CHAPTER II
MATERIAS AND METHODS
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AFLATOXIN PRODUCTION

A toxigenic strain of *Aspergillus parasiticus var. globosus* (MTCC 411) obtained from Institute of Microbial Technology, Chandigarh, India was maintained on potato dextrose agar medium.

**Potato dextrose agar medium**

- Sliced potato : 200.00 gm
- Glucose : 20.00 gm
- Agar : 40.00 gm
- Distilled water : 1000 ml

*Aspergillus parasiticus* was grown on sucrose-magnesium sulphate-potassium nitrate- yeast extract (SMKY) liquid medium at 28 ± 2°C for 10 days (Diener and Davis, 1966).

**SMKY liquid medium**

- Sucrose : 200.00 gm
- Magnesium sulphate : 0.5 gm
- Potassium nitrate : 3.0 gm
- Yeast extract : 7.0 gm
- Distilled water : 1000 ml
Fifty ml of SMKY liquid medium was taken in a 500 ml Erlenmeyer flask and sterilized at 15 lb pressure for 20 min. The sterilized medium was inoculated with 0.5 ml spore suspension of *Aspergillus parasiticus* having $10^8$ conidia/ml aseptically (under laminar flow) and incubated at 28 ± 2°C for 10 days. The contents of the autoclaved flasks were filtered through Whatman filter paper No 1. Pooled culture filtrates were extracted twice with chloroform (1:2, v/v) in a separating funnel and the lowermost chloroform layer was passed through the bed of anhydrous sodium sulphate ($\text{Na}_2\text{SO}_4$). The chloroform extract was subsequently evaporated to dryness and stored in vials.

**Qualitative detection of aflatoxins**

Thin-layer chromatography (TLC) technique was used for qualitative analysis of aflatoxins. Glass plates of uniform sizes (20 x 20 x 0.25 cm) were used for coating of silica gel. Five plates previously washed and dried in a dust free atmosphere, were placed on a 5 ft. long plate leveller which was kept on a table. The margin and level of each plate was adjusted. A slurry with 30 gm silica gel G and 60 ml distilled water (1:2) was prepared and shaken vigorously for 1 min before pouring it into a plate spreader placed over the first plate. The thickness in spreader was adjusted at 0.5 mm. The spreader with slurry was drawn across the plates smoothly. If the coating was uniform, the plates were left to dry up. In semi-dry condition the coated plates were transferred to an air oven fixed at 120°C for 1 h. The activated plates were cooled at room temperature before spotting with toxin extracts.

With the help of TLC guide, 10-14 straight spot marks were made at 2 cm from the bottom edge of the plate. A fixed amount of aflatoxin extract (dried extract dissolved
in fresh 1 ml of chloroform) was spotted on each spot mark using micropipette. The standard aflatoxin obtained from the International Agency for Research on Cancer, Lyon, France was also spotted at 2 – 3 places on each plate.

The chromatoplate spotted with aflatoxin extract was developed in a solvent system as described by Reddy et al. (1970).

Toluene : 90 ml
Isoamyl alcohol : 30 ml
Methanol : 2 ml

Before putting the plate in solvent system, an upper limit scribbling the solvent stop was marked at about 12 cm from the bottom edge. The solvent system was put in a glass tank well before use for homogeneous saturation. The developed plates were air-dried and observed under long-wave UV light (360 nm). Different components of aflatoxins were initially identified visually by comparing the colour and intensity of fluorescence as well as polarity of the sample spots with standards. Aflatoxin B₁ and B₂ showed blue fluorescent spots while aflatoxin G₁ and G₂ showed bluish-green fluorescent spots. The order of appearance from lesser to greater Rf was aflatoxin G₂, G₁, B₂ and B₁. The Rf values of aflatoxin B₁ and B₂ was seen as 0.56 and 0.48 respectively.

Chemical confirmation of aflatoxins

Trifluoroacetic acid (TFA) and 25% sulphuric acid were used for the chemical confirmation of aflatoxin B and G (Stack and Pohland, 1975). The aflatoxin positive
extracts (confirmed through visual observation) were spotted on another TLC plate. A very little amount of TFA was directly applied on to sample spots. The standard aflatoxin was also treated similarly and plate was developed in the solvent system as described earlier. Derivatives of aflatoxin B₁ and G₁ fluoresced at reduced Rf value than the untreated spots.

Sulphuric acid is used in case the amount of aflatoxin B₁ is very low. For this purpose, the developed plate was sprayed with 25% sulphuric acid through glass atomizer which changed the blue fluorescence to yellow under UV light.

Analysis of standard as well as mixture of aflatoxin were also done by HPLC in the beginning while initiating this work.

Quantitative determination of aflatoxin

The spots of aflatoxin on chromatograms were marked out with the help of a needle under UV light. Each spot was subsequently scrapped separately and dissolved in 5 ml spectrophotometric methanol. After centrifugation at 1,000 g for 10 min, the UV absorption spectrum of methanolic solution was determined using UV-Vis Spectrophotometer (Nabney and Nesbitt, 1965). The optical density was measured and the amount of aflatoxin was calculated using the following formula:

\[
\mu g \text{ aflatoxin/ml} = \frac{D \times M \times 1000}{\varepsilon \times L}
\]

Where  \( D = \) optical density  
\( M = \) molecular weight of aflatoxin  
\( \varepsilon = \) molar extinction coefficient  
\( L = \) path length (1 cm cell used)
Molecular weight and molar extinction coefficient of different aflatoxins at respective wavelength are as follows:

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Molecular weight</th>
<th>Wavelength</th>
<th>Molar extinction coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₃</td>
<td>312</td>
<td>360</td>
<td>20,600</td>
</tr>
<tr>
<td>B₂</td>
<td>314</td>
<td>362</td>
<td>20,900</td>
</tr>
<tr>
<td>G₁</td>
<td>328</td>
<td>362</td>
<td>17,100</td>
</tr>
<tr>
<td>G₂</td>
<td>330</td>
<td>362</td>
<td>18,200</td>
</tr>
</tbody>
</table>

Known amount of aflatoxin extract containing B₁, B₂, G₁ and G₂ in the ratio of 8:3:2.1 respectively in olive oil carrier was used for feeding animals.

ANIMAL EXPERIMENTATION

Young adult inbred Swiss strain male albino mice (*Mus musculus*) weighing approximately 37-40 gm were obtained from Alembic Ltd., Baroda, India. Animals were provided with animal feed and water *ad-libitum* and maintained under 12 h light/dark cycles at 26 ± 2°C. Animal feed was prepared as per the formulation given by the National Institute of Occupational Health, Ahmedabad, India and was confirmed to be free of mycotoxins. Guidelines for care and use of animals in Scientific Research 1991 published by Indian National Science Academy, New Delhi, India, was followed.

Seventy animals were divided into seven groups and caged separately. Group I (untreated control) animals were maintained without any treatment. Animals of Group 2 and 3 received olive oil (0.2 ml/animal/day) and curcumin (2.0 mg/0.2 ml olive oil/animal/day) (50 mg/kg body weight) respectively for 45 days and served as
pretreatment controls. Animals of Group 4 and 5 were orally administered with 25 and 50 μg aflatoxin/0.2 ml olive oil/animal/day (750 and 1500 μg/kg body weight) respectively for 45 days. Group 6 and 7 animals were orally treated with aflatoxin as mentioned for Group 4 and 5 animals along with curcumin as in Group 3 for 45 days (Table 21).

Olive oil was obtained from Figaro, Madrid, Spain. Curcumin was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. For *in vitro* experiments, dry yellow turmeric (*Curcuma longa* L.; Family-Zingerberaceae), bulbs (rhizomes) were purchased from local market. Aflatoxin and curcumin were dissolved in olive oil, hence it was administered as a vehicle alone in Group 2. As different isomers of aflatoxin exists together in the food-stuffs, we preferred to carry out the experiment with mixed aflatoxins. The dose of aflatoxin was based on LD$_{50}$ value of aflatoxin, i.e. 9 mg/kg body weight for male mice (Smith and Moss, 1985). The dose of curcumin (50 mg/kg body weight) was based on earlier work (Ammon and Wahl, 1991).

Turmeric was powdered by mechanical grinder and 5 gm of this was added to 100 ml of 50% ethanol and shaked well. Allow it to set for overnight. Then it was filtered through Whatman filter paper No.1 and filtrate was evaporated to dryness in vaccum and stored in vials. The aqueous extract of turmeric was prepared by the method as mentioned above, instead of ethanol, water was used.

All the treatment were given orally using a feeding tube attached to a hypodermic syringe for 45 days. Oral route was selected as aflatoxin and curcumin/turmeric extracts enter the human body from foodstuffs by oral route. All chemicals used in the present study were of analytical grade.
To study the effect of feeding aflatoxin alone and aflatoxin plus curcumin on body weight, each group of mice were weighed individually and mean weights were calculated. Behavioural and clinical changes throughout the experiments were also recorded.

Table 2.1: Experimental Protocol

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Treatments</th>
<th>Duration (Days)</th>
<th>Day of autopsy</th>
<th>No. of animals used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control (untreated)</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Control olive oil (0.2 ml/animal/day)</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>Curcumin (2 mg/0.2 ml olive oil/animal/day)</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>4.</td>
<td>Low dose aflatoxin (25 μg/0.2 ml olive oil/animal/day) treated</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td>High dose aflatoxin (50 μg/0.2 ml olive oil/animal/day) treated</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>6.</td>
<td>Low dose aflatoxin (25 μg/0.2 ml olive oil/animal/day) + curcumin (2 mg/0.2 ml olive oil/animal/day) treated</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>7.</td>
<td>High dose aflatoxin (50 μg/0.2 ml olive oil/animal/day) + curcumin (2 mg/0.2 ml olive oil/animal/day) treated</td>
<td>45</td>
<td>46</td>
<td>10</td>
</tr>
</tbody>
</table>
Percent gain in body weight was calculated by the formula:

\[
\frac{W_1 - W_0}{W_0} \times 100
\]

Where \( W_1 \) = Mean body weight at 45 days of the treatment.

\( W_0 \) = Mean body weight at 0 day of the treatment

On completion of the treatment, the animals were sacrificed by cervical dislocation. Blood samples collected in non-anticoagulant added bulbs by cardiac puncture were allowed to clot and centrifuged at 1,000 g for 10 min to obtain serum. Serum samples were stored under cold refrigerated condition and used within 24 h.

Morphological alterations in internal organs and tissues were recorded. Selected organs such as liver, kidney, testis, caput and cauda epididymides, seminal vesicle and ventral prostate gland of all controls and treated groups of animals were quickly isolated, blotted free of blood and weighed to the nearest mg on a balance. Relative organ weight was calculated by the formula:

\[
\text{Relative organ weight} = \frac{\text{Absolute weight}}{\text{Body weight}} \times 100
\]

**HISTOPATHOLOGICAL STUDIES**

Histopathological studies were carried out using the standard technique of hematoxylin and eosin staining. Liver, kidney, testis, caput and cauda epididymides, seminal vesicle and ventral prostate gland of controls and all treated groups of animals were dissected out, blotted free of blood and fixed (for 18 h) in alcoholic Bouin’s fixative. A pinch of lithium carbonate was added to remove excess of picric acid. The
tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58 to 60°C mp). Transverse sections of 5 µm thickness were cut on a rotary microtome. These sections were stained with Ehrlich’s hematoxylin and eosin (alcohol soluble), dehydrated in alcohol, cleared in xylene, mounted in DPX and examined microscopically.

BIOCHEMICAL ANALYSIS

Protein content

Protein content in liver, kidney, testis, caput and cauda epididymides and ventral prostate gland as well as serum of controls and all treated groups of animals was estimated by the method of Lowry et al. (1951). Protein containing preparation reacts with the Folin & Ciocalteu’s phenol reagent to give a coloured complex. This colour development is due to two reactions occurring simultaneously i.e. the reaction of alkaline copper sulphate solution with peptide bonds and reduction of phosphotungstic acids by aromatic amino acids like tyrosine and tryptophan present in the protein. The blue colour developed is quantitatively proportional to the total protein, which is measured colorimetrically.

A known weight of tissue was homogenized in a definite volume of glass distilled water. In the sample tube, 0.2 ml of tissue homogenate, 0.6 ml of distilled water and 4 ml of alkaline copper sulphate solutions were added. The contents of the tubes were vortex mixed. In the blank tube, instead of the sample, 0.2 ml of distilled water was taken. The tubes were incubated at room temperature for 15 min. Then 0.4 ml of Folin & Ciocalteu’s phenol reagent (diluted 1:1, phenol reagent : distilled water) was added to each tube.
which was thoroughly mixed. The tubes were allowed to stand at room temperature for
30 min. The optical density (O.D.) of the blue colour developed was read at 540 nm on a
Systronics 106 colorimeter.

Protein concentration in the tissue was calculated by using the following
regression formula:

\[ X = 807.35149 \cdot (Y) - 74.8043 \]

Where,

\[ X = \text{concentration obtained from regression formula} \]
\[ Y = \text{O.D. of unknown sample} \]

The protein content was then calculated by substituting the value of \( X \) in the
formula.

\[
\text{Protein content} = \frac{\text{Concentration (X) x dilution}}{\text{Weight of tissue (in mg) x aliquot volume}} \times 100
\]

Where Dilution = 5 ml

Aliquot volume = 0.2 ml

The protein content was expressed as mg protein/100 mg tissue weight and mg
protein/100 ml serum.

Cholesterol content

The concentration of cholesterol was estimated in the testis of controls and all
treated groups of mice by the method of Zlatki et al. (1953). In the presence of
concentrated sulphuric acid and glacial acetic acid, cholesterol forms a coloured complex
with ferric chloride (FeCl₃) that can be measured colorimetrically at 540 nm.
A known weight of tissue was homogenized in 2 ml of glacial acetic acid. 0.2 ml of this homogenate was added to the test tube containing 5 ml of working FeCl₃ solution (1 ml of stock 5% FeCl₃ solution diluted to 100 ml with glacial acetic acid). 0.2 ml of standard cholesterol (100 μg in 100 ml acetic acid) was added to the standard tube instead of homogenate. Standard and blank tubes contained same volume of FeCl₃ solution. 3 ml of concentrated sulphuric acid was then added to all the test tubes and mixed thoroughly. After incubating the tubes for 20 min at room temperature, the optical density was read at 540 nm on a Systronics 106 colorimeter. Concentration of cholesterol was calculated by using the formula:

\[
\text{Cholesterol conc.} = \frac{\text{O.D. of sample} \times \text{conc. of std.} \times \text{dilution} \times 100}{\text{O.D. of std.} \times \text{tissue weight (mg)} \times \text{aliquot volume}}
\]

Where, Dilution = 2 ml

Aliquot Volume = 0.2 ml

The cholesterol concentration was expressed as mg/100 mg tissue weight.

**Extraction of nucleic acid**

A known weight of fresh tissue was homogenized in 5 ml of cold 5% trichloroacetic acid (TCA) in a cooled pestle and mortar and the homogenate was kept at 0-4°C for 30 min. The precipitates were obtained after centrifugation (10 min at 1,000 g) which were dissolved again in 5 ml of cold 5% TCA and left for 30 min at 0-4°C. Thereafter centrifugation was carried out and the precipitates obtained were dissolved in 3:1 alcohol-ether mixture and left for 30 min at 50°C. This process was repeated once again and precipitates were formed. These tubes were centrifuged and the supernatant was discarded. The pellet obtained finally was lipid free. It was dissolved in 5 ml of 0.1 N
KOH and incubated at 37°C for 16-18 h. Then 0.17 ml of 6N HCl and 5 ml of 10% TCA were added to the incubated suspension and precipitates were allowed to be formed at 4°C for 30 min. After centrifugation the supernatant and pellet was separated. The supernatant was used for RNA estimation. The pellet containing DNA and protein was heated at 90°C for 15 min after adding 5 ml of 5% TCA. The supernatant was then separated by centrifugation (10 min at 1,000 g) after cooling at 4°C for 30 min and used for DNA estimation.

**Estimation of deoxyribonucleic acid (DNA)**

The estimation of DNA in the liver, kidney and testis of controls and all treated groups of animals was carried out by the method of Giles and Meyer (1965). The DNA in the supernatant reacts with diphenylamine to give a blue coloured complex whose optical density is measured colorimetrically.

One ml of supernatant was added to 2 ml of diphenylamine reagent, in a test tube, vortex mixed and incubated at 37°C for 16-18 h. The blank was run with 1 ml of double distilled water instead of the supernatant. The optical density of the resultant blue colour was read at 620 nm on a Systronics 106 colorimeter.

The DNA content was calculated by the following regression formula:

\[ X = 908.514 \times (Y) - 0.032 \]

Where \( X \) = DNA concentration obtained from the regression formula

\( Y \) = O.D. of unknown sample

The DNA concentration was determined by substituting the value of `X` in the formula:
DNA concentration = \( \frac{X \text{ (concentration)} \times \text{ dilution} \times 100}{\text{tissue weight} \times \text{ aliquot volume}} \)

Where, Dilution = 5 ml

Aliquot Volume = 1 ml

The DNA content was expressed as μ moles/100 mg tissue weight.

**Estimation of ribonucleic acid (RNA)**

The estimation of RNA in the liver, kidney and testis of controls and all treated groups of animals was carried out by the method of Mejboum (1939). The RNA in the supernatant reacts with orcinol reagent to give a greenish colour which is measured colorimetrically.

To 1 ml of supernatant, 3 ml of working orcinol reagent (Stock solution: 13.5 gm ferric ammonium sulphate, 20 gm orcinol in 500 ml double D/W; Working reagent: 5 ml of stock solution added to 85 ml of conc. HCl and volume made to 100 ml with double D/W) was added. The solutions were mixed thoroughly using a cyclomixer. The tubes were kept in a boiling water bath for 20 min. The blank was run with 1 ml of double distilled water instead of the supernatant. After cooling, the optical density of the green colour developed was read at 670 nm on a Systronics 106 colorimeter.

The concentration of RNA was calculated by the following regression formula:

\[ X = 133.995 \times Y - 0.232 \]

Where \( X \) = RNA concentration obtained from the regression formula:

\( Y \) = O.D. of unknown sample
The RNA content was determined by substituting the value of 'X' in the formula:

\[
\text{RNA concentration} = \frac{X \times \text{concentration} \times \text{dilution} \times 100}{\text{tissue weight} \times \text{aliquot volume}}
\]

Where, Dilution = 5 ml

Aliquot Volume = 1 ml

The concentration of RNA was expressed as \( \mu \) moles/100 mg tissue weight.

**Adenosine triphosphatase (ATPase) (E.C.3.6.1.3) activity**

The ATPase activity was assayed in the liver, kidney, testis, caput and cauda epididymides of controls and all treated groups of animals by the method of Quinn and White (1968). The enzyme adenosine triphosphatase hydrolyses the substrate ATP into adenosine diphosphate (ADP) and inorganic phosphate (i.p.).

The reaction mixture contains 0.3 ml substrate buffer (3 mM ATP disodium salt in Tris HCl buffer, pH 7.4), 0.1 ml MgCl\(_2\) (3 mM), 0.1 ml NaCl (150 mM), 0.1 ml KCl (30 mM), 0.2 ml tris sucrose buffer (pH 7.4) followed by 0.2 ml tissue homogenate. In the blank tube 0.3 ml of distilled water instead of substrate buffer was taken. The contents of the tubes were thoroughly mixed and incubated at 37°C for 30 min. For termination of the reaction, 0.5 ml 10% TCA was added to the tubes and kept at 4°C for 5 min for precipitation. The solution was centrifuged at 1,000 g for 15 min. The clear supernatant fluid was used for the determination of inorganic phosphate by the method of Fiske and Subbarow (1925). The optical density was read at 660 nm on a Systronics 106 colorimeter. The enzyme activity was calculated using the formula:

\[
\text{ATPase activity} = \frac{\text{O.D.} \times 2.09 \times \text{dilution}}{\text{mg protein}}
\]
Where $2.09 = \text{conversion factor}$

The protein levels were estimated by the method of Lowry et al. (1951).

The enzyme activity was expressed as $\mu$ moles i.p. released/mg protein/30 min.

**Succinate dehydrogenase (SDH) (E.C.1.3.99.1) activity**

The SDH activity was measured in the liver, kidney, testis, caput and cauda epididymides of controls and all treated groups of animals by the method of Beatty et al. (1966) using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl - 2H - tetrazolium chloride (INT) as an electron acceptor. The electrons released by the enzyme SDH from the substrate are taken up by an electron acceptor, i.e. INT which is reduced to a red coloured formazan. After extracting it in ethyl acetate, the colour intensity was measured at 420 nm.

A known weight of tissue was homogenized in a known volume of distilled water. To each sample tube, 1 ml of 0.2 M phosphate buffer (pH 7.6), 1 ml of 0.1 M sodium succinate (substrate), 1 ml of INT (mg/ml) and 0.4 ml of tissue homogenate were added. 1 ml of distilled water was added to the blank tube instead of INT solution. All the tubes were incubated for 15 min at 37°C and thereafter 0.1 ml of 30% TCA was added to each tubes to terminate the reaction. The formazan formed was extracted into 7 ml of ethyl acetate by vigorous shaking for 30 seconds. All the tubes were centrifuged for 3-5 min at 1,000 g and the supernatant was used to measure the colour intensity on a Spectronic 20 colorimeter at 420 nm against the blank.

Succinic dehydrogenase activity was calculated using the regression formula:

$$ X = 1.87 + 152.21 \ (Y) $$
Where, \( X \) = Enzyme activity obtained from the regression formula

\[
Y = \text{O.D. of unknown sample}
\]

The enzyme activity was determined by substituting the value of \( X \) in the formula:

\[
\text{SDH activity} = \frac{X \text{ (activity)} \times \text{dilution}}{\text{mg protein}}
\]

Where, Dilution = 5 ml

The protein levels were estimated by the method of Lowry et al. (1951).

The enzyme activity was expressed as \( \mu g \) formazon formed/mg protein/15 min.

**Acid phosphatase (E.C. 3.1.3.2) activity**

The acid phosphatase activity was assayed in the ventral prostate gland of controls and all treated groups of mice by the method of Bessey et al. (1946). Acid phosphatase (orthophosphoric monoester phosphohydrolase) catalyses the hydrolysis of p-nitrophenyl phosphate (disodium salt) at pH 4.8 and liberates p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol combines with sodium hydroxide to form a yellow coloured complex which was measured colorimetrically at 420 nm.

A known amount of tissue was homogenized in a definite volume of cold distilled water. In each test tube containing 0.6 ml of buffered substrate, 0.2 ml of homogenate was added. In the blank tube, instead of homogenate, 0.2 ml of distilled water was added. The tubes were incubated at 37°C for 30 min. Then 4 ml of 0.1 N NaOH was added to all the tubes. The colour intensity was read at 420 nm using a Systronics 106 colorimeter. The enzyme activity was calculated using the formula:
Acid phosphatase activity = \frac{\text{O.D.}}{3.8} \times \frac{1}{\text{mg protein}}

Conversion factor = 3.8

The protein levels were estimated by the method of Lowry et al. (1951)

The enzyme activity was expressed as \mu \text{ moles p-mtrophenol released/mg protein/30 min.}

**Lipid peroxidation (LPO)**

The lipid peroxidation in the liver, kidney and testis of controls and all treated groups of animals was determined by the method of Ohkawa et al. (1979). The method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other breakdown products of peroxidised lipid collectively called as thiobarbituric acid reactive substances (TBARS)

A known amount of tissue was washed with 0.1 M phosphate buffered saline (PBS) (pH 7.4) several times. The tissue was then blotted dry, weighed and analysed immediately or stored at 20°C for not more than 24 h. The tissue was then homogenized in PBS (pH 7.4) to prepare 10% homogemate. 0.2 ml of 10% tissue homogenate was then added to the tube containing 0.2 ml of 8.1% sodium dodecylsulphate (SDS), 1.5 ml of 20% acetic acid solution (adjusted to pH 3.5 with 1N NaOH) and 1.5 ml of 1% thiobarbituric acid (TBA) solution. The blank for each sample was prepared by substituting the TBA solution with distilled water. The final volume was made to 4.0 ml with distilled water. The solution was mixed and heated in a water bath at 95°C for 60 min. The tubes were then immediately cooled and 2 ml of the aliquot was transferred to a
centrifuge tube to which an equal volume of 10% TCA was added. The solution was mixed and centrifuged at 1,000 g for 15 min. The aliquot of the resulting supernatant fraction was read against blank on a Systronics 118 UV-Vis spectrophotometer at 532 nm. The lipid peroxidation was calculated using the formula:

\[ X = \frac{O.D.\text{ of sample} \times \text{dilution factor}}{\varepsilon \times \text{tissue weight (mg)}} \]

Where, Dilution factor = 0.5 ml

Extinction coefficient \( \varepsilon = 1.56 \times 10^5 \) of MDA

The lipid peroxidation was determined by substituting the value of 'X' in the formula:

Concentration of MDA = \[ \frac{X}{\text{mg protein}} \]

The protein levels were estimated by the method of Lowry et al. (1951)

The results were expressed as n moles MDA/mg protein / 60 min.

Glutathione (GSH) content

The concentration of glutathione in liver, kidney and testis of controls and all treated groups of mice was assayed by the method of Grunert and Philips (1951). Glutathione (GSH) present in the tissue reacts with sodium nitroprusside to give a red coloured complex in saturated alkaline medium. A mixture of sodium carbonate (\( \text{Na}_2\text{CO}_3 \)) and sodium cyanide (\( \text{NaCN} \)) is added to the contents to stabilize the reaction.

A known amount of tissue was homogenized in 3 ml of 3% metaphosphoric acid and 1 ml of distilled water saturated with salt solution (1.5 gm NaCl crystals) and
centrifuged (15 min at 1,000 g). 2 ml of aliquot was added to the sample tube containing
6 ml of saturated NaCl solution and allowed to stand for 10 min at 20°C for equilibrium.
The blank tube was run with 2 ml of 2% metaphosphoric acid instead of the aliquot. Then
1 ml of each 0.067 M sodium nitroprusside and 1.5 M NaCN mixture were added to
blank and sample tubes respectively. The coloured complex developed was measured at
520 nm on a Bausch and Lomb Spectronic 88 spectrophotometer within one min.

The concentration of glutathione was calculated using the regression formula:

$$X = 272.01 \times (Y) - 2.32$$

Where $X =$ Concentration of glutathione obtained from the regression formula

$Y =$ O.D. of the sample

The value of $X$ was substituted in the formula:

Glutathione concentration = \frac{\text{Concentration of sample (X)} \times \text{dilution} \times 100}{\text{tissue weight (mg)} \times \text{aliquot volume}}

Where Dilution = 3 ml

Aliquot volume = 2 ml

Glutathione content was expressed as μg/100 mg tissue weight.

Glutathione peroxidase (GSH-Px) (E.C.1.11.1.9) activity

The glutathione peroxidase activity in the liver, kidney and testis of controls and
all treated groups of mice were assayed by the modified method of Pagila and Valentine
(1967).

A known amount of tissue was homogenized in a known volume of 0.01% digitonin and centrifuged at 4,000 g for 30 min at 0-1.5°C The supernatant was used as
an aliquot. The reaction mixture consisted of 0.3 ml 50 mM potassium phosphate buffer
(pH 7.0), 0.1 ml 1 mM ethylenediaminetetra acetic acid (EDTA), 0.1 ml 1 mM sodium azide (NaN₃), 0.1 ml 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 ml E.U./ml oxidized glutathione reductase (GSSG), 0.1 ml 1 mM reduced glutathione (GSH), 0.1 ml 25 mM H₂O₂ and 0.1 ml aliquot. Standard tube and blank tube contains 0.1 ml of standard glutathione and distilled water instead of aliquot. The tubes were then incubated for 5 min at room temperature. Decrease in absorbance at 340 nm was recorded for 3 min on a Systronics UV-Vis spectrophotometer model 118. The blank tube contained complete reaction mixture without the enzyme source. The blank readings were subtracted from the test readings for obtaining the correction factor for the spontaneous reaction. The enzyme activity was calculated using the formula:

\[ X = \frac{\text{Sample O.D.} \times \text{standard concentration} \times \text{dilution factor}}{\text{standard O.D.}} \]

Where, standard concentration = 1.5 units/mg.

\[ \text{Dilution factor} = 20 \]

Glutathione peroxidase activity = \[ \frac{X}{\text{mg protein}} \]

The protein levels were estimated by the method of Lowry et al. (1951)

The enzyme activity was expressed as units/mg protein/min, where 1 unit of GSH-Px equals to n moles of NADPH consumed/mg protein/min.
Catalase (E.C.1.11.1.6) activity

The catalase activity was assayed in the liver, kidney and testis of controls and all treated groups of mice by the modified method of Luck (1963).

A known amount of fresh tissue was homogenized in a known volume of 0.01% chilled digitonin and centrifuged at 3,000 g for 30 min at 4°C. The supernatant was used as an aliquot. The assay mixture consisted of 0.5 ml 50 mM phosphate buffer (pH 7.0) and 1.0 ml aliquot. The decrease in absorbance was noted every 5 seconds at 240 nm on a Systronics UV-Vis spectrophotometer (Model 118). The blank tube contained complete reaction mixture without the enzyme source. The non-enzymatic readings were subtracted from the test readings for obtaining the correction factor for the spontaneous reaction. The enzyme activity was calculated using the formula:

\[ X = \frac{\text{O.D. of sample} \times \text{total volume of assay}}{\text{aliquot volume} \times \varepsilon} \]

Where, \( \varepsilon \) = Extinction coefficient = 0.0041 m mol\(^{-1}\) x mm\(^{-1}\).

Aliquot Volume = 1ml
Total volume of assay = 2 ml

Catalase activity = \[ \frac{X}{\text{mg protein}} \]

The levels of protein were estimated by the method of Lowry et al. (1951).

The enzyme activity was expressed as \( \mu \) moles H\(_2\)O\(_2\) consumed/mg protein/min.
Superoxide dismutase (SOD) (E.C.1.15.1.1) activity

The activity of superoxide dismutase in the liver, kidney and testis of controls all treated groups of animals was assayed by the modified spectrophotometric method of Kakkar et al. (1984). In this method, the formazone formed at the end of the reaction indicates the presence of the enzyme. The activity was calculated wherein one unit of the enzyme concentration required to inhibit 50% of the optical density of chromogen formed in one min at 560 nm under the assay condition.

A known amount of the tissue was homogenized in cold normal saline. The tubes were then centrifuged. The supernatant was discarded and to the pellet 2 ml of hypotonic solution (0.56% KCl) was added to yield the enzyme extract. After 15 min incubation this was centrifuged at 1,000 g for 10 min and the supernatant was used as the sample. In the assay mixture, the control consisted of 2.4 ml pyrophosphate buffer (0.052 M sodium pyrophosphate buffer at a pH 8.4 adjusted with 0.052 M NaH₂PO₄), 0.1 ml of freshly prepared phenazine methosulphate (186 µ moles phenazine methosulphate in double distilled water), 0.3 ml of nitroblue tetrazolium chloride (30 µ moles freshly prepared) and 0.2 ml of fresh NADH (780 µ moles in double distilled water). To the sample tube 0.2 ml of enzyme was added prior to the addition of NADH. In blank tube, 0.2 ml pyrophosphate buffer was added instead of homogenate. Then the reaction was stopped by the addition of 1 ml of acetic acid, exactly 90 seconds after the addition of NADH. 4 ml of n-butanol was added to the tubes and shaken vigorously to extract the formazone. Then the tubes were centrifuged for 10 min at 1,000 g and supernatant was used for the measurement of optical density at 560 nm against butanol on a Systronics 118 UV-Vis spectrophotometer. The enzyme activity was calculated by the formula:
Where, Accuracy factor = 1.0

Dilution = 10.0 ml

Standard enzyme unit = 3.0

SOD activity = \( \frac{X}{\text{mg protein}} \)

The levels of protein were estimated by the method of Lowry et al. (1951).

The enzyme activity was calculated and expressed as units/mg protein.

**Total ascorbic acid (TAA) contents**

Concentration of total ascorbic acid was estimated in the liver, kidney and testis of controls and all treated groups of mice by the method of Roe and Kuether (1943). Total ascorbic acid was oxidized to dehydro ascorbic acid (DHA) by Norit reagent in the presence of trichloroacetic acid (TCA). This couples with 2, 4-dinitrophenyl hydrazine to yield a red coloured complex by the action of sulphuric acid which was measured colorimetrically.

For total ascorbic acid, the homogenate was prepared in 10 ml Norit reagent (prepared by dissolving 2 gm of activated charcoal in 100 ml 6% TCA) and filtered through Whatman filter paper number 42. To 4 ml aliquot, 1 ml of 2% 2,4-dinitrophenyl hydrazine reagent was added followed by a drop of 10% thiourea to activate the reaction. The blank tube was run with 4 ml of 6% TCA instead of homogenate and the standard
tube contained 4 ml of ascorbic acid solution (10 μg/ml). The contents of the tubes were mixed well and kept in a boiling water bath for 15 min and thereafter cooled in an ice bath. Then 5 ml of 85% sulphuric acid was added along the side of the tube kept in an ice bath. The tubes were allowed to stand for 30 min and the optical density was read at 540 nm against blank on a Systronics 103 colorimeter. The concentration of ascorbic acid was calculated by the formula:

\[
\text{Total ascorbic acid concentration} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \frac{\text{conc. of standard}}{\text{tissue weight (mg)}} \times \frac{\text{dilution}}{\text{aliquot volume}} \times 1000
\]

Concentration of total ascorbic acid was expressed as mg/gm tissue weight.

Fructose

The concentration of fructose in seminal vesicle of control and all treated groups of animals was determined by the method of Foreman et al. (1973).

A known weight of tissue was homogenized in 5 ml of 5% perchloric acid (0.9 N). To the sample tube, 0.2 ml homogenate, 1.8 ml of perchloric acid, 1 ml of 0.1% (0.09 M) resorcinol (dissolved in 95% alcohol) and 3 ml of 30% HCl (0.76 N) were added. In the blank tube, instead of homogenate same amount of perchloric acid was added. The tubes were heated in a water bath at 80°C for one h and cooled at room temperature. The colour intensity was read on Systronics 106 colorimeter at 410 nm. The concentration of fructose was calculated by the regression formula:

\[
X = 781.097 (Y) - 10.99
\]
where,

$X =$ concentration obtained from formula

$Y =$ optical density of unknown sample

\[
\text{Fructose concentration} = \frac{\text{Concentration of unknown sample (X) x dilution}}{\text{tissue weight x aliquot volume}}
\]

where,

Dilution $= 5 \text{ ml}$

Aliquot volume $= 0.2 \text{ ml}$

The concentration of fructose was expressed as $\mu\text{g/mg tissue weight}$.

**Sialic acid content**

The sialic acid concentration was estimated in the testis, caput and cauda epididymides of control and all treated groups of animals by the method of Jourdian *et al.* (1971) The sialic acid was oxidized by periodic acid prior to heating with resorcinol. Free sialic acid gave chromogens which were stable at $0^\circ\text{C}$. The chromogen was treated with an organic solvent which extracted the colour and was measured spectrophotometrically.

A known amount of tissue was homogenized in known volume of distilled water. To a sample tube containing $0.5 \text{ ml}$ of homogenate, $0.1 \text{ ml}$ of $0.04 \text{ M}$ periodic acid solution was added. The blank was prepared by taking $0.5 \text{ ml}$ distilled water instead of a sample. Standards of $0.04 \text{ mg/ml (40 }\mu\text{g/ml)}$ concentration were run along with the sample. The solutions were thoroughly mixed and allowed to stand in an ice-bath for 20 min. After addition of $1.25 \text{ ml}$ of $0.06\%$ resorcinol, all the tubes were mix and kept in ice
bath for 5 min. Thereafter the tubes were kept in boiling water bath (100°C) for 15 min and cooled. After this, 1.25 ml of tertiary butyl alcohol was added. Vigorous mixing gave a single-phase solution. The tubes were placed in a 37°C water bath for 3 min to stabilize the colour, cooled to room temperature and optical density was measured at 630 nm on a Systronics 106 colorimeter.

Concentration of sialic acid was calculated by using the formula:

\[
\text{Concentration of sialic acid} = \frac{\text{O.D. of sample} \times \text{conc. of std.} \times \text{dilution}}{\text{O.D. of standard} \times \text{tissue weight (mg)} \times \text{aliquot volume}}
\]

Where,

- Dilution = 2.5 ml
- Aliquot Volume = 0.5 ml

The concentration of sialic acid was expressed as μg/mg fresh tissue weight.

3β-HYDROXYSTEROID DEHYDROGENASE (3β-HSD; E.C.1.1.51)

The testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was assayed by the method of Talalay (1962) in testis of control and all treated groups. The 3β-hydroxysteroid dehydrogenase acts on substrate epiandrosterone (Sigma Chemical Co., U.S.A.) in the presence of nicotinamide adenine dinucleotide (NAD) which is reduced to NADH and androstenedione was formed. The absorbance was measured at 340 nm on a UV-Vis Spectrophotometer.
A known amount of tissue was homogenized in 0.02 M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8,000 g for 30 min at 4°C. The supernatant was used for the assay. To 2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate [3 mg epiandrosterone (Sigma Chemical Co., U.S.A.), in 2 ml of N,N-dimethyl formamide] and 0.2 ml of homogenate were added. Then 0.2 ml of NAD and 0.4 ml of glass distilled water were added. The blank was prepared by adding 2 ml of buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of the assay was 3 ml. The reduction in absorbance of NAD was read at every 15 second interval against blank at 340 nm on a Systronics UV-visible spectrophotometer (Model 118). The standard curve was prepared using 5α-androstane-3,17-dione. The enzyme activity was calculated by using the formula:

\[
X = \frac{\text{Sample O.D.}}{\text{Standard O.D.}} \times \frac{\text{standard conc.}}{\text{aliquot volume}} \times \frac{\text{total volume of assay}}{\text{dilution}}
\]

Activity of 3β-HSD = \[
\frac{X}{\text{mg protein}}
\]

The levels of protein were estimated by the method of Lowry et al. (1951). The enzyme activity was expressed as n moles of androstenedione formed/mg protein/min.

**17β-HYDROXYSTEROID DEHYDROGENASE (17β HSD; EC.1.1.1.51) activity**

The testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) activity was assayed by the method of Talalay (1962) in testis of control and all treated groups. The
enzyme 17β-hydroxysteroid dehydrogenase acts on substrate testosterone in the presence of nicotinamide adenine dinucleotide (NAD) which is reduced to NADH and androstenedione was formed. The absorbance was measured at 340 nm on a UV-Vis Spectrophotometer.

\[
17\beta\text{-HSD} \\
\text{Testosterone} + \text{NAD} \quad \longrightarrow \quad \text{Androstene-3,17-dione} + \text{NADH}
\]

A known amount of tissue was homogenized in 0.02 M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8,000 g for 30 min at 4°C. The supernatant was used for the assay. To 2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate (1.5 mg testosterone dissolved in 20 ml methyl alcohol) and 0.2 ml of homogenate was added. Thereafter 0.2 ml of NAD and 0.4 ml of glass distilled water were added. Blank was prepared by adding 2.0 ml of phosphate buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of assay was 3.0 ml. The reduction in absorbance of NAD was read at interval of every 15 seconds against control at 340 nm on a Systronics UV-Vis spectrophotometer (Model No. 118). The enzyme activity was calculated by using the formula

\[
x = \frac{\text{Sample O.D.}}{\text{Standard O.D.}} \times \frac{\text{standard conc.}}{\text{aliquot volume}} \times \frac{\text{total volume of assay}}{\text{dilution}}
\]

Activity of 17β-HSD = \( \frac{X}{\text{mg protein}} \)

The levels of protein were estimated by the method of Lowry et al. (1951).

The enzyme activity was expressed as n moles of androstenedione formed/mg protein/min.
Total Erythrocytes (RBC) count

Erythrocytes (RBC) in the blood of all controls and treated groups animals were counted by haemocytometric method using Neubauer's chamber by method of Mukherjee (1988).

Venous blood was drawn up to 0.5 mark in RBC pipette. No air bubble was trapped inside while drawing blood and excess blood around the tip of the pipette was wiped off. RBC diluting fluid was drawn up to 101 mark. The pipette was rotated well for several times holding it in a horizontal position. First few drops of fluid in the pipette stem were discarded. Then the cover slip was placed over ruled area in center of Neubauer's chamber. Tip of RBC pipette was put at an angle of 25° with horizontal surface of chamber at the point where the edge of cover slip and surface as ruled area meet. By capillary action the diluted blood flows under the cover slip into ruled area of the chamber. Precaution was taken to ensure that no air bubbles get trapped in the chamber during the charging and the ruled area was filled up without overspreading into groove around it. Then the chamber was placed on the stage of light microscope. The light was adjusted so that RBC appears as round. The cells were counted in RBC counting chamber and RBC count per mm³ was calculated.

Measurement of Haemoglobin (Hb)

Haemoglobin content in all control and treated groups was measured by Sahli's method (Mukherjee, 1988).

The graduated tube was filled up to 20 mark with 0.1 N HCl. Venous blood was drawn up to 0.02 ml mark in Sahli's pipette. No air bubble was trapped while drawing
blood and excess blood from outside the pipette was wiped with absorbent paper. The blood level was checked. Then blood from the pipette was blown into graduated tube containing acid solution. Pipette was rinsed by drawing in and blowing out acid solution 3 to 4 times. The mixture of blood and acid gave brownish colour. Allow it to stand for 5 to 10 min at room temperature to obtain a complete conversion of haemoglobin into acid haematin. The graduated tube containing mixture was placed in haemoglobinometer and faced a window for better lighting from the back. The mixture was diluted by adding distilled water drop by drop. Stirring was done with glass rod after adding each drop. Dilution was carried until same intensity of colour as the standard colour of comparator was obtained. The mark on the tube at the bottom of the meniscus was noted.

**Serum creatinine**

Creatinine present in the serum reacts with picric acid in alkaline medium to form an orange red colour which can be measured colorimetrically (Jaffe reaction). Estimation of creatinine was performed by the alkaline picrate method of Bonsnes and Taussky (1945) as described by Varley (1988).

Serum was diluted in known amount of distilled water. 0.4 ml of diluted serum was added to all the sample tubes containing 1.2 ml picric acid. Blank was run with 0.4 ml distilled water instead of serum. 0.4 ml of standard (2 mg/dl) was added to the standard tubes instead of serum. Then 0.1 ml of NaOH solution was added to all the tubes and allowed it to stand for 20 min. The optical density of the orange red colour developed was read at 520 nm on a Systronics 106 colorimeter against blank. Concentration of creatinine was calculated by using the formula:
Creatinine concentration = \( \frac{\text{O.D. of sample}}{\text{O.D. of std.}} \times \text{concentration of std.} \times \text{dilution factor} \)

The creatinine concentration was expressed as mg/dl serum.

**Serum glutamate pyruvate transaminase (SGPT) (E.C.2.6.1.2) activity**

The photometric determination of SGPT activity was carried out by the method of Reitman and Frankel (1957). The serum was allowed to act in a buffered solution of α-ketoglutaric acid and alanine. The quantity of pyruvate formed was measured photometrically as the corresponding 2, 4-dinitrophenyl hydrazine in an alkaline medium. As the α-ketoglutarate present in the sample also forms a hydrazone, the measurements were conducted in the 500-550 nm range where the absorbance of different hydrazone differs maximally.

One ml of buffered substrate (phosphate buffer 0.05 M, \( \text{K}_2\text{HPO}_4 \) 0.05 M \( \text{KH}_2\text{PO}_4 \), pH 7.4 and 0.2 M DL-alanine and 0.002 M α-ketoglutarate in 50 ml distilled water) was pipetted into a test tube which was kept in a water bath at 37°C for 5 min. Thereafter 0.2 ml fresh non-haemolytic serum (1:4 diluted with physiological saline) was added, mixed and incubated at 37°C for exactly 30 min. One ml of colouring reagent (0.001 M 2,4-dinitrophenyl hydrazine in 100 ml of 1N HCl) was added. It was allowed to stand exactly for 20 min at room temperature and then 10 ml 0.4 N NaOH (16 gm NaOH in 1000 ml of distilled water) was added, mixed thoroughly and allowed to stand for 3 min. The measurements of the absorbance of sample against that of the blank was carried out at 546 nm on a Systronics 106 colorimeter. The concentration was determined from the measured absorbance by reading them off from calibration curves. The curves were
drawn by plotting the tabulated value. The enzyme activity was expressed as 
mU/ml

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Serum glutamate oxaloacetate transaminase (SGOT) (E.C.2.6.1.1) activity

The SGOT activity was assayed by the method of Reitman and Frankel (1957). The diluted serum (1:4 with physiological saline) was used for estimation. The procedure was same as SGPT. However, SGOT assay consisted of L-aspartate instead of alanine (phosphate buffer: 0.05 M K$_2$HPO$_4$; 0.05 M KH$_2$PO$_4$, 0.1 M L-aspartate; 0.002 M α-ketoglutarate in 50 ml of double distilled water) and the tubes were incubated for exactly one h after addition of serum. For the calculation, calibration curves were drawn by plotting the tabulated values. The enzyme activity was expressed as mU/ml.

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Sperm Count

Cauda epididymal sperm count of control and treated groups of animals were determined by the method of Prasad et al. (1972) using the Neubauer chamber of a haemocytometer. The cauda epididymal sperm suspension was sucked upto the 0.5 mark in the WBC pipette. The suspension is then diluted upto the 11 mark with 5% sodium bicarbonate (NaHCO₃) and mixed thoroughly. Sodium bicarbonate acts as a spermicide and kills the spermatozoa to facilitate counting. A drop of suspension was transferred to the Neubauer chamber and gently covered with cover slip. Spermatozoa were counted in 64 subsquares of the white blood cell counting regions.

The sperm count (density) was calculated by the formula:

\[
\text{Sperm Density} = \frac{N \times \text{Dilution} \times 1000}{\text{Volume of 64 Squares}}
\]

Where, \( N \) = total number of sperms counted in 64 subsquares.

\( \text{Dilution} = 20 \text{ times.} \)

The sperm density was expressed in terms of million spermatozoa/ml of sperm suspension.

Sperm motility

The percentage of motile spermatozoa was measured by the method of Prasad et al. (1972). Cauda epididymis of known weight (100 mg/2 ml) was teased gently in a known volume of physiological saline (0.9% NaCl) to release the spermatozoa from the
epididymal tubules. The tissue components were removed and sperm suspension was used for evaluating sperm motility and count. A drop of sperm suspension was placed on a Neubauer chamber of a haemocytometer and observed under low magnification (10 X) in a microscope. The chamber is focused on the four squares (A,B,C,D) where WBCs are usually counted.

The motile spermatozoa are counted along with the total number of spermatozoa in each small sub-square. The sperm motility was determined by counting the number of motile and total number of spermatozoa in 20 separate random fields.

The percent motility is calculated as follows:

\[
\text{Percent Motility} = \frac{\text{No. of Motile Spermatozoa}}{\text{Total Number of Spermatozoa}} \times 100
\]

The sperm motility was expressed as percent motile sperms.

**Sperm viability**

The ratio of live:dead spermatozoa were determined using 1% trypan blue (supravital stain) as described in the method of Talbot and Chacon (1981). Live spermatozoa retain the selective semi-permeability of their cell membrane and hence do not take up stains such as trypan blue and therefore remain unstained. Dead spermatozoa on the other hand loose their membrane selective permeability and are permeable to stain and hence appear stained under the microscope. The ratio of unstained to stained spermatozoa gives the live:dead ratio.
An aliquot of 0.2 ml of cauda epididymal sperm suspension was incubated with 0.2 ml of 1% trypan blue stain prepared in physiological saline. Incubation was carried out for 15 min at 37°C. A drop of the suspension was placed on a pre-cleaned slide and allowed to settle for one min after placing a cover slip. Observations were made under 40X magnification of a Nikon microscope. The number of stained/unstained spermatozoa were scored in 10-20 separate visual fields. The live, viable spermatozoa remained unstained, while dead sperms took up the supravital stain. This technique makes it possible to differentiate the live spermatozoa from immotile dead spermatozoa. In each sample, the ratio of live: dead spermatozoa was determined.

**Sperm Morphology**

Cauda epididymal sperm morphology of control and treated groups of animals were determined by the method of Chinoy et al. (1992). 0.1 ml of cauda epididymal sperm sample was added to 0.1 ml of Hank’s balanced salt solution. The smear was made from the suspension and passed through 70% and 90% alcohol for fixation. To each slide 3 drops of alcoholic acidic silver nitrate reagent was added, after which 1-2 drops of 1% gelatin formic acid solution was added. The slides were covered with a coverslip and placed in a moist chamber at 4°C for 24 h. After 24 h, the slides were washed in 5% alcoholic ammonia for differentiation. Slides were dehydrated, cleared in xylene, mounted in DPX and observed under a light microscope at 40X magnifications.

The percent sperm morphology is calculated as followed by method of Jayendran et al. (1984)

\[
\text{Percent abnormal sperm morphology} = \frac{\text{No. of abnormal spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100
\]
Hank's Balanced Salt Solution

(Magnesium free)

- KH$_2$PO$_4$ - 60.0 mg/dl
- Na$_2$HPO$_4$ - 60.0 mg/dl
- KCl - 400.0 mg/dl
- NaCl - 8.0 mg/dl
- CaCl$_2$ (anhydrous) - 140.0 mg/dl
- NaHCO$_3$ - 350.0 mg/dl
- D-Glucose - 100 mg/dl
- Phenol red - 10.0 mg/dl
- Formalin (3%) - 2-3 drops

Fertility rate

The fertility rates of controls and all treated groups of animals were assessed according to the WHO MB-50 Protocol (WHO, 1983).

Male mice of proven fertility used in the experiments were allowed to mate with proestrous female mice in 1:2 ratio 5 days prior to the end of the respective treatment. The vaginal smear was checked daily in the morning to observe the presence of sperm or vaginal plug to ascertain positive mating and was considered as day one of the pregnancy. The females were then separated from the male and allowed to remain on normal diet for 16 days after which they were autopsied. The presence of implantation sites and their number were taken as a criteria for successful insemination and fertility test was considered positive. The absence of implantation sites indicated negative fertility rate.
IN VITRO STUDIES

Effect of ethanolic and aqueous extracts of turmeric/curcumin on aflatoxin-induced toxicity on human RBC

Random venous blood samples from healthy adult human beings (25-30 years age group) having normal RBC counts were collected in EDTA (anti-coagulant) vials. After dilution with saline, the samples were centrifuged at 1000 g for 10 min. Supernatant was discarded and the RBC pellet was further washed twice with saline by centrifugation. Final RBC suspension was prepared in saline to have $2 \times 10^4$ cells/ml. For examining the effect of aflatoxin on RBC and its amelioration by antioxidants such as ethanolic and aqueous extracts of turmeric/curcumin, four sets of the tubes were prepared as follows:

a) Control tubes containing 2.0 ml of RBC suspension.

b) Antioxidants control tubes containing 100 µg/ml ethanolic and aqueous extracts of turmeric/curcumin added to 2.0 ml of RBC suspension.

c) Treated tubes containing different concentrations of aflatoxin (0.5 µg/ml to 2.0 µg/ml) added to 2.0 ml of RBC suspension.

d) Tubes containing different concentrations of ethanolic and aqueous extracts of turmeric/curcumin (1 µg/ml to 100 µg/ml) added to RBC suspension treated with 2 µg/ml of aflatoxin.

Aflatoxin solutions and extracts of turmeric and curcumin were prepared in normal saline (0.9% NaCl). Final volume of each tube was made up to 4.0 ml by adding saline. All the tubes were incubated at 37°C for 4 h. Morphological alterations in RBC were
observed after staining with Leishman’s stain. Tubes were centrifuged at 1000 g for 10 min and colour density of supernatant was measured spectrophotometrically at 540 nm.

Percent haemolysis was calculated by the formula as:

\[
\text{Percent haemolysis} = \frac{\text{Absorbance of individual tubes}}{\text{Absorbance with 100% haemolysis}} \times 100
\]

100% haemolysis was obtained by adding 2 ml of distilled water to 2 ml of RBC suspension.

Percent retardation with different concentration of antioxidants was calculated with the following formula (Raval and Verma, 1993).

\[
\text{Percent Retardation} = \left(\frac{A - B}{A}\right) \times 100
\]

where, A = aflatoxin-induced haemolysis

B = haemolysis caused by concurrent addition of aflatoxin and antioxidant

**Effect of ethanolic and aqueous extracts of turmeric/curcumin on aflatoxin-induced toxicity on human spermatozoa**

The effects of different concentrations of aflatoxin with and without turmeric extracts and curcumin on human spermatozoa was studied in accordance with the method described in WHO laboratory manual for the examination of human semen (1999).

Semen samples (n=10) obtained from normal healthy adult donors (25-30 years age group, fathered children) after a minimum 48 h of sexual abstinence, were subjected to routine semen analysis following liquefication at 37°C. Semen samples with sperm counts above 50 million/ml with normal morphology, rapid, linear, progressive motility and viability above 50% was considered for the *in vitro* test.
The semen sample was diluted (1:10 dilution) with normal saline (0.9% NaCl) and used. Turmeric extracts/curcumin solutions were also prepared in normal saline (0.9% NaCl). Tubes were prepared as follows:

a) Control tubes containing diluted semen sample (0.5 ml)

b) Experimental tubes containing 0.5 ml diluted semen sample with different concentrations of aflatoxin (2-10 µg/ml) prepared in normal saline.

c) Antioxidants (turmeric extracts/curcumin) control tubes containing concentration (10 mg/ml) with 0.5 ml diluted semen samples

d) Tubes containing aflatoxin (10 µg/ml) with different concentrations of antioxidant turmeric extracts/curcumin (2-10 mg/ml) with 0.5 ml diluted semen samples.

Sperm motility was assessed immediately after the addition of semen samples and thereafter every 15 min for up to 1 h. The percentage of motile sperms was calculated per unit area and expressed as the percent motility (Prasad et al., 1972). The ratio of live/dead spermatozoa was determined using 1% trypan blue (supravital stain) as described in the method of Talbot and Chacon (1981).

Following completion of the experiment, morphological assessments were carried out by papanicolaou staining method (Belsey et al., 1980).

A thin, wet smear of the semen sample was prepared in such a way that all the sperms lie in a single focal plane. Semi-dried smear were fixed in ether:alcohol (1:1) mixture for 20 min. The slides were then subjected to the staining method as mentioned below.
Slides cleared in xylene were mounted in DPX. Examination of the stained slides was carried out under light microscope. Various morphological abnormalities were identified and counted.

**Effect of curcumin on aflatoxin-induced lipid peroxidation (LPO) in liver, kidney and testis homogenates**

The effect of curcumin on aflatoxin-induced lipid peroxidation in vitro was measured in liver, kidney and testis homogenates by quantification of thiobarbituric acid
Liver, kidney and testis homogenates (10%) were prepared in 0.1 M phosphate buffered saline (pH 7.4).

(a) Reaction mixture contained 0.2 ml of homogenate, 10 mM \( \text{H}_2\text{O}_2 \) and varying concentrations of aflatoxin solution (2-10 \( \mu \text{g/ml} \)) in saline.

(b) Other tubes containing curcumin control (200 \( \mu \text{g/ml} \)).

(c) Set of tubes containing curcumin of different concentrations (25-200 \( \mu \text{g/ml} \)) along with aflatoxin (6 \( \mu \text{g/ml} \)).

Reaction was initiated by addition of \( \text{H}_2\text{O}_2 \) and the mixture was incubated at 37°C for 30 min with occasional shaking. Lipid peroxides (TBARS) were estimated using 8.1% sodium dodecyl sulphate, 20% acetic acid and 1% thiobarbituric acid solution. Care was taken to adjust the pH of 20% acetic acid to 3.5 using 1 N NaOH. The blank for each sample was prepared by substituting the TBA solution with distilled water. The final volume was adjusted by 0.1 M phosphate buffered saline. The solution was mixed and heated in a water bath at 95°C for 60 min. The tubes were then immediately cooled and aliquot was transferred to a centrifuge tube to which an equal volume of 10% TCA was added. The solution was mixed and centrifuge at 1000 \( \text{g} \) for 15 min. The absorbance of the resulting supernatant fraction was read at 532 nm against blank on a Systronics 118 UV-Vis spectrophotometer at 532 nm. The results were expressed as TBARS formed/mg protein/60 min.
STATISTICAL ANALYSIS

For each parameter at least 10 replicates were done. Results are expressed as means ± S.E.M.

Percent change in means of each parameter for controls and all treated groups of animals from vehicle control (Group 2) expressed in the form of Bar diagram, was calculated by the formula:

\[
\frac{M_a - M_b}{M_b} \times 100
\]

Where, \(M_a\) = Mean of other groups (Groups 1, 3-7)

\(M_b\) = Mean of vehicle control (Group 2)

Standard deviation

The standard deviation was calculated by following formula:

\[
SD = \sqrt{\frac{\sum X^2 - nX^2}{n - 1}}
\]

Where \(\sum X^2\) = sum of squares of individual observation

\(n\) = Number of replicates

\(X^2\) = Square of the mean value of the observation

Standard error

Standard error was calculated by

\[
SE = \frac{SD}{\sqrt{n}}
\]
ANOVA

The data (in vivo studies) were statistically analysed using one way Analysis of Variance (ANOVA) followed by Tukey Test. The levels of significance was accepted with p<0.05. Comparisons of P-values between different groups were performed.

STUDENT’s ‘t’ TEST

Student’s ‘t’ test was used (only in vitro studies) for statistical analysis of the data (Ispen and Feigh, 1970).

Student’s ‘t’ test

\[ t = \frac{d}{D} \]

where, \( d = X_1 - X_2 \)

\[ D = \sqrt{(SE_1)^2 + (SE_2)^2} \]

and degree of freedom \( (n_1 + n_2 - 2) \)

\( X_1 = \) Mean of control value

\( X_2 = \) Mean of treated value

\( SE_1 = \) Standard error of control group

\( SE_2 = \) Standard error of treated group

\( n_1 = \) Number of variables in control group

\( n_2 = \) Number of variables in treated groups

The values are less than p < 0.05 were considered significant.