Materials & Methods
3.1 CHEMICALS

Dulbecco's Modified Eagle's Medium, Nutrient Mixture, F-12 Ham (DME/F-12 1:1 mixture), collagenase (type IV), Percoll, bovine serum albumin (BSA), fetal bovine serum (FBS), penicillin-streptomycin solution, amphotericin-B solution, deoxyribonuclease, trypsin inhibitor, RNase ZAP, propranolol, testosterone and androstenedione were purchased from Sigma Chemical Co., USA. Recombinant human chorionic gonadotrophin (hCG) (iodination grade, Lot No. CR-127), and ovine luteinizing hormone (oLH) (biological grade, Lot No. AFP5551B) were provided by NIDDK, USA as a gift. Agarose and ethidium bromide were purchased from US Biologicals, USA. Total RNA isolation reagent (TRIR) was obtained from AB gene, UK. One step RT-PCR kit was purchased from Qiagen, Germany. Primers for human and rat LH receptors and housekeeping genes (human β-actin and rat RPS-16) were procured from Alpha DNA, Canada. Sephadex G-25 column was obtained from Supelco, USA. Radioactive iodine-[^{125}I] was obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai, India. \[^{3}H\]-cAMP assay system, \[^{1},2,6,7\]^{3}H]-androst-4-ene-3, 17β-dione, \[^{1},2,6,7\]^{3}H]-testosterone and 2-keto-[1-^{14}C]-isocaproic acid were purchased from Amersham Biosciences, UK. \[^{26,27-3}H\]-25-Hydroxycholesterol was obtained from NEN Life science products, USA. Testosterone and estradiol radioimmunoassay (RIA) kits, follicle-stimulating hormone (FSH) and
luteinizing hormone (LH) immunoradiometric assay (IRMA) kits were purchased from Diasorin, Italy. All other chemicals and reagents used were of analytical grade obtained from Sisco Research Laboratory (SRL), Mumbai, India.

### 3.2 SOURCE OF HUMAN TESTIS

After getting ethical committee clearance and consent from the patients, testicles removed from men aged between 60 and 70 years for prostatic carcinoma, when indicated for the treatment were obtained from Madras Medical College and Hospitals and Government Stanley Medical College, Chennai. During the period of study, thirty pairs of testes from 30 patients were collected and used for the isolation of Leydig cells. Prior to orchidectomy, blood was collected from the patients, sera separated and used for the assay of serum hormones such as LH, FSH, testosterone and estradiol to evaluate testicular function. Testis from the patients who have not received any treatment and not had any endocrine or metabolic diseases or systemic illness known to affect Leydig cell function only were used.

### 3.3 ASSAY OF SERUM HORMONES

Serum LH and FSH were assayed using immunoradiometric assay (IRMA) kits, and testosterone and estradiol were assayed using solid phase radioimmunoassay (RIA) kits obtained from Diasorin, Italy.
3.3.1 Luteinizing hormone (LH)

Principle

This assay is based on the use of antibody-coated tubes, employing two mouse monoclonal antibodies directed against two different epitopes on the LH molecule. Monoclonal IgG to LH is coated on the inner surface of the tubes and labeled monoclonal IgG to LH is used as tracer. The assay features a single incubation during which LH contained in standards or samples to be assayed binds to the solid phase anti-LH antibody. The LH/anti-LH complex thus formed allows the $[^{125}\text{I}]$-tracer to bind to the solid phase. After the incubation, the amount of labeled antibody bound to the solid phase is proportional to the amount of LH present in standards or samples. At the end of the incubation, the unbound material is removed by aspiration and washing.

Reagents

1. Tracer: A vial contains 5.5 ml of buffered $[^{125}\text{I}]$ labeled IgG to LH (mouse monoclonal).

2. LH standards: A set of seven vials containing LH standards ranging in concentration from 0 to 180 IU/L, serum and preservatives was provided. Each vial was reconstituted with 1 ml of double distilled water 30 min before use.
3. Antibody coated tubes: The tubes coated with IgG to LH (Mouse monoclonal antibody directed against LH epitope that is different from the one against which the IgG used for the tracer is raised) in the inner surface were provided.

4. Wash solution: The 50 ml saline solution containing detergent was made up to 1000 ml with distilled water.

5. Control serum: The vial containing LH, human serum and preservatives was reconstituted with 1 ml distilled water 30 min before use.

Procedure

All the reagents were brought to room temperature at the time of assay. The assay was carried out in duplicate with total count, non-specific binding, maximum binding, standards and unknown serum sample tubes.

1. Plain 12 x 75 mm polypropylene tubes in duplicate were used for total count and non-specific binding.

2. LH antibody coated tubes were used for maximum binding, standards and unknown serum samples.
The reagents were added as shown below.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Status</th>
<th>0 Standard</th>
<th>Different concentration of standards</th>
<th>Control serum</th>
<th>Unknown serum</th>
<th>[³⁵S]-LH antibody (Tracer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Total counts (Plain tubes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>3-4</td>
<td>Non-specific binding (Plain tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>5-6</td>
<td>Maximum binding (Antibody coated tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>7-16</td>
<td>Standards (Antibody coated tubes)</td>
<td></td>
<td>100 µl</td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>17-18</td>
<td>Control serum (Antibody coated tubes)</td>
<td></td>
<td>100 µl</td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>19 onwards</td>
<td>Unknown (Antibody coated tubes)</td>
<td></td>
<td></td>
<td></td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Except total count tubes, all other tubes were vortexed and then incubated for 90 min at room temperature in a shaker (300 - 350 rpm). Then the content was aspirated from the tubes and the tubes were washed twice with 2 ml of wash solution. The radioactivity was measured in a gamma counter for 1 min.

Using semilog co-ordinates, the percent value of each standard on the Y-axis versus the LH amount expressed as IU/L on the X-axis were plotted. From the calibration curve, concentrations of LH in the samples were read and expressed as IU/L. The sensitivity of the assay was 0.2 IU/L. Intra and
inter-assay coefficients of variations were 2.7 - 6.7 and 0.45 - 3.70, respectively.

3.3.2 Follicle-stimulating hormone (FSH)

Principle

This assay is based on the use of antibody-coated tubes, employing two mouse monoclonal antibodies directed against two different epitopes on the FSH molecule. Monoclonal IgG to FSH is coated on the inner surface of the tubes and labeled monoclonal IgG to FSH is used as tracer. The assay features a single incubation during which FSH contained in standards or samples to be assayed binds to the solid phase anti-FSH antibody. The FSH/anti-FSH complex thus formed allows the \[^{125}\text{I}^\]-tracer to bind to the solid phase. After the incubation, the amount of labeled antibody bound to the solid phase is proportional to the amount of FSH present in standards or samples. At the end of the incubation, the unbound material is removed by aspiration and washing.

Reagents

1. Tracer: A vial contains 5.5 ml of buffered \[^{125}\text{I}^\] labeled IgG to FSH (mouse monoclonal).

2. FSH standards: A set of seven vials containing FSH standards ranging in concentration from 0 to 180 IU/L, serum and preservatives was
provided. Each vial was reconstituted with 1 ml of double distilled water 30 min before use.

3. Antibody coated tubes: The tubes coated with IgG to FSH (Mouse monoclonal antibody directed against FSH epitope that is different from the one against which the IgG used for the tracer is raised) in the inner surface were provided.

4. Wash solution: The 50 ml saline solution containing detergent was made upto 1000 ml with distilled water.

5. Control serum: The vial containing FSH, human serum and preservatives was reconstituted with 1 ml distilled water 30 min before use.

Procedure

All the reagents were brought to room temperature at the time of assay. The assay was carried out in duplicate with total count, non-specific binding, maximum binding, standards and unknown serum sample tubes.

1. Plain 12 x 75 mm polypropylene tubes in duplicate were used for total count and non-specific binding.

2. FSH antibody coated tubes were used for maximum binding, standards and unknown serum samples.
The reagents were added as shown below.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Status</th>
<th>0 Standard</th>
<th>Different concentration of standards</th>
<th>Control serum</th>
<th>Unknown serum</th>
<th>[{}^{3}H] FSH (Tracer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Total counts (Plain tubes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>3-4</td>
<td>Non-specific binding (Plain tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
</tr>
<tr>
<td>5-6</td>
<td>Maximum binding (Antibody coated tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>50 µl</td>
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<td>Standards (Antibody coated tubes)</td>
<td></td>
<td></td>
<td></td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>17-18</td>
<td>Control serum (Antibody coated tubes)</td>
<td></td>
<td></td>
<td></td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>19 onwards</td>
<td>Unknown (Antibody coated tubes)</td>
<td></td>
<td></td>
<td></td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Except total count tubes, all other tubes were vortexed and then incubated for 90 min at room temperature in a shaker (300 - 350 rpm). Then the content was aspirated from the tubes and the tubes were washed twice with 2 ml of wash solution. The radioactivity was measured in a gamma counter for 1 min.

Using semilog co-ordinates, the percent value of each standard on the Y-axis versus the FSH amount expressed as IU/L on the X-axis were plotted. From the calibration curve, concentrations of FSH in the samples were read
and expressed as IU/L. The sensitivity of the assay was 0.2 IU/L. Intra and inter-assay coefficients of variations were 1.4 - 2.6 and 4.2 - 6.3, respectively.

Serum testosterone and estradiol were assayed using RIA kits as described in the chapters 3.12.1 and 3.12.2, respectively.

3.4 ISOLATION AND PURIFICATION OF HUMAN LEYDIG CELLS

Human Leydig cells were isolated by the procedure described by Lejeune et al. (1993) with little modifications.

Reagents

1. Dulbecco’s Modified Eagle’s Medium, Nutrient Mixture, F-12 Ham (DME/F-12 1:1 mixture) (pH 7.4): 12 g DME/F-12 and 1.2 g sodium bicarbonate were dissolved in 800 ml of autoclaved Milli Q water. 10 ml of penicillin-streptomycin solution and 1 ml of amphotericin-B solution were added and pH of the medium was adjusted to 7.4 with 1 N NaOH and the volume was made upto 1 litre.

2. Collagenase (Type IV) (0.5 mg/ml): 50 mg Collagenase was dissolved in 100 ml of DME/F-12 (pH 7.4).

3. 100% Percoll: 100% Percoll solution was prepared by dissolving 3.9 mg DME/F-12, 75 mg bovine serum albumin (BSA) and 0.75 mg
sodium pyruvate in 22.5 ml of Percoll (113%). The pH was adjusted to 7.4 with 0.01 N HCl and finally made up to 25 ml with autoclaved Milli Q water.

4. Preparation of Percoll gradient: Different Percoll gradients were prepared by mixing 100% Percoll and DME/F-12 (pH 7.4) in different ratios: i.e. 60% (6 ml + 4 ml), 40% (4 ml + 6 ml), 34% (3.4 ml + 6.6 ml), 26% (2.6 ml + 7.4 ml), 21% (2.1 ml + 7.9 ml). 2 ml of 60% Percoll gradient was added to a graduated centrifuge tube and above this layer, 40%, 34%, 26%, and 21% gradients of Percoll (2 ml each) were laid gently one over the other, taking care to avoid mixing.

Procedure

As soon as testicles were removed from the patients, they were transported to the laboratory in an ice-cold condition and decapsulated aseptically. Decapsulated testes were incubated for 1-2 h with constant agitation at 37°C in 200 ml Dulbecco’s Modified Eagle’s Medium (DME) with 0.5 mg/ml collagenase, 5 μg/ml deoxyribonuclease and 2 μg/ml soyabean trypsin inhibitor. At the end of the incubation period, the digested tissue was filtered through nylon gauze (400 mesh) and the cells were pelleted by centrifugation at 200 x g for 5 min. The cell pellet was resuspended in DME and loaded onto five-layer discontinuous Percoll gradient (21, 26, 34,
40 and 60% Percoll in F-12 DME/F-12). After centrifugation at 1500 x g for 30 min, three cell bands were seen; ‘L1’ at 26% Percoll, ‘L2’ at 34% and ‘L3’ at 40%. Cell bands ‘L2’ and ‘L3’ were collected and diluted with two volumes of DME and centrifuged at 200 x g for 10 min. Cells were then resuspended in fresh medium and counted using haemocytometer. The percentage of Leydig cells in air-dried cell smear was determined histochemically by staining for 3β-hydroxysteroid dehydrogenase as described in the chapter 3.5.1. Viability of the purified Leydig cell was determined by trypan blue exclusion method as described in the chapter 3.5.2. The purity of the human Leydig cell was 80 - 85% and the viability was about 90 - 95%.

3.5 ISOLATION AND PURIFICATION OF RAT LEYDIG CELLS

A total of 224 adult male albino rats (90 days old) of Wistar strain (Rattus norvegicus) weighing approximately 200 g were used for this study. Leydig cells were isolated from testes by enzymatic digestion and purified on a discontinuous Percoll gradient (Lefevre et al., 1983).

Reagents

1. Dulbecco’s Modified Eagle’s Medium, Nutrient Mixture, F-12 Ham (DME/F-12 1:1 mixture) (pH 7.4): 12 g DME/F-12 and 1.2 g sodium bicarbonate were dissolved in 800 ml of autoclaved Milli Q water.
10 ml of penicillin-streptomycin solution and 1 ml of amphotericin-B solution were added and pH of the medium was adjusted to 7.4 with 1 N NaOH and the volume was made upto 1 litre.

2. Collagenase (Type IV) (0.25 mg/ml): 25 mg Collagenase was dissolved in 100 ml DME/F-12 (pH 7.4).

3. 100% Percoll: 100% Percoll solution was prepared by dissolving 3.9 mg DME/F-12, 75 mg bovine serum albumin (BSA) and 0.75 mg sodium pyruvate in 22.5 ml of Percoll (113%). The pH was adjusted to 7.4 with 0.01 N HCl and finally made upto 25 ml with autoclaved Milli Q water.

4. Preparation of Percoll gradient: Different Percoll gradients were prepared by mixing 100% Percoll and DME/F-12 (pH 7.4) in different ratios: i.e., 75% (7.5 ml + 2.5 ml), 60% (6 ml + 4 ml), 45% (4.5 ml + 5.5 ml), 30% (3 ml + 7 ml), 15% (1.5 ml + 8.5 ml) and 5% (0.5 ml + 9.5 ml). 2 ml of 75% Percoll gradient was added to a graduated centrifuge tube and above this layer, 60%, 45%, 30%, 15% and 5% gradients of Percoll (2 ml each) were laid gently one over the other, taking care to avoid mixing.
Testes were decapsulated aseptically with fine forceps without breaking the seminiferous tubules and incubated in polypropylene tubes with culture medium containing HEPES and collagenase (0.25 mg/ml). Incubation was performed by shaking the tubes in their long axis in thermostated shaking water bath at 34°C for 15 min. After the incubation, tubes were gently shaken and 10 ml of DME/F-12 (pH 7.4) without collagenase was added and allowed to stand for 10 min. The supernatant was then aspirated using a Pasteur pipette and transferred to sterile centrifuge tubes. This procedure was repeated once again to improve the yield of Leydig cells. The supernatants were combined and centrifuged at 200 × g for 15 min at 4°C. After discarding the supernatant, the pellet obtained was resuspended in 1 ml of DME/F-12 representing a crude testicular interstitial cell suspension.

Discontinuous Percoll gradients were used to obtain purified Leydig cells from this crude preparation. 1 ml of crude Leydig cell suspension was applied on top of the discontinuous gradient and centrifuged at 800 × g for 20 min at 4°C. After centrifugation, most of the Leydig cells were observed in between 30% and 45% gradients. These Leydig cells were aspirated carefully using a Pasteur pipette and transferred to centrifuge tubes containing 5 ml medium. After mixing thoroughly, the tubes were centrifuged at 200 × g for 10 min at 4°C and the supernatant obtained was discarded. To remove
excess Percoll, the cell pellets were washed thrice with excess medium and then finally suspended in 1 ml of medium and counted using haemocytometer.

3.5.1 Identification of Leydig cells

The purified human and rat Leydig cells were identified by histochemical localization of 3β-HSD performed according to the method of Aldred and Cooke (1983).

Reagents

1. Phosphate buffer (pH 7.2)

Solution-A: 1.56 g monobasic sodium phosphate (0.1 M) was dissolved in 100 ml distilled water.

Solution-B: 1.78 g dibasic sodium phosphate (0.1 M) was dissolved in 100 ml distilled water.

28 ml of solution-A and 72 ml of solution-B were mixed well, pH was adjusted to 7.2 and the final volume was made upto 200 ml with distilled water.

2. Phosphate buffer containing nicotinamide adenine dinucleotide (NAD: 3 mg/ml) and nitroblue tetrazolium (NBT: 1 mg/ml).

3. Dehydroepiandrosterone (DHEA): 2 mg/ml.
**Procedure**

1. To 20 μl of Leydig cell suspension, 490 μl NAD-NBT solution and 10 μl DHEA were added and incubated at 37°C for 60 min.

2. After incubation, cells were washed with phosphate buffer (pH 7.2).

3. The percentage of positively stained cells was counted using a Neubauer haemocytometer under the microscope.

The Neubauer haemocytometer has a grid containing 5 major squares, A, B, C, D and E. In the E square, the four small squares in the corner are called E₁, E₂, E₃, E₄ and the central small square is E₅. 10 μl of 3β-HSD stained Leydig cell suspension was added to both sides of the haemocytometer. While performing the count, all the Leydig cells within the designated squares and those cells, which fall over the lines, were included.

Major square is 1 mm long, 1 mm wide and the thickness of the fluid between the cover slip and the haemocytometer is 0.1 mm. Leydig cells in the squares E₁, E₂, E₃, E₄ and E₅ were counted. The basic formula applied to obtain the Leydig cell concentration using the haemocytometer is given below.

\[
\text{Leydig cell concentration} = \frac{\text{Number of Leydig cells} \times \text{Multiplication factor (50,000)}}{1 \text{ mm}^2 \times 0.1 \text{ mm}} = \text{"x" cells/ml}
\]

Approximately 80 – 85% of the cells stained positive for 3β-HSD by haemocytometer counts, reflecting the purity of Leydig cell preparation.
3.5.2 Viability of Leydig cells

Viability of purified human and rat Leydig cells was assessed by trypan blue exclusion method (Aldred and Cooke, 1983).

Reagent

0.4% trypan blue solution containing 0.1% BSA.

Procedure

1. 100 μl trypan blue solution was mixed with 100 μl Leydig cell suspension (~ 50,000 cells) and incubated for 5 min at 37°C.

2. The cells were then washed twice with saline and 10 μl suspension was placed in the haemocytometer and viewed under the microscope.

3. The number of unstained and stained cells represents viable and damaged cells, respectively. The cells were counted and the percentage of viable cells was calculated using the formula:

\[
\frac{\text{Number of cells unstained}}{\text{Total number of cells}} \times 100 = \% \text{ of viable cells}
\]

The viability of purified Leydig cells was 90 - 95%.
3.6 LEYDIG CELL CULTURE AND TREATMENT

Purified human and rat Leydig cells were plated (16 h) in culture tubes containing DME/F-12 nutrient mixture with fetal bovine serum (FBS) (10% for human and 1% for rat Leydig cells) at the density of 1 x 10^6 cells/tube. After the incubation period, the medium was replaced with FBS free fresh medium and the cells were exposed to different doses of gamma radiation (2, 4, 6, 8 and 10 Gy) (1.5 Gy/min) (Theratron Phoenix, Cobalt-60 source, Ontario, Canada). Each dose was delivered in three fractions for three consecutive days and then the cells were used for LH receptor quantification or total RNA isolation or LH (100 ng/ml)/cAMP (3 mM) stimulation test along with control cells. LH-stimulated cells were used for the assay of cAMP while LH and cAMP-stimulated cells were used for the estimation of steroidogenic enzymes and, testosterone and estradiol production. Viability of the Leydig cells was tested by trypan blue exclusion method following radiation exposure.

3.7 RADIOIODINATION OF hCG

The iodination procedure involves the substitution of radioactive iodine into tyrosine moieties in peptides. Highly purified iodination grade hCG (NIDDK- CR 127) obtained from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), USA was radioiodinated using
[\textsuperscript{125}I] by lactoperoxidase method (Thorell and Johansson, 1971) and purified by gel filtration on a column of Sephadex G-25.

**Principle**

Peroxides have the ability to oxidize iodide and have been used to label proteins with iodine. Enzymatic radioiodination of peptide hormones using lactoperoxidase is a gentle, simple and rapid method. Exposure of antigen (hormone) and [\textsuperscript{125}I] for a defined period to the oxidizing agent lactoperoxidase and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), facilitates the incorporation of [\textsuperscript{125}I] atoms into tyrosine residues in the hormone.

**Reagents**

1. [\textsuperscript{125}I] Carrier free sodium iodide with radioactivity of about 2.5 mCi/25 μl.

2. Sodium phosphate buffer (0.05 M, pH 7.4): 780 mg sodium dihydrogen phosphate and 889 mg disodium hydrogen phosphate were dissolved in 90 ml distilled water, pH was adjusted to 7.4 with 0.1 N NaOH and final volume was made upto 100 ml with distilled water.

3. Sodium acetate buffer (0.1 M, pH 4.6): 820 mg anhydrous sodium acetate was dissolved in 75 ml distilled water, pH adjusted to 4.6 and made upto 100 ml with distilled water.
4. **Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (0.88 mM):** 1 ml of stock-1 H\textsubscript{2}O\textsubscript{2} (8.82 M) was made upto 10 ml with deionized water to prepare H\textsubscript{2}O\textsubscript{2} stock-2 (0.88 M). 1 µl of stock-2 was further diluted to 1 ml with deionized water to prepare H\textsubscript{2}O\textsubscript{2} stock-3 of 0.88 mM concentration.

5. **Lactoperoxidase (4 µg/µl):** 4 mg lactoperoxidase was dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 4.6).

6. **Hormone:** 100 µg hCG was dissolved in 1 ml of 0.05 M phosphate buffer to get a concentration of 0.1 µg/µl.

7. **Barbitone buffer (0.07 M, pH 8.6):** 7.216 g sodium barbitone was dissolved in 490 ml distilled water. The pH was adjusted to 8.6 and final volume was made upto 500 ml with distilled water.

8. **Bovine serum albumin 1%:** 500 mg was dissolved in 50 ml barbitone buffer.

**Preparation of Sephadex G-25 column**

Prepacked Sephadex G-25 (PD-10) column (Supelco, USA) was coated with 1% BSA in 0.07 M barbitone buffer (pH 8.6) and then eluted with 25 ml of the same buffer to equilibrate the column.
**Procedure**

Iodination was performed in glass tubes at 20 - 22°C. The following steps were performed in a sequence rapidly.

1. 0.5 mCi (5 μl) carrier free $^{125}$I was taken in the reaction vial containing 25 μl 0.05 M phosphate buffer.

2. 2.5 μg hCG (25 μl) was added to the vial and mixed gently.

3. The iodination reaction was initiated by adding 1 μl (4 μg) lactoperoxidase and then 1 μl of 0.88 mM H$_2$O$_2$.

4. The contents were mixed gently by mild tapping of the vial and incubated for 60 sec.

5. At the end of incubation, the reaction was stopped by adding 500 μl of phosphate buffer and mixed well.

6. The reaction mixture was immediately transferred into an equilibrated prepacked Sephadex G-25 column to remove unreacted iodide.

7. The column was then eluted with barbitone buffer. 10 drops of elutant were collected in polypropylene tubes precoated with 1% BSA.
8. The radioactivity in the tubes (about 50) was counted in a gamma counter for 60 sec.

Calculation of specific activity

To determine the specific activity of iodinated hormone, the following procedure was followed.

\[
\text{Specific activity of the hormone} = \frac{\text{Counts incorporated into protein}}{\text{Total counts}} \times \frac{\mu\text{Ci of } [^{125}\text{I}]}{\mu\text{g of hormone}}
\]

Total counts (TC) include the radioactivity contained in all the polypropylene tubes as well as in the pipette tips and reaction vial after transferring the reaction mixture into the Sephadex column (residual count). The specific activity of iodinated hormone was 60-80 \(\mu\text{Ci}/\mu\text{g}\).

3.8 QUANTIFICATION OF CELL SURFACE LH/hCG RECEPTOR

Leydig cell surface LH/hCG receptor was quantified according to the method described by Rigaudiere et al. (1988).

Reagents

1. Sodium hydroxide (NaOH) (1 N): 4 g of NaOH was dissolved in 100 ml of distilled water.
determine the receptor concentration and expressed as fmol/10^6 cells.

surface bound radioactivity. The data were subjected to Scatchard analysis to
count. Specific binding was calculated by subtracting NSB from the total
cell surface bound radioactivity was determined by counting in LKB 

Gamma counter. Remove unbound labeled hormone and wash twice with 0.1 M HCl and the

After incubation, the Leydig cells were washed twice with PBS (pH 7.4) to

Specific binding (NSB) was determined with excess unlabeled hormone. Non-

Presence of absence of increasing concentrations of unlabeled hormone.

With saturation concentrations of labeled hormone at 4°C for 16 h in the

The cell surface receptor concentration was quantitated by incubating the cells

concentrations of [1,251-1,251-3H] cholesterol. In the presence or absence of unlabeled hormone.

For saturation analyses, Leydig cells were incubated with increasing

Procedure

Volume was made up to 200 ml with distilled water.

of sodium chloride was added. pH was adjusted to 7.2 and final

28 ml of solution-A and 72 ml of solution-B were mixed well and 1.8 ml

100 ml distilled water.

Solution-B: 1.78 g dibasic sodium phosphate (0.1 M) was dissolved in

Sodium phosphate (0.1 M) was dissolved in 100 ml distilled water.

2. Phosphate buffered saline (pH 7.4): Solution-A: 1.56 M monobasic

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3.9 ASSAY OF cAMP

Cyclic AMP concentration in cultured Leydig cells and media were assayed using [³H]-cAMP assay kit purchased from Amersham Biosciences, UK.

Principle

The assay was based on the competition between unlabeled cyclic AMP and a fixed quantity of the tritium labeled cAMP for binding to a protein, which has high specificity, and affinity for cAMP. The amount of labeled cAMP-protein complex formed is inversely related to the amount of unlabeled cAMP present in the assay sample. Measurement of the protein bound radioactivity enables the amount of unlabeled cAMP in the sample to be calculated. Separation of the protein bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide onto a coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of the unlabeled cAMP in the sample is then determined from a linear standard curve.

Reagents

1. Tris EDTA buffer: The bottle containing tris EDTA was reconstituted with 25 ml double distilled water to get 0.05 M tris and 4 mM EDTA (pH 7.5).
2. Binding protein: The bottle containing cAMP binding protein was reconstituted with 15 ml double distilled water by gentle swirling.

3. [8-\(^3\)H] Adenosine 3', 5'-cyclic phosphate tracer: The bottle containing tracer was dissolved in 10 ml double distilled water by swirling and inversion.

4. Adenosine 3', 5'-cyclic phosphate standard: The bottle containing standard was reconstituted with 5 ml double distilled water by swirling and inversion.

5. Charcoal adsorbent: The bottle containing charcoal adsorbent was dissolved in 20 ml ice cold distilled water and placed in an ice bath and mixed using magnetic stirrer.

Procedure

Extraction of cAMP

2 ml of ice-cold ethanol (65%) was added to 1 ml of media with sonicated cultured Leydig cells, vortex mixed, allowed to stand for 5 min at room temperature and then centrifuged. The supernatant was collected in test tubes, precipitate was washed again using 1 ml of ice-cold ethanol and then centrifuged. The supernatants were combined and evaporated to dryness at 55°C under a stream of nitrogen. The residue was dissolved in 200 µl of assay buffer prior to analysis.
Assay was carried out in duplicate as per the procedure provided by the
manufacturer.

The reactants were added as given below.

<table>
<thead>
<tr>
<th></th>
<th>Charcoal blank</th>
<th>Total counts</th>
<th>Zero standard</th>
<th>Standards</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>150 µl</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standards</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
</tr>
<tr>
<td>Tracer</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Binding Protein</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

The tubes were vortex mixed for 5 sec and incubated in an ice-bath (2 – 8°C) for two hours.

<table>
<thead>
<tr>
<th>Charcoal suspension</th>
<th>-</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
</tr>
</thead>
</table>

Tubes were vortexed and centrifuged in a cooling centrifuge to sediment the charcoal. 200 µl of supernatant was taken from each tube and placed in scintillation vials containing scintillation cocktail and then counted in a liquid scintillation counter.

The concentration of cAMP in the sample was determined from standard curve and values are expressed as pmol/mg protein. Protein content in the extract was estimated by the method of Lowry et al. (1951) as described in the chapter 3.11.
3.10 ASSAY OF STEROIDOGENIC ENZYMES

Steroidogenic enzymes such as cytochrome P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were assayed in control and irradiated cells under basal, LH and cAMP-stimulated conditions.

3.10.1 Cytochrome P450 side chain cleavage enzyme

P450scc enzyme activity was determined radiometrically as per the method of Georgiou et al. (1987).

Principle

Cholesterol is converted into pregnenolone by the action of P450scc enzyme. During this reaction, 4-hydroxy-4-methyl-pentanoic acid (Isocaproic acid) is cleaved off from the cholesterol, which is water soluble. Therefore, the amount of 4-hydroxy-4-methyl-pentanoic acid produced is directly proportional to the enzyme activity. When P450scc acted upon the substrate [26,27-3H]-25-hydroxycholesterol, [3H]-labeled, 4-hydroxy-4-methyl-pentanoic acid is produced. Therefore, by measuring the conversion of [26,27-3H]-25-hydroxycholesterol to [3H]-labeled, 4-hydroxy-4-methyl-pentanoic acid, the enzyme activity can be determined.
Reagents

1. [26,27-\textsuperscript{3}H]-25-hydroxycholesterol.

2. [\textsuperscript{14}C]-Isocaproic acid.

3. Sodium hydroxide (NaOH) (1 N): 4 g of NaOH was dissolved in 100 ml of distilled water.

Procedure

1. Cultured Leydig cells were washed twice with fresh medium.

2. The cells were incubated for 1 h with saturating concentration of [26,27-\textsuperscript{3}H]-25-hydroxycholesterol (5 \mu M; 0.5 \mu Ci) in 100 mM dimethylsulfoxide in 1 ml culture medium at 32°C with 19% O\textsubscript{2}.

3. After the incubation period, the enzyme reaction was stopped by the addition of 0.1 ml of 1 N NaOH.

4. [\textsuperscript{14}C]-Isocaproic acid (3000 cpm) was added to the tubes as recovery standard.

5. Then the medium was removed to an extraction tube, and the culture tube was washed with 1 ml alkalinized medium, which was combined with 1 ml original medium and extracted with 10 ml chloroform.

6. 1 ml of extracted aqueous phase was vortexed with 0.8 g neutral alumina for 1 min, followed by centrifugation at 1200 × g for 25 min.
7. The supernatant aqueous phase (0.4 ml) was transferred to a scintillation vial containing cocktail toluene and radioactivity was measured in a liquid scintillation counter.

8. The enzyme activity is expressed as cpm of $[^1H]^{-}$isocaproic acid formed/h/10⁶ cells.

3.10.2 3β-Hydroxysteroid dehydrogenase (3β-HSD)

The activity of 3β-HSD in control and irradiated Leydig cells was determined by the method described by Bergmeyer (1974).

Principle

3β-HSD catalyses the oxidation of 3β-hydroxyl groups of C₁₉ and C₂₁ steroids in the presence of coenzyme NAD. The activity was determined by the optical measurement of the rate of conversion of NAD to NADH.

$$\Delta^5 \text{Pregnenolone} \xrightarrow{3\beta\text{-HSD}} \text{NAD} \rightarrow \Delta^4 \text{Progesterone + NADH}$$

Reagents

1. Tris- HCl buffer (0.1 M) (pH 7.2):

Solution-A: 1.21 g Tris was dissolved in 100 ml distilled water.
Solution-B: 0.9 ml concentrated HCl was made upto 100 ml with distilled water.

50 ml of solution-A was mixed with 44.2 ml of solution-B and made upto 200 ml with distilled water. The pH was adjusted to 7.2.

2. Sodium pyrophosphate buffer (100 μM) (pH 9.0).

3. NAD (0.5 μM).

4. Pregnenolone (0.1 μM).

Procedure

1. Leydig cells were homogenized and sonicated in ice-cold tris-HCl buffer (0.1 M) (pH 7.2) and centrifuged at 16,000 x g for 5 min at 4°C. The supernatant was taken as enzyme extract.

2. The reaction mixture in a final volume of 3.0 ml contained the following ingredients

| Pyrophosphate buffer (100 μM) (pH 9.0) | 0.6 ml |
| NAD | 0.2 ml |
| Distilled water | 2.0 ml |
| Substrate (pregnenolone) | 0.1 ml |
| Enzyme extract | 0.1 ml |
3. The absorbance at 340 nm was measured immediately after the addition of enzyme extract at 20 sec intervals for 5 min in a spectrophotometer against blank containing all the components except the substrate.

4. The specific activity of the enzyme is expressed as nmol of NAD reduced/min/mg protein.

3.10.3 17β-Hydroxysteroid dehydrogenase (17β-HSD)

17β-HSD activity was determined based on the radiometric method described by Murono (1990).

**Principle**

The enzyme 17β-HSD catalyzes the conversion of androstenedione to testosterone, which is the terminal step in testosterone biosynthesis and dehydroepiandrosterone to androstenediol. This assay is based on the principle that when Leydig cells are incubated with [1,2,6,7\(^3\)H]-androst-4-ene-3, 17β-dione, the product [1,2,6,7\(^3\)H]-testosterone will be formed by the action of the enzyme 17β-HSD. Therefore, by estimating the amount of product formed in a given point of time, the enzyme activity can be determined.
Reagents

1. $[1,2,6,7\, ^3\text{H}]$-androst-4-ene-3, 17$\beta$-dione.
2. $[1,2,6,7\, ^3\text{H}]$-testosterone.
3. Diethyl ether.
4. Methanol.
5. Chloroform.
6. Anisaldehyde.

Procedure

1. Leydig cells were pre-incubated for 30 min with fresh culture media and then washed twice with fresh medium.

2. Then the cells were incubated with $[1,2,6,7\, ^3\text{H}]$-androst-4-ene-3, 17$\beta$-dione (10 $\mu$M/0.5 $\mu$Ci) dissolved in culture media containing 0.3% dimethyl sulfoxide (final concentration) for 1 h at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$.

3. The reaction was terminated by the addition of 0.1 ml of 1 mol/L NaOH. One hundred $\mu$g each of androstenedione and testosterone was added as carriers.

4. To estimate recovery, 4000 cpm of $[1,2,6,7\, ^3\text{H}]$-testosterone was processed separately.
5. Samples were extracted with 5 volume of diethyl ether and the residues of ether extracts were chromatographed by TLC using chloroform : methanol (99.5 : 0.5, v/v) as solvent system.

6. The product testosterone was localized using anisaldehyde spray, which was cut out and counted in a liquid scintillation counter.

7. The enzyme activity was expressed as pmol of [3H]-testosterone formed/h/10^6 cells.

3.11 ESTIMATION OF TOTAL PROTEIN

Protein concentration in Leydig cell extract was determined as per the method of Lowry et al. (1951) with crystalline bovine serum albumin (BSA) as the standard.

Principle

In alkaline solution, proteins form a complex with copper ions. Amino acids tyrosine and tryptophan containing phenolic hydroxyl group present in the protein-copper complex reacts with Folin-Ciocalteau reagent to give a blue colour due to reduction of phosphomolybdate. The intensity of the colour is proportional to the concentration of protein being estimated.
Reagents

1. Sodium hydroxide (0.1 N NaOH): 400 mg of NaOH was dissolved in 100 ml of distilled water.

2. Reagent A: Sodium carbonate (2%) in 0.1 N NaOH: 2 g of Na₂CO₃ was dissolved in 100 ml of 0.1 N NaOH.

3. Potassium - sodium tartarate (1.35%): 135 g of potassium-sodium tartarate was dissolved in 100 ml of distilled water.

4. Reagent B: (0.5%) 500 mg of copper sulphate in 1 ml of 1.35% potassium-sodium tartarate.

5. Reagent C (Lowry’s reagent (or) Alkaline copper reagent): Prepared freshly at the time of estimation by mixing 50 ml of reagent A and 1 ml of reagent B.

6. Folin-Ciocalteau (1 N): Commercially available 2 N reagent was made to 1 N solution by mixing the reagent with equal volume of distilled water.

7. Standard: Standard bovine serum albumin (BSA) containing 1 mg/ml was prepared with 0.1 N NaOH in a standard flask. This solution contained 1000 μg of protein/ml.
Procedure

1. Leydig cell extract (0.01 ml) was taken in a clean test tube and made up to 1 ml with distilled water.

2. To this, 5 ml alkaline copper reagent was added and the contents were mixed well and allowed to stand at room temperature for 10 min.

3. 0.5 ml of 1 N Folin-Ciocalteau reagent was then added and mixed well.

4. After 20 min, the intensity of the blue colour developed was read at 720 nm against reagent blank containing all the reagents except the Leydig cell extract.

For plotting the standard graph, a set of standards (25, 50, 75, 100 and 125 µg) were taken in a series of test tubes, and made up to 1 ml with distilled water and processed as the samples. The standard graph was drawn by plotting the concentration of standards on the X-axis and the optical density on the Y-axis. The concentration of protein in sample was calculated by referring the standard curve and expressed as mg/10^6 cells.
3.12 ESTIMATION OF TESTOSTERONE AND ESTRADIOL PRODUCTION BY LEYDIG CELLS

Control and irradiated human and rat Leydig cells were incubated in DME/F-12 medium with LH (100 ng/ml) or cAMP (3 mM) at 34°C under 5% CO₂ for 24 h. At the end of incubation, cells were sonicated with media and 2 ml of cold medium containing 0.1% BSA was added to each vial and transferred to glass tubes before placing in a boiling water bath for 5 min. The tubes were then cooled and centrifuged for 20 min to remove denatured protein. Supernatants were frozen and stored for subsequent testosterone and estradiol assays. RIA kits were provided by Diasorin (Italy), wherein the antibody is immobilized on the inner wall of the tubes.

3.12.1 Assay of testosterone

Principle

The principle of the assay is based on the competition between labeled testosterone [¹²⁵I] and testosterone present in the standards or samples to be assayed for fixed and limited number of antibody binding sites. They are allowed to reach equilibrium during incubation. After incubation, antibody bound and free antigens are separated by decanting the tubes. The amount of labeled testosterone bound to the antibody is inversely related to the amount of testosterone present in the sample or standard.
Reagents

1. Buffered $[^{125}\text{I}]-\text{testosterone}$: One vial of iodinated testosterone in liquid form (52 ml) contained not more than 1.6 $\mu$Ci of radioactive testosterone.

2. Testosterone standards: A set of seven vials, labeled A through G was provided. The zero standard A contains 1.0 ml and the remaining standards B through G contain 0.5 ml each. The standards contain 0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng of testosterone per ml (ng/ml) in processed serum.

3. Antibody coated tubes: The tubes coated with testosterone antiserum in the inner surface were provided.

4. Control serum: The vial containing control serum was reconstituted with 1 ml distilled water.

Procedure

The assay was carried out in duplicate with total count, non-specific binding, maximum binding, standards and unknown serum or medium sample tubes.

1. Plain 12 $\times$ 75 mm polypropylene tubes in duplicate were used for total count and non-specific binding.

2. Testosterone antibody coated polypropylene tubes were used for maximal binding, standards and unknown serum or medium samples.
The reagents were added as shown below.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Status</th>
<th>0 standard</th>
<th>Different concentration of standards</th>
<th>Control serum</th>
<th>Unknown serum or medium</th>
<th>Buffered [^{125}\text{I}^]\text{-Testosterone}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Total counts (Plain tubes)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>3-4</td>
<td>Non-specific binding (Plain tubes)</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>5-6</td>
<td>Maximum binding (Antibody coated tubes)</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>7-18</td>
<td>Standards (Antibody coated tubes)</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>19-20</td>
<td>Control serum (Antibody coated tubes)</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>21 onwards</td>
<td>Unknown (Antibody coated tubes)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Except total count tubes, all other tubes were vortexed and then incubated for 3 h at 37°C. At the end of incubation, the tubes were thoroughly decanted, dried and counted in a gamma counter for 1 min.

Using logit log representation of the calibration curve, testosterone concentration in the sample was calculated and expressed as ng/ml or ng/10^6.
cells. The maximum binding was 47% and the sensitivity of the assay was 0.05 ng/ml. The % cross reactivity of testosterone antibody to other steroids such as 5α-dihydrotestosterone and androstenedione is 6.9% and 1.1%, respectively. Intra- and inter-assay coefficient of variations were 6.9 - 13.2 and 10.3 - 13, respectively.

3.12.2 Assay of estradiol

Principle

The principle of the assay is based on the competition between labeled estradiol [125I] and estradiol present in the standards or samples to be assayed for fixed and limited number of antibody binding sites. They are allowed to reach equilibrium during incubation. After incubation, antibody bound and free antigens are separated by decanting the tubes. The amount of labeled estradiol bound to the antibody is inversely related to the amount of estradiol present in the sample or standard.

Reagents

1. Buffered [125I]-estradiol: One vial of iodinated estradiol in liquid form (55 ml) contained not more than 8.1 μCi of radioactive estradiol.

2. Estradiol standards: A set of seven vials, labeled A through G was provided. The standards were reconstituted with 0.5 ml distilled water
30 min before use. The standards contain 0, 10, 40, 140, 500, 1500 and 5000 pg of estradiol per ml (pg/ml) in processed serum.

3. Antibody coated tubes: The tubes coated with estradiol antiserum in the inner surface were provided.

4. Control serum: The vial containing control serum was reconstituted with 0.5 ml distilled water.

Procedure

The assay was carried out in duplicate, with total count, non-specific binding, maximum binding, standards and unknown serum or medium sample tubes.

1. Plain 12 × 75 mm polypropylene tubes in duplicate were used for total count and non-specific binding.

2. Estradiol antibody coated polypropylene tubes were used for maximal binding, standards and unknown serum or medium samples.
The reagents were added as shown below.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Status</th>
<th>0 standard</th>
<th>Different concentration of standards</th>
<th>Control serum</th>
<th>Unknown serum or medium</th>
<th>Buffered (^{125}I)- Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Total counts (Plain tubes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 µl</td>
</tr>
<tr>
<td>3-4</td>
<td>Non-specific binding (Plain tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>500 µl</td>
</tr>
<tr>
<td>5-6</td>
<td>Maximum binding (Antibody coated tubes)</td>
<td>100 µl</td>
<td></td>
<td></td>
<td></td>
<td>500 µl</td>
</tr>
<tr>
<td>7-18</td>
<td>Standards (Antibody coated tubes)</td>
<td></td>
<td>100 µl</td>
<td></td>
<td></td>
<td>500 µl</td>
</tr>
<tr>
<td>19-20</td>
<td>Control serum (Antibody coated tubes)</td>
<td></td>
<td></td>
<td>100 µl</td>
<td></td>
<td>500 µl</td>
</tr>
<tr>
<td>21 onwards</td>
<td>Unknown (Antibody coated tubes)</td>
<td></td>
<td></td>
<td></td>
<td>100 µl</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Except total count tubes, all other tubes were vortexed and then incubated for 3 h at room temperature in a shaker. At the end of incubation, the tubes were thoroughly decanted, dried and counted in a gamma counter for 1 min.

Using logit log representation of the calibration curve, estradiol concentration in the sample was calculated and expressed as pg/ml or pg/10^6 cells. The sensitivity of the assay is 4.0 pg/ml. The % cross reactivity of estradiol antibody to other steroids is minimal i.e. 1.3% with estrone and 0.65% with estriol. Intra- and inter-assay coefficient of variations were 3-15 and 4-14, respectively.
3.13 HUMAN AND RAT LEYDIG CELL LH RECEPTOR mRNA EXPRESSION

Control and irradiated human and rat Leydig cells were used for the isolation of total RNA to quantify the LH receptor mRNA levels by RT-PCR.

3.13.1 Isolation of total RNA

Control and irradiated human and rat Leydig cells were used for isolation of total RNA using Total RNA Isolation Reagent (TRIR, AB gene, 1 K) kit. The total RNA isolated by this method was undegraded and free of protein and DNA contamination. The total RNA isolation was performed at 25°C.

Reagents

1. Total RNA isolation reagent.
2. Chloroform (Molecular biology grade).
3. Isopropanol (Molecular biology grade).
4. Ethanol (Molecular biology grade).
5. Autoclaved Milli Q water.
**Procedure**

**Preparation of cell lysate**

Cultured Leydig cells (10 x 10^6 cells/ml of TRIR reagent) were lysed directly by adding the RNA isolation reagent and passing the cell lysate several times through a pipette and then transferred immediately to autoclaved microfuge tubes.

**RNA extraction**

1. The cell lysate was stored for 5 min at 4°C to permit the complete dissociation of nucleoprotein complexes.

2. 0.2 ml of chloroform was added to the lysate (0.2 ml per ml of RNA reagent), vortexed vigorously for 15 sec and placed on ice at 4°C for 5 min.

3. Then it was centrifuged at 12,000 x g for 15 min at 4°C. The lysate formed two phases: the lower organic phase and the upper aqueous phase. DNA and protein were in the organic phase while RNA was in the aqueous phase.

**RNA precipitation**

1. The aqueous phase was carefully transferred to a fresh tube and an equal volume of isopropanol was added and stored for 10 min at 4°C.
2. Then centrifuged at 12,000 x g for 10 min. RNA precipitated as a white pellet at the bottom of the tube.

RNA wash

1. The supernatant was removed and RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 x g at 4°C.

2. At the end of this procedure, RNA pellet was mixed with 50 μl of autoclaved Milli Q water and then incubated at 60°C for 10 min in a water bath to ensure the solubility.

Yield and purity of RNA

Then the concentration and purity of RNA were determined spectrophotometrically at A_{260}/A_{280} nm. The yield of RNA was expressed in micrograms. The purity of the isolated total RNA was 1.7 – 1.8.

3.13.2 Quantification of human and rat LH receptor mRNA expression by RT-PCR

Principle

RT-PCR is one of the most powerful techniques in molecular biology. RT-PCR selectively amplifies the first strand of cDNA that has been synthesized in vitro from mRNA templates by reverse transcription. The
cDNA is first denatured by heating in the presence of a large molar excess of the two oligonucleotide primers and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerases. The cycle of denaturation, annealing and DNA synthesis is then repeated many times.

**Primers**

**Oligonucleotide primers for human LH receptor**

The primers used for RT-PCR of human LH receptor were gene specific primers selected according to Dirnhofer *et al* (1998). The sequence of forward primer is 5'-CTT GGA TAT TIC TIC CAC CAA A-3' corresponding to the nucleotides 678 to 699 and the reverse primer is 5'-TGG CAT GGT TAT AGT ACT GGC-3' corresponding to the nucleotides 1271-1291 of the published cDNA sequence of hLH receptor (Minigishi *et al.*, 1990). These primers will amplify the length of 614 bp, that would cover LH receptor mRNA encoding the part of extracellular and transmembrane domains and thus it represents mRNA encoding the membrane bound receptors. RT-PCR was performed using Qiagen one step RT-PCR kit to assess the LH receptor mRNA expression. Human β-actin mRNA was co-amplified as an internal control to normalize the amplified LH receptor cDNA. Primers for β-actin were selected based on the published
Reagents

1. RNAse free water.
2. 5x Qagen one step RT-PCR buffer
3. dNTP Mix.
4. RT-PCR Enzyme mix
5. Template RNA (Total RNA isolated from human or rat Leydig cells).
6. Forward primer for rat or human L.H receptor
7. Reverse primer for rat or human L.H receptor
8. Forward primer for rat (RPS 16) or human (β-actin) house keeping gene.
9. Reverse primer for rat (RPS-16) or human (β-actin) house keeping gene

Procedure

1. Template RNA, primers, dNTP mix, 5x Qagen one step RT-PCR buffer and RNAse free water were thawed and placed on ice.
2. A master mix was prepared containing all the components required for RT-PCR except the template RNA as shown below.
Preparation of master mix for ten RT-PCR reactions of 50 μl each

Master mix was prepared 5% excess than required quantity in order to avoid the shortage.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 5x Qiagen one step RT-PCR buffer</td>
<td>105 μl</td>
<td>1x</td>
</tr>
<tr>
<td>b) dNTP Mix</td>
<td>21 μl</td>
<td>400 μM of each dNTP</td>
</tr>
<tr>
<td>c) Forward primer for rat or human LH receptor</td>
<td>52.5 μl</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>d) Reverse primer for rat or human LH receptor</td>
<td>52.5 μl</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>e) Forward primer for rat (RPS-16) or human (β-actin) house keeping gene</td>
<td>52.5 μl</td>
<td>0.3 μM (h) 0.1 μM (r)</td>
</tr>
<tr>
<td>f) Reverse primer for rat (RPS-16) or human (β-actin) house keeping gene</td>
<td>52.5 μl</td>
<td>0.3 μM (h) 0.1 μM (r)</td>
</tr>
<tr>
<td>g) RT-PCR Enzyme mix.</td>
<td>21 μl</td>
<td>5-10 units/reaction</td>
</tr>
</tbody>
</table>

3. The master mix was mixed by pipetting up and down for a few times.

4. Then 34 μl of master mix was dispensed into PCR tubes.

5. Template RNA [2 μg/reaction (2-5 μl)] was added to individual PCR tubes.

6. The final volume of the RT-PCR mixture was made upto 50 μl using RNAse free water (11-14 μl).
The thermal cycler was programmed as outlined below.

<table>
<thead>
<tr>
<th>RT reaction</th>
<th>Human I.H receptor</th>
<th>Rat I.H</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT reaction at</td>
<td>50°C for 30 min</td>
<td>80°C for 30 min</td>
</tr>
<tr>
<td>Initial PCR activation at</td>
<td>98°C for 15 min</td>
<td>98°C for 15 min</td>
</tr>
</tbody>
</table>

3-step cycling

- Denaturation: 15 min at 98°C
- Annealing: 15 min at 57°C
- Extension: 1 min at 72°C
- Number of cycles: 40 cycles
- Final Extension at: 72°C for 10 min

The RT-PCR programme was started by allowing the thermal cycler to reach 50°C. Then the PCR tubes were placed in the thermal cycler. The RT-PCR products obtained were subjected to agarose gel electrophoresis for identification of the cDNA bands.

3.13.3 Agarose gel electrophoresis of RT-PCR products and densitometric scanning

Agarose gel electrophoresis is simple and highly effective method for separation, identification and purification of DNA.
Reagents

1. Tris Boric acid EDTA buffer (TBE 5x). Tris (5.4 g) and boric acid (27.5 g) were dissolved in 800 ml of autoclaved double distilled water and 20 ml of 0.5 M EDTA (pH 8.0) was added and the final volume was made up to 1000 ml with autoclaved double distilled water.

2. Preparation of 1x TBE buffer (500 ml) (pH 8.0). 60 ml of 5x TBE buffer was mixed with 200 ml of autoclaved double distilled water. pH was adjusted to 8.0 and made up to 300 ml with autoclaved double distilled water.

3. Lithium bromide (10 µg/ml).

4. Gel loading buffer

   0.1 M EDTA 20 ml
   0.01 M Tris HCl 0.1 ml
   0.25% Bromophenol blue 25 mg
   50% Glycerol 50 ml

2.9 ml of autoclaved double distilled water was added to make the final volume of 10 ml.

Preparation and casting of 2% agarose gel

1 g of standard agarose was dissolved in 50 ml of 1x TBE buffer and placed in microwave oven for 50 sec to get a clear homogeneous of agarose
mix. The solution was cooled to 55°C and ethidium bromide was added (0.5 μg/ml). The content was mixed gently to dissolve ethidium bromide without forming air bubbles. Then it was poured into the assembled gel casting tray (Amersham Biosciences, UK) and comb was placed properly.

**Procedure**

1. After allowing it to settle for 30 min, the boat containing the gel was taken from the tray and placed in the electrophoresis tank containing 1x TBE buffer.

2. The comb was removed carefully from the gel and 1x TBE buffer was added until the gel was completely submerged.

3. The electrodes were connected and allowed to run at 20 mA as a pre run for about 15 min and then the power supply was switched off.

4. 10 μl of PCR mixture was taken from each reaction tube, mixed with gel loading buffer and loaded to each well of agarose gel. Suitable molecular weight marker (100 bp ladder) was simultaneously loaded in the first lane.

5. The power supply was turned on and the current adjusted to 20 mA. The gel was run till the bromophenol blue dye reaches the end of the gel. Then the gel was visualized under UV transilluminator to identify the bands.
The intensity of the human or rat LH receptor cDNAs was quantified and normalized against respective housekeeping genes using Bio Rad gel documentation system. The levels of LH receptor mRNA are expressed as OD units of LH receptor mRNA levels relative to housekeeping gene.

3.14 STATISTICAL ANALYSIS

The data were subjected to one-way analysis of variance (ANOVA) followed by Student-Newman-Keul (SNK) test for multiple comparisons using a computer-based software, Statistical Package for Social Sciences (SPSS).