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Studies on the Cytotoxic Effect of Benzopyrene in Liver of Swiss Albino Mice

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ABSTRACT

The most important problem that humanity is facing in this century is environmental pollution. Polycyclic aromatic hydrocarbons (PAHs) are abundant pollutants and many of them are carcinogenic. The most important PAH is Benzo(a)pyrene [B(a)P] which is formed by the incomplete combustion of organic substances, cigarette smoke, charcoal and grilling of food. Benzo(a)pyrene [B(a)P] has been shown to cause mutagenic, carcinogenic and cytotoxic effects in various species and tissues. The present study was aimed to divulge the cytotoxic effect of B(a)P induced oxidative damage in liver of male Swiss albino mice. Animals were divided into 3 groups of which Group I served as control and were given corn oil, Group II animals were administered with B(a)P (100 mg/kg body weight) dissolved in corn oil orally thrice a week for three successive weeks for an induction period of 6 weeks, Group III animals were administered with B(a)P (100 mg/kg body weight) dissolved in corn oil orally thrice a week for three successive weeks for an induction period of 12 weeks. At the end of the experimental period, the extracted liver tissue was investigated biochemically for cytotoxic markers, oxidative stress markers, lipid peroxidation and antioxidant enzymes .The evaluation of these enzymes and their activities reflect the severity of damage caused to the membrane or to the organ itself. The data suggests that the difference in morphology and cellular changes in liver on exposure to B(a)P is time dependent.

Key words: PAH, B(a)P, toxicity, swiss albino mice, liver, oxidative stress

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INTRODUCTION

Each puff of cigarette smoke forms over trillion free radicals, which may contribute to both initiation and promotion of various forms of human tumor because of repeated ROS attacks on cellular macromolecules.[1,2] Tobacco smoke has been shown to depress antioxidant defense system leading to oxidative damage to DNA, proteins and lipids which may act as an initiator and promoter in multistage chemical carcinogenesis.[3,4,5]

Though B (a) P has its selectivity for the lung, the liver is considered to be main target for chemically induced toxicity and several factors contribute it to being particularly susceptible. Firstly, it is the organ with the highest complement of Cyt P-450 in terms of quantity as well as number of isoenzymes. Secondly, the liver is the first site for the metabolism of carcinogens in gastrointestinal tract. It is the largest organ and gland, since it plays an important role in maintaining the energy level and structural stability of the body.[6]

Oxidation and production of free radicals and reactive oxygen-containing species (ROS) are an integral part of life and metabolism. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, these free radicals can wreak havoc on a broad range of macromolecules.[7]Complex antioxidants prevent the oxidative damage by removing or inactivating chemical intermediates that produce free radicals. Failure of antioxidants to prevent oxidative damage leads to faulty disposal of free radicals and its accumulation. These ROS are responsible for oxidation of tissues leading to lipid peroxidation and tissue damage. They are also responsible for oxidation of bases in cellular DNA, making them mutagenic, cytotoxic and cross linking agents which in turn causes uncontrolled expression of certain genes causing increased multiplication of cells leading to cancer .[8]

The current study is an attempt to investigate the B(a)P induced toxicity to liver during varied induction periods. Hence the present investigation has been focused to evaluate the oxidative stress mediated damage caused by B(a)P administration. Enzymes are highly specialized proteins that accelerate biochemical reactions, which otherwise would proceed at a much slower rate. They are usually confined to a specific cellular membrane, an organelle such as cytosol or mitochondria and sometimes more specific to an organ as well. Hence the present study is aimed to study the alterations in the marker enzymes, oxidative stress markers and antioxidant status due to excess free radical generation.

MATERIALS AND METHODS

Chemicals

Benzo(a)pyrene was purchased from sigma chemical company St. Louis, MO, USA. All other chemicals used were of analytical grade.

Animals

Healthy male Swiss Albino Mice (6-8 weeks old) weighing 25-30g were used throughout the study. The animals were purchased from Central Animal House Facility, Dr.ALM PG IBMS, University of Madras, Taramani campus, Chennai-600113 and were maintained in polypropylene cages in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor feed, Ms. Hindustan Lever Ltd., Mumbai) and water ad libitum. The experimental designs were approved by the institutional animal ethical committee. (IAEC No.01/027/2010).

Experimental design

The animals were divided into three groups containing six animals in each group.

Group I: Control animals were given corn oil orally

Group II: Animals were induced with B(a)P (100mg/Kg body weight) dissolved in corn oil orally thrice a week for three successive weeks for a period of 8 weeks.

Group III: Animals were induced with B(a)P (100mg/Kg body weight) dissolved in corn oil orally thrice a week for three successive weeks for a period of 12 weeks.

After the experimental period, the animals were sacrificed by cervical decapitation. Liver tissues were collected and washed in ice cold saline (0.89%). The tissue was then blotted to dryness and a 10% homogenate was prepared immediately using Tris-HCl buffer 0.1M (pH-7.4) using a Potter Elvejhem glass homogenizer, which was used for the following biochemical estimations.

Total protein was estimated by the method of Lowry et al, 1951.[9] The activities of alanine aminotransferase (ALT)and aspartate aminotransferase(AST) were measured by the method of King .[10] The activity of lactate dehydrogenase(LDH) was assayed using the method of King.[11] The activity of gamma glutamyl transpeptidase(GGT) was estimated according to the method of Orlowski and Meister.[12] 5'-Nucleotidase (5'-ND) was assayed using the method of Luly *et al*.[13] Thioredoxin reductase(TrXR) activity was assessed by the methodology adopted by Holgren(1977).[14]Lipid peroxidation (LPO) was determined by the method of Okhawa et al. (1979).[15]The activity of superoxide dismutase (SOD) was assayed based on Marklund and Marklund (1973).[16] Catalase (CAT) activity was assayed by the method of Sinha (1972).[17] The

activity of glutathione peroxidase (Gpx) was determined by the method of Rotruck et al. (1973).[18] Reduced glutathione (GSH) was assayed by the method of Moron et al. (1979). [19]

Statistical analysis

Results are expressed as mean ± standard deviation (S.D). One-way analysis of variance (ANOVA) was used to detect the significant changes between the groups. The student least significant difference (LSD) method was used to compare the means of different groups and the significance was denoted by 'P' value.

RESULTS AND DISSCUSION

Laboratory findings have suggested that damage to liver can be diagnosed by estimating the levels and activities of the enzyme specifically secreted in liver of B(a)P induced mice.

Transaminases - sensitive cellular integrity markers

Figure 1 elicits the activities of marker enzymes in liver of control and experimental animals. In the present study the activities of transaminases were increased. There was a significant increase (p<0.01) in the activities of ALT and AST GROUP II animals, whereas in GROUP III the increase in significance (P<0.001) was observed as compared to GROUP I animals .

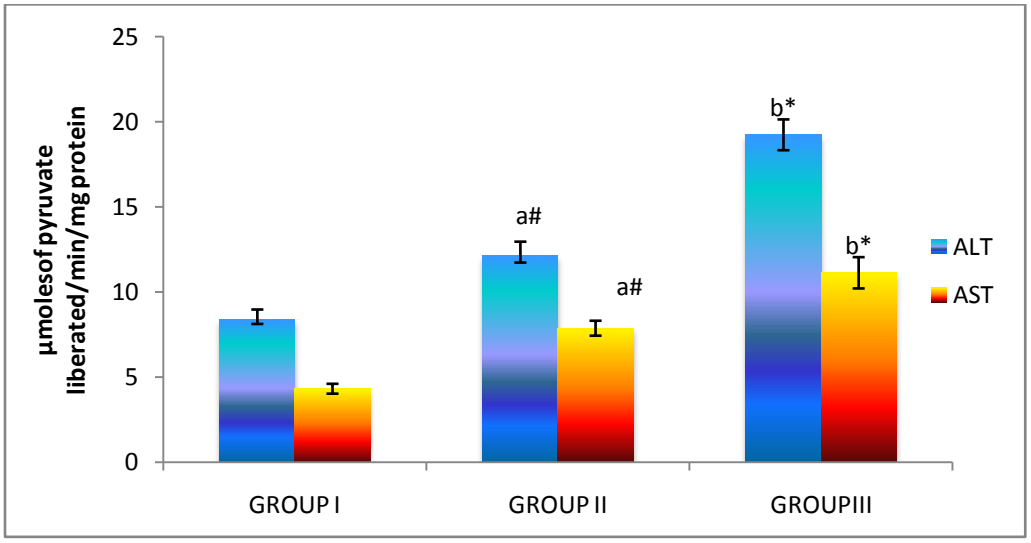

Figure 1: Changes in activities of cellular integrity markers in the liver tissue of control and experimental animals

Each value is expressed as mean ± S.D. for six mice in each group
a: compared with Group I; b: compared with Group I
Units: LDH – µmoles of pyruvate liberated/min/mg protein
Statistical significance: *p<0.001, #p<0.01, $$p$<0.05, NS-Not significant.

ALT and AST are two closely related transaminases involved in the reversible transfer of an amino group to a ketoacid. Tissue levels of these enzymes are highest in the heart, liver, kidney, pancreas, RBCs and GI tract.[20] These are often called markers of liver function. In clinical

diagnosis, neoplastic patients show approximately eight fold increase over normal persons .[21]ALT is predominantly found in liver and very low concentration are found in muscle and kidney .[22]An increase is therefore more specific for liver damage. In liver, ALT is localized mainly in cytoplasm, whereas AST in both cytosol and mitochondria.[23] Damage to liver cells due to acute infection or chronic injury releases these enzymes into circulation.

Cytotoxic markers

Figure 2 illustrates the alterations in the activities of cytotoxic marker enzymes in Liver of control and experimental animals. In the present study increased activities of marker enzymes such as LDH, GGT and 5'ND in B(a)P induced animals. A significant increase in the activities of these cytotoxic marker enzymes were observed in GROUP II ($p<0.01$) and GROUP III ($p<0.001$) animals when compared with control animals.

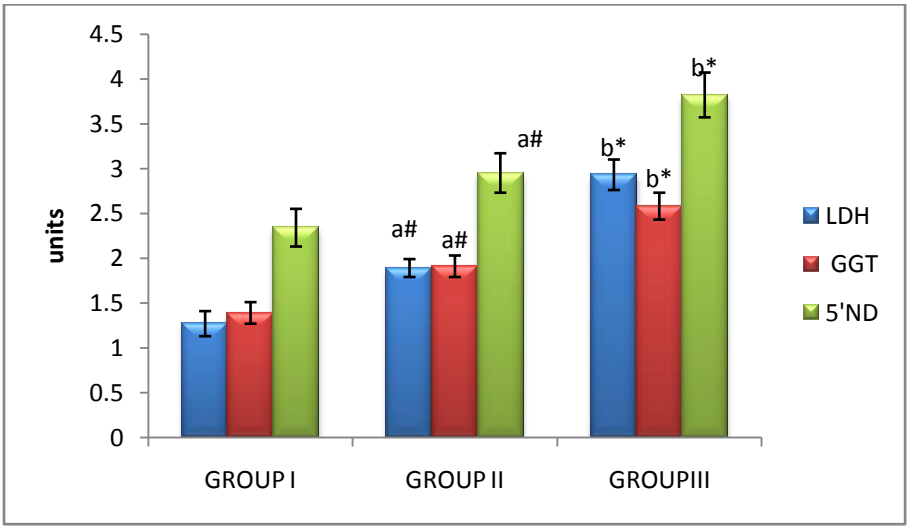

Figure 2: Effect of B(a)P on cytotoxic marker enzymes in the liver tissue of control and experimental animals

Each value is expressed as mean ± S.D. for six mice in each group
a: compared with Group I; b: compared with Group I
Units: LDH – micro-moles of pyruvate liberated/min/mg protein; GGT – nano-moles of p-nitroaniline formed/min/mg protein; 5'N -n moles of p-inorganic phosphorus formed/min/mg protein
Statistical significance: *$p<0.001$, #$p<0.01$, \$$p<0.05$, NS-Not significant.

Elevated activity of LDH may be due to the over production and release of isoenzymes from the destroyed tissue. A significantly increased serum LDH activity was reported in dimethylnitrosamine-induced chronic liver injury in dogs.[24]Elevated activities of LDH4 and LDH5 enzymes were noticed in patients with neoplastic liver disease. [25, 26] Since LDH4 and LDH 5 are plentiful in liver, an increased activity of these isoenzymes denotes damage of the hepatic tissue and leakage of the enzyme into the surroundings.[27] In the present investigation

also, the increased LDH activity was observed in B(a) P induced animals at different time intervals.

GGT activity is affected by genetic and environmental factors. Glutamyl transpeptidase activity serves as a specific marker for the prognosis of carcinogenic events. Moderate smoking causes a 10% increase in glutamyl transpeptidase activity, where as heavy smoking produces 20% rise in values.[28] Chemical carcinogens that enter liver may initiate some systemic effects that induce glutamyl transpeptidase synthesis. GGT activity is affected by genetic and environmental factors.[29]The present study is also in accord with the above statement since B(a) P is a most prevalent environmental carcinogen.

5'-Nucleotidase , an intrinsic membrane glycoprotein present as an ectoenzyme in a wide variety of mammalian cells, hydrolyzes 5'-nucleotides to their corresponding nucleosides.[30]Despite its ubiquitous distribution, serum concentrations of 5'ND appear to reflect hepatobiliary disease with considerable specificity.[31]Its activity inversely correlates with T- and B- lymphocyte function , intracellular zinc concentration , and rate of RNA and DNA synthesis [32.] 5'-nucleotidase has been successfully used to distinguish extrahepatic liver obstructions from liver failure, viral hepatitis and portal cirrhosis .[33]A significant elevation of 5'-nucleotidase activity is seen in liver failure produced by high dose cytostatic treatment .[34] In the current study a significant increase in the activities of these enzymes suggests that cells losses its membrane integrity due to toxic effects of B (a) P.

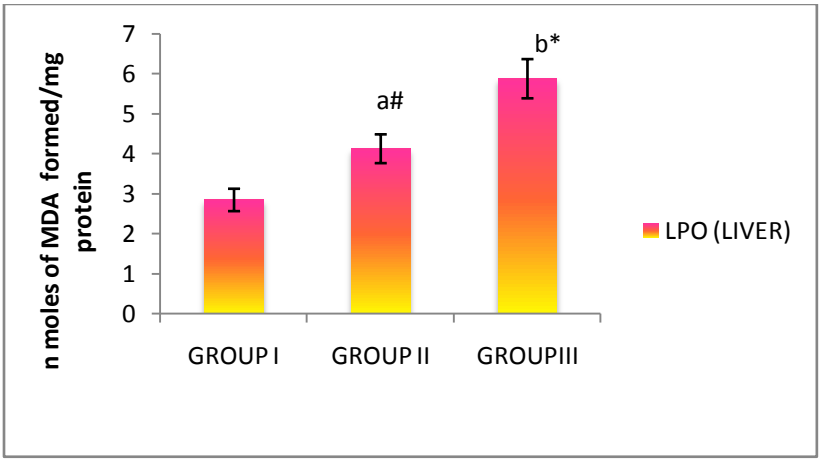

Figure 3: Level of Lipid Peroxidation in the liver tissue of control and B(a)P induced animals

Each value is expressed as mean ± S.D. for six mice in each group
a: compared with Group I; b: compared with Group I
Units: n moles of MDA formed/min/mg protein
Statistical significance: *p<0.001, #p<0.01, $$p$<0.05, NS-Not significant.

Macromolecular damage

Figure 3 depicts the effect of B (a) P on the levels of lipid peroxidation in the liver of control and experimental animals. There found to be a significant increase in the levels of lipid peroxidation in group II ($p<0.01$) and group III ($p<0.001$) animals when compared with control group I control animals.

LPO is regarded as one of the basic mechanisms of cellular damage caused by free radicals. B (a) P is a very effective carcinogen in interacting with membrane lipids and consequently inducing free radical formation [35]. Free radicals react with lipids causing peroxidation, resulting in the release of products such as malondialdehyde, hydrogen peroxide and hydroxyl radicals. Byproducts of lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane bound enzymes and loss of essential fatty acids .[36] An increase in lipid peroxides indicates serious damage to cell membranes, inhibition of several enzymes, cellular function and cell death .[37] It is known that LPO products , both chemically reactive and stable compounds, exert cytotoxic effects. In the present study increased levels of LPO in liver of group II and group III animals may be due to the excessive free radicals produced by administration of B (a) P.

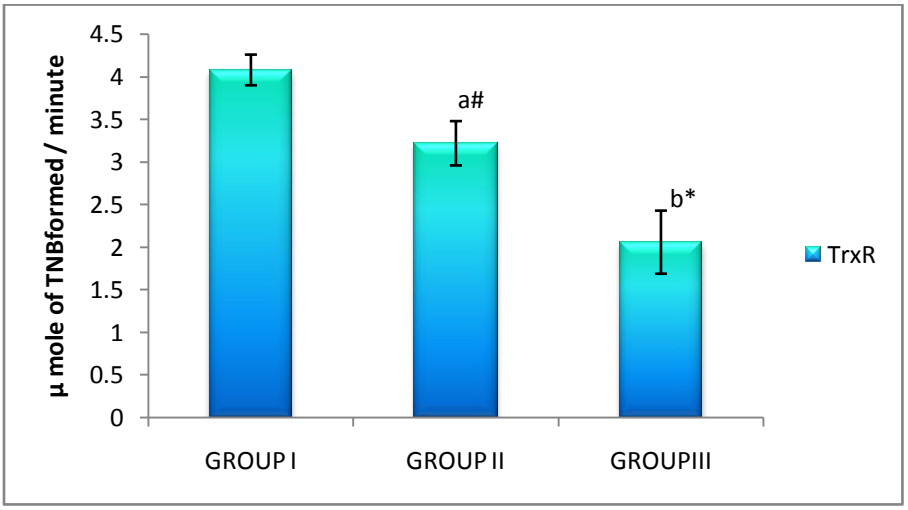

Figure 4: Level of oxidative stress marker enzyme in the liver tissue of control and experimental animals

Each value is expressed as mean ± S.D. for six mice in each group
a: compared with Group I; b: compared with Group I.
Units:µ moles of TNB formed / minute/ mg of protein
Statistical significance: *$p<0.001$, #$p<0.01$, \$$p<0.05$, NS-Not significant.

Oxidative stress marker

Figure 4 shows the level of oxidative stress marker, thioredoxin in the liver of control and experimental animals.A significant decline was noted in the level of TrxR ($P<0.01$) in GROUP II animals and GROUP III ($P<0.001$) B(a)P induced experimental animals compared with control animals. Thioredoxin reductase play an important role in multiple cellular events such as ROS detoxification , oxidoreductase activities and cytokine effects related to carcinogenesis including cell proliferation, apoptosis and cell signaling.[38,39] Over expression of TrxR facilitates protection against a wide variety of oxidative stress including cytokines, UV irradiation and ischemic injury.[40] The liver might have been prone to more oxidative damage due to the cytotoxic effects of benzo(a)pyrene which would have been the reason for decline in the level of thioredoxin.

Enzymic antioxidants

Figure 5(a) and 5(b) and shows the activities of enzymic antioxidants such as SOD, CAT, GPx and GSH in the liver of B (a)P induced animals. The levels were profoundly decreased ($p<0.01$ and $p<0.001$) in GROUP II and GROUP III animals respectively when compared with Group I control animals.

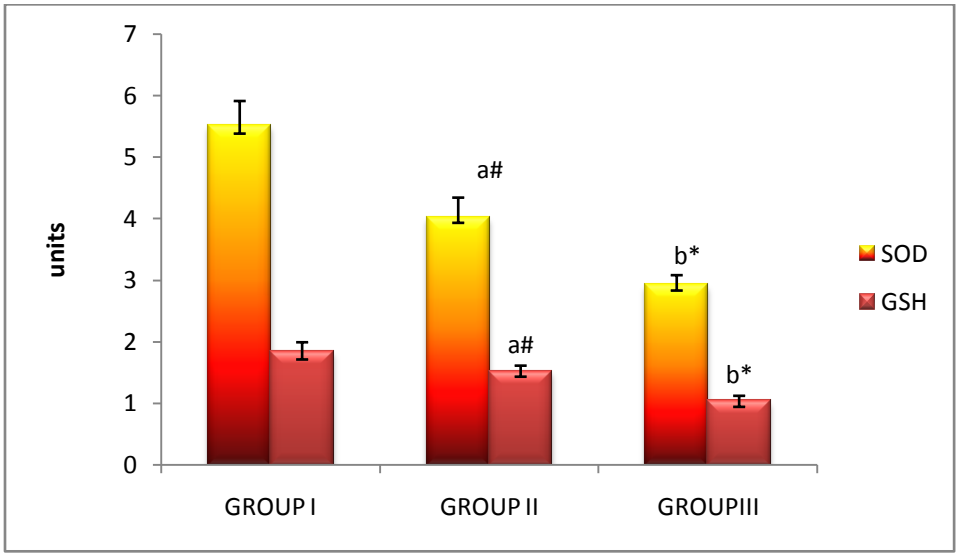

Figure 5(a): Activities of antioxidant enzymes in the liver tissue of control and induced animals.

Each value is expressed as mean ± S.D. for six mice in each group.
a: compared with Group I; b: compared with Group I
Units: SOD – one enzyme unit = amount of enzyme required to prevent 50% auto oxidation/min/mg protein; GSH- µg/mg protein
Statistical significance: *$p<0.001$, $^{\#}p<0.01$, \$$p<0.05$, NS-Not significant.

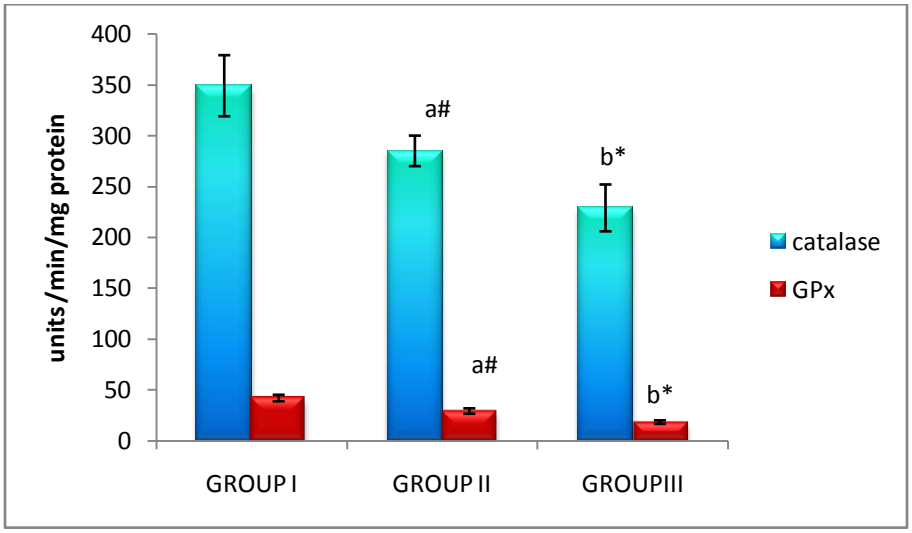

Figure 5 (b): Alterations on antioxidant enzymes in the liver tissue of control and experimental animals

Each value is expressed as mean ± S.D. for six mice in each group.
a: compared with Group I; b: compared with Group I
Units: CAT – n moles of H_2O_2 consumed/min/mg protein; Glutathione Peroxidase- µmoles of GSH oxidized/min/mg protein
Statistical significance: *p<0.001, #p<0.01, $$p$<0.05, NS-Not significant.

SOD has been reported as one of the most important enzymes in the enzymic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effect caused by free radical. [41]The increased superoxide radical levels in tumour cells as compared with normal cells may explain the decrease of the enzymic activity in malignant than normal tissues.[42] In the present study, the decreased level of SOD as observed in benzo(a)pyrene induced GROUP II and GROUP III animals may be due to the utilization of the enzyme to scavenge H_2O_2 radicals. Catalase decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals and it is thought to be the first line of defense against oxidative damage caused by hydrogen peroxide and other free radicals induced by carcinogens.[43] The decreased level of CAT activities in Group II & III animals may be due to the utilization of this enzyme in the removal of hydrogen peroxide radicals caused by carcinogen administration.

GPx is also considered to be an important H_2O_2 removing enzyme in mammalian cells and is more important than catalase for removing H_2O_2 .[44]GPx is involved in the defense mechanism against oxidative damage in reducing the H_2O_2 and hydroperoxide levels. The present study reveals that the activity of GPx in liver was significantly decreased in B(a)P induced animals which might be due to excessive production of lipid hydroperoxides. GPx levels are also relatively low in hepatoma .[45] The reduction in GPx is found to be more deleterious than SOD.

The greater relative importance of GPx over SOD can be attributed to the ability of GPx to detoxify H_2O_2 formed by the activity of SOD.[46] Liver is the main organ with the highest content of GSH and supplies it to other extra- hepatic tissues. It plays a major role in the inter organ homeostasis of glutathione.[47] GSH acts directly as a free radical scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radical. In the present study a significant decrease was observed in Group II & III animals due to oxidative stress mediated effect of B(a)P.

CONCLUSION

To conclude, many biochemical and molecular changes were observed in the tissues of liver in B(a)P induced cancer animals of different periods of induction. The biochemical alterations were evidently seen from the results of activities of marker enzymes, oxidative stress marker and antioxidant enzymes in liver of control and experimental animals. The excess production of free radicals might act as a threat in causing many diseases .The oxidative damage and cytotoxic effect of B(a)P to liver is evident from this study. Thus to prevent oxidative stress mediated toxicity to various organs, the most pivotal action would seem to be minimize exposure to endogenous and exogenous sources of oxidative stress by the elimination of environmental carcinogens as far as possible. Prevention as in all threatening aspects of life, being better than cure.

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