MATERIALS AND METHODS
2.1 Animal

Female Swiss albino mice of 7 - 8 weeks were used in the present study. They were random bred and kept in the airconditioned (at 25 ± 3° C) Central Animal House of the Jawaharlal Nehru University, New Delhi. Food and water were provided ad libitum. Food pellets were obtained from Hindustan Lever Ltd., New Delhi. The studies were conducted according to ethical guidelines of Indian National Science Academy (INSA) on the use of laboratory animals for scientific research.

2.2 Chemicals

Xanthine, Tris - HCl, NAD⁺, LDH (Lactate Dehydrogenase), Thiobarbituric acid (TBA), DTNB and EDTA, PMSF, DTT and allopurinol were obtained from Sigma Chemical Co., USA. Pyruvate, H₂O₂ and Brilliant Blue G-250 was from E.Merck Co., Germany. All other chemicals were of analytical grade and were from BDH, Mumbai and SRL, Mumbai.

2.3 Preparation of Samples

Animals were killed by cervical dislocation. The liver of mice was excised out and washed in normal saline. The 10% homogenate was prepared by homogenising the tissue with the help of Potter Elvehjem homogeniser. 1mM dithiothreitol (DTT) was added in the buffer before homogenisation. The homogenate was then centrifuged at 4°C for 10 min at 40,000 g using refrigerated centrifuge (Hitachi, Japan). The pellet was discarded. Then the supernatant was again centrifuged for 1 hr at 4° C at 105,000 g using refrigerated ultracentrifuge (Beckman L8-70). The final supernatant was used for enzyme assays.
2.4 Isolation of Serum

The serum was isolated from the blood of from Swiss albino mice by the method as described by Choudhary et al., 1998. The blood was taken out from the animals by eye puncture with the help of heparinised capillary. Then the blood was kept at room temperature for 2 hr and then left at 4°C for about 8 hr. After the incubation, the blood samples were centrifuged at 3000 rpm for 10 mins at 4°C. Then, the serum was pipetted out from the samples and kept in ice for enzyme assays.

2.5 Assay of Xanthine Oxidoreductase

The enzymes of xanthine oxidoreductase were assayed according to the method of Kaminski and Jezewska (1979). The reaction mixture contains 50 mM Tris-HCl (750 μl), pH 8.0, 50 μM xanthine (75 μl), enzyme preparation (400 μg of protein) with or without NAD (150 μM, 500 μl) in a final volume of 3 ml. The blank contained no xanthine in the mixture. In some reactions 0.5 mM sodium pyruvate and 1 unit of LDH was added to prevent the inhibition of XDH by NADH formed in the reaction as and when required as mentioned below. The enzyme activity was measured as formation of Uric acid and NADH at 302 and 340 nm respectively and expressed in international units using the molar absorbance coefficient 7.12 × 10³ mole⁻¹ cm⁻¹ for uric acid at 302 nm and 6.22 × 10³ mole⁻¹ cm⁻¹ for NADH at 340 nm. The different form can be worked by taking the absorbance changes as follows:

1) The activity measured at 302 nm in presence of O₂, NAD⁺, LDH and pyruvate gives the activity of uninhibited dehydrogenase activity of D-Form and D/O and activity of O-Form.

2) The absorbance change at the 302 nm in the presence of O₂ only comprises the activities of O-Form and oxidase activity of D/O-Form.
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3) The enzyme activity recorded at 302 nm in the presence of O₂ and NAD⁺ and in the absence of LDH and pyruvate consists of dehydrogenase activities of D-Forms and D/O (partly inhibited) and the activity of O-Form.

4) The enzyme activity measured at 340 nm in the presence of O₂ and NAD⁺ and in the absence of LDH and pyruvate gives the dehydrogenase activities of D-Form and D/O-Form. The third and fourth readings can be taken in the same sample reaction mixture. The amount of different forms can be calculated by following formula:
The dehydrogenase activity of D-Form = (1) - (2)
The oxidase activity of O-Form = (3) - (4)
The oxidase activity of D/O-Form = (2) - [(3)-(4)]
The total dehydrogenase activity of the sum of the D + D/O-Forms = (1) - [(3)-(4)]

2.6 Measurement of GSH content

The GSH levels were determined by the method of Moron et al., 1979, in the samples using dye binding reaction of GSH with DTNB. The reaction mixture contains 48 mM phosphate buffer pH 8.0, 0.1 ml of sample. The reaction was started by adding DTNB. Before the addition of DTNB the samples were treated with 25 % TCA (100 μl / 400 μl of sample) at ice cold temperature. The absorbance was taken at 412 nm. The blank contains no sample instead 5 % TCA was added. The amount of GSH was expressed in terms μM/mg protein in the sample.

2.7 Lactate dehydrogenase assay

The LDH activity was assayed according to the method of Littauer and De-Groot (1988) by measuring the disappearance of NADH at 340 nm. The principle behind the procedure is:
Lactate + NAD$^+$ -------- Pyruvate + NADH + H$^+$

The equilibrium is more towards left. So in order to estimate the LDH activity the disappearance of NADH was followed. In brief the reaction mixture contains 0.1 ml of pyruvate (0.01 M), 0.1 ml of NADH (0.002 M), 1 ml of phosphate buffer(0.1 M pH 7.6) and sample (100 μg). The change in absorbance was determined at 340 nm in Hitachi 2000 spectrophotometer and change was followed for 3 min. The extinction coefficient used in the calculation was $6.22 \times 10^6$ cm$^2$ M$^{-1}$. The enzyme activity was expressed in terms of unit/mg protein. One unit of enzyme was defined as the amount which was required to oxidise one micromole of NADH/min.

2.8 Preparation of Microsomes

Microsomes were prepared from the liver of mice according to the method described by Varshney and Kale (1990) with some modifications. Mice were killed by cervical dislocation and a 10 % homogenate was prepared with the help of Potter Elvehjem homogeniser. The homogenate was centrifuged at 4°C for 10 min at 15000 g and pellet was discarded. The supernatant was centrifuged at 105,000 g for 1 hr at 4°C. The pellet which has the microsomal fraction was dissolved in 0.15 M KCl and 10 mM tris-HCl buffer pH 7.4 and a homogeneous suspension was prepared. Freshly prepared microsomes were used for lipid peroxidation studies.

2.9 Determination of Lipid peroxidation

Lipid peroxidation in microsomes was measured by the method as described by Varshney and Kale (1990). 0.5 ml of microsomal suspension was mixed with 1.5 ml of suspension medium (10mM Tris-HCl+0.15 M KCl) to which 0.5 ml of 30 % TCA was added followed by 0.5 ml of 52 mM TBA.
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The tubes were placed in water bath at 80 °C for 45 min. Then allowed to cool at ice bath for 10 min and then centrifuged for 10 min at room temperature at 3,000 rpm in REMI T-8 table top centrifuge. The absorbance of supernatant was measured at 531.8 nm against reference blank of distilled water in spectrophotometer (Hitachi 2000). The extent of lipid peroxidation was expressed in terms of nM MDA formed/mg of protein.

The Calculation of MDA concentration

The amount of MDA formed can be estimated according to the formula:

\[ n \text{ moles of MDA} = \frac{V \times OD}{0.152} \]

where,

- \( V \) = Final volume of test solution
- \( OD \) = optical density
- \( \varepsilon = 1.52 \times 10^5 \)

2.10 Protein Estimation

The amount of protein in the samples were estimated by method described by Bradford, 1976. The samples were mixed with the Bradford's reagent and the absorbance was taken at 595 nm. The amount of protein was evaluated with the help of standard curve prepared with Bovine serum albumin and expressed in mg/ml.

2.11 Statistical analysis

The statistical significance of difference between data pairs was evaluated by analysis of variance (ANOVA) followed by Mann-Whitney test.
2.12 Irradiation of animals

The animals were irradiated with gamma rays in the gamma chamber (TBq $^{60}$Co Model 4000A) obtained from Bhabha Atomic Research Centre, Mumbai. The irradiation was performed at room temperature with required doses of radiation. Radiation dose was determined by Fricke’s dosimetry.

Determination of Dose rate

The dose rate was determined by the Fricke’s chemical dosimetry. The solution of dosimetry has 0.01 M Ferrous ammonium sulfate, 0.8 N H$_2$SO$_4$ and 0.01 M NaCl. The solution was prepared as follows:

Sulfuric acid (4.4ml) was added to the water (200 ml) first and allowed to cool at the room temperature. Ferrous ammonium sulfate (80 mg) and NaCl (12 mg) were then added and the solution was shaken vigorously with a glass rod to dissolve the salts completely as soon as possible. The dosimeter solution was irradiated for small time periods (1, 2, 3, 4, 5 min) duration. The optical density of Fe$^{3+}$ formed was measured spectrophotometrically at 305 nm. At this wavelength the molar extinction coefficient of ferrous ions is particularly zero and that of ferric ions is 2197 M$^{-1}$ cm$^{-1}$ at 25°C. Another Fe$^{3+}$ peak is located at 244 nm where the molar extinction coefficient is about twice i.e. 4565 M$^{-1}$ cm$^{-1}$. However, at this wavelength the absorption of the ferrous ions cannot be neglected (about 20 M$^{-1}$ cm$^{-1}$). The optical density of the sample after irradiation is compared with that of the blank (Unirradiated solution). A graph of O.D. (O.D. irr - O.D. unirr) vs time is plotted and the slope (O.D./min) was calculated.
According to the definition of G value

\[
G = \frac{\text{Molecules/ml} \times 100}{D \text{ (ev)}},
\]

Where \( D \) (ev) is the dose in ev/ml. Therefore,

\[
\text{molecules/ml} \times 100 \quad \frac{D \text{ (ev)}}{(G)}
\]

In case of spectrophotometry, we have

\[
\text{OD} \times N \times 10^{-3} = \frac{\text{molecules/ml}}{D \text{ e}^1}.
\]

The conversion of \( D \) (ev) to \( D \) in rad is

\[
D \text{ (ev)} = 6.245 \times 10^{13} D \text{ in rad}
\]

and the dose equation can be now written as

\[
\text{OD} \times N \times 10^{-3} \times 100 = \frac{\text{D rad}}{G \text{ d e}^1 \times 6.245 \times 10^{13}}
\]

Where,

- OD = difference in the optical density between the irradiated sample and the blank.
- \( N \) = Avogadro's number (= 6.02 \times 10^{23} \text{ molecules/mole})
- \( G \) = no. of ferrous ions oxidised per 100 ev of absorbed energy. (G Fe^{3+} = 15.6)
- \( d \) = specific density of the dosimetric solution
  \( (= 1.024 \text{ mg/cm}^3 \text{ for } 0.8 \text{ N (NH}_4)_2\text{SO}_4)\)
- \( e \) = molar extinction coefficient ( 2197 M^{-1} \text{ cm}^{-1} \text{ at } 25 ^\circ \text{C})
- \( l \) = optical path length of the spectrophotometric cell
  \( (= 1 \text{ cm})\)
After substituting these values

Dose rate = $2.75 \times 10^4 \times \text{OD in rad}$

Where,

OD is a slope i.e. OD/min

Rad being an old unit of absorbed dose of radiation, it was converted into Gray by the following relation:

$100 \text{ rad} = 1 \text{ Gy (Gray)}$