3. Materials and Methods

3.1 Maintenance of laboratory animals

Healthy, adult, fertile Wistar strain albino rats were considered for the conduction of the whole experiment. The albino rats were about 3 months and weight about 120 ± 10 g. All the animals were maintained in standard laboratory condition. The room was airy, ventilated, clean and sterilized. The temperature was maintained up to 25 ± 2˚C and the humidity of the room was about 45-60%. To maintain the normal circadian cycle, 12 hrs light: 12 hrs dark was maintained in the laboratory. All the animals were given standard animal chew and water *ad libitum*. Animals were acclimatized in laboratory condition before the initiation of the experiment to minimise the stress due to animal handling during drug administration. Permission regarding the animal handling, animal treatment, drug administration was taken from Institutional Ethical Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] prior the experiment and the total experiment was followed as per the guideline provided by of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

3.2 Drug and reagent

Cyproterone acetate, commercially known as 'Androcur' was procured from Schering AG, Berlin, Germany. 'Lakshyam herbs located at Shalimar Bagh, New Delhi' provided lycopene. Activities of GOT, GPT and kits of serum urea, uric acid and creatinine were procured from Span Diagnostic Ltd. Surat, India. Testosterone kit was purchased from Meril diagnostics, Muktanand Marg, Gujrat, India. Immuno-histochemistry was assessed by ISEL which obtained from TACS Tdt-DAB in Situ Apoptosis Detection Kit from Trevigen Inc., Gaithersburg, MD, U.S.A.

Rabbit polyclonal primary antibodies such as anti-Δ5, 3β-HSD, anti-17β-HSD, Anti-SOD, anti-catalase, anti-Bax, anti-Bcl-2, anti-caspase-3 and anti-β-actin and goat anti rabbit /

mouse-HRP conjugate which was used as secondary antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Primers of specific genes were required for qRT-PCR study. Different primers such as -Δ5, 3β-HSD, 17β-HSD, SOD, catalase, peroxidase, Bax, Bcl-2, caspase 3, cytochrome-c were obtained from Roche Diagnstic, Mannheim, Germany.

3.3 Spermatogenic profile

3.3.1 Sperm motility

Assessment of sperm motility was performed by following the standard method of **(Zemjanis, 1977). Data was** evaluated under the microscope just within 2-4 minutes of the separation from the cauda part of the epididymis and result was expressed in terms of percentages **(WHO, 1999).**

3.3.2 Sperm count

Sperm count was done by mincing the four pairs of cauda of the epidydimis in distilled water. Total content of the sample was then and filtered through a nylon mesh. The spermatozoa were quantified by using Neubauer chamber (Deep 1/10mm, LABART, Germany) as per standard method **(Pant and Srivastava, 2003).**

3.3.3 Sperm viability

Percentage of viable sperm was analysed by eosin-nigrosin staining. The sample was collected from cauda part of epididymis. Thin film of the sample was drawn on the slide and after drying the slides, the samples were observed under the microscope and values were represented in terms of percentage **(WHO, 1999).**

3.3.4 Acrosomal status

Slides were coated with gelatin coat which was prepared by dissolving gelatin in 100 ml water followed by warming of water at 60º C. After 24 hours of coating, the slides were fixed by using glutaraldehyde solution (0.05%). At the day of sacrifice, the cauda part of

epididymis was dissected and the sperms were diluted with the help of PBS-D-glucose. A smear was drawn by 20 ml of sample on gelatin coated slide. The prepared slide was then allowed for incubation at 37ºC for 2 hours in a wet chamber. Holes were developed on the gelatin coated slide as acrosomal enzyme dissolved the gelatin that created holes **(Gopalkrishnan et al., 1995).**

3.3.5 Nuclear chromatin decondensation (NCD) test

Assessment of NCD was conducted according to the standard method **(Rodriguez et al., 1985).** Sperm was obtained from cauda part of epididymis. Sperm was diluted with ethyl enediamine tetra acetic acid (EDTA) and sodium dodecyl sulfate (SDS) mixture. The mixture was then incubated with glutaraldehyde/ borate buffer at equal volume of at 37º C for one hour. From the incubated mixture, about 10 µl was taken in a glass slide. Then about 10 microliters of the incubated mixture was taken over a slide and the slide was examined under a microscope at 40X.

3.3.6 Hypo-osmotic swelling test

Membrane integrity of sperm was analysed by measuring the hypo-osmotic swelling (HOS) test. Sperm was separated by cauda epididymis. About 50µl of sperm suspension was added with 1 ml hypo-osmotic solution. Data was obtained by calculating the percentage of live sperm having coiled tail **(Jeyendran et al., 1992).**

3.4 Assessment of androgenic key enzymes

3.4.1 Assessment of testicular Δ5, 3β-hydroxysteroid dehydrogenase (HSD) activity

Activity of testicular Δ^5, 3β-HSD was assessed by following the standard method **(Talalay, 1962).** Testes were decapsulated and allowed for homogenization in a cold centrifuge at 4ºC in a homogenizing mixture containing spectroscopic grade glycerol (20%), 1mM EDTA and 5mM of potassium phosphate. Tissue concentration was maintained at 100 mg/ml. The total

mixture was allowed for centrifugation at 10,000×g for 30 min followed by collection of the supernatant. About 1 ml of supernatant was taken and added with about 1 ml of 100 μM of sodium pyrophosphate buffer having pH 8.9, 960 μl bovine serum albumin (BSA) and 40μl of ethanol. The total volume of the mixture was 3 ml. Total mixture was allowed for measuring the optical density (OD) after placing the supernatant in the cuvette by adding 100 μl of 0.5 μM nicotinamide adenine dinucleotide (NAD) against blank. Optical density was taken at 30 sec interval for 3 min.

3.8.5 Measurement of 17β hydroxysteroid dehydrogenase (HSD) activity

Testicular 17β-HSD activity has been assessed biochemically by specific method **(Jarabak, 1962).** Preparation of tissue sample was same as Δ5, 3β-HSD. About 1 ml supernatant was mixed with sodium pyrophosphate buffer of about 440 μM having pH 10.2, 0.3 μM testosterone provided by Sigma Chemical Company, St Loius, MO. USA and 40 μl ethanol and the total volume eof incubation mixture was 3 ml. Enzyme activity was analysed after adding about 100 μl NAD (0.5 μM) with the supernatant and OD was measured at 340 nm against a blank. OD was measured at 30 sec of interval for 3 min.

3.9 Measurement of testicular cholesterol

Testicular cholesterol has been measured by a standard protocol **(Plummer, 1995).** Tissue homogenate was prepared by using phosphate buffer (pH 7.0) having tissue concentration 50mg/ ml. 10 ml of alcohol – acetone mixture was added along with 0.2 ml tissue homogenate was added in a centrifuge tube. Tubes were then placed in a hot water bath at boiling temperature and it was allowed for boiling. The mixture was then cooled and allowed for centrifugation. The supernatant was subjected for evaporation. The residue was then dissolved in chloroform. Series of cholesterol standards were prepared. 2 ml of chloroform was taken in a blank marked test tube. About 2 ml of acetic anhydride sulfuric acid was incorporated in each test tube marked as sample standard and blank. All the tubes were kept

for 15 minutes in a dark place. Reading was noted at 680 nm. Ultimately, the cholesterol concentration was determined after preparation of standard curve.

3.10 Assay of serum testosterone

Level of serum testosterone was assessed by a testosterone kit of solid phase conjugate assay **(Srivastava, 2001)**. OD of standard and unknown samples were determined at 480 nm wavelength.

3.11 Measurement of seminal vascular fructose

Sperm was separate from cauda epididymis. Then sample was allowed for centrifugation for 10 min at 1500 rpm. Seminal plasma was separated from the supernatant. From the sample, about 20 µl of seminal plasma was diluted in 220 µl of distilled water. 50 µl of $ZnSO_4$ and 50 µl of NaOH were added with it in order to deproteinize the sample. The test tubes were stabilized for 15 min and then allowed for centrifugation at 2500 rpm for 15 min. About 200 µl of supernatant was used for analysis and reading was taken in 470 nm **(Lu et al., 1974)**.

3.12 Oxidative stress profile

3.12.1 Catalase activity

Testicular catalase activity was assessed by homogenizing the testes in solution of 0.05 M Tris-HCl buffer having pH-7.0. 50 mg/ml was considered as the tissue concentration. After that the homogenized tissue samples were allowed for centrifugation for 10 min at 10000 × g at 4 ºC. About 0.5 ml of H2O2 (0.00035 M) and distilled water were added about 2.5 ml in spectrophotometer cuvette and reading was taken at 240 nm. Then, supernatant was incorporated about 40 µl to the cuvette and OD was measured at 30 sec interval. Finally the result was expressed in terms of µM of H2O2 consumed / mg of tissue / min **(Beers and Sizer, 1952)**.

3.12.2 Activity of SOD

Superoxide dismutase activity in testicular tissue was analysed by standard protocol **(Marklund and Marklund, 1974)**. 2.04 ml (50 mM) Tris buffer (pH-8.2), 20 µl of pyrogallol and 20 µl of sample were added in a cuvette,. Reading was taken in a having absorbance 420 nm.

3.12.3 Testicular GST activity

Activity of testicular GST was assessed by standard protocol **(Habig et al., 1974).** In this purpose, 1-chloro-2, 4-dinitrobenzene (CDNB) was used as substrate. Total 3 ml of assay mixture was prepared that contained 0.1 ml (1 mM) CDNB dissolved in ethanol, 0.1 mM of 0.2 ml GSH, 2.7 ml of 100 mM potassium phosphate buffer having pH-6.5. About 0.1 ml of tissue supernatant was added with the assay mixture at the tissue concentration of 50 mg/ ml. CDNB, S-2, 4-dinitrophenylglutathione adduct was formed which was monitored by analysing the elevation in absorbance at 340 nm in respect to the blank. The result was expressed in terms of unit/mg of tissue.

3.12.4 Peroxidase activity assessment

Testicular peroxidase activity was analysed as per standard method **(Sadasivam and Manickam, 2008)**. The tissue concentration was 50 mg/ ml. Testicular tissue was allowed for homogenization in 0.1 M phosphate buffer saline having pH -7.0 in ice-cold temperature. In the next step, the sample was centrifuged at 4ºC at 10000 × g rpm for 10 minuted. About 0.1 ml supernatant was collected and 20 mM guiacol and 0.3 ml of 12.3 mM H2O2 were added with it. The absorbance was taken at 436 nm.

3.12.5 Estimation of conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) levels

Testicular tissues were allowed for homogenization at 50 mg/ ml in 0.1 M phosphate buffer having pH-7.4 in ice-cold condition. The total content of the homogenate was subjected for

centrifugation at 10000 × g at 4º C for 5 min. TBARS level was assessed by mixing 0.5 ml sample with 0.5 ml of normal saline and about 2 ml of TBA-TCA mixture that contains 0.392 gm thiobarbituric acid. 95% ethanol was added to that mixture to make the volume 100 ml and then allowed for boiling for 10 min. The mixture was then allowed for centrifugaton at 4000 × g for 10 min after it was cooled. Optical density was measured and read at 535 nm **(Okhawa et al., 1979)**.

Level of CD was analysed by standard protocol **(Slater, 1984)**. In this concern, mixture of Chloroform-methanol (2:1) was used to extract the lipid and allowed for centrifugation at 1000 × g for 5 min. The extracted chloroform was allowed for evaporation under nitrogen stream until it was dried. The residue was then dissolved in 1.5 ml cyclohexane and amount of the hydro peroxide that was formed was quantified by taking the optical density at 233 nm.

3.13 Toxicity profile

3.13.1 Activities of SGOT and SGPT

About 1 ml of substrate DL aspartate (200 mM/L) and α-keto glutarate (2 mM/L) were taken in a test tube. The reagent mixture was kept at room temperature at 37º C for 5 min. After that 0.2 ml of serum sample, 1 ml of 2, 4-dinitro-phenyl hydrazine were added to the test tub containing reagent mixture. Test tubes were kept in water bath and the temperature was fixed at 37ºC. The absorbance was taken at 505 nm using green filter against blank after it cooled down **(Henry et al., 1960).**

Activity of GPT was assessed by incubating the sample for 60 min. DL-alanine (200 mM/L) and α-ketoglutarate (2 mM/L) were used as substrate. Rest of the protocol was assessed as per the previous protocol **(Henry et al., 1960)**.

3.13.2 Activities of ALP and ACP

Tissues were homogenized with homogenizing medium (0.22 M Tris-HCL buffer, pH 7.5) in ice-cold condition. The tissue concentration was maintained by 20 mg/ ml. Then about 0.25

ml of homogenized tissue sample was kept in a centrifuge tube and 1 ml buffer (1 mM p-nitrophenol phosphate in 1 M Tris buffer, pH 8.0) was added with it. Total mixture was kept in water bath at 30ºC for 30 mins. The enzyme was assessed by spectrophotometer at 420 nm **(Malamy and Horecker, 1996)**.

Acid phosphatase activity was determined by using similar homogenizing medium and same tissue concentration as mentioned above. p-nitrophenol phosphate (pH 4.5) is used as a substrate. Optical density was measured similar as mentioned above **(Vanha –perttula and Nikkanen, 1973)**.

3.13.3 Assessment of serum urea, uric acid, creatinine and blood urea nitrogen (BUN) levels

In the test tubes marked as blank, test and standard, serum urea was added. 0.01 ml sample was added to the test marked test tubes. Working urea standard was added at about 0.01 ml and about 0.01 ml water was included to the test tubes labelled as blank. All the test tubes were mixed well and about 0.25 ml of Diacetylmonoxime was incorporated to those test tubes. All the test tubes were kept in hot water bath for10 minutes. The colour intensity of test tube containing sample was measured against standard and blank and the OD was taken at 525 nm in spectrophotometer (**Tiffany et al., 1972).**

Test tubes were marked as blank, standard and sample were taken. About 20 µl of uric acid standard was taken in that test tubes as provided in the kit and about 20 µl serum was also added in the sample marked test tubes. Uric acid monoreagent was incorporated at about 1000 µl. Total content of the test tubes were allowed for incubation at 37º C for about 5 min. Absorbance was taken at 550 nm against blank **(Kabasakalian et al., 1973).**

Serum creatinine and BUN were measured in spectrophotometer. Creatinine developed a yellow-orange complex in alkaline solution. The colour intensity which was developed was

directly proportional to the creatinine concentration. The OD was then measured at 520 nm **(Junge et al., 2004)**.

3.13.4 Serum total protein, serum albumin and serum globulin level

All the test tubes were marked as test, standard and blank. About 5 ml protein reagent was added to all the test tubes. Serum was added at about 0.05 ml to the test tube marked as test. Standard marked test tubes were added with 0.5 ml protein standard and then same amount of deionized water was added to the blank labelled test tube. All the test tubes were kept at room temperature for 10 minutes. The colour intensity of the test and standard was assessed with help of spectrophotometer at 530 nm in respect to blank **(Rodkey, 1965).**

Serum albumin was measured by following the standard protocol **(Reinhold, 1980).** Test tubes were taken marked as blank, test and standard. About 5 ml of albumin reagent was added to all the test tubes. 0.5 ml serum was included in test marked test tube. 0.5 ml of albumin and deionized was incorporated to the test tube marked as blank. All the content of the test tube was mixed thoroughly and allowed to keep it in room temperature. Reading was taken in 630 nm by using spectrophotometer.

Serum globulin was also measure with same procedure by the following the standard method (**Reinhold, 1980)**.

3.13.5 Levels of triglyceride, low density lipoprotein (LDL), very low density lipoprotein (VLDL), and high density lipoprotein (HDL)

Estimation of lipid profile was assessed by following the standard method **(Friedwald et al., 1972).**

Serum level of triglyceride, LDL, VLDL and HDL were assessed by following:

$$\text{Low density lipoprotein cholesterol (mg/dl)} = TC - \frac{(HDL + TG)}{5}$$

$$\text{Very low density lipoprotein cholesterol (mg/dl)} = \frac{TG}{5}$$

TC stands for total cholesterol and TG stands for triglyceride.

3.14 Estimation of androgenic, oxidative stress and apoptotic profile by reverse transcription polymerase chain reaction (qRT-PCR) analysis

Testicular tissue was considered for qRT-PCR analysis. 'High Pure Tissue RNA Kit' (Roche Diagnostic, Germany) guideline was followed for the extraction of mRNA from testicular tissue. About 2 µg of extracted total RNA was used for the synthesis of complementary DNA (cDNA) by following the instruction provided by 'Transcriptor First Strand cDNA Synthesis Kit' (Roche Diagnostic, Germany)**.**

Testicular Δ5, 3β-HSD, 17β-HSD, SOD, catalase, peroxidase, Bax, Bcl-2, caspase-3, caspase-8, caspase-9, cytochrome c gene expression was carried out by using Light Cycler 480 II (Roche Diagnostic, Mannheiem, Germany) and β- actin was considered as reference gene for this study

3.15 Protein expression of different markers by western blot analysis

Western blot analysis of Δ5, 3β-HSD, 17β-HSD, SOD, catalase, Bax, Bcl-2, caspase-3 protein expression in testicular tissue was as performed according to the standard protocol **(Maheshwari et al., 2009)**. Testes were brought out in the frozen condition and allowed for thawing in 3 ml ice-cold RIPA buffer which contains about 0.5% sodium deoxycholate, 1%

NP-40, 1% SDS in PBS with protease inhibitor at the concentration of one gram of tissue. Then tissue was subjected to homogenization by Dounce homogeniser and incubated on ice for half an hour. The lysates were centrifuged at 10000 × *g* for 10 min at 4°C. Bradford method was followed for the assessment of protein concentration. Gel electrophoresis was performed by resolving protein in 12.5% SDS polyacrylamide gel at 100 V. The gel was then transferred into nitrocellulose membrane with the help of transfer buffer (190 mM glycine, 25 mM Tris base, 20% methanol) for 1 h at 100V in the icy cold chamber. PBS (5% non fat milk and 0.05% Tween-80 (PBST) was used to block the membranes and then the membranes were subjected for overnight incubation at 4°C. Next day the membranes again allowed for incubation with horseradish peroxidase-conjugated goat anti-rabbit which was a secondary antibody at the ratio of 1 : 2000 dilutions followed by washing with phosphate buffer saline with Tween-80. β-actin was considered as control for the confirmation of equal protein loading.

3.16 In-Situ End Labelling Study

Testes were considered for the immunohistological study. In this purpose, testicular tissues were fixed with 10% formaldehyde solution. Testicular tissues were cut at 5μm thickness by 'Leica Semi auto microtome'. The tissue sections were deparaffinised followed by rehydration by using xylene and graded alcohol. Protocol for the imminohitochemical staining was conducetd as per the guideline provided by Travigen, Inc (Gaithersburg, MD) **(Shikone et al., 1994).** The quantification of ISEL positive cells was performed per field at 400X magnification. Ten fields were considered for counting the ISEL positive cell from each section. Then from that data, average of ISEL positive cells were calculated. Ultimately, the values were represented in percentage after the statistical analysis.

3.17 Flow cytometric analysis of sperm viability and sperm mitochondrial integrity

The protocol was followed as per the guideline provided in the kit from BD Accuri. Sperm viability was assessed by a fluorescent probe, propidium iodide, which binds with DNA. Propidium iodide can not enter the cell if the plasma membrane was intact and to stain the nucleus. Cells with damaged plasma membrane allowed propidium iodide to enter the cell and bind with the DNA that exhibited fluorescence red colour of the cells **(Graham et al., 1990)**.

Percentage of polarized and depolarized sperm was amalysed by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, BD Cat no 551302) staining provided in the kit. The procedure of sperm mitochondrial membrane integrity was determined by following the manual provided by BD **(Garner et al., 1997).**

3.18 Comet assay

About 25 ml sperm suspension was incorporated to the 75 ml of agarose (low melting point) in PBS at 37ºC. Total mixture was placed on a glass slide which was previously coated with 1 % agarose. Cover slip was placed on the top of the slide to trigger the solidification. Then the slides were allowed to soak in lysis buffer in icy cool condition. The slides were subjected to wash thrice in PBS buffer in room temperature The slides were incubate at 37ºC for 40 min. Again the slides were washed for two times and then placed in gel electrophoresis chamber (submarine, Bio-Rad, USA) containing alkaline electrophoresis buffer. The electrophoresis was conducted at 25 V for 30 minutes and the current was set to 300 mA. After the completion of the run, PBS was used in order to neutralise the slides. Slides were then stained by using 10 mg/ ml ethydium bromide. Finally, the slides were placed under fluorescence microscope (Nikon, Eclipse LV100 POL) **(Sing et al., 1988)**.

3.19 Determination of seminiferous tubular diameter (STD)

Testes were allowed for fixation in Bouin's solution followed by dehydration by using alcohol and xylene. The dehydrated tissues were then embedded in paraffin wax. Tissues were sectioned at 5 µm thickness and hematoxylin-eosin (HE) was used for staining purpose. The software named "Dewinter calliper pro 3.0" was used to determine the STD at 400 X **(Ghosh et al., 2014).**

3.20 Different generation of germ cell quantification

The slides containing testicular tissue, stained with haematoxylin–eosin were placed under light microscope. Quantification of different stages of spermatogenesis at stage VII of seminiferous epithelial cell cycle was performed as per the established protocol **(Leblond and Clermont, 1952)**.

3.21 Statistical analysis

All the collected data were subjected for the computation of mean, standard deviation and standard error. Analysis of variance (ANOVA) followed by "Multiple Comparison Two tail *t*-test" was conducted for statistical analysis **(Sokal and Rohle, 1997).** Result of the statistical analysis was expressed in mean ± SEM. Significant difference was considered at $p < 0.05$.