ABSTRACT

Mutagenic and carcinogenic effects resulting from DNA damage are long term sequelae of genotoxicity. The genotoxic agents can be defined functionally as having the ability to alter DNA replication and genetic transmission. The necessity of monitoring genotoxic agents using mice as a model has been forced by ever increasing use of industrial and agricultural chemicals, health hazardous compounds and carcinogens. One such group of compounds that qualify for much attention is phenoxy acid derivatives, viz. Pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid (2,4-D). In mouse liver PCP is metabolized to tetrachlorohydroquinone and tetrachlorocatechol which can be further oxidized to tetrachloro-1,4-benzoquinone and tetrachloro-1,2-benzoquinone via the corresponding semiquinones. Redox cycling associated with the oxidation of tetrachlorohydroquinone and/or the reduction of tetrachloro-1,4-benzoquinone to semiquinones generate oxygen radicals. The 2,4-D is a chlorophenoxy herbicide, it exhibits a variety of mechanisms of toxicity including dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation, and disruption of acetylcoenzyme A metabolism.

PCP whose wide spread use are mainly in food protection, pulp and paper mills, has led to a substantial environmental contamination and continuous exposure of organisms and ecosystems. Its pronounced biocidal activity has increased its use in a great number of applications such as fungicide, molluscicide, insecticide, herbicide, slimicide and preservative. The 2,4-D is a common herbicide employed as post emergence foliar spray used in grassland and turf areas and for the control of aquatic weeds.

In the present study, genotoxic effects of PCP and 2,4-D were tested in chromosomes and DNA. Further biochemical investigations were carried out in sera and liver homogenate. Three different concentrations of PCP and
2,4-D was selected on the basis of their LD50 (separate for each chemical). The effect of these concentrations was seen at 4 different time intervals.

The peripheral blood was withdrawn and the animals were sacrificed by cervical dislocation. The whole liver (about 1 to 2 gm) was removed and subjected to homogenization. Furthermore the contents of femur were extracted and subjected to cytogenetic preparations. For chromosomal aberrations (CA) study cytogenetic preparation from bone marrow cells was made in exposed specimen against appropriate controls. In making the chromosome preparation, the colchicine-hypotonic aceto-alcohol air / flame dry Giemsa staining method was used. Time and concentration dependent increase in total observation was evident for both chemicals. Various types of aberrations like breaks, exchanges, multiple aberrations, stickiness, pulverization and C-metaphases were recorded.

For both the chemicals, the increase in all CA frequencies for 24hr was found statistically significant when compared with relevant controls (Student’s ‘t’ test, P < 0.05), whereas for 48hr, the effect of higher concentration showed statistically significant values except the concentration I of 2,4-D, where the damage was repaired by repair enzymes. The highest frequency of CA recorded by PCP was 9.70% (ex-gaps) and 17.25% (in gaps) for 24hr of the highest concentration as against the level of 8.25% (ex-gaps) and 13.50% (in gaps) at the highest concentration and duration.

As a supporting evidence of induced chromosome aberrations, preparation and observation on micronucleus test (MNT) was performed in bone marrow cells of the mice. The Giemsa May Gruenwald staining technique (ID Adler) was followed. The PCP induced micronuclei in cells was statistically significant at two higher concentrations and duration, whereas 2,4-D induced micronuclei at each concentration, but the statistically significant level was found only at the highest concentration. The incidence of micronuclei formation showed a positive dose response of
effects over the entire dose range tested. Besides, P/N ratio also showed a
decline for both the chemicals when compared to relevant control.
Furthermore, a slight increase in normochromatic erythrocytes was also
observed as compared to the controls.

Mitotic index studies indicated an inhibition of cell division in a dose
and time dependent manner. The observed values for PCP and 2,4-D were
significant when compared with relevant controls. Observations proved the
susceptibility of mammals to genetic toxicity of PCP and 2,4-D, PCP being
the most toxic and 2,4-D reasonable toxic, but both were mutagenic to
mammalian system.

The liver is oftai the target organ of chemically induced tissue injury
due to its role in biotransformation of xenobiotics and its unique position in
circulatory system. A plethora of biochemical changes occurred in blood and
liver of the investigated organism after the administration of PCP and 2,4-D.
These changes were recorded through various biochemical parameters.

Lipid peroxides in the liver homogenate was determined
spectrophotometrically. One molecule of malonaldehyde (MDA) reacts
stoichiometrically with extinction co-efficient of 156 mM^-1-Cu^-1 at 532 nm.
The PCP and 2,4-D did induce lipid peroxides in the liver in a concentration
and dose dependent manner. PCP being more potent produced larger
quantities of LPO reaching the maximum value of 747.43 n mole against the
676.29 n mole for 2,4-D. For both chemicals, maximum damage was
recorded at 24h of the treatment except for the maximum concentration of
PCP, where the damage was recorded at 48h of treatment.

Lipid peroxidation phenomenon is the oxidative breakdown of
unsaturated fatty acids. The formation of lipid peroxidation products leads to
spread of free radical reactions. The general process of lipid peroxidation
consists of three stages: initiation, propagation and termination (Catala,
2006). Peroxidation of lipids can disturb the assembly of membrane, causing
changes in fluidity and permeability, alterations of ion transport and
inhibition of metabolic processes (Nigam and Schewe, 2000). Damage to mitochondria induced by lipid peroxidation can direct further ROS generation (Green and Reed, 1998). In addition LOOH can be broken down, frequently in presence of reduced metals or ascorbate to reactive aldehyde products including malondialdehyde (MDA), 4-hydroxy-2 nonenal (NHE), 4 hydroxy 2-hexenal (4-HHE) and acrolein (Esterbauer et al., 1991). Lipid peroxidation is one of major outcomes of free radical mediated injury to tissues. Peroxidation of lipids can greatly alter the physiochemical properties of membrane lipid bilayer resulting in severe cellular dysfunction.

Estimation of reduced glutathione was another aspect of biochemical investigation through which the damage to antioxidants was recorded. Glutathione (r-glutamylcyteinylglycine GSH) is a sulphhydryl (-SH) antioxidant, antitoxin and an enzyme co-factor. It also provides protection to mitochondria against endogenous oxygen radicals. Glutathione is most concentrated in the liver where the “P450 phase II” enzymes are required to convert the fat soluble substances into water soluble GSH conjugates in order to facilitate their excretion. GSH depletion may be the ultimate factor for determining vulnerability to oxidant attack and its depletion can lead to cell death. Reduced glutathione in liver homogenate was measured by the assay based on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by-SH group of glutathione to form 2 nitromercaptobenzoic acid. The product was measured spectrophotometrically at 412 nm, using the extinction co-efficient of 137 nM\(^{-1}\) cm\(^{-1}\). PCP and 2,4-D did show their effect on glutathione. PCP showed a remarkable effect on reduction where the value declined to 365 µm /g liver from 8.75 µm/g liver of respective control. The 2,4-D also depleted the glutathione. Maximum effect was seen upto 5.71 µmole/gm of liver, against the relevant control noticed at 24 hour of the treatment, hence once again the dose and time dependent concentration relationship was established.
The damage caused by PCP and 2,4-D was further assessed by determining certain liver enzymes in blood. In normal conditions, these enzymes reside within hepatocytes. After damage to liver, these enzymes are spilled into the bloodstream. Alanine aminotransferase (ALT) is one such enzyme. It is markedly elevated in acute liver damage. The enzyme aspartate aminotransferase (AST) has similar role but it is also found in other tissues. The amount of ALT and AST in the blood is directly related to the extent of liver damage. AST and ALT were calculated by the method of Frankel and Reitman (1954). The pyruvate produced by the transamination by ALT reacts with 2,4-dinitrophenyl hydrazine (DNPH) to give a brown-coloured hydrozone, which is measured colorimetrically at 510 nm. The AST decarboxylates spontaneously to pyruvate, which is again measured by hydrazone formations. In both the estimations the concentration of the substrate are suboptimal to reduce the background colour given in the reaction by α-ketoglutarate with DNPH. PCP and 2,4-D did raise AST and ALT enzymes above control levels. Furthermore, the AST/ALT ratio also supported the results that 24hr treatment produced maximum damage. The damage noted at 48hr was also significant. The PCP raised the AST upto 56.25 IU/liter against the 30.25 IU/liter for respective control.

The effects of PCP and 2,4-D were further analyzed by various DNA scanning profiles. The hepatic DNA was extracted by Sambrooke and Russel’s (2001) method. DNA denaturation, also called DNA melting, was the first scanning profile, where the unwinding and separation of deoxyribonucleic acid into single stranded strands (through the breakdown of hydrogen bonding between the bases) was induced by PCP and 2,4-D. The structural changes in modified hepatic DNA was further characterized by measuring the progress of denaturation of PCP and 2,4-D preparations at 260nm as a function of temperature. Melting behaviour of normal and PCP and 2,4-D modified hepatic DNA samples were monitored over a temperature range of 30-90°C at a rate of 1.5C/min and increase in
absorbance at 260 nm was taken as a measure of melting under our experimental conditions. Both PCP and 2,4-D modified DNA showed early melting, with the peak response at 24hr time interval. Here again, dose and time dependent relationship was obtained. The overall profile of Tm of normal and modified hepatic DNA depicted that the structure of the DNA has been damaged by the test chemicals, and this seems to be the reason for observed the lowering of Tm values of modified DNA. The UV absorption spectrum of hepatic native DNA showed characteristic maximum absorbance at 260 nm (A_{max}) The purity was determined from absorbance ratio (A_{260/280}) of 1.8 and electrophoresis in agarose gel. Native hepatic DNA and PCP and 2,4-D modified forms were subjected to alkaline gel electrophoresis in 0.8% agarose gel. Maximum damage was recorded for 24hr of highest concentration i.e. 40mg/1000gm b.w. of PCP, whereas 2,4-D recorded the maximum damage at 24 h at 90 mg/100 gm b.w. At 48h of every concentration, the repair systems repaired the DNA of some of the repairable damage. Maximum repair was seen for lower concentration, where the damage brought by test chemical was not severe. The greater amounts of both oxidative and direct DNA damage by PCP together with increased hepatotoxicity and cell proliferation, may provide, thus, the critical events necessary for hepatic carcinogenesis in mouse.

Neither hepatic DNA nor PCP or 2,4-D, has their own fluorescence and therefore an extrinsic fluorophore, ethidium bromide was use to look into the structure of normal hepatic DNA and its PCP and 2,4-D modified conformers. Normal hepatic DNA and its modified conformers were incubated with ethidium bromide and emission profiles were recorded using the excitation wavelength of ethidium bromide at 325nm.

The fluorescence profiles were recordea and the calculated loss in fluorescence was determined.
The loss of fluorescence was maximum at highest concentration of 40mg/1000gm of PCP at 24h i.e. 63.01% whereas 2,4-D showed the maximum of 40.30% at 90mg/1000gm of 24h time interval.

Above studies are expected to provide excellent understanding for monitoring genotoxic effects of various environmental and man made mutagenic agents, some of which reportedly show carcinogenic effects in human beings. Information extracted are likely to be useful in management and genetic background of cancers. PCP being the most toxic and 2,4-D reasonable toxic both are seen to be genotoxic in mammalian system and their minimum permissible exposure or even discontinuation to humans is recommended herewith.