CHAPTER 3

Chemical Carcinogenesis
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Carcinogenesis, an active phenomenon induced by one or several agents is the process that determines the evolution of cancer and is triggered from mutations in the DNA of normal cells. The resulting alteration in the equilibrium between proliferation and programmed cell death leads to uncontrolled cell division and, therefore, tumor formation. A compound that has the capacity to unchain the process of cancer development in man and animals by acting on one of several organs or tissues is defined as a carcinogen (Gomes et al., 1997, Huff 1999). From an experimental point of view, a compound is considered carcinogenic when its administration to laboratory animals induces a statistically significant rise in the incidence of one or more histological types of neoplasia, compared with the animals in the control group that are not exposed to the substance (Gutiérrez and Salsamendi 2001).

Carcinogenesis is generally preceded by a latent period from the time of the first administration of a carcinogenic agent to the development of visible preneoplastic lesions (Pitot and Dragan, 1991). Morphological and biochemical characterization of these preneoplastic lesions has improved the understanding of carcinogenic development and related cellular alterations. The control of responses to carcinogenesis through the application of several chemical, biochemical and biological techniques facilitates the identification of those basic mechanisms involved in neoplastic development; pre-initial structural and antioxidant profile alteration due to carcinogenic insult. Experimental assays with laboratory animals, epidemiological studies and quick tests enable the identification of carcinogenic compounds, the dissection of many aspects of carcinogenesis, and the establishment of effective strategies to prevent the cancer that results from exposure to chemicals (Paula, et al., 2007).

The neoplastic tissue shows several characteristic features and thus differs radically not only from the normal tissue but also from the so-called “preneoplastic tissue”. The history of chemical carcinogenesis is a record of the observations of physicians and epidemiologists of the relation between the occurrence of uncommon cancers in humans and the exposures of those people to certain chemical agents (Williams, 1982; Luch, 2005). In parallel with some of these findings, experimental animal models were developed to imitate the findings in humans. These experimental studies helped us extracting most of the information about the mechanisms of chemical carcinogenesis.
3.1. Liver and Liver Cancer

The multistage nature of neoplasia has been demonstrated in several mammalian organ systems, most notably in rat liver (Farber, 1984a). Liver is the unique organ and has got several advantages for studying chemical carcinogenesis and pathological changes in vivo during the early stages of initiated cells as altered foci, because (i) liver provides a more sensitive indication of a distant neoplasm in rats (Herzfeld and Greengar, 1977), (ii) it proves helpful and simultaneously feasible in that histomorphological analysis of preneoplastic lesions and hepatic nodules can be done on the same tissue sample by examination of the serial sections or counter staining of the histological slides (Pitot, 1990), (iii) it further provides a potential experimental model for studying pathological, cellular and subcellular changes all through, from the time of the first administration of an initiating dose of a carcinogenic agent to the development of visible preneoplastic lesions (Pitot, 1990), and (iv) it is the ultimate organ for activation and deactivation of almost all chemical carcinogens due to its unique enzyme profile, and as such a frequent target site for the development of chemically induced cancer in rodents (Farber, 1984b). The liver plays a key role in metabolism of nutrients and various xenobiotics, such as food additives, drugs or environmental pollutants. It is the largest gland in human body, harboring important processes associated with e.g. regulation of carbohydrate, lipid, amino acid and hormone metabolism, the synthesis and degradation of plasma proteins, the storage of vitamins and metals, the secretion of bile and finally with xenobiotics metabolism (Baynes and Marek, 2005; Sherlock and James, 2002; Kmiec, 2001). Liver is the main target or at least one of the target organs for about half of all carcinogens tested (Gold et al., 1991).

The liver has a unique architecture and its functions are performed by several types of cells with differing phenotypic characteristics. The smallest functional unit of the liver is called the acinus, comprising the liver parenchyma supplied by a terminal branch of the potential vein and hepatic artery. The lobule is a structural unit of the liver that consists of hepatocytes and sinusoids radiating from the central vein. Sinusoids are vascular channels lined with fenestrated endothelial cells and bounded by hepatocytes in hexagonal arrangements (Fig. 4). Hepatocytes are arranged with one side to the sinusoids and one to form canaliculi, the first channel in the biliary system. At the vertices of the lobule are portal triads, with bile duct, terminal branch of hepatic artery and portal vein.
The liver is made up of different cell types (for example, bile ducts, blood vessels, and fat-storing cells). However, liver cells (hepatocytes) make up 80% of the liver tissue. Thus, the majority of primary liver cancers (over 90 to 95%) arises from liver cells and is called hepatocellular cancer or carcinoma. Liver cancer (hepatocellular carcinoma) is a cancer arising from the liver carcinoma. On a worldwide scale, hepatocellular carcinoma (HCC) is one of the most frequent cancers in humans, ranking fifth in all cancer-related deaths (Thorgerisson and Grisham, 2002) with no reliable diagnosis prior to late stages of disease and no cure except surgery (Luo et al., 2007). It is also known as primary liver cancer or hepatoma. The development of HCC is invariably associated with liver damage resulting from chronic hepatitis, extensive alcohol intake, or toxins, sequentially leading to liver cirrhosis, dysplastic lesions, and finally invasive liver carcinoma (Farazi and DePinho, 2006). However, the mechanism of HCC initiation and progression and how specific lesions interact to produce its aggressive characteristic remain poorly understood (Luo et al., 2007). Thus there is some urgency to deepen understanding of the process of neoplasia and to elucidate biomarkers for early diagnosis as well as to identify potential targets for early therapeutic intervention. When patients or physicians speak of liver cancer, however, they are often referring to cancer that has spread to the liver, having
originated in other organs (such as the colon, stomach, pancreas, breast, and lung). More specifically, this type of liver cancer is called metastatic liver disease (cancer) or secondary liver cancer. Thus, the term liver cancer actually can refer to either metastatic liver cancer or hepatocellular cancer.

Liver plays a key role in xenobiotic metabolism facilitating excretion of chemicals from body. However, detoxification processes may be also accompanied with increased toxicity of reactive metabolites. This is for example the case of activation of procarcinogens to carcinogens, where more reactive diols, quinones and/or epoxides are produced during the promutagen metabolism thus leading to generation of e.g. DNA adducts and/or oxygen species-related genotoxic stress (Josephy and Mannervik, 2006). Drugs, food additives or numerous environmental pollutants are xenobiotics daily ingested with food, inhaled or absorbed through skin, thus leading to exposure of various body organs to their toxic metabolites. Xenobiotics enter the cells by either passive or protein-assisted membrane transport and they are metabolised by 2 principal groups of enzymes (Nebert and Gonzalez, 1987; Nebert and Dalton, 2006): 1) phase I enzymes, catalyzing reactions of hydroxylation, deamination, dehalogenation, epoxidation or peroxidation, which include monoxygenases, such as of cytochrome P450 (CYP) enzymes; and ii) phase II (also called conjugation phase) enzymes, which catalyze conjugation of phase I metabolites with the donors like uridine diphosphate (UDP)-glucuronate (glucuronosylation), adenosine-3'–phosphate-5’-phosphosulfate (PAPS) (sulfatation) or glutathione, thus creating water-soluble complexes, which are more efficiently excreted from the body (Nebert and Dalton, 2006). A large body of evidence supports the possibility that lifetime exposure to chemicals causes parts of the liver tumors developed in humans, either alone or In combination with other factors (Gold et al., 1997). Rodent liver models have long been employed for experimental studies of chemically induced cancer. In this regard, exposure to a wide range of different chemicals results in development of putative precursors of cancer in rodent livers (Bannasch et al., 2003). It is not known to what extent exposures to any particular chemical carcinogens are responsible for the liver cancer in humans and detailed list of agents responsible for hepatic cancer and their precise mechanism of action has not been fully worked out. It is probable that many noncarcinogenic chemicals act as promotors of carcinogenesis, and among these alcohol can be included as an important contributor for liver cancer (Lijinsky, 1979). Earlier researchers have shown that preneoplastic alterations in human liver share many characteristics of rodent preneoplasia (Su and Bannasch, 2003; Su, et al., 1997; Thorgeirsson and Grisham, 2002).
3.1.2. **Mechanisms Underlying Liver Carcinogenesis**

The number of carcinogens inducing liver tumors of similar type in rats is large and diverse including nitrosopyrrolidine, nitrosodi-n-propylamine, dimethylaminoazobenzene, aflatoxin B1, and acetylaminofluorene. It is difficult to imagine that these could act by a common mechanism (Butterworth, 1990). High rate of biochemical alterations is reasonably etiologically associated with the initiation of the carcinogenic process, including that of hepatocarcinogenesis (Luch, 2005).

The development of cancer usually proceeds over many years and is considered to be a multistage process involving several distinct steps. It was recently suggested that malignant transformation require six essential alterations. These alterations include self-sufficiency in growth signals, intensity of growth-inhibitory signals, escape of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. The six capabilities are probably shared by almost all tumors (Hanahan and Weinberg, 2000).

Prolonged cellular damage due to chronic inflammation is one of the reasons leading to the development of tumours (Caballero et al., 2004). The mechanisms by which carcinogens induce their effects have been studied extensively for over half a century (Klaunig et al., 2010). Using the rodent liver model as an example, the modes of action by which carcinogens induce hepatic cancer can be categorized based upon molecular targets and cellular effects that include genotoxic (DNA reactive) and nongenotoxic mechanisms. Nongenotoxic agents can be cytotoxic or mitogenic and may act through specific receptor-mediated pathways.

It has long been known that tumor suppressor genes often are inactivated during cancer development, whereas proto-oncogenes are activated (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 1993). Tumor suppressors normally control cellular growth and cell death. Consequently, mutations in such genes may result in loss of cell growth regulation. Proto-oncogenes are normal genes that can be converted to oncogenes by mutation. This often involves a gain of function. By stimulating mitogenic signal transduction pathways activation of oncogenes often leads to uncontrolled cell growth and proliferation (Oliveira et al., 2007).

Cancer development can also involve modifications of the methylation pattern (Wachsman, 1997). This includes silencing of tumor suppressor genes or overexpression of
oncogenes. A permanent alteration of the phenotype without any changes in the DNA base sequence is often called an epigenetic change; methylation of DNA, the methyl group acting as chemical signal for overexpression of oncogenes. Hypomethylation has been found to be an early event in carcinogenesis (Goelz et al., 1985; Goodman and Watson, 2002).

3.1.3. \textit{p}-dimethylaminoazobenzene as hepatocarcinogen

In the past, azo colorants based on benzidine, 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine (o-tolidine), and 3,3'-dimethoxybenzidine (o-dianisidine) have been synthesized in large amounts and numbers. The azo-dye \textit{p}-dimethylaminoazobenzene (\textit{p}-DAB) was commonly used as food additive, "butter yellow" before its toxicity was recognized. Butter yellow or methyl yellow is also used for coloring polishes, other wax products, polystyrene, petrol, soap and for determination of free hydrochloric acid in gastric juice. Earlier works reporting azo reduction of benzidine-based dyes occurring in man (Badger and Lewis, 1952) has been reconfirmed in recent years (Golka et al., 2004).

It is well known that feeding of carcinogenic azo-dye produces liver damage followed by regeneration of parenchymal cells, proliferation of bile ducts and connective tissue, and at later stages development of tumors from liver parenchyma that end up with neoplastic characteristics. \textit{p}-DAB as a cancer initiator has been reported to have various toxic effects (IARC, 1945; Kitagawa and Sugano, 1977; Manjeswar et al., 1994; Aydinlik et al., 2001; Biswas and Khuda-Bukhsh, 2004; Pathak and Khuda-Bukhsh, 2007) and its chronic feeding for a very long time 45-61 weeks (Akamatsu and Ikegami, 1968) has been reported to induce liver tumors in rodents. But the chronic feeding of \textit{p}-DAB (Initiator) and Phenobarbital (PB, promoter) together has been successfully used to develop liver tumors (and subsequently cancer) (Nesn et al., 1987; IARC, 2003; Biswas and Khuda-Bukhsh, 2005; Pathak and Khuda-Bukhsh, 2007) in a reasonable time period.

\textit{p}-DAB has been demonstrated to bind covalently to cellular macromolecules such as proteins and undergo microsome-catalysed biotransformation \textit{in vitro} and is effective as a liver carcinogen when tested in rats (Miller and Miller, 1947, Stevenson et al., 1942, Mueller and Miller, 1948, Conney et al., 1956). It is a potent hepatocarcinogen in rats influencing the Initiation stage of carcinogenesis. Used occasionally as a food additive, is known to act as initiator of liver cancer when used chronically (Biswa and Khuda-Bukhsh, 2005) and is known to cause hazard to humans and animals (Siemiatycki et al., 2004). Studies by Caballero et al., (2004) in experimental mouse model had shown that
metabolization of p-DAB along with acetylsalicylic acid (ASA) carcinogen generates an oxidative process leading to inflammation and consequent liver injury identified by early preneoplastic lesions (altered hepatic foci of necrosis with specific marker protein expression).

The p-DAB mice model of experimental hepatocarcinogenesis is so well defined that it makes itself a potential tool for studying mechanisms of chemical carcinogenesis (Farber, 1984a,b). Since its carcinogenicity was discovered in 1930's, addition to food is prohibited. This compound is a member of the azo-dye family of carcinogens and has been classified as a Group-2B carcinogen by the International Agency for Research on Cancer (IARC, 1975). The compound has been listed as a hazardous substance (Akamatsu and Ikegami, 1968, NTP, 2002, Siemiatycki et al., 2004, Niosh, 2004) posing a risk to humans. Carcinogenic effects of p-DAB have been reported, particularly in the liver of mice and rats when fed chronically for a long time (Nesn, 1987, IARC, 2003). The teratogenicity and mutagenicity of this dye has also been quite extensively studied earlier including its dose-response, which suggested a positive dose-effect relationship. p-DAB is metabolized to monoaminoazobenzene (MAB) by N-demethylation, and MAB is subsequently metabolized to aminoazobenzene (AAB) through demethylation or to N-hydroxy-N-methyl-4-aminoazobenzene (NOH-AAB) (Tsuda et al., 2001, Haseman et al., 1994).

Though metabolic activation of p-DAB to its ultimate carcinogenic metabolites was extensively studied by Miller and Miller and others (Miller and Miller, 1947, 1949, 1953; Litman and Litman, 1974), the whole path of its cytotoxic potential is ill defined (Biswas and Khuda-Bukhsh, 2005). Further, the perturbation of internal homeostasis with special reference to elemental composition (macro and micro), their interaction during the stage wise progression and development of hepatic neoplasia has not been worked out. The present chapter describes morphometric alterations at initial stage of p-DAB induced hepatocarcinogenesis In association with biochemical alterations viz. marker enzymes, oxidants, antioxidant system under the current experimental regime.

3.2. Materials and methods
3.2.1. Animals

Swiss albino mice (Mus musculus) of both sexes were caged on corn cob bedding and bred in a controlled environment at 23-25°C, 60% humidity and a 12-hour light cycle. Water and food was available ad libitum. The mice were maintained on standard basal diet fed twice a day. During pre weaning period, mice pups were caged with their mother.
Food cups were changed as needed. At the age of 4 weeks, animals were caged 2-3 per cage and were weighed 3 times a week.

The recommendations of the NIH guide for the Care and Use of Laboratory Animals were followed for the maintenance, treatment and sacrifice of the animals used in this study (NRC, 1985).

3.2.2. Experimental protocol

The animals were divided into three experimental groups (n=10) and were given dietary p-DAB at a dose of 0.05 mg/kg body weight via daily gavage (single dose per day) for 3, 5 and 7 weeks respectively. The intoxication protocol was designed based on previous studies (Biswas and Khuda-Bukhsh, 2005) and the dose was selected as recommended (Palekar and Srsat, 1966; Daoust and Molnar 1962; Akamatsu and Ikegami 1968; IARC, 1987) for positive initiation of liver tumors within a reasonable time frame (60 days) of chemical carcinogen administration.

3.2.3. Tissue preparation; Homogenization of tissues and cells

At the end of 3rd, 5th and 7th week, the treated animals along with the respective control set of animals were sacrificed. The liver was promptly removed, washed to remove blood, and finely chopped with scissors. The tissue was then destructed using a mechanical shear and then homogenized in 1.5% KCl using Teflon pestle. Tissue and cell debris was removed by low speed centrifugation (200xg) (Chambers and Rickwood, 1978).

3.2.4. Fractionation of Liver

Centrifugation at successively higher speeds yielded the following fractions: crude nuclear fraction at 1,000 x g for 10 min; heavy mitochondria at 3,000xg for 10 min; light mitochondria at 20,000xg for 20 min; and microsomes at 144,000xg for 90 min. The final supernatant was the cytosolic fraction (Okado-Matsumoto and Fridovich, 2001).

3.2.5. Liver pathology

Tissue damage inflicted because of chronic feeding of p-DAB was assessed by optical, scanning, and transmission electron microscopy of liver.
3.2.6. Light Microscopy

At different times (3, 5, 7 weeks), slices (4-5 mm in thickness) of the three main lobes of the liver were fixed in 10% neutral buffered formalin overnight prior to processing and embedding in paraffin wax. Microtome sectioning was followed and 4 μm thin sections were prepared. Routine processing protocol was followed and processed sections were stained with haematoxylin and eosin (H&E) and observed by light microscopy (Leica, DM R with Qwin Image analyser, Germany,) for gross morphological alterations.

3.2.7. Electron Microscopy

For transmission electron microscopy, the ultrathin sections (60-90 nm) were stained with uranyl acetate and lead citrate (Sigma, USA). For visualization of ultrastructural alterations at day 60, glutaraldehyde fixed liver tissues (1 mm cubes) were post fixed with osmium tetroxide. The ultrathin sections (60-90 nm) were stained with uranyl acetate and lead citrate and visualized using FEI Philips Morgagni 268D Transmission Electron Microscope at 28,000-x magnification.

3.2.8. Liver damage profiling

Before doing the enzyme assays, quantitative determination of total protein of liver homogenate was done following the method of Lowry et al., (1951).

To study liver damage, activity of several toxicity biomarkers such as, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and lipid peroxidation were assayed in liver of treated and control mice. Mice were sacrificed and their liver, blood spleen, and kidneys were quickly isolated. The tissues were separately homogenized with cold (4°C) 0.87% normal saline, followed by centrifugation at 3000×g for 20 min at 4°C in a cooling centrifuge.

Glutamic transaminase enzymes; Glutamic oxaloacetate transaminase (GOT) (EC 2.6.1.1) and Glutamic pyruvate transaminase (GPT) (EC 2.6.1.2) were assayed by the method of Reitman (Reitman and Frankel, 1957). The enzymes catalyze the transfer of the amino group of glutamic acid to oxalacetic acid and pyruvic acid in reversible reactions. The transaminase activity is proportional to the amount of oxaloacetate pyruvate formed over a definite period of time and is measured by a reaction with 2.4-dinitrophenylhydrazine (DNPH) in alkaline solution.
For glutamate oxaloacetate transaminase GOT (also known as aspartate aminotransferase, AST) 0.1 ml of tissue homogenate was mixed with 0.5 ml of substrate solution (L-aspartate) and incubated for 60 min at 37°C. This was followed by addition of 0.5 ml of dinitrophenylhydrazine (DNPH) and 5.0 ml 0.4N NaOH. The absorbance was measured at 510 nm / yellow green filter.

For the analysis of glutamate pyruvate transaminase GPT (also known as alanine amino transferase, ALT) 0.1 ml of tissue homogenate was mixed with 0.5 ml of substrate solution (L-alanine) and incubated for 30 min at 37°C. The rest of the procedure was same as that for GOT; the absorbance was measured at 405nm.

3.2.9. Test for Lipid peroxidation

Lipid peroxidation was measured in the supernatant by the method of Buege and Aust (Buege and Aust, 1978). One milliliter of sample (homogenate containing 0.1-0.2 mg of protein) was mixed thoroughly with 2ml of trichloroacetic/thiobarbituric acid-HCl (15%, w/v TCA and 0.375%, w/v TBA in 0.25N HCl). Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) at 535 nm in a double-beam spectrophotometer against a suitable blank. The malondialdehyde (MDA) concentration of the sample was calculated using extinction coefficient £=1.56x10^5 M⁻¹ cm⁻¹.

3.2.10. Test for antioxidant enzymes

Catalase (EC 1.11.1.6) decomposes the H₂O₂ and forms water and molecular oxygen. H₂O₂ absorbs maximum light at 240 nm. When H₂O₂ is decomposed by catalase, the absorbance decreases. Determination of catalase activity was assayed by monitoring the rate of decomposition of H₂O₂ spectrophotometrically at 240 nm following the procedure of Aebi (Aebi, H., 1984). The difference in absorbance (Δ Aₘₚ) per unit time is a measure of catalase activity. The assay mixture contained 2.0 ml of homogenate and 1 ml 30 mM H₂O₂ at 20°C with the final volume of 3.0 ml against a blank containing 1 ml 50 mM phosphate buffer, pH 7.0, instead of substrate and 2.0 ml homogenate. The reaction was started by the addition of H₂O₂. The decrease in absorbance was measured with a recorder at an interval of 30 seconds for 3 minutes. The value of absorbance of the reference was subtracted from that of the test cuvette before units of activity were calculated. The activity of catalase was evaluated and expressed as kU/mg. protein.
Superoxide dismutase (SOD) (EC 1.15.1.1) catalyzes the dismutation of superoxide radicals \(O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2\). Its activity was measured according to the method of Paoletti et al., (1986) based on the inhibitory action of the enzyme on the rate of NADH oxidation. One SOD activity unit is defined as the amount of SOD capable of inhibiting 50% rate of NADH oxidation as determined by linear regression of a standard curve of rate of NADH oxidation (% of control) versus the concentration of pure SOD (ng). Analyses were performed in control and treatment animals from the crude homogenates. A decrease in absorbance after 15 min was measured at 340 nm in a reaction mixture containing 3 mM NADH, 25 mM/12.5 mM EDTA/Mn, 10 mM B-mercaptoethanol, and 50 µl of the homogenate in 300 µl of triethanolamine-diethanolamine buffer (pH 7.4). The specific activity has been expressed as units/mg of protein.

3.2.11. Statistical Analysis

Analysis of variance (ANOVA) test was used to assess the degree of significance. Data’s were presented as Mean±SD. Differences were judged as statistically significant for \(P \leq 0.05\).

3.3. Results and Discussions

The general appearance of the p-DAB-treated or untreated mice groups were not changed throughout this study. In addition, no macroscopic alterations were perceptible in the liver of p-DAB-injected animals, as the normal lobular structure was maintained and no visible macroscopic intralobular inflammatory reaction was seen in the successive weeks of analysis viz. 3rd, 5th and 7th week after first administration of p-DAB. Furthermore, no significant differences \((p > 0.05)\) in body weight were found between p-DAB-Injected animals and the control group as presented in Table-1. However, the percentage of liver wet weight per animal body weight showed a significant difference \((p < 0.05)\) between p-DAB-Injected animals and the control group. An increase in hemolysis as measured by the decrease in hematocrit in addition to increase in relative spleen weight was observed in a dose dependent manner (Table-II).
Table II: Body weights, Hematocrit, Relative liver weights and Relative spleen weights in control and p-DAB-treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Relative liver weight*</th>
<th>Hematocrit (%)</th>
<th>Relative spleen weight*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>p-DAB</td>
<td>Control</td>
<td>p-DAB</td>
</tr>
<tr>
<td>3 Wks</td>
<td>25.8 ± 0.4</td>
<td>28.7 ± 0.5a</td>
<td>1.46 ± 0.1</td>
<td>4.5 ± 0.2b</td>
</tr>
<tr>
<td>5 Wks</td>
<td>28.6 ± 0.6</td>
<td>28.6 ± 0.8a</td>
<td>1.56 ± 0.1</td>
<td>4.5 ± 0.2b</td>
</tr>
<tr>
<td>7 Wks</td>
<td>35.5 ± 0.8</td>
<td>31.6 ± 0.8a</td>
<td>1.68 ± 0.1</td>
<td>3.6 ± 0.1b</td>
</tr>
</tbody>
</table>

*Relative liver weight = [(liver wt. / body wt.) x 100]. **Relative spleen weight = [(spleen wt. / body wt.) x 100]. aNo significant differences (p > 0.05) between p-DAB-treated and control groups. bSignificant differences (p < 0.05) between p-DAB-treated and control groups.
Light microscopy and Electron microscopic study of liver sections from p-DAB-administered mice (after 3 weeks of p-DAB treatment); showed changes in histological features. Appearance of bi-nucleated cells, sign of necrosis and inflammation, alteration in endoplasmic reticular network and nuclear pore were observed in light microscopy (Fig. 5). As compared to normal histological structures observed in liver of normal control mice, a careful study of liver sections in the p-DAB fed mice revealed some destructive changes which include: excessive fibrosis in hepatic parenchyma, rapidly proliferating small cells, presence of vacuolated cells, nucleoplasm either condensed or absent and more ramification of blood capillaries. The results are in agreement with the findings of Daoust & Molnar (Daoust & Molnar, 1964).

Similarly, compared to the liver sections of normal control mice, liver sections of the p-DAB fed mice showed several distinct changes in their subcellular organelles as observed under transmission electron microscopy (Fig. 6). These included: i) mitochondria and lipid droplets were numerous; ii) some mitochondria with altered orientation viz. deformed and irregular arrangement of cristae were present; iii) nucleus was round with diverse nucleoplasm; iv) smooth endoplasmic reticulums (ERs) were broken at many places; and v) Kupffer cells were numerous and were activated probably due to increased secretion of lymphokines. More glycogen granules and lipid droplets were present. In the liver section from control group, mitochondria were fewer in number with distinct orientation of the cristae, Kupffer cells were less activated.

Chronic feeding of the carcinogen produced liver damage with neoplastic characteristics without formation of liver nodules. The damage to the liver tissue as was observed by microscopic study clearly reflected possible initiation of alterations in metabolic parameters in the target tissue. Earlier reports on “precancerous liver” have revealed that the phenomenon of damage and repair is accompanied by various histologic and histochemical changes occurring in a gradual manner, whereas tumor formation is associated with discontinuous changes (Daoust and Molnar, 1964, Bannasch, 1986 Bannasch, et al., 2003). Histologic changes are often utilized to study underlying mechanisms of genotoxic and nongenotoxic chemical carcinogenesis (Barrett et al., 1984).

The formation of morphological transformation is an early event in the multistep process of chemically induced carcinogenesis in vivo. The effects of azo-dyes in early and advanced stages of hepatocarcinogenesis have been extensively studied earlier (Caballero et al., 2001).
Fig. 5. Histological liver features of mice after 3 weeks of p-DAB treatment (magnification 40x). 2A. Control 2B. Treated. Histological changes in treated liver are shown by arrows: → Bi-nucleated cells ← Necrosis and Inflammation
Fig. 6. Section of liver under TEM; (3A-B): Untreated control showing intact liver ultrastructure; mitochondria are fewer in number with distinct orientation of the cristae, Kupffer cells are less activated in contrast to p-DAB fed mice (3C-F): showing morphometric alterations; increase mitochondria, lipid droplets, glycogen granules and Kupffer cells, broken smooth endoplasmic reticulums (ERs).
Generally, high doses of p-DAB (50-250 mg/kg bw) in rats cause hepatic degeneration within 24 h, followed by focal degeneration of parenchymal cells around portal spaces (IARC, 1987). However, most investigators used 0.05-0.06% p-DAB in the diet, which was given chronically in order to induce hepatic tumors (IARC, 1987, Akamatsu and Ikegami, 1968, Daoust and Molnar, 1964, Palekar and Sirsat, 1966). It is generally accepted that covalent binding of the metabolites of p-DAB (e.g. MAB, AAB, etc.) with DNA is a major carcinogenic factor (Ohnishi et al., 2001).

Based on these findings, it may be conjectured that the morphological alterations at the early phase of carcinogenesis in the mice treated with p-DAB may be mediated through a direct toxic action of one of the metabolites of p-DAB on liver as proposed by Timbrell (Timbrell, 1991). Additionally, p-DAB may also have cumulative effect by increasing reactive oxygen species and inducing oxidative stress (Klaunig et al., 1998) in bringing about DNA damage. In tune with the report of Klaunig et al., the occurrence of morphologic aberrations, specially distortions in the subcellular organelles including mitochondria and ER noted in the present study, possibly indicates that p-DAB might have acted through oxidative stress mediated cellular pathways.

Mean activities of toxicity marker enzymes, Glutamic oxaloacetate transaminase (GOT) and serum glutamic pyruvate transaminase (GPT) appeared to increase remarkably in mice fed with p-DAB at all fixation intervals compared to values in normal controls (p < 0.05 to < 0.001). Maximum increase in activity of both the enzymes was noted on 5th week; while GOT activity after 5th week was increased by 92% (Fig. 7.) when compared to the normal untreated counterparts, GPT activity at the end of 5th week of DAB administration was noted to be increased by 95% (Fig. 8.) with respect to that of the control group. Increase in GPT activity was proportionally more in comparison to that of GOT activity at the other fixation intervals also i.e. after 3rd and 7th week. The fact that GPT is more sensitive towards hepatic toxicity is reflected in the observed data showing increase in GPT activities by 93% after 3rd week and by 89% after 7th week in contrast to increase in GOT activities by 82% and 87%, at these two successive fixation interval respectively.

In conditions of normal metabolism, GOT and GPT are found in significant quantities in different tissues like liver, kidney, and skeletal muscles. When liver cells are damaged, GOT and GPT levels rise especially early in the disease (Iwata, et al., 1968; Ikegwuonu, et al., 1980; Mukai, et al., 2002). It has been established that in hepatitis, transaminase levels rise several days before jaundice begins (Kuntz and Kuntz, 2008).
Fig. 7. Mean Glutamate Oxaloacetate Transaminase (GOT) Activities (activity±SE) in (mmol/L per min per mg protein) in different tissues of control and treated mice at different fixation intervals. *p < 0.05; **p < 0.01; ***p < 0.001. Statistical analysis done between series p-DAB and cont.; SE, standard error.

Fig. 8. Mean glutamate pyruvate transaminase (GPT) activities in (mmol/L per min per mg protein) in different tissues of control and treated mice at different fixation intervals. *p < 0.05; **p < 0.01; ***p < 0.001. Statistical analysis done between series p-DAB and cont.; SE, standard error.
The enzyme levels are especially useful in assessing subtle and early changes in biliary obstruction and active cirrhosis. In the present study, the marked elevation in the serum GPT and GOT after treatment with p-DAB indicates liver dysfunctions which is correlated with the histological damages in the liver induced by its toxic components. Elevation of serum GOT and GPT has been reported by Vinitha, et al., (1995) to be an indicator of loss of liver cell integrity. Increase of both the transaminases are a common finding in liver diseases with GPT often being higher than GOT (Jeena, et al., 1999). Similar to the data observed in the present study, elevation of liver enzymes has been reported by earlier workers (Rahman and Siddiqui, 2005; Siddiqui, 2004). As cited by Plaa, et al., (1991) GOT and GPT are known to have significant role during the carcinogenesis process. In hepatoma, both GOT and GPT levels are elevated and the ratio of GOT and GPT showed more than 1.0. In metastatic liver tumors, a few cases also show high GOT and GPT levels and their ratios were more than 1.0. As reported much earlier by Iwata (1968) the larger the tumor, GOT elevation is higher. Administration of nimesulide, an anti-inflammatory drug that acts via oxidative stress and mitochondrial injury induces severe liver damage by imposing massive enhancement of the marker enzymes (GPT, GOT and ALP) (Chatterjee and Sil, 2007). The changes observed in the liver enzymes in the present investigation may be attributed to the ability of p-DAB to induce severe physiological and histological alterations in the liver (Becker and Nakatsugawa, 1990). The hypothesis proposed from the present data is based on the fact that, the repeated exposure of the hepatocytes to the toxic compounds might cause severe histological alterations in these cells including rupture of the hepatocytes membranes, causing the release of the toxicity marker enzymes from the cytoplasm of the damaged cells into the blood stream which explain the elevation of these enzymes (Farrell, 1995). GPT activity being a relatively sensitive indicator of hepatic damage, its release from the cytosol can occur secondary to cellular necrosis or as a result of cellular injury with membrane damage and bleb formation (Stockham and Scott, 2002). Thus, in the present study, elevation of GPT in the serum indicates the necrosis of the hepatocyte similar to the findings reported by (Biswas et al., 2011).

The changes observed in the liver cytology is also reflected in the data of lipid peroxidation (LPO) study. The process of lipid peroxidation is initiated by the attack of a free radical on unsaturated lipids and the resulting chain reaction is terminated by the production of lipid breakdown products, lipid, alcohols, aldehydes and malonaldehyde (MDA), which is a low-molecular weight aldehyde. Active oxygen species generated by xenobiotics interact with unsaturated fatty acids to initiate LPO, which in turn influence
the breakdown and turnover of bio-membrane (Biswas et al., 2008). Lipid peroxidation is associated with tissue damage and necrosis and can lead to improper liver function, thereby causing hepatotoxicity and is probably the most extensively investigated free radical-induced process (Sodergren, 2000). Free radicals are electrophilic species that can react with cellular components. Incidentally, reactive oxygen species (ROS) are produced by metabolism of various endogenous and exogenous compounds. It is now being established that there is a cascade of peroxidative reactions leading to increased formation of ROS which may play an important role in carcinogenesis, atherosclerosis, diabetes, emphysema, cataracts, and neurodegenerative diseases, ultimately resulting in the destruction of lipid (Salganik, 2001).

Increased oxidative stress and lipid peroxidation are also implicated in carcinogenic processes (Khanzode et al., 2004; Go‘ncenc et al., 2001; Halliwell and Gutteridge, 1999; Huang et al., 1999) and are associated in the pathogenesis of different diseases including liver diseases such as cirrhosis and hepatocellular cancer (Balasubramanian and Kowdley, 2005). In recent years, using MDA as a marker of oxidative stress, there has been a growing interest in studying the role played by lipid peroxidation in cancer progression (Go’ncenc, et al., 2006). The liberation and quantification of malonaldehyde reflects the state of tissue toxicity, which may affect membrane structure by altering fluidity of the membrane.

The present study clearly reflected a time-dependent increase in membrane lipid peroxidation in hepatic tissues of the p-DAB treated mice when compared to that of the normal untreated group of animals (Fig. 9). The data shows that NADPH-dependent lipid peroxidation was greater in liver of mice during early stages of ingesting a p-DAB containing diet than that of the normal counterparts; there was a remarkable increase in malondialdehyde concentration; MDA conc. was 83% more w.r.t control animals on 3rd week and it showed a time-dependent increase upto 89% on the 7th week as a function of p-DAB exposure (through 86 % on 5th week). The differences between treated mice and corresponding controls were statistically significant at all fixation intervals (p < 0.05).

In concordance to the present evaluation, the elevation in LPO has also been reported in different types of cancer. Elevated LPO in tissue of benign breast disease was observed to be related to an imbalance in the antioxidant system and depression of antioxidant scavenger status that promoted production of MDA (Sodergren, 2000). The antioxidant system is damaged severely and the MDA level rises in cirrhotic and cancer
tissues (Balasubramanian and Kowdley, 2005; Jungst et al., 2004; Dreher and Junod, 1996). LPO has been demonstrated to be a main mechanism of renal carcinogenesis (Dominguez et al., 2005).

![Lipid Peroxidation Graph](image)

Fig. 9. Lipid peroxidation in liver of control and treated mice at different feeding intervals. Values are expressed as the mean±S.E.

Pérez et al., (2005) has indicated about the correlation between LPO and appearance of preneoplastic lesions, pointing towards ROS participation in carcinogenesis. In contrast to this the present study shows virtual absence of pre-neoplastic lesions after initiation with p-DAB with gradual rise of LPO activity from 3rd week to 7th week. This gives an indication of increased mitochondrial respiration which is the important source of active oxygen and might possibly increase leakage of free electrons leading to oxidative stress-induced enhancement in toxicity. In short the present data establishes rise in liver LPO levels in rats initiated with p-DAB is actually mediated through oxidative stress. Thus, a correlation between LPO and hepatocarcinogenicity and the important role of oxidative stress on liver carcinogenesis is reflected. Incidentally, a moderate/strong relation could be substantiated between incidence of morphometric alterations and activities of GOT, GPT and LPO in the p-DAB-fed mice.
The findings have raised concerns on mechanisms underlying tumor initiation in liver and to critically scrutinize the relevance of carcinogenic agents acting during this step of carcinogenesis in rodents with the central tenet being the identification of mode of action as already proposed by some earlier research groups (Williams, 1997, Klaunig et al., 2003; Holzapple et al., 2005).

Liver being the major target site for the metabolism of carcinogens, which generates circulating oxidants and xenobiotics, require a specific antioxidant defense system. The free radicals generated during the carcinogenic process are controlled by a large number of antioxidant systems. The antioxidants detoxify oxidants, free radicals, and electrophilic compounds, thus in a way protect the organism as a whole against oxidative stress. Trace elements such Fe, Cu, Zn, Se, Mn being an integral part of antioxidant enzyme system seems to play an important role in this defense system against oxidants and free radicals.

The disturbance of the pro-oxidant/antioxidant balance, resulting from increased free radical production, antioxidant enzyme inactivation or excessive antioxidant consumption, is a causative factor in oxidative damage. Antioxidant enzymes are preventive antioxidants, because they eliminate species involved in the initiation of free radical chain reaction. Catalase (CAT) acts to control hydrogen peroxide (H$_2$O$_2$) concentration in cells (Radi, et al., 1991). Super oxide dismutase (SOD) mainly acts by quenching of superoxide (O$_2^-$), an active oxygen radical (Kizaki, et al., 1993). Therefore, measurement of the total antioxidant capacity in biological samples shows that total antioxidant capacity of the concerned system.

In the present investigation, activities of catalase (CAT) (Fig.10) and super oxide dismutase (SOD) (Fig.11) were observed to be altered in the p-DAB treated group when compared to that of the control group of animals. At the end of the 3$^{rd}$ week, both CAT and SOD showed a slight increase in activity. CAT activity was increased by 4.5% during 3$^{rd}$ week of treatment, which was decreased with subsequent treatment when compared with the control groups; 43% decrease in activity was observed during 5$^{th}$ week of treatment, indicating profound effect of p-DAB metabolites on antioxidant status of the treated mice. The SOD activity shown initial 5.5% increase in activity during 3$^{rd}$ week of treatment which could be due to the result of a higher production of ROS, when the antioxidant defense system tries to combat the stress induced by carcinogenic administration. With further increase in carcinogenic insult SOD activity was decreased by 20% when compared with the
Fig. 10. Catalase activity expressed as rate of H2O2 decomposition at 240 nm.

Fig. 11. SOD activity expressed as rate of NADH oxidation.
normal counterparts, indicating that the antioxidant system seems to fail to combat further oxidative injury. Thus the carcinogen p-DAB effectively produced alterations in oxidant - antioxidant profile in the treated animals. Parallel to the present data, decrease in activity of these enzymes in cancerous patients has been reported by several other investigators (Liu et al., 2003; Gonenc et al., 2006; Rajneesh et al., 2008).

The diminution of catalase and SOD activities in treated animals is often corroborated with the sensibility of the antioxidant enzymes to at least one of the ROS generated under oxidative stress. It has been reported that the lowered antioxidant capacity and the oxidant-antioxidant imbalance have been considered to play a role in multistage carcinogenesis (Oberley & Oberley 1997). The cellular antioxidant defenses are bound to play a role in oxidant carcinogenesis. The progress made in the understanding of the role of the antioxidant enzymes Cu-Zn-superoxide dismutase (SOD), catalase (CAT), and Se-glutathione peroxidase (GPx) in oxidant carcinogenesis has been reviewed by Cerutti et al., 1994. In their study, the difference between two cell line clones was described; two antioxidant enzymes SOD and CAT are increased coordinately in clone 41. Because the product of the action of SOD is $\text{H}_2\text{O}_2$, an increase in its activity is only beneficial to the cell if it is counterbalanced by a sufficient capacity for the destruction of $\text{H}_2\text{O}_2$. This is apparently accomplished in clone 41 by an increase in CAT (Crawford et al., 1989). Inhibition of both enzymes in the present study could be the consequence of an irreversible autocatalytic process, in which the sustained increase of ROS would finally lead to cellular death as has been observed by Caballero, et al., (2001) who studied long term effect of Tamoxifen (TMX), a breast cancer treatment drug in the liver of mice treated with p-DAB.

Studies on SOD expression in cancer cells revealed that SOD expression is variable but often high in human tumours compared to their normal counterparts (Nishida et al. 1993, Oberley et al. 1994, Coursin et al. 1996, Cobbs et al. 1996, Landriscina et al. 1996, Van Driel et al. 1997, Baker et al. 1997). The levels of the other antioxidant enzymes also have been highly variable, but the CuZnSOD and catalase activities have often been low (Sun 1990). Most of these studies, however, have been conducted on homogenised tumour tissues, and only a few reliable comparisons to the parental cell type are available. CuZnSOD activity was higher in Wilms’ tumour tissue compared to adjacent normal tissue (Gajewksa et al. 1996), but lower in hepatocellular carcinoma than in normal liver cells (Liaw et al. 1997). Punnonen et al. (1994) investigated cancerous and non-cancerous tissue samples from 23 patients with breast cancer and found that the CuZnSOD activities were
higher in cancer tissue, whereas catalase activity was lower. In a study by Jaruga et al. (1994), the total SOD and catalase activities were lower in five samples of lung cancer tissue compared to the surrounding normal tissue. Catalase was negative for the neoplastic cells of all tumour samples, whereas CuZnSOD showed variable immunostaining. Diminished amounts of manganese-containing superoxide dismutase have been found in all the tumors examined. Since some tumor cells have a diminished number of mitochondria, it was possible that the loss of SOD activity could be due to a loss of mitochondria which was also supported by works of Oberley and Bueftner (1979). SOD overexpression in transgenic mice has been shown to increase rather than decrease skin susceptibility to carcinogen-induced tumours (Lu et al. 1997). Comprehensive analysis of all these reports indicate that the expression of antioxidant enzyme expression in cancer are highly variable.

Tumour cells generate superoxide and other ROS, and this generation, if it also occurs in vivo, might have effects on tumour cell proliferation and invasion (Bize et al. 1980, Shaughnessy et al. 1989, Szatrowski & Nathan 1991). It has been hypothesised that the production of ROS combined to a decreased antioxidant enzyme level may be characteristic of tumour cells (Toyokuni et al. 1995, Oberley & Oberley 1997).

The DNA theory has been given added force in recent years with the finding that most activated carcinogens are indeed mutagens (Miller and Miller, 1971). The 2 theories can now easily be reconciled if one realizes that the gene for Mn SOD is thought to be located in the nuclear genome (Weisiger, A. A., and Fridovich, 1973). Thus, a defect in DNA or its expression could easily lead to a loss of Mn SOD which could in turn lead to mitochondrial damage from oxygen-derived radicals. The mitochondrial damage then could lead to increased use of glycolysis for energy. This theory does not necessarily explain the origin of cancer, but it does explain many of the observed properties of the cancer cell. Compounds such as Benzopyrine (B(a)P) are known to alter DNA, protein, and antioxidant enzymes oxidatively in rats and this might be associated with B(a)P carcinogenesis (Kim and Lee, 1997). Thus, SOD and catalase activities in organs of rats were inversely correlated with oxidative damages to DNA and protein.

Studies of liver catalase depression by cancers of men were pioneered in 1910 by Blumenthal and Brahn (Mason et al., 1960). They reported that the liver catalase activity was very low in human beings who had died as a result of various forms of cancer. Experimental work prior to 1941 has been summarized by Greenstein (Greenstein et al.,
and has amply demonstrated that the peroxide-splitting enzyme, catalase, is considerably reduced in the liver of some strains of mice and rats bearing tumors. The decrease in liver catalase in susceptible animals is progressive with the growth of the tumor. There have been reports that in some strains of animals, depression of catalase did not occur despite rapid tumor growth to a large size (Avllemnxn et al., 1950).

The present study revealed that chronic feeding of mice with p-DAB for various periods of time leads to biochemical alterations and cytotoxic effects, as was evident from the modulations in toxicity marker enzymes. The results reflect the alteration of biochemical markers during initiation phase of carcinogenesis, which is possibly associated with functional involvement of certain specific elements in enzymatic reactions leading to metabolic deregulation.

3.4. Conclusion

Change in activities of toxicity biomarker enzymes like glutamate oxalo transaminase (GOT), glutamate pyruvate transaminase (GPT) and lipid peroxidation (LPO) activity can be corroborated with corresponding changes in histomorphological abnormalities of liver cells during early initiation phase of carcinogenesis in mice fed with p-DAB chronically, which have positive association with the progression of tissue necrosis and cellular toxicity (Plaa, 1991).

The results of the present study support the proposal of ROS participation in the initiation of p-DAB carcinogenesis. The results reflect that alteration of biochemical markers during initiation phase of carcinogenesis is possibly associated with functional involvement of certain specific elements in enzymatic reactions leading to metabolic deregulation.

In conclusion, the results of the present study clearly indicate that chronic feeding of p-DAB induced a plethora of changes, of which the morphometric and biochemical changes appeared to be positively time-dependent. In addition, there was a corresponding modulation in enzymatic activities, although this did not rigidly follow a time dependent increase/decrease pattern at specific fixation intervals. The study opens the possibility of further studies with this model to shed light on the mechanism of ROS participation in mice hepatocarcinogenesis.