CDNB</th>
<th>EPNP</th>
<th>pNPA</th>
<th>pNBC</th>
<th>BSP</th>
<th>CHP</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>21.14±3.1</td>
<td>0.58±0.50</td>
<td>0.072±0.008</td>
<td>0.428±0.015</td>
<td>0.090±0.006</td>
<td>0.625±0.018</td>
<td>0.303±0.030</td>
</tr>
<tr>
<td>1mgAC</td>
<td>54.11±6.5</td>
<td>0.92±0.62</td>
<td>0.138±0.042</td>
<td>0.710±0.42</td>
<td>0.23±0.016</td>
<td>0.801±0.24</td>
<td>0.514±0.065</td>
</tr>
<tr>
<td>2mgAC</td>
<td>63.24±6.8</td>
<td>1.23±0.82</td>
<td>0.161±0.056</td>
<td>0.938±0.55</td>
<td>0.46±0.025</td>
<td>1.240±0.28</td>
<td>0.828±0.090</td>
</tr>
<tr>
<td>3mgAC</td>
<td>79.30±7.1</td>
<td>1.68±0.92</td>
<td>0.194±0.062</td>
<td>1.251±0.79</td>
<td>0.63±0.046</td>
<td>1.623±0.52</td>
<td>1.381±0.233</td>
</tr>
<tr>
<td>4mgAC</td>
<td>81.00±7.2</td>
<td>1.69±0.97</td>
<td>0.199±0.068</td>
<td>1.301±0.82</td>
<td>0.78±0.053</td>
<td>1.623±0.52</td>
<td>1.381±0.233</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as micromoles of GSH conjugate formed/min/mg protein (CDNB, EPNP, pNPA, pNBC and BSP).

One unit is defined as micromoles of NADPH oxidized/min/mg protein (CHP, H₂O₂). Values are average of three separate experiments of three samples.

Mean ±SD significant (t-test).
FIGURE-21: Effect of acrylamide (24hours interval) on levels of GSTs of mice testis with battery of substrates

(A) CDNB

(B) EPNP
FIGURE-22: Effect of acrylamide (24 hours interval) on levels of Gpx of mice testis with CHP and H2O2

(A) CHP

(B) H2O2
WESTERN BLOTTING

Antisera raised against affinity purified GSTs of mice kidney and testis on transblot analysis showed immunoprecipitin bands with both control and acrylamide treated mice kidney tissue homogenates. The kidney GST antibodies had precipitation bands with control and acrylamide treated kidney tissue homogenates. On 24 hours of treatment of acrylamide 1-4mg revealed the elevation of expression of GST proteins in kidney tissues. Similar to this result testis tissue homogenates were also showed cross reactivity with GST proteins after transblot and immunopreipitin analysis (Figure-23). The results of acrylamide treatment (Figure-24and lanes 2-5) showed the elevated expression of Yb subunit of μ-class GST and two GSTs were almost disappered on gel. The disappeared proteins are Yβ and Yδ protein of testis (lanes 4-5).
Figure-23: Immuno blot analysis of mice kidney of both control and acrylamide induced GSTs probed with GST specific antibodies (BCIP/NBT staining)

Lane: 1-4 AC induced mice testis GSTs
Lane: 5-6 HE active principle extract treated mice testis GSTs against AC

Figure-24: Immuno blot analysis of mice testis of both control and acrylamide induced GSTs probed with GST specific antibodies (BCIP/NBT staining)
DISCUSSION

The effects of pollutants in the ecosystem have attracted a great deal of interest, on the production of biomarkers and use of them as bioindicators. The principle of the biomarker approach is to analyze the organism's physiological and biochemical response to a pollutant exposure. The phase II system and endogenous cellular glutathione (GSII) as environmental indicators have received relatively little attention (Stein et al., 1992). The toxicity of acrylamide as well as its potential and dependent on the concentration and duration in the target organ, which is determined by the quantitative description of absorption, distribution, metabolism, and excretion of the chemical. Over the course of evolution, a detoxification system for eliminating both xenobiotics and endogenous molecules from the organism has developed. This comprises a large group of enzymes, which participate in the excretion process. These include two phases to modify the structure of the xenobiotics from a hydrophobic to hydrophilic form and for easier excretion after conjugation with a glutathione molecule.

The mechanism of action of environmental agents often considered the domain of toxicology and can also be addressed in humans using gene by environment, drug and phenotype which are having relation with environment interactions. Although there are limitations to this approach, the ability to study the species of interest in the exposure range of interest makes it a valuable tool for examining mechanisms of environmental toxins.

The activity of GST is known to increase in rats when exposed to poly chlorinated biphenols (PCBs). However, Gallagher et al., (1996) reported that GST detoxifies a number of environmental carcinogens and epoxide intermediates. Thus, the GST assay was suggested as a useful tool for biomonitoring oxidative stress (Di Giulio et al. 1993). The status of other antioxidant systems was variably affected by PAHs exposure.

GSTs, as a family of soluble and insoluble isozymes are involved in cellular detoxification of plenty of chemicals. They prevent the build up of potentially toxic substances by catalyzing the conjugation of reduced glutathione with various electrophilic substances thereby protecting cellular components such as DNA, proteins,
and lipids. In addition to the function of detoxification, they also play an important role in steroid isomerization (Benson et al. 1979); glutathione peroxidation (Prohaska and Ganther, 1976); leukotriene C biosynthesis (Pemble et al. 1986); and binding noncovalently to a number of nonsubstrate ligands, including steroids (Homma et al. 1986).

The mice kidney and testis GSTs showed increased activity with substrates of CDNB, EPNP, pNPA, pNBC and BSP (Table-12 & 13) on the treatment with acrylamide. These substrate specificity studies indicate that the kidney and testis to protect itself from the drugs. Due to this the mice can avoid teratological changes. There by the biological systems adapt defense for various types of molecules.

In rodents almost all tissue acquire defense from oxidative stress and there by expression of new GSTs elucidated using the analysis of the above said substrates. In the mice kidney and testis the GSTs showed substrate specificities increase for CDNB, pNPA, pNBC indicate the induction of all types of $\alpha$ and $\mu$. However reduction in the expression of $\pi$ of Y$\delta$ and $\mu$ of Y$\beta$ as shown in transblot. It indicate that these two proteins can't serve as transporting proteins for acrylamide. The $\alpha$ and $\mu$ proteins are directly involved in conjugation, peroxidase function and transporting agents of various xenobiotics (Hayes, J. D., and D. J. Pulford. 1995).

A few reports on non-neurological effects have been reported following both acute short and long-term acrylamide administration. Congestion of the lungs and kidneys was reported by after administration of a lethal dose (200 mg/kg body weight) of a acrylamide to a monkey. found that administration of 50mg acrylamide/ kg body weight/ day, to rats caused a significant increase in heart rate and cystolic arterial blood pressure. The major metabolite formed is both rat and mouse is N-acetyl-S- (3-amino-3-oxopropyl cysteine).

Acrylamide with 1mg for 4 doses with 24hrs administration caused remarkable expression of GSTs in mice kidney and testis. The effect of inducers on the expression of phase II enzymes revealed that animal systems on exposure to chemical carcinogens have evolved various defence mechanisms to protect them selves from the oxidative damage.
Similar to the function of Gpxs associated with GST and Se dependent Gpx play a role in the protection of all organisms organelles from the free radicals damage produce on the contact of xenobiotics to the body. These two enzymes, selenium dependent and selenium independent, scavenge the free radicals and avoid the propagation of free radicals. In the present study on acrylamide (Table-12 & 13, Figure- 20 & 22) treatment to mice observed the decreased amounts GSH peroxidase activities. These studies indicate that the free radicals generated in the kidney and testis must have used of GSH as substrate to reduce the oxygenated molecules to convert them into hydrophilic compounds. Due to the available GSH absence in the mice Gpx must have stopped in mice and the mice must have induced GST proteins (Figure- 19 & 21) to scavenge the molecules to protect various cell types of kidney, testis and other organs.

In conclusion in mice kidney and testis the acrylamide have induced only GSTs and not Gpxs for to protection of cells from these insults. The immunoblot analysis of mice kidney and testis cytosols suggested the elevated expression of GSTs due to the effect of acrylamide on exposure to 24 hours.