Chapter 3

Materials and Methods
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A. Experimental Animals

Inbred Balb/c male mice of two months age, weighing 20-25 g, purchased from Veterinary College, Mannuthy, Thrissur, were used for the study. The mice were obtained from the stock inbred colony, which was maintained by mating brothers and sisters. They were housed in ventilated cages maintained on dry pellets of rat feed (Gold Mohur mouse chow, Hindustan Lever (Ltd.) and water *ad libitum*.

B. Aflatoxin-B₁ Preparation

Experimental hepatoma in Balb/c mice induced by Aflatoxin-B₁ (Purchased from Sigma Chemical Co., USA). Aflatoxin-B₁ was dissolved in DMSO (10 mg/ml) and further diluted with sterile distilled water to get appropriate concentrations. An aliquot of 0.1 ml preparation containing 20 μg Aflatoxin-B₁ was injected per mouse intraperitoneally for a period of 10 days. Normal control animals received same amount of the vehicle.

C. Tumour Cell Line and Their Maintenance

Dalton's lymphoma ascites tumour cell line, kindly provided by Amala Cancer Research Institute, Thrissur, was used for the study. The tumour cell line was maintained by serial intraperitoneal (IP) transplantation in mice. Full-grown tumour cell line were aspirated from the mouse peritoneum, washed thrice with 0.9% saline and suspended in saline. About $1 \times 10^5$ cells were injected intraperitoneally into a new healthy mouse. All operations were done aseptically.
D. Preparation of Retinyl Acetate

Retinyl acetate used for the study was purchased from SRL Chemical Co. Appropriate concentration of retinyl acetate (500 μg/kg, 2.5 mg/kg and 5 mg/kg Body wt.) was mixed with the feed and fed to animals till the end of the experiment.

E. Study of Survival Period

a) Survival period of mice in Aflatoxin-B₁-induced hepatoma

The mice were divided into five groups, consisting of six mice in each group. Group I was treated as normal control with normal feed and group II was administered with a dose of 20 μg Aflatoxin- B₁/day by intraperitoneal injection for a period of 10 days. The groups III, IV and V were given the same amount of Aflatoxin- B₁ (as in the case of group II) and retinyl acetate at different concentrations i.e. 10, 50, and 100 μg of retinyl acetate.

The death rate was noticed and the survival period was also taken.

b) Survival period in Dalton’s lymphoma bearing mice

Mice were grouped into five as in the study of survival period in Aflatoxin-B₁-induced hepatoma bearing mice. Group I treated as the normal control and fed with normal feed, the group II given a intraperitoneal injection of Dalton’s lymphoma ascites cells consisting of $1 \times 10^5$ cells, and not treated. Group III, IV and V were also given the same amount of ascites cells and were treated with retinyl acetate at a dose level of 10 μg, 50 μg and 100 μg/day/mouse respectively. The death rate was noticed and the survival period was also taken.
**F. Grouping of Animals for Hepatoma Study**

Mice of average weight of 22.7 ± 1.15g were divided into five groups, containing of twelve mice in each group. They were divided as follows:

**G1** – Normal control: Maintained with normal laboratory feed (G1).

**G2** – Hepatoma control: 20 µg of aflatoxin B1, injected into the peritoneal cavity of each mouse daily for a period of 10 days. Sacrificed six mice on the 60th day and 120th day of aflatoxin B1 injection (G2a & G2b).

**G3** – Retinyl acetate treated group I: Hepatoma-induced mice, each consumed 10 µg of retinyl acetate per day. Sacrificed six mice on 60th and 120th day of aflatoxin injection (G3a & G3b).

**G4** – Retinyl acetate treated group II: Hepatoma-induced mice, each consumed 50 µg of retinyl acetate per day. Sacrificed six mice on 60th and 120th day of aflatoxin injection (G4a & G4b).

**G5** – Treated group III: Hepatoma-induced mice each consumed Retinyl acetate 100 µg of retinyl acetate per day and six mice were sacrificed on the 60th and 120th day of aflatoxin injection. (G5a & G5b).

The gain in body weight and liver weight were noted on the 120th day of the experiment.
G. Grouping of Animals for Dalton's Lymphoma Study

Mice of average weight of 21 ± 1.5 were divided into five groups containing 12 mice in each group as in the case of hepatoma study. The animals were grouped as follows:

G₁ — Normal control: Maintained with normal laboratory feed (G₁).
G₂ — Disease control: For inducing Dalton's lymphoma about $1 \times 10^5$ tumour cells were injected into the peritoneal cavity of each mouse as a single dose. These mice were not treated and six mice were sacrificed on 8th and 15th day of tumour implantation (G₂a & G₂b).
G₃ — Retinyl acetate treated group I: Tumour implanted mice, each consumed 10 μg of retinyl acetate per day. Six mice were sacrificed on 8th and 15th day of tumour implantation (G₃a & G₃b).
G₄ — Retinyl acetate treated group II: Tumour implanted mice, each consumed 50 μg of retinyl acetate per day. Six mice were sacrificed in 8th and 15th day of tumour implantation (G₄a & G₄b).
G₅ — Retinyl acetate treated group III: Tumour implanted mice, each consumed 100 μg of retinyl acetate per day. Six mice were sacrificed in 8th and 15th day of tumour implantation (G₅a & G₅b).

The gain in body weight were noted on the 8th & 15th day of the experiment.

H. Methods Used for the Biochemical Analysis

Antioxidants such as Ascorbic acid, Glutathione (reduced), and antioxidant enzymes such as superoxide dismutase, catalase and detoxifying enzyme, glutathione-S-transferase. Liver enzymes like $\gamma$-glutamyl transpeptidase, lactate dehydrogenase, aspartate and alanine transaminases,
Alkaline and acid phosphatases has been estimated using UV spectrophotometer (UV-1601, Shimadzu).

I. Estimation of Ascorbic Acid

Ascorbic acid level in tissues were determined by using the method of Roe\textsuperscript{27}.

Reagents

1. Trichloro acetic acid (TCA) – 6%.
2. Thiourea reagent – 50% in alcohol.
3. 2,4 dinitrophenyl hydrazine – 2% in 9 N H\textsubscript{2}SO\textsubscript{4}.
4. Conc. H\textsubscript{2}SO\textsubscript{4} – 85%.
5. Ascorbic acid standard
   (a) Stock – 100 mg ascorbic acid in 100 ml of 6% TCA
   (b) Working standard – 100 \mu g ascorbic acid in 1 ml 6% TCA
6. Activated charcoal.

Procedure

Weighed sample was homogenised in 5 ml ice cold 6% TCA in a pre-chilled mortar. The extract was shaken well in a test tube and added activated animal charcoal and allowed to stand for 15 min. The clear supernatant was filtered through Whatman filter paper. To 4 ml of supernatant, added a drop of thiourea reagent (50% in alcohol) and 1 ml of 2%, 2,4 dinitrophenyl hydrazine in 9 N H\textsubscript{2}SO\textsubscript{4} and incubated for 3 h at 37°C in a water bath. At the end of the incubation, placed the test tubes in an ice bath and added carefully 4 ml of 85% conc. H\textsubscript{2}SO\textsubscript{4}. Kept for 30 min in refrigerator. Centrifuged and OD measured at 540 nm in a spectrophotometer. The values are expressed in mg/100 g tissue.
II. Estimation of Glutathione (Reduced)

Glutathione level in tissue was determined by using the method of Patterson and Lazarro.\textsuperscript{22x}

Reagents

1. Alloxan 0.1 M
2. Phosphate buffer – 0.5 M (pH 7.5).
3. NaOH (0.5 N)
4. NaOH (1 N).
5. GSH Standard – 3mg GSH/5% 25ml metaphosphoric acid.

Procedure

Tissues were homogenized in phosphate buffer. The reaction mixture containing 50 µl tissue extract, 50 µl alloxan, 50 µl phosphate buffer and 50 µl NaOH (0.5 N) was incubated at 25°C for 6 min. The reaction was stopped by the addition of 50 µl IN NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1 cm length path in a spectrophotometer. A control tube also maintained using phosphate buffer instead of extract. The glutathione (reduced) level was expressed as mg of GSH/g tissue weight. The values are expressed in mg/g tissue.

III. Estimation of Superoxide Dismutase

Superoxide dismutase in tissues were determined using the method of Kakkar et al.\textsuperscript{22y}

Reagents

1. Sodium pyrophosphate buffer – 0.052 M (pH 8.3).
2. Tris-HCl buffer – 0.0025M (pH 7.4).
3. Sucrose – 0.25M
4. Phenazine methosulphate – 186 µM.
5. Nitro blue tetrazolium – 300 µM.
6. NADH – 780 µM.
7. Glacial acetic acid.
8. n-butanol.

Procedure

100 mg tissue was homogenized in 2 ml 0.25M sucrose and differentially centrifuged to get cytosol fraction. This fraction was then dialysed against 0.0025M Tris-HCl buffer (pH 7.4) overnight before using for enzyme assay. Assay mixture contained 1.2 ml of the sodium pyro phosphate buffer and 0.1 ml of phenazine methosulphate, 0.3 ml nitro blue tetrazolium 0.2 ml NADH and 1.2 ml of the enzyme source. Reaction was initiated by the addition of NADH. Incubated at 30°C for 90 sec. and stopped the reaction by the addition of 1 ml glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min and centrifuged and the butanol layer was taken out. Colour intensity of the chromogen in butanol was measured at 560 nm, against n-butanol blank. A system devoid of enzyme served as control. Protein estimation was carried out on the same enzyme source by the method of Lowry et al. The values are expressed as 50% inhibition of nitroblue tetrazolium/min/mg/protein.
IV. Estimation of Catalase

Catalase level in tissues were determined using the method of Cohen et al.\textsuperscript{230}.

Reagents

1. Phosphate buffer 0.067 M (pH 7.0).
2. H\textsubscript{2}O\textsubscript{2} - 30 \(\mu\)l/10 ml buffer.

Procedure

100 mg was tissue homogenized in 2 ml phosphate buffer and centrifuged. To 30 \(\mu\)l of the supernatant, added 3 ml of buffer and 0.75 ml of H\textsubscript{2}O\textsubscript{2}. Change in OD was measured at 240 nm at 0 sec, 30 sec and 60 sec respectively. The control system devoid of tissue extract was used as blank. Values are expressed in \(\mu\) moles of hydrogen peroxide consumed/min/mg/protein.

V. Estimation of Glutathione-S-Transferase

Glutathione-S-transferase in tissues were determined using the method of Butler et al.\textsuperscript{231}.

Reagents

1. Phosphate buffer : 0.5 M (pH 6.5).
2. CDNB : 25 mM in 95% Ethanol
3. GSH : 20 mM

Procedure

Tissues were homogenized in phosphate buffer. The reaction mixture containing 200 \(\mu\)l phosphate buffer, 20 \(\mu\)l CDNB, 730 \(\mu\)l distilled water was taken in the control tubes and 200 \(\mu\)l phosphate buffer, 20 \(\mu\)l CDNB, 680 \(\mu\)l distilled water was taken in the test sample tubes. Then the tubes were incubated at 37°C for 10 min. After the incubation added 50 \(\mu\)l of GSH in both the set of
tubes. After mixing well, added 50 μl of tissue extract in the test sample tubes. Absorbance was noted at 340 nm for five min. in a quartz cuvette of 1 cm length path in a spectrophotometer. Values are expressed in n moles of CDNB complexed/min/mg/protein.

VI. Estimation of Protein

Protein content of the serum and tissues were assayed by the method of Lowry et al.\textsuperscript{232}.

Reagents

1. Sodium hydroxide solution – 0.1 N.
2. Sodium carbonate solution – 2% in 0.1 N sodium hydroxide.
3. Copper sulphate solution – 0.5%
4. Sodium potassium tartrate solution – 1%
5. Alkaline copper reagent – A mixture of 50 ml sodium carbonate solution and 0.5 ml of each of copper sulphate solution and sodium potassium tartrate solution.
6. Folin’s phenol reagent – 1:1 dilution with distilled water.
7. Standard protein solution. – 100 mg % in 0.1N NaOH

Procedure

Pipetted out 0.2 ml of serum or extract to the test tube and added 1.8 ml of sodium hydroxide solution and 5 ml of alkaline copper reagent. Shaken well and kept the mixture for 10 min. After 10 min, 0.5 ml of Folin’s phenol reagent was added and mixed well. The mixture was kept for another 30 min. The optical density was measured at 675 nm in a spectrophotometer. The system devoid of sample was used as the blank.
VII. Estimation of γ-Glutamyl Transpeptidase

GGT in serum was assayed by the method of Gowanlock.²³³

Reagents

1. Buffer – Tris 120 mmol/l MgCl₂ 12 mmol/l and glycyl glycine 90 mmol/l at pH 7.8 (37°C).

Dissolve 14.54 g Tris 2.44 g MgCl₂.6H₂O and 11.89 glycyl glycine in about 800 ml distilled water and adjusted pH to 7.8 at 37°C and made up to 1 litre. This was stable for 24 h to 8 weeks at 4°C.

2. Substrate

Dissolved 1.28 g L-γ-glutamyl 4-nitro anilide (anhydrous) in 0.15 ml/l HCl made up to 100 ml with the acid. Constant stirring was required to dissolve to the substrates. Stored at -20°C and it was stable for several weeks (The reagent was stable for only a few days at room temperature).

Technique

100 μl serum and 1 ml buffer were incubated at 37°C. Started the reaction by adding 0.1 ml substrate, mixed and monitored the reaction continuously at 405 in 1 cm cuvette so as to obtain the change in absorbance per minute. Values are expressed in μg/min/100ml serum.

VIII. Estimation of Serum Lactate Dehydrogenase

The serum lactate dehydrogenase was determined by the method of King.²³⁴

Reagents

1. Glycine buffer 0.1 M – 7.505 g glycine (SRL) and 5.85 g sodium chloride (AR) in 1 litre distilled water.
2. Buffered substrate – To 125 ml glycine buffer and 75 ml 0.1 N sodium hydroxide added 5 ml of 75% sodium lactate (Merck) solution.

3. NAD⁺ – 10 mg NAD⁺ (Sigma) in 2 ml distilled water. Kept at 0°C–4°C.

4. 2,4 dinitrophenyl hydrazine reagent – Dissolved 200 mg DNPH (Merck) in 100 ml hot 1 N Hydrochloric acid and made up to 1 litre with the acid.

5. Sodium hydroxide – 0.4 N.


7. NADH – 1 mmol/ml of buffered substrate

**Procedure**

Pipetted out 1 ml of buffered substrate and 20 μl serum into each of two tubes, designated as test and blank, added 0.2 ml of distilled water into the blank tube and place both the tubes in a water bath at 37°C and allowed to reach the temperature. Then added 0.2 ml of NAD solution to the test and shaken well. After 15 min added 1 ml DNPH solution to both the tubes and mixed and kept in the water bath for another 20 min. After removal from the bath, to each tube, added 10 ml of 0.4 N sodium hydroxide and the absorbance was read at 440 nm within 5 min. Sodium pyruvate solution containing 1 μM of pyruvate/ml was used as the standard. Values are expressed in IU/100 ml serum.

**IX. Estimation of Serum Transaminases**

Serum levels of alanine and aspartate transaminase were determined by the method of Mohun and Cook \(^{235}\).
Reagents

(a) Potassium phosphate buffer, 0.075 M (pH 7.5)

(b) Buffered substrates.

1. Aspartate transaminase – 300 mg L-aspartic acid (Sigma) and 50 mg alpha ketoglutaric acid (Sigma) in 100 ml phosphate buffer and the pH was adjusted to 7.5 with sodium hydroxide.

2. Alanine transaminase – 5 g DL-alanine (Sigma) and 20 mg alpha ketoglutaric acid (Sigma) in 100 ml phosphate buffer and the pH was adjusted to 7.5 with sodium hydroxide.

3. Aniline-citrate reagent – Dissolved 50 g citric acid (SRL) in 50 ml distilled water and to this an equal volume of redistilled aniline (Merck) was added.

4. Dinitrophenyl hydrazine reagent – Dissolved 200 mg of 2,4-dinitrophenyl hydrazine (Merck) in 85 ml of con. Hydrochloric acid and made up to one litre with distilled water.

5. Sodium hydroxide, 0.4 N.

Procedure

Pipetted out 1 ml of substrate solution into two tubes and placed in water bath at 37°C for few minutes. To one of the tubes (test), added 0.2 ml of serum and shaken gently to mix. Exactly after one hour, in the case of aspartate transaminase and half an hour in the case of alanine transaminase. With the tubes still in the bath added two drops aniline-citrate reagent to both and added 0.2 ml of serum in control. Tube left undisturbed for 20 minutes. Then into both the tubes added 1 ml of dinitrophenyl hydrazine reagent. Left the tubes for another 20 minutes in room temperature and then added 10 ml of 0.4N sodium hydroxide.
and read at 520 nm after 10 minutes. A solution of sodium pyruvate containing 10 mg pyruvate per ml was used as standard. Values are expressed in IU/100 ml serum.

X. Estimation of Serum Phosphatases

Serum phosphatases were assayed following the method of King and Jagatheesan, using 4-aminoantipyrine.

1. Alkaline phosphatase

Reagents

1. Disodium phenyl phosphate (Merck) – 0.01 M in distilled water.
2. Sodium carbonate and bicarbonate buffer (0.1M) – 3.18 g anhydrous sodium carbonate and 1.68 g sodium bicarbonate dissolved in 500 ml distilled water.
3. Buffered substrate for use: Prepared by mixing equal volumes of reagent (1) and (2).
4. Sodium hydroxide – 0.5 N.
5. Sodium bicarbonate – 0.5 M.
6. 4-aminoantipyrine (Sigma)- 0.6% in distilled water.
7. Potassium ferricyanide – 2.4% in distilled water.

Procedure

2 ml of buffered substrate was incubated at 37°C for few minutes in a water bath. 0.1 ml of serum was added in test tube designated with test and further incubated to 15 min. At the end of incubation, into the tube added 0.8 ml of sodium hydroxide and 1.2 ml of sodium bicarbonate. To control tubes, serum was added only after adding sodium bicarbonate. This tube was used as the blank. To both the tubes then added 1 ml aminoantipyrine reagent and 1 ml potassium
ferricyanide solution. For standard 1.1 ml of the buffer and 1 ml of phenol solution containing 0.01 mg of phenol, and for the standard blank 1.1 ml buffer and 1 ml distilled water were taken, instead of buffered substrate and serum. All other procedures are same as in the case of test samples. The absorbance was read at 520 nm. Values are expressed in King Amstrong units/100 ml serum.

2. Acid phosphatase

Reagents

1. Citric acid – Sodium citrate buffer 0.1 M (pH 4.9).

All other reagents were same as for alkaline phosphatase.

Procedure

Procedure was same in the case of alkaline phosphatase except that the citric acid -sodium citrate buffer was used for the preparation of buffered substrate and incubation as 1 hour. For developing the colour with aminoantipyrine, 1 ml sodium hydroxide and 1 ml sodium bicarbonate were added. Values are expressed in King Amstrong units/100 ml serum.

1. Statistical Analysis

All statistical analysis have done using a computer software created by Russel E. Freed.