Chapter 2

Comparative Evaluation of Follicle Stimulating Hormone and/or Testosterone Responsiveness of Sertoli Cells Cultured from 5-days, 18-days and 60 days old Rat Testes
2.1. INTRODUCTION

Sertoli cells (Sc) are indispensable for sex determination and spermatogenesis in males. The number of Sc in adult testis determine final testicular size and total sperm output (Orth et al. 1988). At 12.5dpc, Sertoli cell epithelialize seminiferous tubule enclosing germ cells and express the male sex determining gene, Sry, which control development of bi-potential gonads into testis by influencing the differentiation of other cell types in the XY gonads (Lovell-Badge 1993; Koopman 1995; Swain et al. 1998). Sc divide until the age of 16 days to increase number of functional niches for germ cell (Oatley et al. 2011). After 16 dpp neighboring Sc form tight junctions with each other such that nothing larger than 1000 daltons can pass from the outside to the inside of the tubule. Tight junctions, also called Blood-Testis-Barrier (BTB), create compartments in testis separating Spermatogonial Stem cells (SSC) and meiotic germ cells (Cheng et al. 2011). A single Sc holds 30-40 Gc in a mature testis (Hofmann 2008). After getting differentiated post puberty Sc do not change any further and are called terminally differentiated cells which are specialized to maintain spermatogenesis. Sc create a specialized, immune protected environment within seminiferous tubules conducive for unhindered development of Germ cells (Gc). The functions of Sc are tightly regulated by FSH and testosterone. Sc structure, physiology and hormone responsiveness behavior changes with the age of animal. Concurrent with development of Sc, germ cell development also takes place. Till postnatal days 6–7, spermatogonia remain quiescent. By days 13–14, leptotene spermatocytes appear; by days 17–18, zygotene spermatocytes are present; by days 19–20 and Days 22–23, early and late pachytene spermatocytes, respectively, are seen. Haploid round spermatids first appear at days 24–25 and elongating spermatids by days 30–31; by day 36, elongated spermatozoa can be found and by day 52, mature sperm are seen in the lumen of seminiferous tubule (Hofmann 2008; Malkov 1998b). During all stages of germ cell development Sc provide factors required to fuel germ cell metabolism (lactate, transferrin, androgen binding protein), growth regulatory factors (stem cell factor, transforming growth factors alpha and beta (TGF-a and TGF-b), insulin-like growth factor-I (IGF-I),
fibroblast growth factor (FGF) and epidermal growth factor (EGF) and hormones and Mullerian-Inhibiting Substance (MIS) which regulate the development of the male reproductive structures or inhibin which feedback to regulate the hormonal signals affecting Sc (Skinner and Griswold 1980; Petersen and Soder 2006).

Most of our knowledge about Sc functions till date is based on studies conducted on pubertal (18-20 days old) Sc as model system. But extrapolation of results obtained from pubertal Sc on adult Sc does not seem logical. Since adult Sc dramatically differ from pubertal Sc in structure, function and regulation of Gc, it is imperative to culture pure population of adult Sc study their physiology. There are few reports describing methods of isolation and culture of Sc from adult animals (Hofmann 2008; Karzai and Wright 1992; Lampa 1999b; Anway et al. 2003; Wright and Luzarraga 1986). These methods are time consuming, inconsistent, very difficult and tedious to perform and do not allow cells to culture (Anway et al. 2003). These procedures involve several digestion steps with multiple enzymes and exorbitant high concentration of enzymes (collagenase 1mg/ml, hyaluronidase 0.5mg/ml and trypsin 0.5mg/ml). Treatment with high concentration of enzymes severely affects functions of Sc (WEISS 1963). These procedures use chemical adherents for making cells adhere to the culture surface. The chemicals used as adherents stimulate various signaling pathways in cells thus making interpretation of results superfluous (Singh et al. 2006). To circumvent difficulties inherent with existing methods of mature Sc cultures we took up this study to develop an efficient procedure of isolation and culture of Sc from adult rats. We have been able to isolate and culture Sc from 60-day old rats. The Sc cultures were assayed for hormone responsiveness in terms of lactate, Estradiol, cAMP production, lactate Dehydrogenase enzyme activity, FSH and T binding and expression of transferring, Inhibin, androgen binding protein, desert hedgehog protein gene etc. Our results shows that Sc cultured from 60-day old rat testis were viable and responsive to hormones and could be used for further studies.
2.2. MATERIALS AND METHODS

2.2.1. Animals

Wistar outbred rats (*Rattus norvegicus*) were procured from colony maintained by Small Animal Facility, National Institute of Immunology, New Delhi, India. Animals were maintained in a standard day night cycle with stable temperature and humidity and provided food and water ad libitum. All the animal experimentations were cleared by Institutional Animal Ethics Committee (IAEC) and performed following standard guidelines of “Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA),” Government of India.

2.2.2. Histological Analysis of Testes

2.2.2.1. Tissue Processing

For evaluating the status of Gc development, testes obtained from 5, 18 and 60-days postnatal ages of rats were fixed in Bouin’s fluid (saturated picric acid: formaldehyde: acetic acid in 15:5:1 ratio) for 24h. Subsequently, two pieces from each tissue were washed under running water for 3 to 4h to remove excess fixative. The tissues were then processed through ascending grades of ethanol (50-70-95%) for 1hr at each grade, and then subjected to two changes of 100% ethanol for 1hr each, following which they were transferred to xylene-ethanol (50% each v/v) for 30min. Tissues were finally processed in 100% xylene for 1hr. The xylene was replaced with xylene-paraffin (50% each v/v) solution followed by further infiltration with melted paraffin (at 58-60°C). Three changes of paraffin of 1hr each were given. The tissues were then embedded in paraffin and blocks were made using the embedding centre (Reichert Jung embedding centre). Sections (4µm thick) were cut with a Reichert Jung microtome 1640 and transferred onto albumin-coated slides.

2.2.2.2. Staining of Tissue Sections

The tissue sections were deparaffinized in xylene and processed through descending grades of ethanol (100-70-50%). Subsequently, the sections were hydrated with distilled water and stained with Harri’s hematoxylin for 10min at room temperature. Excess stain was removed with 1% acid ethanol (1% HCl in 95% ethanol) and washed.
briefly under running tap water. For staining with eosin, the slides were processed in ascending grades of ethanol and then stained with 1% eosin (1% ethanolic eosin) for 30 sec. Excess stain was removed with ethanol and the slides were treated with xylene for 5min. After mounting in DPX mountant, the sections were studied under bright field illumination with an upright microscope (Nikon, MICROPHOT-FX).

2.2.3. Chemicals and Reagents

Hanks Balance Salt Solution (HBSS), Dulbecco’s Modified Eagles Medium (DMEM) HAM F-12, ITS (Insulin, Transferrin, sodium Selenite), Insulin, Epidermal Growth Factor (EGF), Collagenase, Pancreatin, ovine Follicle Stimulating Hormone (oFSH) (NHPP, NIDDK, Torrance, CA, USA), Testosterone, Fetal Bovine/Calf serum, TRI Reagent, Chloroform, Isoprpyl Alcohol, Ethanol, RNA Polymerase, Lactate Dehydrogenase, βNAD, Glycine Buffer, Lactate Dehydrogenase Assay Kit (BioAssay Systems, USA), Estradiol antibody, cAMP antibody, R1881, RNase, DNase, Sybr Green (Applied Biosystems), Agarose, Loading Dye, Ethidium Bromide, Vimentin Antibody, αSmooth Muscle Actin Antibody, Secondary Antibody, Oil Red O, Fast Blue RR, Haematoxylin, Eosin, RNALater (Life Technology), Microarray Chips (Agilent Technologies), Trypsin EDTA, Naphthol ASB1 phosphate, DMSO. All the chemicals were from Sigma chemicals, USA, unless otherwise stated.

2.2.4. Isolation of Sc from 5-days old Rat Testes

20-25 rats of 5 days post partum age were used for a culture of Sc. Animals were euthanized by CO₂ gas and castrated. Castrated testes were washed twice with ice cold 10 ml HBSS before de-capsulation by tearing off the tunica albuginea with sterile forceps. Decapsulated testes were kept in a petri dish and chopped with sterile and sharp surgical blade. Minced tissue was transferred to a 15 ml tube and reconstituted in 10 ml ice chilled HBSS. All procedures were carried out on ice unless otherwise mentioned. Chopped tissue was washed twice with HBSS by inverting 5-6 times by hand. After washing, tissue was transferred to an autoclaved 100 ml glass flask and 25 ml pre-warmed HBSS containing 5 mg collagenase was added to this flask. Flask was kept for digestion in shaking water bath at 120 oscillations per
minute at 34°C for 20-25 minutes. Immediately after digestion, flask was kept on ice to quench enzymatic reaction. A cluster of digested peritubular and other interstitial cells forms after the digestion which was removed by a pair of long forceps. The content of the flask was poured in two 15 ml tubes and centrifuged at 1200 rpm at 4°C for 5 minutes. Supernatant was discarded and pellet was resuspended in 10 ml HBSS at 1200 rpm at 4°C for 5 minutes. This wash step was repeated twice. After washing, pellet was reconstituted in 10 ml HBSS and kept on ice for 5 minutes. Partially digested seminiferous tubules and larger clusters of Sc settles down at the bottom of the tube while small, culture ready, clusters of Sc remain suspended in supernatant. Supernatant was separated and stored with 1-2% serum on ice. The pellet of undigested tubules and larger Sc clusters was subjected to pancreatin digestion. For this digestion, pellet was resuspended in 25 ml pre warmed HBSS containing 5 mg pancreatin. Digestion was given by inverting slowly by hand at room temperature for 5 minutes. Clumps of digested cells and debris were discarded and serum was added immediately to the digestion solution to stop the enzymatic reaction. The content of the tube were transferred to two 15 ml tubes and spun at 1000 rpm at 4°C for 5 minutes. Supernatant was discarded and pellet was washed twice with 10 ml HBSS at 1000 rpm at 4°C for 5 minutes. After washing pellet was resuspended in 5 ml HBSS and pooled in with small clusters of Sc obtained after collagenase digestion and filtered through 80µm mesh before centrifuging at 1000 rpm at 4°C for 5 minutes. Supernatant was discarded and pellet was further washed twice with DMEM HAM F-12 at 1000 rpm at 4°C for 5 minutes each. After washings, pellet was reconstituted in DMEM HAM F-12 with 1% FBS/FCS as such that 1-2x10⁴ clusters were there in 1 ml volume and plated in 6 or 24 well plate.

2.2.5. Isolation of Sertoli Cells from 18 day old Rat

For isolation and culture of Sc from pubertal rats, 6-8 animals aged 18-20 days old were used and Sc were isolated as described previously (Welsh and Wiebe 1976) with slight modifications (Devi et al. 2006). Decapsulated testes were minced thoroughly by sharp and sterile surgical blade. Chopped tissue was washed thrice with 10 ml HBSS by inverting. The pellet was resuspended in 20 ml HBSS in an autoclaved 100 ml flask and 6 mg collagenase enzyme was added to this flask. The tissue was kept
for digestion in shaking water bath at 120 oscillations per minute at 34°C for 25-30 minutes or until a cluster of digested peritubular and other interstitial cells is formed. The cluster of digested cells was removed by a pair of long forceps and discarded. The content of flask was poured equally in two 15 ml tubes and spun at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded and pellets were washed twice with 10 ml HBSS at 1200 rpm for 5 minutes at 4°C. After washings, pellets were resuspended in 10 ml HBSS and kept on ice at unit g for 5 minutes. At this step, undigested seminiferous tubules and larger Sc clusters settle down at the bottom of the tube while small culture ready clusters remain suspended in supernatant. Supernatant was pooled in a 50 ml tube and stored on ice with 1-2% serum. The pellet consisting of undigested seminiferous tubules and larger Sc clusters was further digested with pancreatin. The pellet was resuspended in 15 ml pre warmed HBSS in a 50 ml tube and 4.5 mg pancreatin enzyme was added to this tube. Digestion was given at room temperature by inverting by hands for 5 minutes or until a transparent clusters of peritubular and other cells were seen. The clusters of digested cells were removed and serum was added immediately to this tube to block enzymatic reaction. The cell suspension was centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded and pellet was washed twice with 10 ml HBSS at 1200 rpm for 5 minutes at 4°C. This pellet was pooled in with the pellet obtained after collagenase digestion and filtered through 80µm filter to remove remaining aggregate of Ptc. Both the pellets were resuspended in 10 ml HBSS and spun at 800 rpm for 5 minutes at 4°C. The pellet obtained after this step was further washed twice with 10 ml DMEM HAM F-12 at 800 rpm for 5 minutes at 4°C. The supernatant was discarded and finally the pellet was reconstituted in DMEM HAM F-12 accordingly and plated with 1% serum.

2.2.6. Isolation of Sertoli cells from 60 day old Rat

Since established procedures (Wilson and Griswold 1979) for isolation and culture of Sc from adult rats are very limited, we used a procedure modified from that described by (Majumdar et al. 1995) for the culture of Sc from adult hamster testes. 4-6 numbers of animals (8-12 testes) were used for this culture. Animals were euthanized and castrated to obtain the testes. Testes were washed twice with 25 ml Ca^{2+}, Mg^{2+}
free HBSS in 50 ml tube to get rid of blood and other contaminants. Tunica albuginea was torn by using fine pointed forceps to de-capsulate the testes. Decapsulated testes were put in a sterile 100 ml flask which contains enzyme solution (3 mg collagenase/30ml pre-warmed HBSS). This flask was kept for digestion at 150-160 oscillation per minute at 37°C for 3 minutes (Digestion I). After digestion the supernatant enzyme solution was separated and testes were washed thrice with 25 ml HBSS. Enzyme solution was purified by spinning at 3000 rpm for 5 minute at 4°C and stored to be reused. The testes were again kept in flask with 25 ml HBSS (without enzyme) and put for mechanical shaking in shaking water bath at 150-160 oscillation/minute at 37°C for 7 minutes. Testes were taken out of flask and washed thoroughly with 25 ml HBSS in 50 ml tube to remove maximum number of Leydig cells and other interstitial cells. Now, the tissue was transferred to a petri dish and chopped into 2-4 mm long pieces. Chopped tissue was distributed equally in 3 tubes of 50 ml and washed thrice with 35 ml HBSS by vigorously shaking by hand and allowed to settle at unit ‘g’ on ice for 2-3 min. This washing step is important as it removes a large number of germ cells without the loss of Sc. The supernatant (containing mostly Germ cell (Gc) and interstitial cells) was discarded. This process was repeated at least 4 times to remove maximum number of Gc, free Leydig cells and RBC. The pellets were pooled and subjected to further digestion. Pellet was reconstituted in 30 ml of enzymatic solution (3 mg/30 ml HBSS, saved from digestion I) and kept for digestion in shaking water bath at 140-150 oscillation per minute at 37°C for 10 minutes (Digestion II). This digestion step strips off Peritubular cells (Ptc) leading to weakening of seminiferous tubules and exposing Sc-Sc and Sc-Gc junctions to collagenase enzyme. The partially digested tubules were settled at unit ‘g’ on ice for 2-3 minutes. Unlike 18 day Sc culture where a single cluster of cell mass formed, many cell aggregates were formed during this digestion. The cell aggregates were removed by a pair of sterile forceps. Supernatant was discarded and pellet of tubules was washed twice with 35 ml HBSS by shaking to get rid of germ cells. Washed tubules were again subjected to digestion with 3 mg collagenase/30 ml HBSS at 37°C for 10 minutes (Digestion III). After this digestion, the content of flask was decanted in a 50 ml tube and tube was kept on ice for 5 minutes. Supernatant, which contains small clusters of Sc, was separated in a tube and put for centrifugation at 600 rpm at
4°C for 5 minutes and pellet, which contains undigested tubules and large Sc clusters, was subjected to further digestion. Pellet obtained after centrifugation was resuspended in 20 ml HBSS and saved on ice with 1-2% serum.

Flow chart of procedure for isolation of Sertoli cells from 60-day old rat testis.
The step of digestion III was repeated several times (4-5 times) to gradually digest seminiferous tubules and obtain maximum number of Sc clusters. After the digestions were completed, all the fractions of Sc cluster suspensions were pooled in and filtered through 80µm mesh. Filtrate was spun at 600 rpm at 4°C for 5 minutes. Supernatant was discarded and pellet was washed twice with 25 ml DMEM HAM F-12 at 500 rpm at 4°C for 3 minutes. After washing pellet was reconstituted in DMEM HAM F-12 as such that 1-2x10^4 clusters of Sc were there in 1ml volume and plated with 5% serum.

2.2.7. Culture Conditions

After the isolation of Sc, culturable Sc clusters were plated in 24-well or 6-well plate(s) and cultured in DMEM/nutrient mixture F-12 Ham (DMEM/F12 HAM) containing 1% Fetal Calf Serum (FCS) (for 5 & 18 days Sc) and 5% FCS (for 60 days Sc) for 24 hr in a humidified 5% CO₂ incubator at 34°C. The next day i.e. day 2 of culture, cells were washed thrice with pre-warmed media (DMEM/F12 HAM) and were cultured further in media containing 5 µg/ml sodium selenite, 10 µg/ml insulin, 5 µg/ml transferrin, and 2.5 ng/ml epidermal growth factor. This media was designated as Growth Factor Media (GF media). On day 3 of culture, residual Gc, if any, were removed by hypotonic shock by incubating Sc with 20mM Tris-HCl, pH 7.4 for 3 to 5 min at 34°C (Galdieri et al. 1983) , then cells were washed thrice with pre-warmed media to remove dead Gc. Hormone treatment and other experiments were performed on day 4 of the culture.

2.2.8. Cytochemical Evaluation of the Cultured Cells

2.2.8.1. Phase Contrast Microscopy of cultured Sertoli cells

To study the morphology of cultured Sc obtained from various age groups, Sc were observed under upright phase contrast microscope (Nikon, DIAPHOT 300) and photographs were taken.
2.2.8.2. Cell Viability

Viability of the cultured Sc was estimated at each termination time points by staining the cells with trypan blue (0.04% in 10mM PBS) for 10min. Cells were washed with PBS and observed under light microscope for blue cells (non-viable cells).

2.2.8.3. Purity of Sc Culture Obtained from Various Postnatal Age Groups of Rats

2.2.8.3.1. Sc specific vimentin and peritubular cell specific α-smooth muscle actin staining

In a 24-well plate, 12-mm$^2$ glass coverslips were coated with poly-L-lysine and allowed to dry. Coverslips were washed with PBS, and isolated Sc obtained from 5-days, 18-days and 60-days -old rat testes were plated on the coverslips inside the culture well. Cells were cultured for 4 days in GF media without any hormonal supplemtations and on day 4 of culture, i.e. 24hr after hypotonic shock Sc and PTc, were identified by immunofluorescence. Briefly, coverslips were washed with PBS and fixed with 2% Paraformaldehyde for 20 min at room temperature. The coverslips were stained with antisera against Sc specific vimentin (Abcam, ab 8978) and Ptc specific α smooth muscle actin antibodies. Bound primary antisera were detected by secondary antibodies tagged with Alexa 488 (Molecular Probes, Invitrogen ; A-11001) and nucleus were visualized using Hoechst 33342. All images were taken on an inverted microscope (Nikon, ECLIPSE TE2000-S). Ten random fields from multiple coverslips were analyzed to determine the total number of nuclei and the number of cells expressing each marker. The mean (±SEM) percentage of cells expressing the cell-specific marker was calculated. More than 500 cells were counted for each detection method.

2.2.8.3.2. Alkaline Phosphatase Activity of the Cultured Sc

PTc contamination in the culture was identified by determining the alkaline phosphatase activity of the cultured Sc as previously described by (Chapin et al. 1987). On day 4 of culture Sc (cultured without any hormonal supplementation) were covered with the staining solution (2.5mg Fast blue RR added in mixture of solution 1 and 2, (where solution 1 is 2.5mg of naphthol ASB1 phosphate dissolved in 10µl
DMSO and solution 2 is 2.5ml of 1:1 distilled water diluted AMP buffer) and kept in the dark at room temperature for 10min. or until blue color appears. The staining solution was removed and cells were washed with diluted AMP buffer. Cells were observed under the microscope for blue color indicating alkaline phosphatase activity of the cells. Ten random fields from multiple wells were analyzed to determine the total number of blue spots indicating alkaline phosphatase activity of the cells. The mean (±SEM) percentage of the spots was calculated.

2.2.8.3.3. Oil Red ‘O’ Staining of Cultured Sc

Media from the cultured Sc was removed and 50% polypropylene glycol was added in enough volume to make a layer on cells for 12 h. After 12 h, the 50% polypropylene glycol was aspirated and cells were treated with 70% polypropylene glycol for 12 h and finally with 100% polypropylene glycol for 12 h. Polypropylene glycol was removed and 1% Oil Red O solution was added to the cells for 12 h. Oil Red O solution was removed and cells were washed twice with 50% polypropylene glycol and observed under microscope.

2.2.9. Hormone Treatments in Sc Culture

Hormone treatments were usually given 24hr after hypotonic shock i.e. day 4 of culture.

2.2.10. Estimation of L-lactate produced by Sc

On day 4 of culture, Sc were treated with i) GF media alone, ii) GF media containing o-FSH (50 ng/ml), iii) GF media containing Testosterone (10⁻⁷) and iv) GF media containing oFSH and T both for 24 h and the Sc exposed media were collected to measure the lactate produced and secreted by Sc. Cells from each well were dislodged by 1x Trypsin EDTA solution and were counted using hemocytometer.

Lactate was estimated in Sc spent media after 24 h of hormone treatment by using Lactate estimation kit (Sigma Diagnostics, USA). Spent media was distributed in 1.5 ml tubes and diluted by double distilled water to make the final volume 400 µl. Standard curve for lactate was obtained in the range of 0.66 to 6.34 µg. Reaction
mixture was prepared by mixing together appropriate amount of Glycine buffer, βNAD and Lactate Dehydrogenase enzyme. 100 μl of reaction mixture was aliqoted in every tube of sample, blank and standard. Tubes were vortexed and kept for incubation at 37°C water bath for 15 minutes. Immediately after incubation, tubes were put on ice to stop the enzymatic reaction. Optical Density (OD) of the tubes was read at 340 nm wavelength using spectrophotometer. The amount of lactate present in unknown samples was calculated using known standard as reference.

2.2.11. Lactate Dehydrogenase Enzyme Activity Assay

Lactate Dehydrogenase Enzyme activity assay was performed following (Meshulam et al. 2011) method. Briefly, Sc were treated with hormones and control media as per experimental requirement for 24 h. After the incubation was over, spent media was removed and discarded. Sc were scrapped by using sterile cell scraper. For one sample, generally Sc from three wells of 6 well plate were pooled. Cells were spun at 800 rpm for 5 minutes at 4°C. Supernatant was discarded and pellet was resuspended in 1 ml of buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA. Cells were then sonicated using cell sonicator for 90 seconds. Sonicated cells were centrifuged at 10,000g for 10 minutes at 4°C. Supernatant, which contains cytoplasmic component of cells, was saved in separate tubes and later used for Lactate Dehydrogenase (LDH) enzyme activity assay and protein estimation. Assay was run as per kit manufacturer’s (QuantiChrom™ Lactate dehydrogenase Kit, BioAssay Systems, USA) instructions in a flat bottom 96 well plate. All the components of reaction mixture were calibrated to room temperature and reaction mixture for one sample was prepared by mixing 14μl MTT solution, 8μl NAD solution, 8μl PMS solution and 170μl substrate buffer. 200μl water and 200μl calibrator were taken as reference. 10μl of cytoplasmic component of Sc from each experimental group was used for the assay. To the 10μl experimental sample, 190μl reaction mixture was added and OD was recorded immediately at 595 nm in ELISA reader. This reading is termed as OD at 0 minute. Reaction was incubated at room temperature for 25 minutes and OD was recorded again at 595 nm. This reading is considered as OD at 25 minutes. The calculation of LDH enzyme activity in the given sample was calculated by following formulae-
2.2.12. Protein Estimation

Protein was estimated in cytoplasmic fraction of sonicated Sc. A fraction of the sample was mixed with 750 µl of Bradford’s reagent. Bradford’s reagent was calibrated at room temperature before use. Incubation was carried out at room temperature for 45 minutes. OD was recorded at 595 nm. Amount of protein present in unknown samples was calculated against a standard curve using BSA standards.

2.2.13. Estimation of Cyclic AMP produced by Sertoli Cells

On day 4 of culture, Sc were treated with i) GF media alone ii) GF media containing 50 ng/ml o-FSH (obtained from National Hormone and Pituitary Program (NHPP), National Institutes of Health; Torrance, CA), GF media containing Testosterone (10^{-7}) and iv) GF media containing oFSH and T both for 1/2hr and the Sc exposed media were collected and stored in -80°C for evaluating the cAMP produced and secreted by Sc. Cells from each well were dislodged by Trypsin EDTA and were counted using hemocytometer. Cyclic AMP concentration in culture medium were analyzed by radioimmunoassay using ^125I-cAMP-TME (2-0’ monosuccinyl cAMP tyrosine methyl ester) and anti-cAMP antibody in accordance to the instructions provided by the National Hormone and Pituitary Programme (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health).

Briefly, cAMP-TME was iodinated by chloramine- T method and the iodinated cAMP-TME was separated by loading on a Sephadex G-10 (Amersham Pharmacia) column and eluting with peptide assay buffer. Various dilutions of cAMP...
standards as well as samples (250µl each) were acetylated by adding 7.5µl acetylation mix (2:1 mix of triethylamine and acetic anhydride) and 100 µl were taken from this (in duplicates) and were transferred in separate RIA tubes. 100 µl cAMP anti serum (in 1:10,000 dilution) and 100 µl $^{125}\text{I}$-cAMP-TME (15,000 cpm) were added to each tube and kept overnight (minimum 18hr) at 4°C. Next day, 1% anti-rabbit gamma globulin (200µl) and 20% polyethylene glycol were added before pelleting the suspension at 3000 rpm, at 4°C for 30min. The supernatant was removed and the radioactivity in the pellet was subsequently detected using a gamma counter (Wizard, 2470, Perkin Elmer). The concentration of cAMP in the sample was calculated from a standard curve plotted using known concentrations of cAMP run in parallel, along with unknown samples.

2.2.14. Estimation of Estradiol produced by Sertoli Cells

On day 4 of culture, Sc were treated with i) GF media alone ii) GF media containing o-FSH (50 ng/ml), GF media containing Testosterone ($10^{-7}$) and iv) GF media containing oFSH and T both for 24 h and the Sc exposed media were collected and were stored in -80°C to measure the estradiol converted by Sc. Cells from each well were dislodged by Trypsin EDTA and were counted using hemocytometer.

Estradiol was estimated using the protocol provided by WHO Matched Reagent Program. Briefly, 200 µl aliquots of culture media were transferred to glass tubes and 2 ml of diethyl ether was added to each tube. The solution was vortex mixed for 1 minute and allowed to settle. The ether extract was transferred to assay tubes and evaporated under a fume cupboard. After evaporation, 500 µl of buffer S was added to each sample and vortex mixed to completely dissolve the extract. To this 500 µl of sample solution, 100 µl of working tracer (100nCi/ml of $E_2$) and 100 µl of working dilution of antiserum were added. The mixture was incubated overnight at 4°C. Next day, 200 µl of the charcoal reagent was added rapidly to each tube, while the charcoal reagent was continuously stirred. The tubes were vortex mixed and allowed to stand for 30 min. at 4°C. The solution was centrifuged at 500g for 10 min. the supernatant was decanted carefully into scintillation vials. This step was done very fast to avoid delay in centrifugation and decanting of supernatant. Scintillation
cocktail mixture (4 ml) was added to each vial. The suspension was equilibrated at RT for overnight and the radioactivity in each sample was counted using a β-counter. The concentration of E₂ in the sample was calculated from a standard curve plotted using known concentration of E₂ run in parallel, along with unknown samples. The intra- and inter-assay coefficient of variation were < 5% and < 9% respectively.

2.2.15. Cell Counting

After the spent media was collected, Sc were washed with 0.5 ml pre-warmed (34°C) HBSS. Then, cells were digested with 200 µl of trypsin-EDTA (0.2% trypsin, 0.05 mM EDTA) solution per well at 34°C for 3 minutes (Janecki and Steinberger, 1987). The cells were removed from the wells and chilled on ice to stop enzyme reaction. The remaining cells in the well were collected by washing the well with HBSS and total volume was made up to 1 ml. Cell suspension was shaken manually and 1% FCS was added. A fraction (10 µl) of cell suspension from each well was used for counting cells on a hemocytometer under 10X magnification. Rest of the cells were pelleted (805g, 4°C, 4 min.), resuspended in 500 µl ice cold HBSS and centrifuged again. The cell pellet resulted from each well were suspended in Trizol and stored at -80°C for RNA isolation.

2.2.16. Androgen Binding Ability Assay of Sc

Androgen binding assay was performed as previously described (Fix et al. 2004). Briefly, on day 4 of culture, plated cells were incubated with 5nM radioactive R1881 or 10,000cpm/ml [3H] T (10⁻⁷ M) in presence or absence of 5µM cold R1881 or cold T (10⁻⁵ M) for 4hr at 34°C. After that, cells were washed 5 times with ice cold HBSS. Cells were treated with ethanol (1ml/well) for 30min to extract the bound steroid. 800µl ethanol from each well was transferred to vial containing Scintillation fluid and kept overnight. After removing the traces of ethanol in each well, cells were treated with lysis buffer for protein estimation by Bradford reagent (Sigma, USA). Next day receptor bound radioactivity was measured using β-counter. Bound androgen levels were expressed as fmol/mg protein indicating the detectable Androgen Receptor concentration in Sc.
2.2.17. $^{125}$I o-FSH Binding Ability Assay of Sc

FSH binding assay was done as described by (Dahia and Rao 2006). Briefly, iodination of 5 µg of o-FSH was carried out with 50 µCi of $^{125}$I-Na by the iodogen method. Cultured Sc from different age groups of rats were dislodged from the wells by scraper (without using any enzymatic treatment e.g. Trypsin-EDTA) and washed twice in ice cold DMEM. 1-2 million Sc were incubated with 100,000 cpm of $^{125}$I-oFSH for 2hr at 34°C. Non-specific binding was evaluated by incubating splenocytes (which lack FSH-Rs) with 100,000 cpm of $^{125}$I-oFSH (radioactive FSH). Specific binding of FSH was evaluated by incubating radioactive FSH in presence of increasing concentration of excessive (minimal 100 fold to 1000 fold) cold FSH for 2hr at 34°C.

2.2.18. RNA Extraction

RNA from Sc was isolated following TRIZOL method (Chomczynski and Sacchi 1987). Briefly, 0.5 million Sc were lysed in 200µl TRI Reagent and mixed thoroughly. 200µl of chloroform per 1 ml of initial volume of TRI reagent was added and mix by vigorous inverting for 25-30 times by hand and left at room temperature for 3-4 minutes before putting for centrifugation at 12000 g at 4°C for 15 minutes. Upper aqueous phase was seen in the tube which consists of total RNA. Aqueous phase was separated in a fresh tube. Phase separation was done very carefully to avoid genomic DNA contamination in RNA. Isopropyl alcohol in half the volume of initial volume of TRI reagent was added to the separated phase and kept on room temperature for 10 minutes and centrifuged at 10000g at 4°C for 5 minutes. Supernatant was decanted and RNA pellet was dissolved in 75% ethyl alcohol in equal the volume of initial volume of TRI reagent and spun at 10000g at 4°C for 5 minutes. Supernatant was decanted and pellet was semi-dried under laminar air flow hood for 10-15 minutes. RNA pellet was dissolved by pipetting up and down in 20 µl RNase free water (Ambion). Dissolved RNA was kept on 80°C in dry bath for 5 minutes and at room temperature for 5 minutes. This cycle was repeated thrice before either quantification or storage of RNA at -80°C.
2.2.19. Semi Quantitative RT-PCR

1µg of Total RNA isolated from each treatment group was first reverse transcribed using Reverse Transcription (RT) System (Promega Corp, USA) with AMV reverse transcriptase and oligo (dT)$_{15}$ for the single-strand cDNA synthesis. Subsequent PCR reactions (10µl reaction volume) were carried out using 1µl of the cDNA as template for checking the expression profile of each gene. For each gene numbers of PCR cycles were standardized to detect an acceptable expression level to confirm the findings. The list of genes along with primer sequences, annealing temperature ($T_m$) and PCR product sizes are given in table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Sequence</th>
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<tr>
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<td>NM_012502.1</td>
<td>F TGCAAGTGCCCAAGATCCTTT&lt;br&gt;R TTGGTTGGCACACACACAGT</td>
<td>70°C</td>
<td>408bp</td>
<td>30</td>
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<tr>
<td>FSHR</td>
<td>NM_199237.1</td>
<td>F ATTGACTGGCAAACAGGAGCA&lt;br&gt;R TTGGCAATCTTGGGTGCCC</td>
<td>67°C</td>
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<tr>
<td>Cyp19</td>
<td>NM_017085.2</td>
<td>F GCTTCTCATCGCAGAGTATCCGG&lt;br&gt;R CAAGGGTAAATTCATTGGGCTTGG</td>
<td>60°C</td>
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<td>LDHa</td>
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<tr>
<td>Transferrin</td>
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<tr>
<td>Dhh</td>
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<td>30</td>
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<td>Cyclophilin A</td>
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<td>F TCACCATTTCGGACTTGAGAC&lt;br&gt;R ACAGGACATTGGCGGAGACAGT</td>
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<td>120bp</td>
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</tbody>
</table>

All the primers mentioned in the table are rat specific and were designed manually.

2.2.20. Data Representation and Statistical Analysis

In all experiments, one treatment group was comprised of 3-4 wells within one culture set. At least 3 such sets of cultures (performed on different calendar dates) were used
to interpret the data. Testes of 25-30, 8-10 and 5-6 male rats were pooled for 5-days old, 18-days-old, and 60-days old rat Sc cultures respectively. Each gel photograph provided for RT-PCR is a representative of three independent experiments carried out on three different culture sets of each age group. One way ANOVA followed by Dunnett’s test was used for statistical analyses of the data.

2.3. RESULTS

2.3.1. Evaluation of the Status of Gc Differentiation in Rat Testis during Postnatal Development

Careful observation of testicular sections obtained from 5-days, 18-days and 60-days old rats enabled to determine the time of the robust initiation of Gc differentiation. Histological analyses of the testis of 5-days-old showed only spermatogonial populations, mainly spermatogonia A (Fig: 1A). Testicular sections of 18-days-old rats showed enlargement of seminiferous tubules and initiation of Gc differentiation associated with the presence of large number of spermatogonia B and appearance of primary spermatocytes (Fig:1B). Histology of 60 day old rat testis showed presence of mature sperm in lumen of seminiferous tubule (Fig: 1C).

2.3.2. Viability and Cytochemical Evaluation of the Purity of Cultured Sc

To check the viability and purity of Sc culture, trypan blue, vimentin staining (Sc specific) and α Smooth Muscle Actin & alkaline phosphatase activity (for PTc contamination) were performed respectively. Viability of Sc on day 4 of culture was found to be more than 98% in Sc from all 3 age groups (5, 18 and 60-days old) of rats. The purity of cultures was assessed by staining for Sc specific vimentin and alkaline phosphatase activity was evaluated to estimate the percentage of PTc contamination (Table 2). Imaging analyses confirmed that the isolation procedure resulted in highly enriched Sc fractions. Approximately 95% of the cells stained with the Sc specific marker vimentin and 1-3% cells stained for contaminating Peritubular cells in 5-days (Fig. 2 A, B, C and D), 18-days (Fig. 3 A, B, C and D) and 60-days (Fig. 4 A, B, C, D, E and F) old Sc cultures.
Table 2

<table>
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<tr>
<th>Name of Age Group</th>
<th>% of PTc contamination</th>
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<tr>
<td>5-days-old Sc</td>
<td>2-4%</td>
</tr>
<tr>
<td>18-days-old Sc</td>
<td>1-2%</td>
</tr>
<tr>
<td>60-days-old Sc</td>
<td>3-5%</td>
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Figure 1: Cross sections of testis. A. cross section of testis from 5-days old rat, B. cross section of testis from 18-dyas old rat and C. cross section of testis from 60-days old rat. Note the presence of spermatogonia A (yellow arrow) and Sc in the seminiferous tubules of 5-days, presence of spermatogonia B (green arrow) in 18-days-old rat testis and presence of all the stages of germ cell along with mature sperm (red arrow) in 60-days old testis. All the sections are at same magnification.
Figure 2: Photographs of Sc cultured from 5-days old rat testis. A. Sc stained with haematoxylin stain, B. Peritubular cell specific α-smooth muscle actin staining to quantitate number of contaminating peritubular cells (encircled) in Sc cultures, C. Sc specific vimentin staining and D. Nucleus stained with Hoechst dye. All the photographs are at same magnification.

Figure 3: Photographs of Sc cultured from 18-days old rat testis. A. Sc stained with haematoxylin stain, B. Sc specific Oil Red ‘O’ staining, C. Sc specific vimentin staining and D. Nucleus stained with Hoechst dye. All the photographs are at same magnification.
2.3.3. Lactate production by Sc Cultured from 5-days, 18-days and 60 days old Rats in Response to FSH and/or T Treatment

Lactate is a very important fuel metabolite produced by Sc and used by germ cells as major energy source. Sc use glucose from blood and stored glycogen to convert into lactate. 18 day old rat Sc are considered to be most responsive to hormones and hence deemed as standard to compare hormone responsiveness of Sc of various age groups. Sc were treated with FSH, Testosterone and FSH and T both. 18 day old Sc produces significantly higher amount of lactate when treated with FSH alone or in combination with T (Fig. 5 b). Testosterone alone does not stimulate lactate production in Sc from any of the age groups i.e. 5 days, 18 days and 60 days old rats.

Infant Sc (5 days old) secrets less amount of lactate as compared to that of 18 days old Sc. Contrasting to 18 days Sc, infant Sc are non responsive to hormone treatments and hence lactate produce by infant Sc is not augmented by either FSH
alone or FSH and T (Fig. 5 a). Although the basal level of lactate produced by Sc from 5 and 18 days were similar.

When stimulated by hormones (FSH and FSH+T), adult (60 days old) Sc behaved in the same manner as does 5 days Sc. Lactate produced by adult Sc was also not augmented by FSH and FSH+T treatment. The amount of lactate produced by adult Sc was approximately 10 times higher than lactate endogenously produced by 18 day Sc (Fig. 5 c). Testosterone did not alter lactate production by Sc from any age group studied.

![Graphs showing lactate production by Sc](image)

**Figure 5**: Lactate production by Sertoli cells in response to FSH and/or T. a. Lactate produced by 5 days old rat Sertoli cell b. Lactate produced by 18 days old rat Sertoli cell and c. Lactate produced by 60 days old rat Sertoli cell. C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH, T- Growth Factor media with Testosterone (10^{-7} M) and FT- Growth Factor media with 50ng/ml oFSH and Testosterone (10^{-7} M). * p< 0.05

2.3.4. **Lactate Dehydrogenase Enzyme activity of Sc Cultured from 5, 18 and 60 Days Old Rats upon FSH Stimulation**

Activity of Lactate Dehydrogenase Enzyme was evaluated in response to FSH treatment in Sc isolated from 5 day, 18 day and 60 day old rats. Sc were lysed by sonication and a part of cytoplasmic fraction was used for measurement of activity of
LDH enzyme. The basal levels of activity of Lactate Dehydrogenase in 5 days, 18 days and 60 days Sc were comparable. FSH did not have any effect on bioactivity of Lactate Dehydrogenase Enzyme in 5 day and 60 day Sc (Fig. 6 a, c). FSH significantly increased activity of Lactate Dehydrogenase Enzyme in 18 days Sc (Fig. 6 b).

![Figure 6: Lactate Dehydrogenase Enzyme activity of Sc in response to FSH. a. Lactate Dehydrogenase Enzyme activity of 5 days old rat Sc b. Lactate Dehydrogenase Enzyme activity of 18 days old rat Sertoli cell and c. Lactate Dehydrogenase Enzyme activity of 60 days old rat Sertoli cell. C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05](image)

### 2.3.5. Expression of Lactate Dehydrogenase Enzyme mRNA by Sc Cultured from 5, 18 and 60 days Old Rats

Expression of Lactate Dehydrogenase A Enzyme mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of LDH-A mRNA was checked in control and FSH treated Sc from
each age group. Expression levels of LDH-A mRNA remained constant in all age groups and was not augmented by FSH treatment in any age group (Fig. 7).

**Figure 7:** Expression of LdhA mRNA in 5-days, 18-days and 60-days old rat Sc in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of LdhA mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05.

### 2.3.6. cAMP production by Sc Cultured from 5, 18 and 60 days old Rats in Response to FSH and/or T treatment

cAMP is an important secondary messenger in Sc. Sc generate cAMP to efficiently transduce extracellular signals. 18 days old Sc, upon FSH and FSH+T stimulation for 30 minutes, produced highly significant amount of cAMP (Fig. 8 b). cAMP generated by 5 day old Sc was beyond the detection range of the assay (Fig. 8 a). Further, the stimulation of 5 day Sc with hormones (FSH and FSH+T) too was unable to produce enough cAMP to be assayed (Fig. 8 a).

Adult Sc produced very little amount of cAMP as compared to that by 18 day old Sc (Fig. 8 c). The basal level of cAMP produced by adult Sc was also less than those of 18 days old Sc. Stimulation of 60 day Sc with hormones marginally increased the amount of cAMP generated but it was way less than cAMP produced by 18 day Sc.
with the similar conditions of hormone treatment (Fig. 8c). Testosterone did not stimulate cAMP production by Sc from any of the above age groups.

![Graphs showing cAMP production by Sertoli cell in response to FSH and T.](image)

Figure 8: cAMP production by Sertoli cell in response to FSH and T. a. cAMP produced by 5 days old rat Sertoli cell b. cAMP produced by 18 day old rat Sertoli cell and c. cAMP produced by 60 days old rat Sertoli cell. C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH, T- Growth Factor media with Testosterone (10^-7 M) and FT- Growth Factor media with 50ng/ml oFSH and Testosterone (10^-7 M). * p< 0.05.

2.3.7. Aromatization Capability of Sertoli Cells Cultured from 5, 18 and 60 days old Rats

Sc have the capacity to aromatize testosterone into Estradiol by the activity of aromatase enzyme. Activity of aromatase enzyme alters with the age of the animal and so the ability to convert T into estradiol. When 18 day old Sc provided testosterone alone they produced a meager amount of Estradiol. But when FSH was also provided along with Testosterone, the production of Estradiol was remarkably very high than in the absence of FSH (Fig. 9b). 5 day old Sc did not produce enough amount of Estradiol to be detectable by the assay and it remained undetectable even in the presence of FSH (Fig. 9a). The ability of adult Sc to convert testosterone into Estradiol was also very weak. The basal
amount of Estradiol produced by 60 day old Sc was similar to that of produced by 18 days old Sc and was not further augmented by FSH treatment (Fig. 9 c).

**Figure 9:** Estradiol production by Sertoli cell in response to FSH and T. a. Estradiol produced by 5 days old rat Sertoli cell b. Estradiol produced by 18 day old rat Sertoli cell and c. Estradiol produced by 60 day old rat Sertoli cell. C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH, T- Growth Factor media with Testosterone (10^{-7} M) and FT- Growth Factor media with 50ng/ml oFSH and Testosterone (10^{-7} M). * p< 0.05.

### 2.3.8. Expression of Cyp19 mRNA by Sertoli Cells Cultured from 5, 18 and 60 Days Old Rats

Expression of Aromatase Enzyme (Cyp19) mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of Cyp19 mRNA was checked in control and FSH treated Sc from each age group. Expression levels of Cyp19 mRNA were significantly higher upon FSH treatment in 5 days and 18 days old Sc (**Fig. 10**). Expression levels of Cyp19 mRNA were not augmented by FSH treatment in 60 days old Sc and remained at the basal levels comparable to control of 5 days and 18 days old (**Fig. 10**).


Figure 10: Expression of Cyp19 mRNA in 5-days, 18-days and 60-days old rat Sertoli cell in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of Cyp19 mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05

2.3.9. Expression of Androgen Receptor (AR) mRNA by Sertoli Cells Cultured from 5, 18 and 60 Days old Rats

Expression of Androgen Receptor (AR) mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of AR mRNA was checked in control and FSH treated Sc from each age group. Expression level of AR mRNA was less in 5 day Sc than in 18 day and 60 day Sc (Fig. 11). But in any of the age group expression of AR mRNA was not altered by FSH treatment (Fig. 11).
Figure 11: Expression of Androgen Receptor (AR) mRNA in 5-days, 18-days and 60-days old rat Sertoli cell in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of AR mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05.

2.3.10. Expression of Follicle Stimulating Hormone Receptor (FSHR) mRNA by Sertoli cells Cultured from 5, 18 and 60 Days old Rats

Expression of Follicle Stimulating Hormone Receptor mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of FSHR mRNA was checked in control and FSH treated Sc from each age group. Expression levels of FSHR mRNA remained constant in all age groups and were not augmented by FSH treatment in any age group (Fig. 12).
Figure 12: Expression of Follicle Stimulating Hormone receptor (FSHR) mRNA in 5-days, 18-days and 60-days old rat Sertoli cell in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of LdhA mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05.

2.3.11. Binding of Testosterone and R1881 with Sertoli Cells Cultured from 5, 18 and 60 Days old Rats

Binding of Testosterone and its non aromatizable synthetic analog R1881 was evaluated in Sc cultured from 5, 18 and 60 days old rat testis. The binding values indirectly shows number of Androgen receptor (AR) protein present in the cell. T and R1881 binding was least in 5 day old Sc, and maximum in 60 day old Sc. Binding was intermediary in 18 days old Sc, however more than 5 days old Sc (Fig. 13).

Figure 13: Testosterone and R1881 binding ability of Sertoli cell from 5-days, 18-days and 60-days old rats. Each bar denotes mean SEM of 3 sets of cultures from respective age groups. * p< 0.05.
2.3.12. Binding of FSH with Sertoli Cells Cultured from 5, 18 and 60 days old Rats

Follicle Stimulating Hormone (FSH) binds to the FSH Receptor (FSH-R) present on the surface of the Sc. Expression levels and bioactivity of FSH-Receptors changes with the age of the animal. Status of binding of FSH to FSH-R was evaluated across the Sc from ages 5, 18 and 60 days old rats. Binding of FSH to FSH-R gradually increased from 5 days to 18 days and it was highest in 18 days old (Fig. 14). The binding was decreased in 60 days old Sc. FSH binding was similar in 5 days and 60 days old Sc (Fig. 14).

![Figure 14: FSH binding ability of Sertoli cell from 5-days, 18-days and 60-days old rats. Each bar denotes mean SEM of 3 sets of cultures from respective age groups. * p< 0.05](image)

2.3.13. Expression of Transferrin mRNA by Sertoli Cells Cultured from 5, 18 and 60 Days old Rats

Expression of Transferrin mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of Transferrin mRNA was checked in control and FSH treated Sc from each age group. Expression level of Transferrin mRNA was relatively significantly lesser in 5 day old Sc than 18 day and 60 day old Sc (Fig. 15). Expression levels remained same in 18
day and 60 day old Sc. In any of the age group studied expression of transferring mRNA was not altered by FSH treatment (Fig. 15).

![Graph showing relative expression of gene](image)

**Figure 15:** Expression of Transferrin mRNA in 5-days, 18-days and 60-days old rat Sertoli cell in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of Transferrin mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * \( p < 0.05 \)

### 2.3.14. Expression of Desert Hedgehog (Dhh) mRNA by Sertoli Cells cultured from 5, 18 and 60 days old Rats

Expression of Desert Hedgehog protein (Dhh) mRNA was evaluated by semi-Quantitative RT-PCR. Sc were isolated from 5-day, 18-day and 60-day old rats and treated with growth factor media alone and growth factor media along with 50ng/ml oFSH. Expression of Dhh mRNA was checked in control and FSH treated Sc from each age group. Expression level of Dhh mRNA was relatively significantly lesser in 5 day Sc than 18 day and 60 day old Sc (Fig. 16). Expression levels remained comparable in 18 day and 60 day old Sc. In any of the age group studied, expression of Dhh mRNA was not altered by FSH treatment (Fig. 16).
Figure 16: Expression of Desert Hedgehog (Dhh) mRNA in 5-days, 18-days and 60-days old rat Sertoli cell in response to FSH treatment for 24 h. by semi-quantitative RT-PCR. The graph depicts the expression of Dhh mRNA relative to expression of housekeeping gene Cyclophilin in respective control and treatment groups. The gel picture is a representative of such analysis carried out in three sets of culture (n=3). C- Growth Factor media alone, F- Growth Factor media with 50 ng/ml oFSH. * p< 0.05.

2.4. DISCUSSION

The number of Sc (Sc) has linear relationship with total sperm output from mature testis (Orth et al. 1988). Sc goes through changes in number, structure, functions and hormone responsiveness from immature to adult age. In the seminiferous tubules of immature (5 days) testis only Sc and quiescent spermatogonia are present. Contrary to this situation, in adult seminiferous tubules (60 day old) along with Sc, meiotic germ cells, spermatocyte, spermatids and mature sperms are present (Malkov 1998a). Germ cell, at various stages of development, affects Sc functions (Jegou 1993). Because of large number of germ cells and their intricate relationship with Sc, it is difficult to isolate and culture Sc from adult testis. As a consequence our understanding of physiology of these cells is poor. Lack of adequate information of adult Sc make extrapolation of results obtained with immature and pubertal cells to the adult potentially misleading.

There are some reports of isolation and culture of Sc from adult testes (Anway et al. 2003; Karzai and Wright 1992; Lampa 1999a; Steinberger et al. 1978).
Despite of efforts made to standardize an efficient method of isolation of Sc from adult testis, no well established protocol is available in literature. Presence of almost 70% germ cells in mature testis makes it tedious to differentially isolate Sc from germ cells. Germ cells are intricately entwined with Sc which make it further difficult to get a pure population of Sc. Existing protocols use high concentration (0.5-1 mg/ml) and a mixture of enzymes (collagenase, hyaluronidase, trypsin, trypsin inhibitor etc.) in order to digest seminiferous tubule to release Sc. But high concentration of enzymes exerts detrimental effect on cell surface receptors leading to poor ability of cells to attach to the surface (Weiss et al. 1992). It is imperative to culture Sc to study effect of hormones and other factors on Sc but some methods of isolation and culture of adult Sc does not allow culturing of Sc (Anway et al. 2003). An important physiological property of Sc is that they behave properly when in clusters. Single Sc does not give actual picture of Sc behavior. Most of the culture methods suggest use of Pea Nut Agglutinin (PNA) or Metrigel to facilitate attachment of Sc to the surface but these agents are known to invoke differentiation and division of epithelial cells (Anway et al. 2003; Hughes et al. 2010; Singh et al. 2006). These problems call for development of a procedure allowing efficient isolation and culture of Sc from adult testis.

We have developed a procedure of isolation and culture of Sc from adult testis that is faster than other available methods. It takes us less than 4 hours to isolate Sc from adult testes. All the cell isolated are not suitable for culture as Sc tend to be in clusters and only clusters of optimum size can be cultured. We are now able to culture approximately 10 million Sc from one adult rat (2 testes). Our procedure differs from others in use of only one enzyme, Collagenase, and that too 10 times lesser concentration (0.1mg/ml). Our method is rapid, Consumes less than 4 hours, requires 10 times less collagenase (1mg/10ml) and needs no other enzyme to isolate cells. We cultured Sc for 4 days only thus minimizing any sort of change in physiology of cells due to culture process. Our procedure involves treatment of Sc with hypotonic medium to get rid of germ cells yielding more than 95% pure culture preparations. Initially, we gave collagenase digestion for 3 minutes to loosen up seminiferous tubules and then gave mechanical shaking for 7 minutes without enzyme.
his mechanical shaking step is unique in our procedure as maximum number of loosely bound Leydig and other interstitial cells got removed due to mechanical shaking. After mechanical shaking we cut seminiferous tubules in smaller (2-4 mm) fragments by sharp blade and washed chopped seminiferous tubules 4-5 times with excess HBSS. This step removed a great number of germ cells from chopped tubules. This step is very critical for a good culture preparation as too long fragments of tubule does not get properly digested and too short fragments get over digested. We then put tubules for digestion with collagenase and terminated digestion after 15 minutes. If there were some Sc clusters in supernatant, we saved them on ice with serum and kept undigested tubules again for digestion. This process of digestion was repeated 5-7 times or until all the tubules got completely digested. At the end Sc clusters obtained after every digestion were pooled and cultured with 5% serum for first 24 h. After 24 h media was replaced with growth factor media which containing epidermal growth factor, insulin, sodium selenite and transferrin. We washed cells every time we changed the media. Hypotonic shock was given on day 3rd of culture with 20mM tris.HCl. Hypotonic shock and extensive washings removed remaining germ cells. These cells on day 4th are used for experimentation. Unlike in other procedures (Karzai and Wright 1992; Wright and Luzarraga 1986) cells were cultured only for 4 days thus avoiding any change in Sc physiology due to long term culture duration. We have evaluated the FT responsiveness of adult Sc in comparison with Sc from less hormone responsive stage (5 days old) and maximum hormone responsive stage (18 days old). We have studied the hormone responsiveness in terms of characteristic features attributed to adult Sc. We have evaluated hormone (FT) responsiveness of mature Sc in terms of lactate, cAMP and Estradiol production and respective gene expression. We have also checked for the expression of marker genes such as transferrin, inhibin, ABP, AR and Dhh associated with mature Sc. Our results shed light on functions of Sc important for maintenance of spermatogenesis.

In our studies, we define the ‘immaturity’ as inability of Sc in supporting spermatogenesis in contrast to ‘maturity’ considered as their ability to support all stages of germ development. Histological examination of sections of seminiferous tubules from 5 days testis showed the presence of somatic Sc and spermatogonia only.
Chapter 2

Histology of testis from 60 day old rats showed the presence of advance germ cell like secondary spermatocyte, spermatid, round spermatid and mature sperms in seminiferous lumen. We used 5 day Sc as model to ‘immature’ Sc and 60 day Sc as their ‘mature’ counterparts. Majority of studies evaluating action of hormones on Sc have been carried out on Sc cultured from 18-21 days old rats (Jutte et al. 1983; Meroni et al. 2003). Sc from 18 days old rats are easy to isolate and maintain in culture, also these cells respond maximally to hormones (Heckert and Griswold 2002). Since the objective of our study was to compare hormone responsiveness of immature and mature Sc, it was imperative to test bioactivity and optimum doses of hormones. For this purpose only we used 18 day old rat Sc to standardize doses of hormones and to ascertain bioactivity of hormones. In all the assays we performed, 18 day Sc were considered as positive control.

FSH induced cAMP production is an important parameter to study hormone responsiveness of Sc. Follicle Stimulating Hormone Receptor (FSHR), present on the surface of Sc, is a typical G-protein coupled receptor which upon binding with FSH leads to cAMP production (Simoni et al. 1997). Adenylate cyclase is responsible for the production of cAMP in the cell whereas cyclic nucleotide dependent phosphodiesterase (PDE) is the enzyme responsible for degradation of cAMP (Means et al. 1980). The report of Means et al establishing, kinetically, that a change in the Vmax of adenylate cyclase is equal to an equivalent change in the Km of PDE, which is inversely related to the Vmax of PDE, indicate dependency of steady-state concentration of cAMP upon the activity of PDE present in the cells (Means et al. 1980). Our results showed that binding of FSH to FSHR increase dramatically from 5 day to 18 day old Sc and then decrease to the basal level of 5 day Sc in 60 day old Sc. However, expression of FSHR remains constant and unaltered by FSH in Sc from 5 day, 18 days and 60 days old rats. In our study, we found that cAMP produced by 18 days old Sc on FSH treatment is much higher that cAMP produced by 5 day and 60 day old Sc. FSH stimulated cAMP production significantly in 5 day old Sc but the amount of cAMP produced was much lesser than what was produced by 18 day old Sc. cAMP produced by 60 day old Sc was equal to basal level cAMP produced by 5 day old Sc but unlike 5 day old Sc, was not augmented by FSH. Our data is in
coherence with published reports showing lesser amount of cAMP produced by 60 day Sc in response to FSH treatment (Steinberger et al. 1978). Low level of cAMP produced by adult Sc could be due to drastic change in PDE activity in Sc as the age of animal increases (Griswold et al. 1993). Using primary rat Sc culture, (Crepieux et al. 2001) have demonstrated that mode of FSH action changes with Sc maturation. FSH induced cAMP production in 5-days-old rat Sc culture is found to be less compared to that of the 19-days-old rat Sc. This change in the level of cAMP generated within Sc decides the fate of downstream signaling cascade. In 5-days-old Sc, FSH mediated low cAMP evokes a PKA dependent ERK activation that ultimately leads to Sc proliferation. However, this elevated level of cAMP in 19-days-old Sc deactivates ERK and stimulates classical PKA dependent pathway (Crepieux et al. 2001). Binding of FSH with Sc and expression of PDE4D isoforms holds the key to variation in cAMP production by Sc from various developmental stages.

L-Lactate, produced by Sc, is an important metabolite and preferred energy source of germ cells, especially meiotic germ cells (Hall and Mita 1984; Mita et al. 1982a). Lactate has been shown to be preventing germ cell apoptosis in human testis tissue culture (Erkkila 2002). 95.8% glucose uptake by Sc in converted in to L-Lactate (Robinson and Fritz 1981). Evidently, lactate is the preferred substrate for round spermatids and energy production is most efficient when this substance is present in high concentrations and pyruvate is present in low concentrations (Mita et al. 1982b). FSH significantly increases lactate production by 16-20 days old Sc (Mita et al. 1982b). Our results also suggest that FSH alone or in combination with Testosterone significantly increases lactate production by 18 days old Sc. As reported earlier, testosterone does not exert any effect on lactate production by Sc from any age group. FSH does not have any stimulatory effect on lactate production by 5 day old Sc. This could be due to unresponsiveness of immature Sc for FSH. Immature Sc does not produce enough cAMP in comparison to 18 day old Sc, when treated with FSH. Although there is adult like circulating hormone (FSH & T) levels in 5 days old rats, Sc from 5 days old rats are not responsive to hormones. Lactate produced by 5 day Sc is comparable to the basal level of that produced by 18 days old Sc. Lactate produced by 60 days old Sc is at least 8-10 times higher than what is produced basally
by 5 and 18 days old Sc. However, unlike 5 days and 60 days old Sc, lactate produced by 18 days old Sc was significantly augmented by FSH treatment. Inability of 5 days and 60 days old Sc to augment cAMP production by FSH also translates to insensitivity of FSH to alter lactate production by 5 days and 60 days old Sc. The basal level of lactate produced by 60-days old Sc was very high. The possible reason for this very high level of basal lactate produced by 60 days old Sc could be the presence of large number of advanced stages of germ cells. Germ cells interact with Sc and affect their physiological responses (Boujrad et al. 1995). Sc at maturity probably becomes self reliant to produce enough lactate that they don’t require any hormonal stimulus. We went on to check mRNA expression of Lactate Dehydrogenase A (LDHa) gene and bioactivity of Lactate Dehydrogenase Enzyme in 5, 18 and 60 days old Sc upon FSH stimulation. LDH is a well characterized isozyme system that is involved in the interconversion of pyruvate and lactate. These isozymes are encoded by three different genes, ldh a (muscle type), ldh b (heart type), and ldh c (testis type) (Li 1989). The latter gives rise to LDH C4 isozyme present only in the mature testis specifically in spermatozoa (Markert et al. 1975). The combinations of the other two gene products result in four tetrameric LDH isozymes, LDH-1 (B4), LDH-2 (A1B3), LDH-3 (A2B2), LDH-4 (A3B1), and LDH-5 (A4), which are present in variable proportions in different somatic tissues including rat Sc (Santiemma et al. 1987). We observed that mRNA expression of LDHa gene was unaltered throughout 5, 18 and 60 days old Sc and it was not affected by FSH treatment. The bioactivity of LDHa enzyme was significantly increased in 18 days old Sc upon FSH treatment but contrasting to 18 days old, LDHa bioactivity was not changed in 5 and 60 days old Sc when treated with FSH. This may give us a clue that why FSH does not augment lactate production by 5 and 60 days old Sc. We have checked the expression of LDHa mRNA by semi-quantitative RT-PCR in Sc from 5-days, 18days and 60-days old Sc rats. Expression of LDHa mRNA remains unchanged in all Sc from above mentioned age groups and the expression is not altered by FSH treatment in any of the age group. Our results indicated that FSH positively augments bioactivity, and not the transcription, of LDH enzyme in 18 days old Sc. FSH does not have any effect either on bioactivity or transcription of LDH enzyme in Sc from 5-days and 60-days old rats.
Aromatase enzyme converts testosterone into estrogen. This enzyme is transcribed by gene Cyp19. Aromatase is expressed in Leydig cells, Sc and in advanced germ cells. Sc are the main source of estrogen in immature testis, whereas Leydig cell and germ cells produce major part of estrogen in mature testis. Estrogen is important for germ cell survival. We could not detect estradiol in Sc spent media from 5 days old Sc. That may be probably because the level of estradiol present in 5 days old Sc spent media was too less to be detected by our assay system. FSH mediated increase in estradiol production by 20 days old Sc is well worked upon (Dorrington and Fritz 1975). In our case also, 18 days old Sc aromatized a good amount of testosterone and produced significantly high level of estrogen when treated with FSH. Contrary to that, estrogen produced by 60 days old Sc was equal to that of basal level produced by 18 days old Sc and it was not increased by FSH treatment. Low levels of Estradiol production by adult Sc affirm the fact that in adult testis, estrogen is produced by Leydig and germ cells. We studied the pattern of Cyp19 gene expression in 5, 18 and 60 days old Sc in response to FSH treatment. Cyp19 mRNA expression was found to be significantly augmented by FSH in 5 and 18 days old Sc but not in 60 days old Sc. Cyp19 expression in 60 days old Sc was at the basal level of 5 and 18 days old Sc and was not augmented by FSH stimulation. The level of expression of Cyp19 gene mRNA correlates well with ability of 5 days, 18 days and 60 days old Sc to convert testosterone in to estradiol. Our data is in coherence with Bois et al where they have observed the same pattern of Cyp19 expression in Sc from 10, 20, 30 and 70 days old old rat (Bois 2010).

Transferrin is an important protein involved in the transfer of iron from serum to the cells (Wada et al. 1979). Transferrin is a major serum glycoprotein synthesized primarily by liver and consisting of a single polypeptide chain (Schreiber et al. 1979). In the seminiferous tubules, Sc are involved in the delivery of transferrin bound iron to developing spermatids (Huggenvik et al. 1984). Low sperm count, region of aspermatogenesis and no sperm in the cauda epididymis results due to mutation in transferrin mRNA in mice (Sylvester and Griswold 1994). Thus transferrin synthesized by Sc is essential for normal spermatogenesis. Higher amount of transferrin secretion is a characteristic of mature Sc (Karzai and Wright 1992).
have checked for the expression of transferrin mRNA by 5 days, 18 days and 60 days old Sc. Expression of transferrin mRNA by 5 day Sc was lower than transferring mRNA expressed by 18 days and 60 days old Sc. The reason for more transferrin secretion by mature Sc could be due to presence of large number of Germ cells which require more transferring for their development (Dym 1970). Transferrin mRNA expression was not found to be regulated by FSH in any of the age group studied.

Hedgehog proteins are involved in a variety of functions during development. There are three types of hedgehog proteins classified till date i.e. Sonic Hedgehog, Indian hedgehog and Desert Hedgehog, of which Desert Hedgehog (Dhh) protein is expressed in Sc (Bitgood et al. 1996). Male mice homozygous null for Dhh gene were sterile (Bitgood et al. 1996). Mutation in DHH gene in human has been shown to cause partial gonadal dysgenesis associated with minifascicular neuropathy. These studies show that Dhh is a key paracrine molecule that plays a crucial role in both male gonadal differentiation and perineural formation in peripheral nerves (Umehara et al. 2000). Johnston et al, 2004 have shown expression of Dhh mRNA by Real Time PCR in Sc from 1, 5, 10, 15, 20, 25, 30, 40 days old and adult mice (Johnston 2004b). Expression of Dhh by Sc increases significantly after 10dpc and maintained at static level throughout till adulthood (Johnston 2004a). Our results show that expression of Dhh is markedly high in 18 days and 60 days old Sc in comparison to 5 days old Sc. Further, expression of Dhh is not regulated by FSH treatment in any of the age groups studied.

The prominent androgens, Testosterone and Dihydrotestosterone (DHT), are thought to mediate their biological effect predominantly through binding to the Androgen Receptor (AR) (Keller et al. 1996). AR, a 110 kD ligand-inducible nuclear receptor present in testicular somatic cells e.g. Sc (Sc), peritubular cells (PTc) and Leydig cells (Lc) but not in developing or mature germ cell (Gc). Binding of T to AR generates a genomic as well as non genomic pathway simultaneously and ultimately augments the expression of certain genes associated with spermatogenesis (Walker and Cheng 2005). Expression of AR in neonatal and infant rat Sc is either low or below the detection limit and the expression level increases as testes develop (Bremner et al. 1994). We have evaluated the expression of AR mRNA in 5 days, 18
days and 60 days old Sc by semi-quantitative RT-PCR. We found that expression of AR mRNA in 5 days Sc was significantly less in comparison to 18 days and 60 days old Sc. The expression of Ar mRNA was not modulated by FSH. Binding of T and R1881 were evaluated in cultured Sc obtained from 5-days, 18-days and 60-days old rats. Bound R1881 and T indicated the functionally active AR present in the culture. Binding ability of T and R1881 (in terms of AR bound T and R1881) to Sc were at the same basal level in 5 days and 18 days old Sc and significantly higher in 60 days old Sc. However, total R1881 bound to Sc in various ages of rats were little more compared to that of the T. Such an observation can be explained on the basis of the higher binding affinity of R1881 for AR than T (Raynaud 1980). Our finding indicated that the AR mRNA was translated properly and further allowed the binding of the ligand (T or R1881) across all the age groups. We inferred that the translation rate of AR mRNA was uniform as we could not find any difference between the AR mRNA and AR protein [expressed by bound ligands (R1881 and / or T)]. AR translation did not alter with the ages of rats.

2.5. CONCLUSION

Unavailability of a suitable procedure for isolation and culture of Sertoli cells from mature testis hampered our knowledge of physiology and regulatory mechanisms of adult Sc. Our understanding of Sc functions relies on information gained by studies conducted on 18-20 days old Sc. Mature Sc drastically differs in their functions from pubertal Sc; in this situation extrapolating data obtained from pubertal Sc on mature Sc is potentially misleading. To circumvent this problem of not having an appropriate method of culturing Sc from mature testis, we developed an efficient procedure allowing us to isolate and culture Sc from adult rats. Our method is rapid and efficient. It takes us less than 4 h to isolate and culture Sc from mature testis preventing loss of Sc functions due to damage of surface receptors on Sc because of high enzyme exposure (William Wright). Contrary to other people who have used exorbitant high concentration and cocktail of enzymes, we have used 10 times lesser enzyme concentration and a single enzyme, collagenase, to prepare Sc cultures. We culture mature Sc in defined media for 4 days avoiding any possible change in Sc physiology due to long duration of cultures. Staining of Sc cultures for Sc specific
vimentin antibody and oil red ‘O’ stain and peritubular cell specific α smooth muscle actin antibody and alkaline phosphatase staining ascertains purity and consistency of our mature Sc cultures. Adult Sc are terminally differentiated cell expressing some characteristics markers including transferrin and Dhh. Our results of higher expression of mRNA of transferring and Dhh correlates well with already published reports. We have studied the hormone responsiveness of adult Sc (60-day old) in comparison to immature Sc (5 days) and maximum hormone responsive Sc (18 days). The cAMP produced by adult Sc was equal to basal levels of cAMP produced by 5 days and 18 days old Sc. Like 5 days but contrasting to 18 days old, cAMP produced by adult Sc was not found to be regulated by FSH. The ability of adult Sc to covert testosterone into Estradiol was at the same level in 5 days and 60 days old Sc and unlike 18 days old was not augmented by FSH. These results were further confirmed by matching patterns of mRNA expression of Cyp19 gene.

Basal lactate produced by 60 day old Sc was 10 times more than what is produced by 5 days and 18 days old Sc but like 5 days and unlike 18 days this was not affected by FSH treatment. The bioactivity of Lactate Dehydrogenase Enzyme affirms the results of lactate production. Expression of Lactate Dehydrogenase A gene mRNA shows no variation in various age groups and on FSH treatment showing that bioactivity and not the gene expression of Lactate Dehydrogenase A enzyme regulates lactate production by Sc. The expression of Androgen receptor (AR) mRNA in Sc increases gradually from infant to adulthood being maximal at adulthood. We have found that androgen binding ability of Sc was seen maximum at 60 days of age in comparison to Sc from 5 days and 18 days old rat. The androgen binding status of Sc correlates with expression of AR mRNA. These results indicate that after attaining maturity Sc becomes independent of hormone signals. Testosterone seems to be more prevalent than FSH in mature Sc. This hormone independent behavior of mature Sc could be due to change in Sc physiology after they become terminally differentiated and largely due to presence of germ cell from various developmental stages. These studies show that the Sc cultures by us are suitable for various studies on mature Sc. Our study will pave the way for further studies on mature Sc helping us to understand insight of maintenance of spermatogenesis.
2.6. REFERENCES


