MATERIALS AND METHODS

4.1 Reagents

Green tea was collected from Institute of Himalayan Bioresource Technology (IHBT), Palampur, Himachal Pradesh. The composition of green tea is EC (Epicatechin) - 1.55%, EGCG (Epigalloy catechin gallate) - 9.00%, ECG (Epicatechin gallate) - 4.8%, EGC (Epigalloy catechin) - 5.0%, Caffeine- 2.38%, as mentioned by the manufacturer.

Black tea was collected from Toklai Tea Research Centre, Jorhat, Assam. The composition of black tea is theaflavins (TF) - 1.56%, thearubigins (TR) - 11.95%, as mentioned by the manufacturer.

NAD, NADPH, β-Estradiol (98%), corticosterone were purchased from Sigma Chemical Company, St. Louis, M. O., USA. EDTA was purchased from E-merck, Mumbai, India. All other reagents were purchased from Sisco Research Laboratories (SRL), Mumbai, India and were of analytical grade. SDS was purchased from LOBA Chemie Pvt. Ltd., Mumbai, India. Goat anti-rabbit γ-globulin, polyethelene glygol and ehloramin T were purchased from Sigma Chemical Company, USA. DMSO was purchased from Sigma Chemical Company, USA.

4.2 Tea Extracts Preparation

Preparation of aqueous extract of green and black tea was done following the method of Wei et al. (1999). Briefly, 2.5g Green tea was added to 100ml of boiling water and was steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 100ml of boiling water and filtered, and two filtrates were combined to obtain a 1.25% tea aqueous extract (1.25% tea leaf / 100ml water). Similarly same procedure as above was performed with 5gm green tea and 10g green tea to prepare 2.5% and 5.0% aqueous green tea extract respectively (GTE). 1.25%, 2.5% and 5.0% aqueous black tea extract (BTE) were prepared in the similar way of GTE preparation. Doses were marked as mild (1.25g% ≈ 5cups of tea/day/ individual), moderate (2.5g% ≈ 10cups of tea/day/ individual) and high (5.0g% ≈ 20 cups of tea/day/ individual). All GTE and BTE were fed orally to animals at a dose of 1ml/ 100g body weight.
4.3 Animal maintenance and treatment

Adult (90±10 days) male albino rats (Rattus norvegicus) of Sprague Dawley strain weighing 200±10g were collected from Indian Institute of Chemical Biology (IICB), Kolkata for the present study. The animals were maintained as per National Guidelines and Protocols, approved by the Institutional Animal Ethics Committee (IAEC). The animals were housed in clean polypropylene cages and maintained in a controlled environment (temperature 22±2°C and relative humidity 40-60%) in an animal house under a photoperiod of 12 h of light and 12 h of darkness. The animals were fed on standardized normal diet (20% protein) which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined til oil and 0.25% shark liver oil and water ad libitum (Chandra et al., 2004).

Each control and experimental group consisted of 8 rats. Body weights were measured every seven days. Treatment for 26 days was selected for all experimental set, because the duration of one seminiferous cycle is 13.2 days in albino rats (Sarkar et al., 2003). All the animals were sacrificed 24h after the last treatment following protocols and ethical procedures. Blood samples were collected from the hepatic portal vein under light ether anesthesia. Serum samples were separated by centrifugation, frozen and stored at -20°C for different hormone assay.

4.4 Body and organ weight

The body weights of the experimental animals were recorded on the first day before treatment with tea extracts and the day of sacrifice. The testicles and accessory sex organs viz. seminal vesicles, ventral prostate, cauda epididymis, coagulating gland were dissected out, trimmed off the attached tissues and weighed. The relative weight of organs was expressed as per 100g body weight. The left testis of each rat was used for other biochemical measurements.

4.5 Histopathological study of testis

Immediately after removal, the testis were fixed in Bouin’s fluid and embedded in paraffin. Section of 5μ thickness were taken from the mid portion of each testis and stained with hematoxylin-eosin and examined under a light microscope. Characterization of the stages of spermatogenesis is based upon the association of cell groupings formed by spermatogonia,
spermatocytes and spermatids components of germinal epithelium. This results in distinct series of cellular association of constant composition which has been considered as stages of a cycle of the seminiferous epithelium. In rat, 14 such stages have been described (Leblond & Clermont, 1952). This cycle has been defined as a complete series of cell associations appearing successively in any given area of the seminiferous epithelium.

In the present investigation, cells were counted only in one of the 14 stages of the cycle i.e. stage VII. This particular stage is composed of type-A spermatogonia (ASg), preleptotene spermatocytes (pLSc), mid pachytene spermatocytes (mPSc) and step 7 spermatids (7Sd). Over the duration of one cycle type-A spermatogonia is transformed to type-B spermatogonia which further divide to produce spertocytes. The young preleptotene spermatocytes may be seen along the basement membrane with their nuclei containing crust-like chromatin. Spermatocytes of the older generation are at the midpachytene stage. Inwardly there were spermatids in which the head cap covers half of the nucleus. These are lined up along the lumen side of the seminiferous epithelium but not regularly. The counts of germ cells at stage VII of the cycle are representative of the condition of spermatogenesis as a whole (Clermont & Harvey, 1967). The nuclei of different germ cells were counted 20 round tubules of each rat.

**Calculation of true count**

All the counts of the different varieties of the germ cells were corrected for differences in their nuclear diameter according to Abercrombie’s formula (Abercrombie, 1946)

\[
TC = \frac{[CC \times S]}{S + D}
\]

Where TC = true count

- **CC** = Crude count, i.e., counts of all identifiable pieces of nuclei of a particular variety of germ cells
- **S** = Section thickness (5 \(\mu\)m in the present investigation)
- **D** = Nuclear diameter of cells (measured with Leika micrometer)
Plate - 1 Photomicrograph of HE-stained rat seminiferous tubule at stage VII of spermatogenesis

ASg = Spermatogonia A

pLSc = Preleptotene spermatocyte

mPSc = Mi pachytene spermatocyte

7Sd = Step 7 spermatid

HE = Hematoxylin Eosin
Correction of tubular shrinkage

Since there was a possibility of variable tubular shrinkage in the testicular sections of various green and black tea extracts and vehicle treated groups, an additional correction for the true count had been performed. The index of tubular shrinkage was obtained from the average number of Sertoli cells containing prominent nucleoli in each seminiferous tubule at stage VII. It appears that the sertoli cell number do not change their by mitosis or degeneration although they may have changed in volume. Therefore any change in their number per tubular cross section is indicative of a change in volume of the tubule, an increase in Sertoli cell number revealing shrinkage of the tubules. Stage VII of spermatogenesis was analyzed because this stage is highly susceptible to testosterone deficiency (Russel et al., 1987) and also reflects the final stages of spermatid maturation and thus provides evidence of spermatogenesis as a whole (Clermont & Harvey, 1967). The index of tubular shrinkage can be obtained by using the formula (Clermont & Morgentalor, 1955; Clermont & Harvey, 1967) as follows

\[
\text{Tubular Shrinkage} = \frac{\text{Average number of Sertoli cell nuclei per tubular cross section in control rats}}{\text{Average number of Sertoli cell nuclei per tubular cross section in experimental rats}}
\]

When
\[
\frac{\text{Average number of Sertoli cell nuclei per tubular cross section in control or in experimental rats}}{\text{Total number of counted tubular cross sections}} = \frac{\text{Total number of Sertoli cell nuclei in all counted tubular cross sections}}{\text{Total number of counted tubular cross sections}}
\]

Calculation of percentage of spermatid degeneration

Analysis of the spermatogenic process revealed that each resting spermatocyte enters into the long lasting prophase of the meiotic division, which after two successive reduction divisions forms four spermatids. Therefore, the ratios of resting spermatocytes to spermatids should theoretically be 1:1 and 1:4 respectively. If there is any alteration in any one or both of the ratios, it is indicative of degeneration of specific germ cell type (Clermont &
Morgentalor, 1955). The percentage of spermatid generation is calculated by the following formula

\[
\text{Percentage of spermatid degeneration} = 100 - \frac{\text{Spermatid count} \times 100}{\text{Pachytene count} \times 4}
\]

**Calculation of effective degeneration of spermatid**

Theoretically, each pachytene spermatocyte, after two successive reduction divisions, forms four spermatids. Therefore the mPSc to 7Sd ratio should be 1:4 (Clermont & Morgentalor, 1955). However, practically this ratio is to be less due to spontaneous degeneration of spermatids for example, if the ratio is 1: x, where x is a figure less than four, then,

\[
\text{Spermatid degeneration} = 100 - \left[\frac{x}{4} \times 100\right]
\]

The percentage of 7Sd degeneration was calculated from this ratio. Subtraction of the percentage of 7Sd degeneration in vehicle-treated rats showed the effective percentage of spermatids degeneration. Therefore,

\[
\text{Effective spermatid degeneration} = \left[\text{Spermatid degeneration in experimental group}\right] - \left[\text{Spermatid degeneration in experimental group}\right]
\]

**4.6 Epididymal Sperm count**

The sperm count was determined by counting mature sperm in a haemocytometer following the method of Majumdar and Biswas (1979). To minimize error, the count was repeated at least five times for each rat by different workers.

**Reagents**

5% fructose solution in phosphate buffer saline (pH 7.4)

**Procedure**

1. Sperm samples were collected from the cauda epididymis.

2. Same weight of cauda epididymis were taken, trimmed off attached fat and placed in a small beaker/ vial containing 2ml of 5% fructose in phosphate buffer saline.
3. The cauda epididymis was minced by fine scissors.

4. The beaker/vial was kept in incubator at 37°C temperature for 1 hour for better release of sperm into the solution.

5. The beaker was shaken gently to give a homogeneous concentration of spermatozoa.

6. A drop of suspension was placed on the hemocytometer and covered with a cover slip.

7. The number of spermatozoa in five small squares was counted.

8. To minimize error each count was repeated at least five times.

4.7 ELISA of serum testosterone

Serum testosterone was assayed using ELISA kit obtained from Dia Metra, S.r.l. Italy (code no. DKO002). This testosterone ELISA kit is based on the competition principle and the micro-titer plate separation. Anti-testosterone antibodies are immobilized on microwell plates. Testosterone in the sample competes with HRP-labeled testosterone for binding to the immobilized antibody. After washing, enzyme substrate is added. The amount of testosterone in the sample is inversely proportional to the enzyme activity. The reaction is terminated by adding stopping solution. Absorbance is measured on a plate reader. The color intensity is inversely proportional to the testosterone concentration in the sample.

Contents of Kit and reagent preparation

1. Micro-well Plate-1 microtiter plate 12x8 wells strips coated with Anti-testosterone IgG.

2. Enzyme conjugate- 1 vial of 12ml proteic buffer solution containing testosterone conjugated to the enzyme Horse-Radish-Peroxidase (HRP).

3. TMB substrate solution- 1 vial of 12ml, containing solution of tetramethylbenzidine (TMB) with activators and stabilizers, diluted in phosphate/citrate buffer.

4. TMB stop solution- 1 vial of 12ml, containing .3M sulfuric acid (H₂SO₄).

5. Standars- 5 vials of 1ml each, the concentration of the standards in ng/ml 0, 0.2, 1.0, 4.0, 16.0.
Assay Procedure

1. 25 µl of standards was dispensed into appropriate wells (Anti-testosterone IgG coated well).

2. 25 µl of sample was dispensed into selected wells. Time between distribution of first standard and last sample was up to 10 min without affecting the result and then incubated for 5 min at room temperature.

3. 100 µl of enzyme conjugate was dispensed into each well and strips were covered with adhesive film.

4. The plate was thoroughly mixed and incubated for 60 min at 37°C.

5. The adhesive film was peeled out and aspirated the reaction solution from all wells.

6. The wells were washed with 300 µl of distilled water. The washing procedure was repeated by draining the water completely.

7. 200 µl of TMB substrate solution was added to each well, at timed intervals.

8. Incubated for 15 min at room temperature, protected from light.

9. The enzymatic reaction was stopped by adding 100µl of TMB stop solution to each well at the same timed intervals as in step 7 and the absorbance of each well was determined at 450nm, within 30 min following step 9 in ELISA Reader (Merck).

10. The sensitivity of the testosterone assay was 0.075ng/ml and intra and inter-run precision had a coefficient of variation of 4.6% and 7.5% respectively.

4.8 Spectrofluorometric assay of serum corticosterone (Glick et al., 1964; Silber, 1966)

Reagents

1. Isooctane (2, 2,4- triethylpentane)
2. Chloroform
3. 0.1N sodium hydroxide
4. Sulfuric acid
5. Dehydrated alcohol

Procedure

1. Preparation of acid alcohol solution. 35ml distilled dehydrated alcohol was taken in a 250ml volumetric flask. The flask was kept in an ice-bath and was allowed to
cool. Then 65ml concentrated sulfuric acid was added slowly and stoppered tightly. It was mixed by slow inversion. This was prepared freshly each day.

2. 0.5ml serum was taken in a 10ml glass-stopper extraction tube.

3. For blank and standard preparation, 0.5ml distilled water and 0.4 and 0.8µg standard/0.5ml distilled water (standard corticosterone solution having 1.6 µg/ml concentrations) respectively were taken in separate extraction tubes.

4. In each tube, 1.5ml iso-octane was added, mixed thoroughly and was centrifuged (3000rpm for 15 min) for separating the layers.

5. The top iso-octane layer was discarded by aspiration.

6. Then 0.25 ml of 0.1N sodium hydroxide was added to each tube and was mixed and centrifuged at 3000 rpm for 15 min.

7. The top aqueous layer was discarded by aspiration.

8. Then 3ml of chloroform was added to each tube, mixed thoroughly and kept for 10 min.

9. From each tube 2.5ml of the above chloroform mixture was taken separately to 5ml glass tube containing 3ml of acid alcohol mixture.

10. It was mixed thoroughly and centrifuged at 3000rpm for 5 min. Then 2.5ml of the bottom layer from each tube was taken in the cuvette and was kept for 45 min.

11. The fluorescence was measured with the spectrofluorometer (Perkin-Elmer MPF-44B Fluorescence Spectrophotometer) at 462 nm (excitation), 518 nm (emission) by setting the instrument at a spectrofluorometric reading 80 with a standard corticosterone solution having 1.6g/ml concentrations.

12. Then the concentration of serum corticosterone was determined by interpolation from the standard curve using the blank corrected transmittance. The obtained result was expressed as µg/dl serum.

4.9 Assay of steroidogenic enzymes activities

4.9.1 Determination of testicular/adrenal Δ⁵ 3β-hydroxysteroid dehydrogenase enzyme activity

Principle

The activity of this enzyme was measured by the method of Talalay (1962) by optical measurement (absorbance at 340nm) of the rate of reduction of pyridine nucleotides (NAD).
Reagents
1. Spectroscopic grade glycerol (20%)
2. Dipotassium hydrogen phosphate (K$_2$HPO$_4$) (0.05mM)
3. Sodium pyrophosphate buffer (100 μM, pH 8.9)
4. Ethylenediamine tetra acetic acid (EDTA) (0.01mM)
5. 17β-estradiol (98%)
6. Nicotinamide adenine dinucleotide (NAD) (0.5μM)

Preparation of Homogenizing Medium
- Spectroscopic grade glycerol : 20ml
- K$_2$HPO$_4$ (0.05mM) : 10ml
- EDTA (0.01mM) : 10ml
- Distilled water : 60ml
The final pH of the medium was adjusted to 7.4 by addition of 0.1N NaOH.

Tissue Extraction
1. Testis/ adrenal was removed from the animal and immediately frozen.
2. Decapsulated testis (in case of adrenal 3-4 of total gland) was taken in icecold homogenizing medium making a tissue concentration of 100mg/ml and was homogenized using Potter-Elvehjem glass homogenizer.
3. The homogenate was centrifuged at 10000rpm for 30min at 4°C in a cold centrifuge (REMI, C 40).
4. The supernatant was used for assay of enzyme activity.

Procedure
1. The assay was performed in spectrophotometer cuvette.
2. The reaction medium contained 1ml of 100μM sodium pyrophosphate buffer (pH 8.9), 20μl of 30μg 17β-estradiol and 200μl tissue supernatant.
3. The reaction was initiated by adding 1ml of 0.5 μM of NAD.
4. The rate of formation of reduced nucleotide was measured in a UV spectrophotometer (UV-1240 Shimadzu, Japan) at 340nm at 15sec intervals for 3min against a blank without NAD.
5. One unit of enzyme activity is defined as the change in absorbency of 0.001 per min at 340nm wavelength and 1cm light path at 25°C.

6. The values obtained with estradiol-17β as substrate was multiplied by 7.5 as estradiol-17β is oxidized at a maximal rate of only 13.3% of that of testosterone.

4.9.2 Determination of testicular 17β-hydroxysteroid dehydrogenase enzyme activity

Principle

The activity of this enzyme was measured by the method of Jarabak et al. (1962) by optical measurement (absorbance at 340nm) of the rate of reduction of pyridine nucleotides (NAD).

Reagents

1. Spectroscopic grade glycerol (20%)
2. Dipotassium hydrogen phosphate (K₂HPO₄) (0.05mM)
3. Sodium pyrophosphate buffer (440 μM, pH 8.9)
4. Ethylenediamine tetra acetic acid (EDTA) (0.01mM)
5. 17β-estradiol (98%)
6. Nicotinamide adenine dinucleotide (NAD) (1.35μM)
7. Crystalline bovine serum albumin (BSA) (5%)

Preparation of Homogenizing Medium

Spectroscopic grade glycerol : 20ml
K₂HPO₄ (0.05mM) : 10ml
EDTA (0.01mM) : 10ml
Distilled water : 60ml
The final pH of the medium was adjusted to 7.4 by addition of 0.1N NaOH.

Tissue Extraction

1. Testis/ adrenal was removed from the animal and immediately frozen.
2. Decapsulated testis (in case of adrenal 3-4 of total gland) was taken in ice cold homogenizing medium making a tissue concentration of 100mg/ml and was homogenized using Potter-Elvehjem glass homogenizer.
3. The homogenate was centrifuged at 10000 rpm for 30 min at 4°C in a cold centrifuge (REMI, C 40).
4. The supernatant was used for assay of enzyme activity.

Procedure
1. The assay was performed in spectrophotometer cuvette.
2. The reaction medium contained 1.5 ml of 440 μM sodium pyrophosphate buffer (pH 8.9), 0.5 ml of 5% BSA (25 mg crystalline BSA) 40 μl of 0.3 μM 17β-estradiol and 200 μl tissue supernatant.
3. The reaction was initiated by adding 1 ml of 1.35 μM of NAD.
4. The rate of formation of reduced nucleotide was measured in a UV spectrophotometer (UV-1240 Shimadzu, Japan) at 340 nm at 15 sec intervals for 3 min against a blank without NAD.
5. One unit of enzyme activity is defined as the change in absorbency of 0.001 per min at 340 nm wave length and 1 cm light path at 25°C.

4.10 Radioimmuneassay (RIA) of Luteinizing hormone (LH), Follicle stimulating hormone (FSH)

Serum levels of FSH and LH were assayed by RIA (Moudgal & Madhawa, 1974)

4.10.1 Assay of serum LH level

Preparation of iodinated LH

Reagents
1. $^{125}$I with specific activity about 500 mCi/mg for hormone iodination
2. 0.05 M phosphate buffer (0.5 M Na$_2$HPO$_4$, 0.5 M NaH$_2$PO$_4$, 0.14 M NaCl; pH 7.5)
3. 0.5 M phosphate buffer (0.5 M Na$_2$HPO$_4$, 0.5 M NaH$_2$PO$_4$; pH 7.5)
4. Chloramines-T solution (1 mg/ml of 0.05 M phosphate buffer)
5. Sodium metabisulfite (Na$_2$S$_2$O$_5$) solution (2 mg/ml of 0.05 M phosphate buffer)
6. Rat LH [NIDDK-r LH-I-10 (AFP-11536B)]
7. Reference of rat LH [NIDDK-r LH-RP-3 (AFP-7187B)]
8. Antisera to LH [NIDDK-anti-r LH-S-11]
9. Second antibody was goat anti-rabbit γ-globulin

[56]
10. Bovine serum albumin (BSA)
11. Transfer solution (bromophenol blue in 16% sucrose-KI solution) and rinse solution (bromophenol blue in 8% sucrose-KI solution)

**Procedure for iodination**

1. 1mCi $^{125}$I was taken in a small polyethylene tubes
2. 25µl of 0.5M phosphate buffer, pH 7.5 was added
3. 20µl (2.5 µg/25 µl) of rat LH-I-10 was added
4. 20µl (1mg/ml) chloramines-T solution was then added and mixed. 20µl (2mg/ml) of Na$_2$S$_2$O$_5$ solution was added after 40 seconds
5. After 30 seconds 3 drops of transfer solution was added and immediately loaded the mixture on Sephadex G-75 column (column length 10cm) and the column was washed with 3 drops of rinse solution
6. Column was eluted with 0.05M phosphate buffer, pH 7.5
7. Aliquots of 0.5ml were collected in a small RIA tubes containing 0.5ml of 2.5% BSA in 0.05M phosphate buffer saline (PBS)

Two peaks of radioactivity were found. The first peak began from 6$^{th}$ tube and ended in 10$^{th}$ tubes while second peak began at tube 11. The iodinated LH was present in the first peak while in the second peak free $^{125}$I was found (Greenwood *et al.*, 1963).

**Procedure for estimation of serum LH by double antibody radioimmunoassay**

1. 0.1ml buffer (0.05M) was taken in 10 x 75mm RIA tubes.
2. 0.1ml serum was added in the tube marked as sample and for standard curve, reference solution (NIDDK-rLH-RP-3 dissolve in 1% BSA phosphate buffer solution) was added in different doses ranging from 25 to 2.5ng per tube and marked as standard.
3. To each tube 0.1ml of iodinated LH (300000cpm) was added.
4. Then in each tube, 0.1ml of 1:75, 000 dilutions of LH antisera (NIDDK-anti-rLH-S-11) was added. Dilution was made with 3% of normal rabbit serum in 0.05M EDTA-PBS (phosphate buffer saline), 0.1% sodium azide.
5. The mixture was mixed with a vortex stirrer and was incubated for 24hr at room temperature.
6. At the end of the incubation, 0.1ml of goat anti-rabbit γ-globulin (sufficient concentration, dissolved in PBS) was added.
7. After 2hr incubation 1ml of 20% polyethylene glycol (PEG) was added. The mixture was mixed properly by vortex mixture and was kept for 1hr incubation at room temperature.
8. At the end of the incubation period all tubes were centrifuged at 4000rpm for 15min at 4°C. The supernatant was discarded and the precipitates were counted in auto gamma counter for 60 sec duration.
9. The unknown samples were compared to percentage counts precipitated with the rat LH.

The intra-assay variation for FSH and LH was 5.0 and 4.5%, respectively. All samples were run in one assay to avoid inter-assay variation.

4.10.2 Assay of serum FSH level

Preparation of iodinated FSH

Reagents
1. $^{125}$I with specific activity about 500mCi/mg for hormone iodination
2. 0.05M phosphate buffer (0.5M Na$_2$HPO$_4$, 0.5M NaH$_2$PO$_4$, 0.14M NaCl; pH 7.5)
3. 0.5M phosphate buffer (0.5M Na$_2$HPO$_4$, 0.5M NaH$_2$PO$_4$; pH 7.5)
4. Chloramines-T solution (1mg/ml of 0.05M phosphate buffer)
5. Sodium metabisulfite (Na$_2$S$_2$O$_5$) solution (2mg/ml of 0.05M phosphate buffer)
6. Rat LH [NIDDK-r FSH-I-9 (AFP-12828B)]
7. Reference of rat LH [NIDDK -r FSH-RP-2 (AFP-4621B)]
8. Antisera to LH [NIDDK-anti-r FSH-S-11]
9. Second antibody was goat anti-rabbit γ-globulin
10. Bovine serum albumin (BSA)
11. Transfer solution (bromophenol blue in 16% sucrose-KI solution) and rinse solution (bromophenol blue in 8% sucrose-KI solution)

Procedure for iodination
1. 1mCi $^{125}$I was taken in a small polyethylene tubes
2. 25μl of 0.5M phosphate buffer, pH 7.5 was added
3. 20μl (2.5 μg/25 μl) of rat FSH-I-9 was added
4. 20μl (1mg/ml) chloramines-T solution was then added and mixed. 20μl (2mg/ml) of Na₂S₂O₅ solution was added after 30 seconds
5. After 30 seconds 3 drops of transfer solution was added and immediately loaded the mixture on Sephadex G-75 column (column length 10cm) and the column was washed with 3 drops of rinse solution
6. Column was eluted with 0.05M phosphate buffer, pH 7.5
7. Aliquots of 0.5ml were collected in a small RIA tubes containing 0.5ml of 2.5% BSA in 0.05M phosphate buffer saline (PBS)

Two peaks of radioactivity were found. The first peak began from 7th tube and ended in 9th tube, while the second peak began at tube 10. The iodinated FSH was present in the first peak while in the second peak free ¹²⁵İ was found (Greenwood et al., 1963).

**Procedure for estimation of serum FSH by double antibody radioimmunoassay**

1. 0.1ml buffer (0.05M) was taken in 10 x 75mm RIA tubes.
2. 0.1ml serum was added in the tube marked as sample and for standard curve, reference solution (NIDDK-rFSH-RP-2 dissolved in 1% BSA phosphate buffer solution) was added in different doses ranging from 25 to 2.5ng per tube and marked as standard.
3. To each tube 0.1ml of iodinated LH (300000cpm) was added.
4. Then in each tube, 0.1ml of 1:50, 000 dilutions of FSH antisera (NIDDK-anti-rFSH-S-11) was added. Dilution was made with 3% of normal rabbit serum in 0.05M EDTA-PBS (phosphate buffer saline), 0.1% sodium azide.
5. The mixture was mixed with a vortex stirrer and was incubated for 24hr at room temperature.
6. At the end of the incubation, 0.1ml of goat anti-rabbit γ-globulin (sufficient concentration, dissolved in PBS) was added.
7. After 2hr incubation 1ml of 20% polyethylene glycol (PEG) was added. The mixture was mixed properly by vortex mixture and was kept for 1hr incubation at room temperature.
8. At the end of the incubation period all tubes were centrifuged at 4000rpm for 15 min at 4°C. The supernatant was discarded and the precipitates were counted in an auto gamma counter for 60 sec duration.

9. The unknown samples were compared to percentage counts precipitated with the rat FSH.

The intra-assay variation for FSH was 5.0%. All samples were run in one assay to avoid inter-assay variation.

### 4.11 Determination of protein

#### Principle

Proteins were estimated by the method of Lowry et al. (1951). When protein is placed in an alkaline solution containing Cu²⁺, a weak colored complex can form between the peptide bonds in the protein and copper atom. It is thought that this binding involves the reduction of Cu²⁺ to Cu⁺. This "Biuret" reaction has been used for several decades to estimate the quantity of protein in samples. The reaction is not very sensitive and large quantities of protein have to be used to get accurate results. Lowry et al. (1951) added Folin's reagent to the Biuret assay, gently improving its sensitivity. The Lowry method combines the biuret reagent with another reagent (Folin-Ciocalteu's phenol reagent) that reacts with tyrosine and tryptophan residues in proteins. This gives a bluish color which can be read somewhere between 500-750nm depending on the sensitivity required.

#### Reagents

1. Bovine serum albumin (BSA) - 10mg BSA in 10ml 0.9% sodium chloride (NaCl) solution was prepared one day prior to measurement.
2. 0.9% sodium chloride (NaCl) solution.
3. Sodium carbonate (Na₂CO₃) - Na₂CO₃ solution was prepared with 10g of Na₂CO₃ dissolved in 100ml of distilled water, 50ml of 0.1N NaOH was added to the solution and then the volume was made upto 500ml.
4. 1% sodium potassium tartarate.
5. 0.5% copper sulphate.
6. Alkaline copper reagent was prepared freshly by mixing 1% sodium potassium tartarate and 0.5% copper sulphate in 1:1 proportion and then 1ml of the above solution was mixed with 50ml of Na₂CO₃ solution.
7. Folin Ciocalteu’s reagent was prepared in 1:1 dilution with distilled water.
Procedure

1. From BSA solution 25, 50, 100, 150 and 200μl standards were taken and mixed with 0.975, 0.950, 0.900, 0.850 and 0.800ml of NaCl solution; in blank 1ml of NaCl solution was taken for the measurement of tissue protein 25μl of sample was taken and mixed with 0.975ml of NaCl solution.

2. 5ml of freshly prepared alkaline copper reagent was added to each sample, standard and blank, mixed well in vortex and kept for 15min.

3. 0.5ml of 1:1 freshly prepared Folin Ciocalteu’s reagent was added to each sample, standard and blank, mixed well in vortex and kept for 30min.

4. After 30 min, optical density was measured at 660nm in a spectrophotometer.

5. Then the concentration of serum protein was determined by interpolation from the standard curve using the blank corrected transmittance. The obtained result was expressed as μg/dl serum.

4.12 Statistical analysis

Results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) test was first carried out to test for any differences between the mean values of all groups. If difference between groups were established, the values of the treated groups were compared with those of the control group by a multiple comparison t-test. A value of P<0.05 was interpreted as statistically significant (Fisher, 1974).
Effects of Green and Black Tea Extracts (GTE & BTE) at different doses for different durations on testicular and adrenocortical activities in in vivo studies
Experimental design of \textit{in vivo} studies

The adult male Sprague Dawley 112 (one hundred twelve) rats were divided into two equal groups with 56 (fifty six) in each group, considering the duration of treatment for 13 days and 26 day respectively. Each group was further divided into three sub-groups – Control, GTE treated and BTE treated.

Sixteen animals (eight for 13days and eight for 26 days treated group) from the Control sub-group were fed sterile distilled water orally daily for 13 and 26 days as vehicle treated.

GTE treated sub-group was further divided into three groups (eight rats in each sub-group), they received oral administration of Green Tea Extracts (GTE) at the doses of Mild (1.25g %), Moderate (2.5g %) and High (5.0g %) at 1ml/100g body weight/day respectively for 13 and 26 days.

BTE treated sub-group was also further divided into three groups (eight rats in each sub-group); received oral administration of Black Tea Extracts (BTE) at the doses of Mild (1.25g %), Moderate (2.5g %) and High (5.0g %) at 1ml/100g body weight/day respectively for 13 and 26 days.

All the animals were sacrificed 24h after the last day of treatment following standard protocols and ethical procedures.

Blood samples were collected from the hepatic portal vein under light ether anesthesia. Serum samples were separated by centrifugation, frozen and stored at -20°C for hormone assay.

The testis and accessory sex organs were dissected out, trimmed off the attached tissues and weighed. The left testis of each rat was fixed immediately for histological study and the right for other biochemical estimations.

All the methods used for studying different parameters have been described in Materials and Methods Section.

Different parameters, which have been studied under \textit{in vivo} section, are described in the following Chapters of this Section (Chapter 5-9).