3 MATERIALS AND METHODS

3.1 Animal

Male rats of Wistar strain were obtained from the Central Animal House, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry, India.

3.2 Environment

The animals were housed in polypropylene cages (38 cm x 25 cm x 15 cm) covered with heavy wire meshes. The cages were lined with paddy husk which was replaced every day and cages were kept under a well-regulated light and dark (12h. 12h) schedule and temperature is maintained at 24 ± 2°C. Rats were fed with pelleted food (Kamedhenu Agencies, Bangalore, India) and water ad libitum. Water was provided through plastic bottles with teats.

3.3 Chemicals

Bisphenol A (2,2-Di (4-hydroxyphenyl) propane) of 97% purity was obtained from S.d. fine-chem, India. Malondialdehyde was obtained from Merck-Schuchardt, Germany. NADPH and glutathione oxidized were obtained from SISCO Research Laboratories, Mumbai, India. Horseradish peroxidase, Ham’s F-12 medium, vitamin C, deoxyribonucleic acid (DNA), thiobarbituric acid and pyrogallol were obtained from Himedia Laboratories, Mumbai, India. For hormone assays, ELISA kits were obtained from Diagnostic Systems Laboratories, Inc. Webster, Texas, USA. All other chemicals were of analytical grade and obtained from local commercial sources.
3.4 **Body weight and growth rate**

All the rats used in the experiments were marked by tail marking. Growth of the animals was monitored regularly and rats showing poor growth rate were discarded from the experiments.

3.5 **Treatments**

Experiments were carried out in pubertal male rats (postnatal days 45). Corresponding groups of control animals, treated with vehicle alone were maintained and killed along with the treated rats. The treatments to various groups of rats are described below

3.5.1 **Group I: Short-term exposure of bisphenol A to male rats**

Bisphenol A was dissolved in acetone and olive oil (see Appendix 1.1) in the ratio of 1:19 and administered daily for 1, 4 and 7 days by oral intubation using micropipette. For administration of dosing solution, the pipette tip was gently placed inside the mouth and the dosing solution was then carefully and slowly expelled from the tip allowing the animal to lick the compound. Corresponding groups of animals were administered with equal volume of vehicle alone and served as control.

- **Subgroup I:** Administration of bisphenol A at the dose of 0.2 mg/Kg body weight/day.
- **Subgroup II:** Administration of bisphenol A at the dose of 2 mg/Kg body weight/day.
- **Subgroup III:** Administration of bisphenol A at the dose of 20 mg/Kg body weight/day.

3.5.1 **Group II: Long-term exposure of bisphenol A to male rats**

Bisphenol A was dissolved in acetone and olive oil (see Appendix 1.1) in the ratio of 1:19 and administered daily for 30, 45 and 60 days by oral intubation by the
method as described previously (see Section 3.5.1). Corresponding groups of animals were administered with equal volume of vehicle alone and served as control.

Subgroup I: Administration of bisphenol A at the dose of 0.2 µg/Kg body weight/day.

Subgroup II: Administration of bisphenol A at the dose of 2 µg/Kg body weight/day.

Subgroup III: Administration of bisphenol A at the dose of 20 µg/Kg body weight/day.

3.5.2 Group III: Co-administration of bisphenol A and vitamin C to male rats

Vitamin C was dissolved along with various doses of bisphenol A (see Appendix 1.1) at the therapeutic dose level of 40 mg/Kg body weight/day for 30, 45 and 60 days. Co-administration of bisphenol A and vitamin C was done by the method as described previously (see Section 3.5.1). Corresponding control animals were administered with equal volume of vitamin C.

Subgroup I: Co-administration of bisphenol A and vitamin C at the dose of 0.2 µg/Kg body weight/day.

Subgroup II: Co-administration of bisphenol A and vitamin C at the dose of 2 µg/Kg body weight/day.

Subgroup III: Co-administration of bisphenol A and vitamin C at the dose of 20 µg/Kg body weight/day.

3.6 Killing of animals

After 24 h of the last treatment the rats were weighed and killed using overdosage of anesthetic ether.
3.7 Collection of serum

Blood samples from control and treated groups of 45 days of treatment were collected in clean glass centrifuge tubes either by cardiac puncture method or decapitation. Blood was centrifuged at 2000 g for 15 min after storing overnight at 4°C. Serum samples were collected and stored at -20°C in microfuge tubes until used.

3.8 Collection of tissues

Testis, epididymis, seminal vesicles, ventral prostate, liver and kidney were dissected out and transferred to a petridish containing cold normal saline.

3.9 Organ weights

Testis, epididymis, accessory sex organs (seminal vesicles and ventral prostate), liver and kidney were cleared from the adhering tissues and weighed in ‘Ohaus Precision Standard’ electronic balance. One side of testis, epididymis, seminal vesicles and ventral prostate from the control and treated groups of 60 days treatment were preserved in Bouin’s fixatives for histological observation. Testis and epididymis of all dosage groups were processed for various assays as given in the respective sections.

3.10 Collection of epididymal sperm and sperm function tests

Epididymal sperm were collected by the method of Gray et al. (1989). Briefly, the epididymis were cut into small pieces in 5 ml of Ham’s F-12 medium at 32°C. The sperm obtained from left epididymis were used for the determination of sperm viability, sperm motility and sperm count. Sperm collected from right epididymis were used for biochemical estimations.
3.10.1 Sperm viability test

Sperm viability test was done by the method as described in the WHO Laboratory Manual (1999). The proportion of live spermatozoa can be determined by using staining techniques that were based on the principle that dead cells with a damaged plasma membrane take up certain stains. An aliquot of 100 µl of epididymal sperm was mixed with 100 µl of 0.5% eosin solution on a microscopic slide covered with a cover slip and examined after 30 sec under light microscope at 200 x magnification. Two hundred spermatozoa were counted, differentiating the live (unstained) and dead cells (unstained). The sperm viability was expressed in percentage as the number of viable sperm of total sperms counted.

3.10.2 Epididymal sperm motility

Epididymal sperm motility was evaluated by the method as described by Linder et al. (1986). Briefly, fluid that oozed out from the cauda epididymides was obtained using a pipette tip and diluted to 2 ml of Ham’s F-12 medium at 32°C. Approximately 10 µl of this solution was placed in Neubauer-type hemocytometer and counted for motile and non-motile sperm. First non-motile sperm were counted followed by motile sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted.

3.10.3 Epididymal sperm count

Epididymal sperm were counted by the method as described in the WHO Laboratory Manual (1999). Briefly, 5 µl of epididymal sperm was diluted with 95 µl of Ham’s F-12 medium. The coverslips on the counting chambers of the improved Neubauer-type hemocytometer were secured. Approximately 10 µl of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer, and was allowed to stand for 5 min in a humid chamber to prevent drying out. The
sperm cells settled during this time were counted with the help of light microscope at 200 × magnification. The complete spermatozoa (head with tail) were counted.

3.11 **Histology of testis, epididymis and accessory sex organs**

Testes, epididymides, seminal vesicles and ventral prostate were cleared from the adhering tissues and were fixed in Bouin's fixative for histological studies (see Appendix 1.2). After dehydration in alcoholic series and cleaning in xylol, the tissue were embedded in paraffin wax. Sections were made with 5 μm thickness The sections were stained with hematoxylin followed by eosin and examined under a light microscope. Photomicrographs were taken using Trinocular Research Microscope with automatic exposurometer – Nikon Optihot Model, Japan.

3.12 **Quantitative determination of serum hormone levels**

Serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, testosterone and estradiol were measured by enzyme linked immunosorbant assay (ELISA) using kits from Diagnostic System Laboratories, Inc. Webster, Texas, USA. The assays were done strictly according to the procedure given along with the kits.

3.12.1 **Quantitative determination of serum FSH**

For assaying FSH in the serum, all the specimens and reagents were allowed to attain room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. Standards, controls and unknowns were assayed in duplicate. First, 100 μl of the standards, controls and unknowns were added to the appropriate wells. The wells were incubated, shaking at a fast speed on an orbital microplate shaker for 60 min at room temperature. Antibody-Enzyme Conjugate solution (100 μl) was added to each well. The wells were incubated, shaking at a fast speed on an orbital microplate shaker for 30 min at room temperature. The wells were aspirated and
washed five times with wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. TMB chromogen solution (100 μl) was added to each well (see Appendix 1.3). The wells were incubated, shaking at a fast speed (500 - 700 rpm) on an orbital microplate shaker, for 10 min at room temperature (~25°C). Stopping solution (0.2 M sulfuric acid; 100 μl) was added to each well and absorbance of the solution in the wells was read within 30 min using a microplate reader set to 450 nm. The absorbance measured is directly proportional to the concentration of FSH present. A set of FSH standards is used to plot a standard curve of absorbance versus FSH concentration from which the FSH concentrations in the unknowns can be calculated.

3.12.2 Quantitative determination of serum LH

For assaying LH in the serum, all the specimens and reagents were allowed to attain room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. Standards, controls and unknowns were assayed in duplicate. First, 50 μl of the standards, controls and unknowns were added to the appropriate wells. Antibody-Enzyme Conjugate solution (100 μl) was added to each well. The wells were incubated, shaking at a fast speed on an orbital microplate shaker for 90 min at room temperature. The wells were aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. TMB chromogen solution (100 μl) was added to each well (see Appendix 1.4). The wells were incubated, shaking at a fast speed (500 - 700 rpm) on an orbital microplate shaker, for 10 min at room temperature (~25°C). Stopping solution (0.2 M sulfuric acid; 100 μl) was added to each well and absorbance of the solution in the wells was read within 30 min using a microplate reader set to 450 nm. The absorbance measured is directly proportional to the concentration of LH present. A set of LH standards is used to plot a standard curve of absorbance versus LH concentration from which the LH concentrations in the unknowns can be calculated.
3.12.3 Quantitative determination of serum prolactin

For assaying prolactin in the serum, all the specimens and reagents were allowed to attain room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. Standards, controls and unknowns were assayed in duplicate. First, 25 µl of the standards, controls and unknowns were added to the appropriate wells. Added 100 µl of assay buffer and the wells were incubated, shaking at a fast speed on an orbital microplate shaker for 60 min at room temperature. The wells were aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. Antibody-Enzyme Conjugate solution (100 µl) was added to each well and incubated by shaking at a fast speed on an orbital microplate shaker for 60 min at room temperature. The wells were aspirated, washed five times with wash solution using an automatic microplate washer and blotted dry by inverting plate on absorbent material. TMB chromogen solution (100 µl) was added to each well (see Appendix 1 5) The wells were incubated, shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, for 10 min at room temperature (~25°C) Stopping solution (0.2 M sulfuric acid, 100 µl) was added to each well and absorbance of the solution in the wells was read within 30 min using a microplate reader set to 450 nm. The absorbance measured is directly proportional to the concentration of prolactin present. A set of prolactin standards is used to plot a standard curve of absorbance versus prolactin concentration from which the prolactin concentrations in the unknowns can be calculated.

3.12.4 Quantitative determination of serum testosterone

For assaying testosterone in the serum, all the specimens and reagents were allowed to attain room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. Standards, controls and unknowns were assayed in duplicate. First, 50 µl of the standards, controls and unknowns were added to the appropriate
wells. Enzyme Conjugate solution (100 μl) and testosterone-antiserum (100 μl) was added to each wells of microplate. The wells were covered, incubated by shaking at a fast speed on an orbital microplate shaker for 60 min at room temperature. Wells were aspirated and washed five times with wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. TMB chromogen solution (100 μl) was added to each well and incubated by shaking at a fast speed (500 - 700 rpm) on an orbital microplate shaker for 10 min at room temperature (~25°C). Stopping solution (0.2 M sulfuric acid; 100 μl) was added to each well and absorbance was read within 30 min using a microplate reader set to 450 nm (see Appendix 1 6). The absorbance measured is inversely proportional to the concentration of testosterone present in the serum. A set of testosterone standards is used to plot a standard curve absorbance versus testosterone concentration from which the testosterone concentrations in the unknowns can be calculated.

3.12.5 Quantitative determination of serum estradiol

For assaying estradiol in the serum, all the specimens and reagents were allowed to attain room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. Standards, controls and unknowns were assayed in duplicate. First, 50 μl of each standards, controls and unknowns were added to the appropriate wells of microplate. Estradiol-Biotin Conjugate solution (100 μl) was added to each well. The wells were incubated by shaking at a fast speed on an orbital microplate shaker for 60 min at room temperature. Wells were aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. Streptavidin-Enzyme Conjugate Solution (200 μl) was added to each well and incubated by shaking at a fast speed on an orbital microplate shaker, for 30 min at room temperature (~25°C). The wells were aspirated and washed five times with the wash solution using an automatic microplate washer. Blotted dry by inverting plate on absorbent material. TMB chromogen solution (100
μl) was added to each well (see Appendix 1.7). The wells were incubated, shaking at a fast speed (500 - 700 rpm) on an orbital microplate shaker, for 10 min at room temperature (~25°C). Stopping solution (0.2 M sulfuric acid; 100 μl) was added to each well and absorbance of the solution in the wells was read within 30 min using a microplate reader at 450 nm.

3.13 Sub-cellular fractionation of testis

Different sub-cellular fractions were obtained by the differential centrifugation method. A 10% (w/ v) homogenate of testis was prepared in ice-cold 0.25 M sucrose solution with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 1000 g for 10 min at 4°C to obtain the nuclear pellet. Mitochondrial pellet was obtained by centrifuging the post-nuclear supernatant at 10,000 g for 10 min at 4°C. The microsomal pellet was prepared by the calcium chloride (CaCl₂) sedimentation method of Karnath and Narayan (1972). Briefly, the post-mitochondrial supernatant was diluted with ice-cold CaCl₂ (1M) so that the final concentration of CaCl₂ was 0.8 molar (see Appendix 1.8). It was incubated at 4°C for 10 min with occasional stirring. Then the sample was centrifuged at 10,000 g for 10 min at 4°C and the microsomal pellet was obtained. All the fractions were washed thrice with ice-cold 1.15% potassium chloride solution and dissolved in the 0.25 M sucrose solution. Mitochondrial and microsomal fractions of testis was used for biochemical studies.

3.14 Preparation of tissue homogenates of epididymis

Epididymis was washed several times in cold normal saline in order to remove maximum number of sperm attached to the epididymal parenchyma. Caput, corpus and cauda regions of epididymis were separated and homogenized separately in cold normal saline with the help of glass Teflon homogenizer. The homogenates
were centrifuged at 800 \( g \) for 20 min at 4°C. The supernatants were collected and used for various biochemical assays.

3.15 **Preparation of homogenates of liver and kidney**

Liver and kidney were isolated and homogenized separately in cold normal saline with the help of glass Teflon homogenizer. The homogenates were centrifuged at 800 \( g \) for 20 min at 4°C. The supernatants were collected and used for various biochemical assays.

3.16 **Determination of deoxyribonucleic acid (DNA)**

DNA was determined by diphenylamine colour reaction following the method of Burton (1956). An aliquot of 1 ml of the test sample was mixed with 2 ml of 1 N perchloric acid and 2 ml of diphenylamine reagent (see Appendix 1.9). The tubes were kept in a boiling water bath for 20 min. The tubes were cooled and the colour developed was read at 600 nm on a Systronics Spectrophotometer. A standard curve was prepared by using known concentrations of calf thymus DNA.

3.17 **Determination of protein**

Protein contents were determined according to the method of Lowry et al. (1951). An aliquot of 0.1 ml of the test sample was mixed with 5.0 ml of alkaline copper reagent and vortexed, they were allowed to stand for 10 min at room temperature. Folin-Ciocalteau reagent 1N (0.5 ml) was added to each of the tubes, vortexed and allowed to stand for 20 min at room temperature. The optical density was read at 610 nm in a Systronics Spectrophotometer. A standard calibration curve was prepared using different concentrations of bovine serum albumin (see Appendix 1.10).
3.18 Determination of antioxidant enzymes in tissues

Activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase and the levels of hydrogen peroxide generation and lipid peroxidation were assayed by the methods as described below.

3.18.1 Assay of superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974). The assay mixture contained 2.4 ml of tris hydrochloric acid buffer (50 mM) containing 1 mM EDTA (pH 7.6), 300 μl of pyrogallol (0.2 mM) and 100 μl enzyme source (see Appendix 1.11). Increase in the absorbance was measured immediately at 420 nm against enzyme blank at 10 sec intervals for 3 min on a Systronics Spectrophotometer. Activity of enzyme was expressed as nmole pyrogallol oxidised/ min.

3.18.2 Assay of catalase

Catalase (EC. 1.11 1.6) was assayed by the method of Claiborne (1985). The assay mixture contained 2.40 ml of phosphate buffer (50 mM, pH 7.0), 10 μl of hydrogen peroxide (19 mM) and 50 μl enzyme source (see Appendix 1.12). Decrease in absorbance was measured immediately at 240 nm against enzyme blank at 10 sec intervals for 3 min on a Systronics Spectrophotometer. Activity of enzyme was expressed as μmole of hydrogen peroxide consumed/ min.

3.18.3 Assay of glutathione reductase

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1985). The assay mixture contained 1.75 ml of phosphate buffer (100 mM, pH 7.6), 100 μl of NADPH (200 mM), 100 μl of EDTA (10 mM), 50 μl of glutathione oxidized (20 mM) and 50 μl of enzyme source (see Appendix 1.13). Disappearance of NADPH was measured immediately at 340 nm against enzyme
blank at 10 sec intervals for 3 min on a Systronics Spectrophotometer. Activity of enzyme was expressed as nmole of NADPH oxidised/ min.

3.18.4 Assay of glutathione peroxidase

Glutathione peroxidase (EC.1.11.1.9) was assayed by the method of Mohandas et al. (1984). The assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 µl of EDTA (10 mM), 100 µl of sodium azide, 50 µl of glutathione reductase, 100 µl of glutathione reduced, 100 µl of NADPH (200 mM), 10 µl of hydrogen peroxide and 10 µl enzyme source (see Appendix 1.14). Disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Systronics Spectrophotometer. Activity of enzyme was expressed as nmole of NADPH oxidised/ min.

3.19 Estimation of hydrogen peroxide generation

Hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981). The assay mixture contained 1.641 ml phosphate buffer (50 mM, pH 7.6), 54 µl of horseradish peroxidase (8.5 units/ ml), 30 µl of phenol red (0.28 nM), 165 µl of dextrose (5.5 nM) and 600 µl of enzyme source was incubated at 32°C for 30 min. Reaction was terminated by addition of 60 µl of 10N sodium hydroxide (see Appendix 1.15). Absorbance was read at 610 nm against enzyme blank on a Systronics Spectrophotometer. The quantity of hydrogen peroxide produced was expressed as nmole hydrogen peroxide generated/ min. For the preparation of standard curve, known amount of hydrogen peroxide and all the above reagents except enzyme source were incubated for 30 min at 32°C and then 60 µl of sodium hydroxide (10N) was added and optical density was read at 610 nm.
3.20 Estimation of lipid peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS), was measured by the method of Ohkawa et al. (1979). The stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25N hydrochloric acid and 2-thiobarbituric acid 0.37% (w/v) in 0.25N hydrochloric acid (see Appendix 1.16). One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min in a boiling water bath. After cooling on ice the precipitate was removed by centrifugation at 1000 g for 15 min and absorbance was measured at 532 nm against enzyme blank. The values were expressed as µmole of malondialdehyde formed/min A standard curve was prepared with the known amount of malondialdehyde and all the above reagents except enzyme source. The optical density was read at 532 nm.

3.21 Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range test. Differences were considered to be significant at p<0.05 against control group. Data are presented as mean ± SD for four animals per group. All biochemical estimations were carried out in duplicate.