CHAPTER II
MATERIALS AND METHODS

HOUSING AND CARE OF EXPERIMENTAL ANIMALS

All animal studies were approved by Institutional Animal Ethics Committee of Gujarat University, Ahmedabad and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg- 167/1999/CPCSEA; 1st December, 1999), New Delhi, India. Healthy young inbred Swiss-strain male albino mice (*Mus musculus*), weighing approximately 30-35 gm were obtained from Cadila Pharmaceuticals, Ahmedabad. The animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India. Animals were maintained under laboratory conditions in a 12-hours light/dark cycle at 25±2°C and relative humidity 50-55%. They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and potable water *ad libitum*. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (1991).

CHEMICALS

Diethanolamine was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and was of analytical grade. Curcumin was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals used in the present study were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India, Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma Aldrich, St. Louis, MO, USA. Olive oil was obtained from Figaro, Madrid, Spain.
INSTRUMENTATION

The optical density was measured on Systronics-Visiscan 167 spectrophotometer and Systronics 118 UV-Vis spectrophotometer.

PLAN OF STUDY

The present study was carried out in two parts:

**PART I:** Ameliorative effect of curcumin on DEA-induced toxicity on human spermatozoa: an *in vitro* study and

**PART II:** Ameliorative effect of curcumin on DEA-induced toxicity in male reproductive system of mice: an *in vivo* study

**PART-I**

AMELIORATIVE EFFECT OF CURCUMIN ON DIETHANOLAMINE-INDUCED TOXICITY ON HUMAN SPERMATOZOA: AN *IN VITRO* STUDY

Healthy adult male donors were recruited to participate in the study after approval of Institutional Ethics Committee of Zoology Department of Gujarat University, Ahmedabad. Semen samples were collected from normal healthy human donors of age group of 23-25 years after two days of abstinence and brought to the laboratory in cold condition. For this study, semen samples with sperm counts above 50 million/ml with normal sperm morphology, rapid, linear, progressive motility and viability above 50% were considered. After liquefaction semen samples were used to prepare sperm suspension in normal saline (0.9% NaCl). Diethanolamine and curcumin solutions were also prepared in normal saline (0.9% NaCl). For evaluation of ameliorative effect of curcumin against DEA-induced toxicity on human spermatozoa following sets of reaction mixtures were prepared:
a) Control tubes containing 0.5 ml sperm suspension
b) Diethanolamine-treated tubes containing 0.5 ml sperm suspension and different concentrations of DEA (100-500 µg/ml)
c) Antidote control tubes containing 0.5 ml sperm suspension and 40 µg/ml curcumin
d) Diethanolamine and curcumin-treated tubes containing 0.5 ml sperm suspension, 300 µg/ml DEA and different concentrations of curcumin (10-40 µg/ml).

In each tubes final volume was made up to 1 ml with addition of normal saline and incubated at 37ºC for 60 min to evaluate sperm parameters such as sperm motility, sperm viability and sperm morphological abnormalities.

Sperm motility was measured at different time interval (0, 15, 30, 45 and 60 min) by counting both motile and non-motile spermatozoa in at least 10 separate randomly selected fields. The percentage of motile sperms was calculated and expressed as the percent motility (Prasad et al., 1972). Sperm viability was determined by counting live and dead spermatozoa using 1% trypan blue (supravital stain) as described in the method of Talbot and Chacon (1981). Sperm morphology was determined by using Giemsa stain (Gupta and Sarakar, 2010). Examination of stained slides were carried out under the light microscope. Different kinds of morphological abnormalities were identified, counted and calculated.

PART II
AMELIORATIVE EFFECT OF CURCUMIN ON DIETHANOLAMINE-INDUCED TOXICITY IN MALE MICE

STUDY DESIGN

Ninety animals were randomly divided in nine groups and caged separately (Table 2.1). Group 1 (untreated control) animals were maintained without any treatment. Animals of group 2 (vehicle control) received olive oil (0.2 ml/animal/day) for 45 days as olive oil
Table 2.1: Experimental protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of Animals</th>
<th>Duration of treatment</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) Control</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>Untreated control</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle control (0.2 ml olive oil/animal/day)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Antidote control (50 mg curcumin/kg bw/day)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>(II) Diethanolamine (DEA)-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Low dose of DEA (DEA-LD) (110 mg/kg bw/day)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
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<tr>
<td>5</td>
<td>Mid dose of DEA (DEA-MD) (165 mg/kg bw/day)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>High dose of DEA (DEA-HD) (330 mg/kg bw/day)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>(III) Diethanolamine (DEA-HD) + curcumin (C)-treated</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td>DEA-HD (330 mg/kg body weight/day) + Curcumin (10 mg/kg bw/day) (C10)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>DEA-HD (330 mg/kg body weight/day) + Curcumin (25 mg/kg bw/day) (C25)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>DEA-HD (330 mg/kg body weight/day) + Curcumin (50 mg/kg bw/day) (C50)</td>
<td>10</td>
<td>45</td>
<td>46&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

was used as vehicle to dissolve curcumin. Animals of group 3 (antidote control) received 50 mg/kg bw/day of curcumin. Animals of group 4, 5 and 6 were orally administered with low dose (110 mg/kg bw/day), mid dose (165 mg/kg bw/day) and high dose (330 mg/kg bw/day) of DEA respectively. Animals of group 7, 8 and 9 were orally administered with 10, 25 and 50 mg/kg bw/day of curcumin respectively along with high dose of DEA.

The three different doses of DEA were selected based on its oral LD$_{50}$ value of 3300 mg/kg bw (Izmerov et al., 1982). Based on the results DEA toxicity studies, the high dose of DEA was chosen for further studies along with curcumin. Three different doses of curcumin were selected based on earlier study (Verma and Mathuria, 2010).

Behavioral and clinical changes throughout the experiment were recorded. The body weight of all control and treated groups of mice were recorded to the nearest gm on weighing balance. At the end of treatment, the animals were weighed and humanly sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture in non - anticoagulant added tubes, allowed to clot for 20-30 min and centrifuged at 1000 x g for 10 min at 4°C. The non – haemolysed serum samples obtained were stored at -4°C and used for analysis of serum testosterone level. The testis, caput and cauda epididymis, vas deferens, seminal vesicle and prostate gland were quickly dissected out, blotted free of blood and weighed to the nearest mg on a balance and used for histopathological and biochemical analysis.

**HISTOPATHOLOGICAL STUDIES:**

Histopathological studies were carried out using the standard technique of hematoxylin and eosin staining. The testis, caput and cauda epididymis, vas deferens, seminal vesicle and prostate gland of all control and treated groups of animals were dissected out, blotted free of blood and fixed in 10% neutral buffered formalin immediately after the autopsy. The preserved tissues were dehydrated by passing through
ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58 to 60°C mp). 5 µm thick sections were cut on a rotary microtome and stained with H & E, dehydrated in alcohol, cleared in xylene, mounted in DPX and examined under a light microscope.

BIOCHEMICAL ANALYSIS

Protein content:

Protein content in testis, caput and cauda epididymis, vas deferens and prostate gland were estimated by the method of Lowry et al. (1951). Protein containing preparation when treated with phenol reagent of Folin-Ciocalteau, a deep blue colour develops. 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 phosphomolybdic acid and phosphotungstic acid by the aromatic amino acids present in the protein. The blue colour developed was read at 540 nm. The protein content was expressed as mg /100 mg tissue weight.

Glycogen content:

The glycogen content in the vas deferens was estimated by the method of Seifter et al. (1950). The glycogen present in tissue is converted to glucose, which reacts with anthrone reagent to give a green colored product which was read at 620 nm. The glycogen content was expressed as µg/100 mg tissue weight.

Total lipid content:

Total lipid content in the testis was estimated according to the method of Fringes et al. (1972) using olive oil as a standard. Lipid on being heated with sulphuric acid followed by addition of vanillin and phosphoric acid produces a pink colour whose optical density is measured at 530 nm. The total lipid content was expressed as mg/100 mg of tissue weight.
Cholesterol content:

The concentration of total cholesterol was estimated in testis by the method of Zlatki et al. (1953). Cholesterol forms a coloured complex with FeCl₃ in the presence of concentrated sulphuric acid and glacial acetic acid which can be measured at 540 nm. The cholesterol content was expressed as mg/100 mg tissue weight.

Sialic acid content:

The concentration of sialic acid was estimated in testis, caput and cauda epididymis 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°C. The chromogen was treated with an organic solvent which extracted the colour whose optical density was measured at 630 nm. The sialic acid content was expressed as µg/mg tissue weight.

Fructose content:

The concentration of fructose was estimated in seminal vesicle by the method of Foreman et al. (1973). Fructose containing preparation when heated with concentrated HCl forms oxymethyl furfural which gives a red coloured complex with resorcinol which was measured spectrophotometrically at 410 nm. The fructose content was expressed as µg/mg tissue weight.

Estimation of nucleic acid:

Extraction:

A known weight of fresh tissue testis was homogenized in 5 ml of cold 5% TCA and the homogenate was kept at 0-4°C for 30 min. The precipitate obtained after centrifugation (10 min at 1000 x g) was dissolved in 5 ml of cold 5% TCA and left for 30 min at 0-4 °C. Thereafter, centrifugation (10 min at 1000 x g) was carried out and the precipitate obtained was dissolved in alcohol: ether (1:3, v/v) mixture and left for 30 min
at 50°C. This process was repeated once again. The tubes were centrifuged at 1000 x g for 10 min and the supernatant was discarded. The lipid free pellet obtained was dissolved in 5 ml of 0.1 N KOH and incubated at 37°C for 16-18 hours. Then 0.17 ml of 6 N HCl and 5 ml of 10% TCA were added to the incubated suspension and precipitate was allowed to be formed at 4°C for 30 min. After centrifugation at 1000 x g for 10 min, the supernatant was separated and used for estimation of RNA. 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 1000 x g) after cooling at 4 °C for 30 min and used for estimation of DNA.

(i) Deoxyribonucleic acid (DNA) content:

The estimation of DNA in the testis 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 was read at 620 nm. The DNA content was expressed as µmoles/100 mg tissue weight.

(ii) Ribonucleic acid (RNA) content

The estimation of RNA in testis was carried out by the method of Schneider (1945). The RNA in the supernatant reacts with the orcinol reagent to give a greenish colour, whose absorbance was read at 670 nm. The concentration of RNA was expressed as µmoles/100 mg tissue weight.

ENZYMATIC ASSAYS

Alkaline phosphatase (E.C.3.1.3.1) activity:

The alkaline phosphatase (ALP) activity in testis was determined by the method of Bessey et al. (1946). Alkaline phosphatase at optimum pH 10.5 catalyzes the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol combines with sodium hydroxide to form yellow coloured
complex which was measured at 410 nm. The ALP activity in testis was expressed as µmoles p-nitrophenol released/mg protein/30 min.

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

The acid phosphatase (ACP) activity was assayed in the testis, caput and cauda epididymis as well as prostate gland by the method as described in Sigma Technical Bulletin (Sigma Technical Bulletin, MO, USA). Acid phosphatase at optimum pH 4.8 catalyzed the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol combines with sodium hydroxide to form a yellow coloured complex which was measured at 420 nm. The enzyme activity was expressed as µmoles p-nitrophenol released/mg protein/30 min.

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

The adenosine triphosphatase (ATPase) activity in the testis, caput and cauda epididymis and vas deferens was assayed by the method of Quinn and White (1968). ATPase causes hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate. The liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925). The optical density was read at 660 nm. The enzyme activity was expressed as µmoles inorganic phosphate released/mg protein/30 min.

**Succinic dehydrogenase (E.C.1.3.99.1) activity:**

The succinic dehydrogenase (SDH) activity in the testis, caput and cauda epididymis was assayed 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 from the substrate are taken up by INT, which was reduced to a red coloured formazan. This was extracted in ethyl acetate and the
absorbance was read at 420 nm. The enzyme activity was expressed as µg formazan formed/mg protein/15 min.

**3β-Hydroxysteroid dehydrogenase (E.C.1.1.1.51) activity:**

The testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was assayed by the method of Talalay (1992). The 3β-hydroxysteroid dehydrogenase acts on substrate epiandrosterone in the presence of nicotinamide adenine dinucleotide (NAD) which is reduced to NADH and androstenedione was formed. The absorbance was measured at 340 nm. The enzyme activity was expressed as nmoles of androstenedione formed/mg protein/min.

**17β-Hydroxysteroid dehydrogenase (E.C.1.1.1.51) activity:**

The testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) activity was assayed by the method of Talalay (1992). The 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. The enzyme activity was expressed as nmoles of androstenedione formed/mg protein/min.

**EFFECTS ON LIPID PEROXIDATION AND ANTIOXIDATIVE DEFENSE MECHANISM**

**Lipid peroxidation:**

The level of lipid peroxidation (LPO) in the testis was measured by the method as described by Ohkawa et al. (1979). This method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with products of lipid peroxidation like malondialdehyde (MDA) and others collectively called as thiobarbituric acid reactive substances (TBARS). The results were expressed as nmoles MDA formed/mg protein/60 min.
Enzymatic antioxidants:

Catalase (E.C.1.11.1.6) activity:

The catalase (CAT) activity was assayed in the testis by the method of Luck (1963). The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), aliquot and 10 mM H$_2$O$_2$ which was added to initiate the reaction. The decrease in absorbance was noted every 5 seconds at 240 nm. The enzyme activity was expressed as µmoles H$_2$O$_2$ consumed/mg protein/min.

Superoxide dismutase (E.C.1.15.1.1) activity:

The superoxide dismutase (SOD) activity in the testis was assayed by the method of Kakkar et al. (1984) with slight modification. The method is based on the NADH-phenazine methosulfate-nitroblue tetrazolium formazon inhibition. The formazon formed at the end of the reaction was extracted into butanol layer, upon inactivation of the reaction with acetic acid. The enzyme activity was expressed as units/mg protein. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density of chromogen production at 560 nm by 50% in 1 min under the assay condition. The enzyme activity was expressed as units/mg protein.

Glutathione peroxidase (E.C.1.11.1.9) activity:

The glutathione peroxidase (GSH-Px) activity in the testis was assayed by the modified method of Pagila and Valentine (1967). The decrease in absorbance was recorded for 3 min at 340 min. The enzyme activity was expressed as units/mg protein/min, where 1 unit of GSH-Px equals to nmoles of NADPH consumed/mg protein/min.
Non-enzymatic antioxidants:

Glutathione content:

The glutathione (GSH) content in the testis was measured by the method of the Grunert and Philips (1951). In saturated alkaline medium, the GSH present in the tissues react with sodium nitroprusside to give a red coloured complex which was measured at 520 nm. The glutathione content was expressed as µg/100 mg tissue weight.

Total ascorbic acid content:

Total ascorbic acid (TAA) content was estimated in the testis by the method of Roe and Kuether (1943). Total ascorbic acid is oxidized to dehydroascorbic acid (DHA) by Norit reagent in the presence of TCA. This couples with 2, 4-dinitrophenyl hydrazine in the presence of thiourea and sulphuric acid to yield a red coloured complex which was read at 540 nm. The TAA content was expressed as mg/gm tissue weight.

SPERM PARAMETERS:

A known amount of cauda epididymis was teased gently in a definite 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 parameters such as sperm count, sperm motility, sperm viability and sperm morphology.

Sperm count:

Cauda epididymal sperm count of all control and treated groups of animals were determined by the method of Prasad et al. (1972) using Neubauer chamber of haemocytometer. The cauda epididymal sperm suspension was sucked upto the 0.5 mark in WBC pipette. The suspension was 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. Then a drop of suspension was transferred
to the Neubauer chamber and gently covered with cover slip. Spermatozoa were counted in 64 subsquares of the WBC counting regions and calculated. The sperm density was expressed in terms of million spermatozoa/ml of sperm suspension.

**Sperm motility:**

The sperm motility was measured by the method of Prasad *et al.* (1972). A drop of sperm suspension was placed on the Neubauer chamber and observed under low magnification (10 X) in a microscope. The chamber is focused on the WBC region. The motile spermatozoa were counted along with the total number of spermatozoa in each small sub-square. A total of minimum 10-12 separate fields were scored and the sperm motility was calculated. The sperm motility was expressed as percent motile sperms.

**Sperm viability:**

The sperm viability was determined by using 1% trypan blue (supravital stain) as described in the method of Talbot and Chacon (1981). Live spermatozoa retain the selective semi-permiability 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 lose 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 for 15 min at 37°C. A drop of the sperm suspension was then placed on a pre-cleaned slide and allowed to settle for one min, after placing a coverslip. The slide was then observed under 40 X magnification of a light microscope. The number of stained and unstained spermatozoa were scored in 10-20 separate high power fields. This technique helps to differentiate live spermatozoa from
immotile dead spermatozoa. In each sample the ratio of live:dead spermatozoa are counted and calculated.

**Sperm morphology:**

The sperm morphological study was done by the method of Gupta and Sarkar (2010). For this study the sperm suspension was smeared on clean slides. Air-dried smears were fixed in methanol for 15 min, stained with Giemsa stain for 30 minutes and observed under the high-power objective of a microscope. In each slide total 150 spermatozoa scored and calculated.

**Serum testosterone level:**

Serum testosterone was estimated by Chemiluminescence Immunoassay (CLIA) method using Advia Centaur XP (Siemens) Immunoassay system.

**Fertility index:**

The fertility index of control and treated groups of animals were calculated by formula (Parker, 2006) as mentioned below:

\[
\text{Male fertility index} = \frac{\text{number of males impregnating female}}{\text{Number of males cohabitated}} \times 100
\]

**Organoprotective index:**

The organ protecting activity of curcumin was expressed as organoprotective percentage (O) (Chandan et al., 2007) which was calculated using the formula as mentioned below:

\[
O = 1 - \left(\frac{T - V}{C - V}\right) \times 100
\]

Where T is the mean value of curcumin along with the DEA, C is the mean value of DEA alone, and V is the mean value of vehicle control animals.
STATISTICAL ANALYSIS:

Statistical analysis was performed by analysis of variance (ANOVA) followed by Tukey’s test using GraphPad Instant software version 5.03. Data are expressed as the means ± S.E.M. The level of significance was accepted with p<0.05. Pearson’s correlation analysis was used to determine the correlation between control and treated.