THREE

Chemopreventive Role of Selenium and Vitamin D₃ either alone or in Combination Against DEN-Induced Neoplastic Transformation in Rat Liver and Reflection in Hepatic Antioxidant Status And Xenobiotic Metabolism
3.1. INTRODUCTION

ANTIOXIDANTS

O$_2$ is a poisonous molecule: Aerobes only survive in its presence because they have evolved antioxidant defenses. Many antioxidants come into play when ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) are generated in vivo in living organisms.

These antioxidants can be broadly categorized as

A. Agents that catalytically remove free radicals and other reactive species.
   1. Enzyme superoxide dismutase  
   2. Catalase
   3. Peroxidase  
   4. Thiol-specific antioxidants

B. Low-molecular mass agents that scavenge ROS and RNS
   1. Glutathione  
   2. $\alpha$-tocopherol synthesized
   3. Bilirubin  
   4. Uric Acid
   5. Ascorbic Acid  
   6. Caronoids

C. Proteins that minimize pro-oxidants such as iron, copper and haem ions.
   1. Tranferrins  
   2. Heptoglobins
   3. Haemopexin  
   4. Metallothionein

D. Proteins that protect biomolecules against damages to other peroxidases
   1. Cytochrome  
   2. NADH peroxidase and oxidase
   3. Non specific peroxidases  
   4. Horseradish peroxidases

A. Enzymes Which Catalytically Remove Free Radicals

1. Superoxide Dismutases

The discovery of superoxide dismutase (SOD) enzymes provided much of the basis for our current understanding of antioxidant defences system. SOD is responsible for the dismutation of oxygen radicals to hydrogen peroxides, which is subsequently determined by glutathione peroxidase.

In 1970, SOD was discovered that the blue-green erythrocyte protein contains zinc as well as copper called as copper-zinc SOD. There was no enzymic function was detected in any of these proteins. So it was often suggested that they served as metal stores. However, in 1969 McCord and Fridovich reported that the erythrocyte protein is able to remove the superoxide radical catalytically.
The SOD first isolated from E.coli was entirely unlike Cu-Zn SOD. It was pink rather than blue-green and contained manganese at its active site called as manganese-SOD (Mn SOD). A third SOD was found to be an iron-containing enzyme (Fe SOD), and similar enzymes were found subsequently in several other bacteria, algae and higher plants.

Physiological importance of SOD

The discovery of SOD enzymes led to the superoxide theory of oxygen toxicity, which proposes that $O_2^-$ is a major factor in $O_2$ toxicity and that SODs are essential defences against it. It is now generally accepted that the biological role of SOD is to scavenge $O_2^-$, which is known to be generated \textit{in vivo} in amounts increasing with $O_2$ exposure.

Superoxide dismutase catalyzes the destruction of $O_2^-$ by converting it to oxygen and $H_2O_2$:

$$2 O_2^- + 2 H^+ \rightarrow O_2 + H_2O_2$$

Superoxide dismutase, however, greatly accelerates the rate of destruction of $O_2^-$, in part by converting a second order reaction to a first order reaction. Because of the effect of superoxide dismutase, steady-state concentrations of $O_2^-$ in tissues are many orders of magnitude lower than they would be if the elimination of $O_2^-$ were solely dependent on its spontaneous dismutation.

Two forms of superoxide dismutase are present in eukaryotic cells: a form that contains Cu$^{2+}$ and Zn$^{2+}$, the former serving as the redox center and the later as a structural element, and a form that contains only one metal, namely Mn$^{2+}$, which functions as the redox center. The Cu$^{2+}$ / Zn$^{2+}$ form, a 32-kDa dimmer, is found in the cytosol, while the Mn$^{2+}$ form, an 80-kDa tetramer, is located in mitochondria. The Mn$^{2+}$ form is also found in bacteria, as is a third form of superoxide dismutase containing Fe$^{2+}$ as its redox element. The concentrations of the Cu$^{2+}$ / Zn$^{2+}$ and Fe$^{2+}$ forms of superoxide dismutase are unaffected by oxidative stresses, but the Mn$^{2+}$ form is inducible in both bacteria and eukaryotic cells, its activity increasing with oxidative stress.

Mutant forms of the Cu$^{2+}$ / Zn$^{2+}$ enzyme appear to explain the familial forms of a fetal neurological disease known as amyotrophic lateral sclerosis, or
motor neuron disease. In this condition, the motor neurons in the patient’s cerebral cortex and spinal cord degenerate over the course of a few years, leading to weakness and eventually paralysis, with death from pneumonia caused by the inability of the patient to clear respiratory secretions. The mutant enzymes dismute superoxide is a normal fashion, but they have excess peroxidase activity, an activity present in normal Cu\(^{2+}\) / Zn\(^{2+}\) dismutase to only a very limited extent. It is presently thought that the oxidative damage inflicted by the increased peroxidase activity of the mutant dismutase is responsible for the early death of these neurons.

2. Catalases

Dismutation of \(O_2^-\) generates \(H_2O_2\) a species also generated by several oxidase enzymes \textit{in vivo}, including xanthine, urate and D-amino acid oxidases. Hydrogen peroxide is usually removed in aerobes by two types of enzyme. The catalase directly decomposition of \(H_2O_2\) to ground state \(O_2\)

\[
2 H_2O_2 \rightarrow 2H_2O + O_2
\]

The peroxidases enzyme removes \(H_2O_2\) by using it to oxidize another substrate (\(SH_2\))

\[
SH_2 + H_2O_2 \rightarrow S + 2H_2O
\]

Table 3.1: Catalase And Glutathione Peroxidase Activities In Human Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Catalase activity mg-1 protein</th>
<th>Glutathione peroxidase activity mg-1 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1300</td>
<td>190</td>
</tr>
<tr>
<td>Kidney cortex</td>
<td>430</td>
<td>140</td>
</tr>
<tr>
<td>Spleen</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>Lung</td>
<td>210</td>
<td>53</td>
</tr>
<tr>
<td>Heart</td>
<td>54</td>
<td>69</td>
</tr>
<tr>
<td>Brain</td>
<td>11</td>
<td>71</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>270</td>
<td>77</td>
</tr>
</tbody>
</table>
Catalase is erroneously said to work only at high concentrations of H$_2$O$_2$, and to serve principally as a backup for the glutathione – dependent system to be discussed below, but the enzyme has a binding site for NADPH, and when this site is occupied, catalase operates at H$_2$O$_2$ concentrations in the vicinity of those at which the glutathione-dependent systems operate. It is therefore likely that some of the H$_2$O$_2$ produced in the cell is destroyed by catalase. Catalase deficiency exists, but is relatively innocuous; the Swiss type is asymptomatic, while the Japanese variety is associated only with ulcers of the oral cavity.

B. Low-Molecular Mass Agents That Scavenge ROS And RNS

1. Glutathione Peroxidase

Glutathione peroxidase (GPx) was first discovered in 1957 (in animal tissue). GPx are not generally present in higher plants or bacteria. Glutathione, (GSH) their substrate is a low – molecular mass thiol – containing tripeptide.

It is present in animals, plants and many aerobic bacteria. GPx structure consists of four protein subunits, each of which contains one atom of the element selenium at its active site as selenocysteine.

The glutathione-dependent antioxidant system, consists of glutathione plus two enzymes: glutathione peroxidase and glutathione reductase. As this system operates, glutathione cycles between its oxidized (GSSG – Oxidized glutathione) and reduced forms. The reactions catalyzed by these enzymes are:

\[
\begin{align*}
2 \text{GSH} + \text{H}_2\text{O}_2 & \xrightarrow{\text{GSH peroxidase}} \text{GSSG} + 2 \text{H}_2\text{O} \\
\text{GSSG} + \text{NADPH} + \text{H}^+ & \xrightarrow{\text{GSSG reductase}} 2 \text{GSH} + \text{NAD}^+ \\
\text{Net: } \text{H}_2\text{O}_2 + \text{HADPH} + \text{H}^+ & \rightarrow 2 \text{H}_2\text{O} + \text{NADP}^+
\end{align*}
\]

Like other enzymes that catalyze the interconversion of sulfhydryl groups and disulfides, the 22-kDa-glutathione reductase uses FAD as its cofactor. Glutathione peroxidase, another 22-kDa protein, is unusual, however, in that the redox element in its active site is selenocysteine. The selenocysteine is introduced into the protein by a special t-RNA that is initially charged with serine but
undergoes a series of reactions that convert it to t-RNA. The triplet UGA, which ordinarily introduces a stop, encodes selenocysteine but in the context of the glutathione peroxidase mRNA is recognized by the selenocysteine-linked t-RNA. The antioxidant properties of selenium are explained by its occurrence in glutathione peroxidase (B.M.Babior et. al., 1997).

Table 3.2: Some Human Disease With Reported Decrease In Glutathione

<table>
<thead>
<tr>
<th>Disease</th>
<th>Tissue/Body fluid</th>
<th>GSH % of normal</th>
<th>GSSH % of normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Blood</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>Alcoholic Liver disease</td>
<td>Blood</td>
<td>64</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>38 – 66</td>
<td>100</td>
</tr>
<tr>
<td>Cigarette Smokers</td>
<td>RBC</td>
<td>126</td>
<td>ND</td>
</tr>
<tr>
<td>Parkinson’s</td>
<td>Blood</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>Wilson’s disease</td>
<td>Liver</td>
<td>13</td>
<td>356</td>
</tr>
</tbody>
</table>

ND: Not Determined (Gower et al., 1988).

The tripeptide glutathione (GSH) [3-Glu-Cys-Cly] is the pivot in various protective systems (Table 3.3) (Bast et al., 1991). Glutathione occurs in all tissues of the body, but in nearly all species the higher concentrations are found in the liver. It was originally believed that glutathione was uniformly distributed throughout the liver cells but investigations using a new quantitative cytochemical method had shown that hepatocytes within 100µm of the central vein contain much less glutathione than other regions of the lobule (Smith et al., 1979).

TABLE 3.3 Concentration of Glutathione in Tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Mg/100gm Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>214</td>
</tr>
<tr>
<td>Spleen</td>
<td>140</td>
</tr>
<tr>
<td>Kidney</td>
<td>125</td>
</tr>
<tr>
<td>Pancreas</td>
<td>120</td>
</tr>
<tr>
<td>Lung</td>
<td>63</td>
</tr>
<tr>
<td>Heart</td>
<td>57</td>
</tr>
<tr>
<td>Adrenal</td>
<td>48</td>
</tr>
<tr>
<td>Blood (RBC)</td>
<td>38</td>
</tr>
</tbody>
</table>
The Glutathione S-Transferase (GST)

Glutathione is also involved in the metabolism of xenobiotic (foreign compounds) herbicide and pesticide generally in both animal and plant tissue. Many xenobiotics supplied to living organisms are metabolized by conjugation with GSH, catalysed by glutathione-S transferase (GST) enzymes.

\[ \text{Rx} + \text{GSH} \rightarrow \text{RSG} + \text{HX} \]

Liver is especially rich in these enzymes and the resulting glutathione conjugates are often excreted into bile using ATP-dependent glutathione S-conjugate ‘efflux pumps’ the same pumps are involved in the export of GSSG when liver is subjected to oxidative stress.

It is now generally accepted that the GSTs are encoded by at least five different gene families (Hiratsuka et al., 1990; Meyer et al., 1991; Ogura et al., 1991 Tsuchida et al., 1992). The active site of GSTs has been shown to contain two binding sites, one binding site for glutathione is G-site and a second site for substrate binding is H-site. Experiments based on kinetic and chemical modification techniques indicated that the active site might contain histidine, cysteine, tryptophan, arginine or aspartic acid (Van Omen et al., 1989; 1991; Tamai et al., 1990; Chang et al., 1991; Ricci et al., 1991). It has been reported that the GSTs are the reliable markers for preneoplastic lesions and neoplastic tissues in the liver as well as in other organs like rat placental form (GT-P or GT 7-7) and the human placental form (GT-r); the two forms are very similar in physiochemical, enzymatic and immunological properties and are therefore grouped together in the class Pi under the species-independent classification of GST (Mannervik et al., 1985).

C. Proteins That Minimize Pro-Oxidant

Metallothioneins are metal-ion sequestration agent in different environment. It include storage of heavy metals in a non-toxic form, regulation of cellular copper and zinc metabolism and control of the absorption of these metals from the gut.
Iron and copper are essential in the human body for the synthesis of a huge range of enzymes and other proteins involved in respiration, $\text{O}_2$ transport, $\text{NO}^-$ formation and other redox reactions. Yet these metals are potentially dangerous, their ability to undergo one-electron transfers enables them to be powerful catalysts of auto oxidation reactions, conversion of $\text{H}_2\text{O}_2$ to $\text{OH}^-$ and decomposition of lipid peroxides to reactive peroxyl and alkoxyl radicals. The proteins like transferrins, heptoglobins, haemopexin and metallothionein minimize pro-oxidants such as iron, copper and haem ions. Transferrin accepts iron released by the destruction of aged red blood cells, e.g. in the spleen.

D. Proteins That Protect Biomolecules Against Damages To Other Peroxidases

Cytochrome

The endoplasmic reticulum of many animal and plant tissue contains cytochromes known collectively as cytochrome P-450. The name P-450 was originally given because the reduced forms of the cytochromes bind carbon monoxide to produce a complex that absorbs light strongly at 450 nm.

Cytochrome P-450 (Cyt.P-450): P-450 s and haem Proteins containing a single polypeptide chain. Four legands to the iron are provided by the haem and the fifth as a thiolate anion ($S^-$) from a cysteine residue. In the resting enzymes the sixth ligand is water.

$$\text{RH} + \text{O}_2 + \text{NADPH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}$$

The steps involved in the P-450 catalyzed reduction of molecular oxygen with incorporation of one oxygen atom into a substrate, RH, to give the corresponding product, ROH. Cyt.P-450s can utilize $\text{H}_2\text{O}_2$ hydroperoxides, and peroxides, and in this regard they have some relationship with the peroxides, which also utilize formal Fe (IV) porphyrin radical intermediates ($\text{FeO}^{3+}$) (Marnett et al., 1986; Ortiz de Montellano, 1987).

Drug metabolism has classically been divided into ‘Phase–I’ (functionalization) and ‘Phase –II’ (conjugation) reactions. Phase I enzymes introduce a functional group, such as $\text{OH}$, into the substrate; Phase – II enzymes then use this functional group as a ‘handle’ for conjugation with such moieties as...
glucuronic acid, sulfate, glutathione (GSH), glucose and cysteine, yielding a hydrophilic product. The cytochrome P-450 (Cyt.P-450) proteins appear to be the most numerous of all drug-metabolizing enzymes and to have the broadest range of substrate specificity, both endogenous and exogenous, including drugs, steroids, prostaglandins, chemical carcinogens and other xenobiotics (Wislocki et al., 1980; Guengerich, 1990).

The following chemical agents are some common inducers and inhibitors of cytochromes P-450:

Inducers; Barbiturates (Pheno barbitone), Phenytoin, Carbamazepine, Griseofulvin, Rifampicin, Glutethi. Inhibitors; Cimitidine, Allopurinol, Isoniazid, Chloramphenicol, Disulfiram, Quinine, Erythromyein.

The Cyt-P450s are a supergene family of enzymes that catalyze the oxidation of lipophilic chemicals through the insertion of one atom of molecular oxygen into the substrate. They are responsible for the metabolism of a wide range of endogenous and foreign compounds, and in man the transformation of many drugs and chemical carcinogens is mediated by the P450 are involved both in the detoxification of many compounds and also, in some cases, production of the ultimate carcinogen (Guengerich, et al., 1987; Miles et al., 1991).

3.2. MATERIALS AND METHODS

Maintenance of Animals

Male Sprague – Dawley rats obtained from the Indian Institution of Chemical Biology (IICB), Kolkata, India weighing 80-100 gm at the beginning of the experiment were acclimatized to standard lab conditions (temperature 24 ± 1°C, relative humidity 55 ± 5% and a 12 hour photoperiod) in Tarson cages (four to five rats per cage) for 1 week before the commencement of the experiment. During the entire period of study, the rats were supplied with a semi purified basal diet (Lipton, India) and water ad libitum. The recommendations of Jadavpur University Institutional Animal Ethics Committee “Committee for the purpose of control and supervision of experimental animals” (CPCSEA Registered Number 0367/01/C/CPCSEA India) for the care and use of lab animals were strictly followed throughout the study.
Experimental Regimen

The rats were randomly divided into eight experimental groups (each group of ten animals) as illustrated in figure 3.1 groups A, C, E and G rats were the DEN (diethyl nitrosamine) treated groups that received a single, necrogenic, intraperitoneal injection of DEN (200 mg/kg body weight in normal saline) at 9 weeks of age i.e. at week 4 of experimentation, whereas groups B, D, F and H rats were the respective controls. The promotion of liver tumor development results from a selective stimulation and outgrowth of hepatocytes harboring activation of mutations in the β gene (Leoppen et al., 2002).

Following a 3 weeks recovery i.e., after week 7, the DEN treated rats were given PB as the carcinogenic promoter in basal diet (0.05%) 5 days a week up to 20 weeks for all rats. Group A rats were the normal vehicle control that received a single intraperitoneal dose of normal saline and 0.2ml of propylene glycol, per os, twice weekly throughout the study, group C and D rats received only 8 ppm Selenium (w/v) as selenomethionine (E.Merck) in drinking water ad libitum starting 4 weeks prior to DEN administration (at week 0) and continued thereafter along with PB till the sacrifice of the animals. Group E and F rats received only 1α, 25 OH Vitamin D3 (Sigma, MO, USA) at a dose 0.3 µg/100 µl propylene glycol, per OS, twice weekly throughout the study. Group G and H received both selenium and Vitamin D3 at the above-mentioned doses.

Group D, F and H served as the respective controls of groups C, E and G rats. Solutions of selenium (pH 7.0) were renewed every 2-3 days. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every second day. All the treatment was withdrawn after week 20 and the rats were sacrificed by decapitation b/w 0900 and 1100 hr under proper light ether anesthesia after week 21 to carry out expomutations. All the animals were fasted over night before sacrificed.

Preparation of Liver Cytosolic Fraction

The animals were sacrificed with proper anesthesia. Liver of either lobes were excised, minced and homogenized with ice-cold
1.15% (w/v) KCl solution (pH 7.4) in a Teflon coated glass homogenizer to make a 10% (w/v) homogenate. The cytosolic fraction was prepared by differential centrifugation. First the homogenate was centrifuged at 9,000 x g for 30 mins. The resultant supernatant fraction was recentrifuged at 105,000 x g (33,000 rpm) for 90 mins in a Sorval – OTD-50B Ultracentrifuge. Supernatant of the 105,000xg represented the cytosolic fraction and was stored at -20°C until further use. While the pellet was resuspended in homogenizing buffer that served as the microsomal fraction. All operations were done at 0-4°C.

**Determination of Enzymes level**

The enzymatic activity of γ-Glutamyl transpeptidase (GGT) in cytosol was measured according to an adaption of the method of Tata and Meister (1974). The cytosolic fraction was preincubated with 1% deoxycholic acid at 25°C for 15 mins. The standard reaction mixture (1ml) contained 0.05M Tris-HCl buffer (pH 8.0), 75mM NaCl, 20mM Glycyl glycine (Sigma) (pH 8.0), 2.5mM L-γ-glutamyl -nitroanilide (Sigma, USA) as the substrate and a suitable amount of the enzyme preparation. The reaction mixture was incubated at 37°C for 5 mins and the reaction was initiated by the addition of the substrate. The rate of release of p-nitro aniline was measured at 410 nm in a Hitachi U-2000 spectrophotometer.

The activity of cytosolic glutathione S-transferase (GST) was determined by an adaption of the method of (Habig et al. 1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) and GSH (Sigma) as substrates. The reaction mixture (1ml) consists of 0.1M phosphate buffer (pH 6.5), 1 mM GSH, 1mM CDNB and a suitable amount of cytosol (1.2 to 1.6 mg protein / ml). The reaction mixture was incubated at 37°C and the reaction was initiated by the addition of the CDNB. The increase in optical density at 340 nm was measured spectrophotometrically with a temperature controlled cell compartment.

The level of cytosolic glutathione (GSH) was quantified by the method of Ellman (1959). For this, 1ml of cytosolic was mixed with 1ml of 4% 5-sulphosalicylic acid and the mixture was centrifuged at 1,500 x g
for 15 mins, the supernatant was allowed to react with 0.1m M 5,5’- dithio-bis (2-nitrobenzoic acid) (Sigma Chemical Co, St. Louis, MO. USA). The solution was kept at room temperature for 10 min and read at 412 nm by a Hitachi U-2000 spectrophotometer.

The total Cytochrome P-450 (Cyt. P-450) in the microsomal fraction were measured by using Na-dithionate as a chemical reductant. The extinction coefficient E at 450 to 490 nm = 91 cm⁻¹ for conventional difference spectroscopy was used as originally described by Omura and Sato (1964).

Total cytosolic and microsomal protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Each enzyme assay and biochemical estimations were performed in triplicate from every treated and control groups under experimental condition.

**Statistical Analysis**

All the data were statistically analyzed by one way ANOVA by using DMRT method of SPSS 11.0 package. The level of significance was set at P<0.05 and P<0.01.
Figure 3.1: Basic Experimental Protocol Design

- ▼ - DEN IP single injection at week 4
- ▲ - Administration of Phenobarbital at week 7
- ★ - Single IP injection of buffer solution
- | - Withdrawal of treatment at week 20
- □ - Sacrifice of animals at week 21
- ★ - Starting of administration of selenium with water *ad libitum*
- ★ - Starting of administration of vitamin D3 with water *ad libitum*
- ★ - Starting of administration of selenium and vitamin D3 with water *ad libitum*
### Experimental Protocol

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Basal diet with DEN IP single injection at week 4 and 0.05% PB at week 7 with drinking water <em>ad. Libitum.</em></td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Basal diet with single IP injection of buffer solution</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>Basal diet with DEN IP single injection at week 4 and 0.05% PB at week 7 with drinking water <em>ad. Libitum</em> and 8PPM of selenium as selenomethionine in drinking water <em>ad. Libitum.</em></td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>Basal diet and 8PPM of selenium as selenomethionine in drinking water <em>ad. Libitum.</em></td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>Basal diet with DEN IP single injection at week 4 and 0.05% PB at week 7 with drinking water <em>ad. Libitum</em> and oral supplementation of Vitamin D₃ at a dose of 0.3 µg/100 µl propylene glycol per OS twice a week.</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Basal diet and oral supplementation of vitamin D₃ at a dose of 0.3µg/100µl propylene glycol per OS twice a week.</td>
</tr>
<tr>
<td>7</td>
<td>G</td>
<td>Basal diet with DEN IP single injection at week 4, 0.05% PB at week 7, oral supplementation of vitamin D₃ at a dose of 0.3µl/100µl propylene glycol per OS twice a week and 8PPM of selenium as selenomethionine in drinking water <em>ad. Libitum.</em></td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>Basal diet with oral supplementation of vitamin D₃ at a dose of 0.3 µl/100µl propylene glycol per OS twice a week and 8PPM of selenium as selenomethionine in drinking water <em>ad. Libitum.</em></td>
</tr>
</tbody>
</table>
3.3. RESULTS

Food and Water Intakes

During the entire period of this study, no difference in food and water consumption could be observed among various groups of rats. Foods and water intakes were 8.7 – 10.8gm/rat/day and 16.6-18.9ml/rat/day respectively for all groups of rats.

Effect of Selenium and/or Vitamin D3 on Hepatic Enzymatic GGT Activity in Different Groups

A significant decrease (P<0.01) in the hepatic GGT activity was observed (Figure 3.2) in DEN control rats (Group A) when compared with vehicle control Group B. However, in all three experimental groups treated with either selenium or vitamin D3 or their combination (Groups C, E and G respectively) reflected a significant (P<0.01) uphill activity in the GGT level in comparison to its DEN control. Interestingly, treatment with selenium offered 32.86%, while vitamin D3 resulted in 26.29% inhibition in the GGT activity over and the normal control rats. In contrast, combination treatment of selenium offered a more pronounced (42.25%) inhibition in treatment groups. Selenium, vitamin D3 and its combination treatment groups were statistically significant at 1% level over DEN and normal control was observed by one-way DMRT –ANOVA method.

Effect of Selenium and/or Vitamin D3, on GST Activity in Different Groups

Figure 3.3 depicts GST activity in different groups against CDNB as substrate. It can be seen that treatment with DEN in group A rats results in a massive 2.5 fold decrease in hepatic cytosolic GST activity over and above the normal controls of group B. Though an increase in GST activity (P<0.01) which observed by either selenium (group C) or Vitamin D3 (group E) were significant and the combination treatment of selenium and vitamin D3 (group G) over DEN control was found to be less effective in the inhibition of rat liver carcinogenesis as reflected by the insignificant effect (P<0.01) in abating hepatic GST activity by more than 40%. In contrast, selenium and Vitamin D3
alone or with combination, was found to reduce GST activity at marginal levels (statistically insignificant) when compared to normal vehicle control (group B).

**Effects of Selenium and / or Vitamin D3 on GSH Content in Different Groups**

A single i.p. injection of DEN in group A rats resulted in a significant decrease (p<0.01) of the total GSH pool in the cytosolic fraction when compared with vehicle control (Table 3.4). It can be seen from the figure 3.4 that, continuous treatment of selenium in group C, vitamin D3 in group E or their combinations in group G for 20 weeks resulted in a significant increase in the total GSH pool at different levels but the most beneficial effect could be observed (p<0.01) in groups D and F. Groups E, G and H resulted only in a marginal increase in the hepatic GSH concentration when compared with DEN control (group B) without offering statistical significance.

**Effect of Selenium and / or Vitamin D3 on Microsomal Cyt P-450 Content in Different Groups**

A significant decrease in the contents of microsomal Cyt. P-450 activity was noted in group A, DEN control rats. The values are depicted in figure 3.5 it was observed that selenium and vitamin D3 alone treatment in group C and E rats respectively could increase significantly (P<0.01) on DEN control, the lowered Cyt. P-450 due to DEN treatment by >23% and >30% respectively. But, the protective effect of selenium and vitamin D3 in-combination (group G) towards elevating (>39%) the above preneoplastic hepatic marker enzyme from DEN control rats were mostly exerted showing activity trends towards near neutral values (P<0.01). Cytochrome P-450 level in all treatment groups except group E were highly significant at 1% level over both DEN and normal control group A and group B.
### Table 3.4: One-Way DMRT -ANOVA Table For Effect of Selenium and / or Vitamin D₃ on Hepatic Antioxidant Enzymatic Activity

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Antioxidant enzymes level –Units per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(GGT)</td>
</tr>
<tr>
<td>DEN Control</td>
<td>5.0 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal Control</td>
<td>42.60 ± 0.98&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN + Selenium</td>
<td>14.66 ± 0.46&lt;sup&gt;c**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Selenium Control</td>
<td>20.90 ± 1.61&lt;sup&gt;d**&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN + Vitamin D₃</td>
<td>11.20 ± 0.50&lt;sup&gt;e**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin D₃ Control</td>
<td>26.46 ± 1.12&lt;sup&gt;f**&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN + Vitamin D₃ + Selenium</td>
<td>18.0 ± 0.63&lt;sup&gt;g**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin D₃ + Selenium Control</td>
<td>28.98 ± 1.36&lt;sup&gt;h**&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD-0.05</td>
<td>2.4300</td>
</tr>
<tr>
<td>0.01</td>
<td>3.3481</td>
</tr>
</tbody>
</table>

- Values are mean ± SD of ten animals.
- Means followed by a common superscript letters are not significant at 1% and 5% level by using DMRT-ANOVA method.
- Statistical significance symbol: -*P < 0.05 and - **P < 0.01.
- CD - Critical Difference Value.
- Significance identification – The difference between normal and compared group value is more than the CD value is considered as significant.
Figure 3.2: Effect of selenium and/or vitamin D3 on hepatic GGT enzyme activity

A- DEN Control                                B- Normal control
C-Vitamin D3                                  D-Vitamin D3 Control
E-Selenium                                   F-Selenium Control
G-Vitamin D3 with Selenium                   H-Vitamin D3 with Selenium Control
Figure 3.3: Effect of selenium and/or vitamin D3 on hepatic GST enzyme activity

A- DEN Control                             B- Normal control
C-Vitamin D3                                   D-Vitamin D3 Control
E-Selenium                                       F-Selenium Control
G-Vitamin D3 with Selenium           H-Vitamin D3 with Selenium Control
Figure 3.4: Effect of selenium and/or vitamin D3 on hepatic GSH enzyme activity

A-DEN Control  
C-Vitamin D3  
E-Selenium  
G-Vitamin D3 with Selenium  
B-Normal control  
D-Vitamin D3 Control  
F-Selenium Control  
H-Vitamin D3 with Selenium Control
Figure 3.5: Effect of selenium and/or vitamin D3 on hepatic cytochrome P-450 enzyme activity

A- DEN Control  B- Normal control
C- Vitamin D3  D- Vitamin D3 Control
E- Selenium  F- Selenium Control
G- Vitamin D3 with Selenium  H- Vitamin D3 with Selenium Control
3.4. DISCUSSION

Hepatic biotransformation enzymes in animals known as drug-metabolizing enzymes are responsible for the detoxification and excretion of foreign compounds. Many foreign compounds, such as drugs and environmental pollutants, are non-polar and dissolve readily in lipid, the principle function of the drug metabolizing enzymes is to convert these chemicals into highly water soluble products that can eliminated.

One area of cancer chemoprevention that has been studied in recent year is the biological modifiers of cancer cells that are designed to retard proliferation (Samid et al., 1993, Novichenko et al., 1995) to induce differentiation of these cells to a quiescent, non dividing state (Liu et al., 1994), and / or to promote apoptosis (Welsh, 1994, Li et al., 1995). Although extensive studies have been made on the anticarcinogenic response of other micronutrients, data relating to the biological influence of selenium and vitamin D3 on hepatic tumorigenesis are very meager.

A number of reports are available on the ability of antioxidants to inhibit chemical carcinogenesis when the antioxidants are administered either prior to or concomitantly with the carcinogen (Novi, 1981; Ames, 1983). An interesting feature of these chemo protectors is that they belong to totally chemical classes such as retinoids, tocopherols, flavones, indoles and selenium compounds. These protective substances act either by scavenging the reactive carcinogenic species or by altering the activity of microsomal mixed –function oxidase system (Prochaska et.al., 1985). Alteration of this enzyme system of microsomal metabolism by antioxidants could prevent carcinogenic derivatives that react with cellular macromolecules in many different cells organelles. The induction of alternative metabolic path ways leading to the formation of non-carcinogenic metabolites are the trapping of reactive species by molecules acting as scavenger may also be involved in anti carcinogenic action of this agents (Prochaska et.al., 1985).

GST- Glutathione S-transferase enzymes substrates like diols and epozides are being converted into more soluble glutathione derivatives which can then be
excreted. This, and other metabolic pathways, such as conjugation with glucuronic acid usually represent detoxification mechanisms. Hence the overall effect of carcinogens \textit{in vivo} depends upon the balance between activation and detoxification mechanisms, as well as upon the efficiency with which any DNA lesions produced by the ultimate carcinogen are repaired. (Barry Halliwell et.al., 1999).

In the present study, a decreased activity of GST enzyme was observed in DEN control rats (Group A). Subsequently, an increased trend of the GST enzyme activity was observed in all treatment groups (Groups C, E and G) those received selenium and vitamin D$_3$ in-combination respectively for the entire period of the experiment. But the most significant increase (>41\%) in enzyme level was found in Group G those received both selenium and vitamin D$_3$ for the entire period of the study. One mechanism that has been proposed to explain anti-cancer drug resistance is the increased and / or differential expression of one or more of the GST isozymes in resistant cells compared with normal cells (Rushmore and Pickett, 1993). Over expression of specific GSTs in mammalian cells can lead to biological resistance to alkylating agents that are used in cancer chemotherapy. However, they do not establish that elevated GST activity in resistant cells correlates with higher rates of conjugation of parent drug to glutathione (Rushmore and Pickett, 1993).

GSH- GSH is not only a co-substrate for transferase enzymes but can also often combine directly with the ions or radicals that attack DNA. Other dietary agents, including vitamin A, \(\beta\)-Carotene and selenium have been suggested to have some anti-cancer effect (Yamasaki et.al., 1996).

GSH occurs primarily in the soluble phase, part of it conjugates with foreign compounds or their metabolites for detoxification and transport from the body (Boyland et.al., 1969). Studies on rats have shown that (\(^{55}\)S) cysteine is rapidly incorporated into liver glutathione, a relatively stable pool containing about 3\(\mu\)mol /g with a half-life of 28.5 hour and a liable pool containing 4-5\(\mu\)mol/g with a half-life of 1.7 hour (Tateishi et.al., 1978).
GSH is believed to perform several different functions in the tissues and the importance of these may vary from one tissue to another. Decreased level of GSH in DEN control rats, in this study Group ‘A’, may be that, GGT catalyses the degradation of extra cellular GSH and is perhaps involve in the production of precursors, that can be used in the intracellular synthesis of GSH (Reiners et al., 1991). Closer investigation reveals that cystenyl glycine; the cleavage product of GSH by GGT is a potent mutagen that gives rise to thiolate anion (Stark et al., 1988). Thus, reduction of GSH level in Group ‘A’ animals may be pictured as an attempt by the host cells to combat the existing oxidative stress. In contrast, Groups C, E and G rats those received selenium, vitamin D₃ or selenium and vitamin D₃ together respectively showed an increased level of GSH but the same is much pronounced in selenium plus vitamin D₃ combinations where it is slightly higher than either in selenium or in vitamin D₃ treatment groups separately. This significant increase of GSH levels in Group G rats reflects an anticancer mechanism, perhaps by promoting the formation of additional pyridine nucleotides to provide the hepatocyte with reducing properties and thereby abate the growth and spread of neoplastic nodules.

It has been well established that selenium exerts its effect largely through the presence in several biologically active selenoproteins. Among about 30 known selenoproteins, GSH-Pxs are the most extensively characterized. Bearing in mind that GSH-Pxs contribute to the transformation of peroxides to less harmful compounds and that peroxidative damage is associated with cancer, it was reasonable to assume that these peroxidases would be involved in the reduction of tumours. It has shown that the red cell GSH-Px activity of PC patients is the same as in healthy subjects and the activity of plasma enzyme is lower than in controls, in prostate tissue of PC patients, the activity of GSH-Px is significantly lower as compared with BPH patients. Yilmaz et al., have also shown that in the red cells of both PC and BPH patients, GSH-Px activity was significantly lower than in controls. Hardell et al. did not find any difference between the red cell GSH-Px activity between PC and BPH patients. Furthermore, Walters et al., have shown that in elderly make beagle dogs supplemented with selenium, the extent of DNA damage in prostate cells and in peripheral blood lymphocytes was significantly lower than among the control dogs, but this damage was not associated with the activity of the antioxidant
enzyme plasma GSH-Px. Since Selenium has anticancer properties it is very likely that its low level in blood may facilitate the development of prostate cancer. A higher level of Selenium in prostate of PC patients has no influence on GSH-Px activity in the gland.

It is interesting to note that 15 kDa selenoprotein, found in the prostatic epithelium by Behine et al., is accounted for about two thirds of the protein-bound selenium. The fact that the formation of protein had priority over that of GSH-Px during insufficient selenium intake is an indication of their biological significance. It is also noteworthy that the prostate and liver in humans express relatively high level of 15 kDa selenoprotein. According to the authors these data raise to possibility that 15 kDa selenoprotein may play a role in the prevention of cancer and possible serve as an agent by which selenium supplementation exerts its chemopreventive effect. *In vivo* studies have also shown that another selenoprotein, namely selenoprotein P, an antioxidant defense protein, highly expressed in prostate epithelium and also binding a large portion of Se, may exert an anti tumorogenic effect.

Selenium is an essential trace nutrient and is widely obtained from grain, fish and meat. It is a key component of a number of functional selenoproteins such as glutathione peroxidase (GSH-Px) and a dozen of other enzymes. GSH-Pxs are the most extensively characterized seleno-enzymes, being found in erythrocytes, cells of different organs, and plasma. The major physiological role of GSH-Px is to maintain a relatively low level of hydrogen peroxide and a variety of organic hydroperoxides within the cell, thus decreasing potential free radical damage of membranes and other cell structures. The activity of GSH-Px is regulated by selenium status.

SOD - Mitochondria from several malignant animal tumors, and from many tumour cells in culture, show low Mn-SOD activity.

Sun, Y et al., 1993 studied the lowered antioxidant enzymes in spontaneously transformed embryonic mouse liver cells in culture. Tranfection of the Mn-SOD gene into cells has been reported to suppress the malignant phenotype in some (but not all) cell lines and also to render the cells more resistant to radiation. For example, transfection of cDNA encoding Mn-SOD into a human breast cancer cell line raised Mn-SOD levels and rendered the cells less malignant when injected into mice.
Over expression of mitochondrial Mn-SOD promotes the survival of tumor cells exposed to IL -1, TNF, selected anticancer drugs, and ionizing radiation. (Hirose K et al., 1993).

Low activities of SOD, catalase and glutathione peroxidase are frequently reported in transformed cell lines and sometimes in biopsies of animal tumours. Studies on human tissues have given variable results, but in general there is no clear evidence for marked decreases in SOD or other antioxidant enzymes reported as elevated. It should be borne in mind that areas within a large tumour mass often have a poor blood supply and so are often anoxic, which may down regulate antioxidant enzymes as a consequence of hypoxia rather than malignancy. (Barry Halliwell et.al.,1999).

Earlier work showed that susceptibility to lipid peroxidation is decreased in certain malignant hepatoma cell lines (eg. Novikoff and Yoshida ascites hepatoma cells). The precise reasons for this decrease depend on the cells studied, but they include a lower content of PUFA side-chains in lipids, an increased accumulation of 2-tocopherol and falls in the activities of cytochromes P-450 and NADPH-cytochrome P-450 reductase.( Slater, T.F. et al., 1990).

Cytochrome P-450

Since chemical carcinogenesis proceeds through the early appearance of hyperplastic nodules, the manifestation of phenotype alteration in the nodules, especially alteration of P-450 isozymes activity, is thought to contribute to the later stage of chemical carcinogenesis (Degawa et al., 1991). Experiments showed that the total content of P-450 is found to lower after exposure of carcinogens (Cameron et al., 1976, Buchmann et al., 1985, Tsuda et al., 1988, Astrom et al., 1983).

Despite the fact that specific chemical mechanisms of DNA damage have been determined for many carcinogens, a frequent observation in carcinogen – treated tissue is a rise in 8-OHdG (8-hydroxy deoxy guanosine) in the neoplastic, and often the pre-neoplastic lesions. The implication is that oxidative stress is involved in carcinogenesis, whatever carcinogen started the process. Of course a decrease in the rate of repair of 8-OHdG could also explain the rise. Some cytochromes P-450 generate ROS, and their induction by carcinogens could make a contribution to oxidative stress. (Barry Halliwell et.al., 1999).
In this study, Group ‘A’ rats showed reduced level of Cyt. P-450 after exposure of DEN. The mechanism by which selenium produces inhibition is altering the Cyt. P-450 related enzymes are known. The induction of Cyt. P-450 may also be due to the alteration of the ATP / ADP ratio by the inhibition of oxidative phosphorylation, thereby increasing the NADPH content rapidly for the mixed-function oxidase system to act (Chakraborty et al., 1995b). The results obtained with vitamin D3 treatment in Group E rats explains that this agent is effective in rising the abnormally decrease level of Cyt. P-450 due to DEN treatment, but how exactly this is brought is not clearly known at present. Studies on the regulation of the mRNA levels of cytochrome oxidase II and I have revealed that these are up regulated by the hormonal from of vitamin D3 (Kessler et al., 1986). However, since vitamin D3 receptor has not been found in the mitochondrion modulation of these genes has been postulated to be post-transcriptional events (Minghetti and Norman, 1988).

Further, P-450 is a constituent of 25-hydroxy vitamin D3-deoxyhydroxylase the enzyme responsible for the activation of vitamin D3 provides added evidence of the possible correlation between vitamin D3 and P-450 content and needs further studies. In the other experimental group, i.e., Group G which received both selenium and vitamin D3 showed the maximum elevation of the level of Cyt.P-450 showing beyond doubt the ability of both the micronutrients to bring about tough resistance against the necrogenic effect DEN. It is possible that both of these micronutrients inhibit the binding of metabolites to DNA. This inhibition of metabolic activation occurs via Cyt. P-450 enzymes along with induction of other detoxification enzymes. Therefore, for preneoplastic nodules, increase of Cyt. P-450 might be favorable for their escape from contact with metabolites derives from an carcinogen and for their growth and progression to cells (Degawa et. al., 1991).

Selenium, vitamin D3 and cancer: a complex relation

Prostate cancer is a frequent type of cancer in old men. Its incidence increase with advancing age and is associated with a wide geographical variation. Even though the age-specific incidence rates remain stable, the problem of PC seems certain to increase because of the population ageing. Although the etiological factors that give rise to PC are not known, an increased
intake of selenium (Se), vitamin E, lycopene and some other antioxidants may have a protective effect. Epidemiological and ecological studies performed internationally over the past several decades have revealed an inverse association between means plasma selenium level and incidence of PC. Brook et al., have shown that plasma selenium before diagnosis was inversely correlated with the risk of prostate cancer. In a prospective cohort study with many prostate cancer cases, statistically significant inverse trend was found between the toenail Se level (a measure of long-term Se intake) and the risk of prostate cancer.

In a study in Linxian, China, approximately 15,000 of 29584 Chinese adults received a mixture of 30mg α-tocopherol per day, 50mg selenium yeast / day and 15mg β-carotene / day for 5.5 year. The subjects who received this mixture had a 13% lower incidence of cancer and a 10% lower mortality from stomach and esophageal cancer than did the subjects who did not receive the mixture. But there are pros and cons to the use of antioxidant. Several studies have shown pro oxidant effect of many naturally occurring antioxidant like vitamin C and selenium at different doses. (Sheikh Arshad saeed et.al., 2005).

Selenium can protect against carcinogenesis induced by certain chemicals that require metabolic activation, selenium deficient animals and more sensitive to certain carcinogens, especially if the animals are fed a diet rich in PUFAs (Poly Unsaturated Fatty Acids). It should be noted that the experimental results obtained vary depending on the species used, the doses of selenium and carcinogen administered and the relative times of administration. It has been suggested that excessive consumption of PUFAs by humans might predispose to cancer. (Gower J.D. et.al., 1988). But the evidence is not clear. Since many carcinogens are lipid – soluble, increased fat intake might lead to increased carcinogen intake. As we have seen, some organic hydroperoxides can act as tumor promoters.

During past 15 years a variety of observations have focused on vitamin D₃ and its relevance to cancer (Clairmont et al., 1996). Despite the intense research that has focused on vitamin D₃ the exact mode of action by which it inhibits cancer cells remains largely unknown. Since vitamin D₃ is the major regulator of calcium homeostasis in the body (Norman et. al., 1979), it is not surprising that administration of this compound leads to unacceptable levels of hypercalcemia.
before antiproliferative responses of vitamin D$_3$ can be achieved (Koeffler et. al., 1985). The dose used in this study does not have any toxic manifestations like hypercalcemia and induction of serum calcium, the calcium levels were measured every 48h following administration of vitamin D$_3$ in rats.

Since, vitamin D$_3$ has already established promise in chemical carcinogenesis (Wood et al., 1981; Noguchi et al., 1989) due to its ability in inducing apoptosis, suppressing cell proliferation and acting as a differentiation- proliferation switch, the present study was undertaken with two basic objectives: First to visualize the anticarcinogenic potential of selenium and vitamin D$_3$ either alone or in-combination on hepatic antioxidant status in order to combat cellular oxidative stress generated by carcinogen insulations. This study may help us further to understand the potentates of the in vivo chemo preventive effect of the interactions of vitamin D$_3$ an essential nutrient and selenium an established antioxidant, on the biochemical aspects of hepatic xenobiotic biotransformation pattern during the development of multistage rat liver carcinogenesis.

Within target cells, vitamin D$_3$ binds to the vitamin D$_3$ receptor (VDR), a nuclear transcription factor that becomes active after binding to its legend, probably subsequent to phosphorylation (Brown and Deluca et al., 1990; Christakos et al., 1996). The active receptor binds as a homodimer or as a heterodimer with the retinoic acid receptor X (RXP) to specific DNA elements, termed VDRE, for regulation of transcription. However, it has become clear that many cellular responses to the hormone are too rapid to be mediated by altered gene expression controlled by the VDR (Clairmont et al., 1996). These effects typical for a membrane receptor –type response, have led to the recognition of a non-genomic role for vitamin D$_3$ in cell regulation.

Regardless of the mechanism, based on the results reported in this chapter, both selenium and vitamin D$_3$ could be considered a potential cancer chemopreventive agents whose effect is presumably based on inhibition of growth of the neoplastic cells by coordinated regulations of different biochemical marker enzymes, studied herein.