Prepubertal Buffalo (*Bubalus bubalis*) Leydig Cells: Isolation, Culture and Characterization

Niranjan Reddy[†], Shikha Sreshtha[†], Muneswarao Katakam, Malla Ashwini, Surabhi Sonam, Vinod Kumar and Sandeep Goel[*]

Testis Biology Group, Laboratory for the Conservation of Endangered Species, Centre for Cellular and Molecular Biology, Council for Scientific and Industrial Research, Uppal Road, Hyderabad, 500 007, India

Abstract: Water buffalo (*Bubalus bubalis*) is an economically important livestock species in India. Male buffaloes display delayed sexual maturity as compared to the bulls (*Bos taurus*). Serum testosterone level, the key regulator of sexual maturity of males, is reported to be low in male buffaloes in comparison to bulls. Testosterone secretion and progression of spermatogenesis is mediated essentially by Leydig cells in the males. Establishment of primary culture for buffalo Leydig cells can provide an excellent tool to investigate the factors which regulate testicular steroidogenesis. Therefore, the objectives of the present study were to isolate, culture and characterize buffalo Leydig cells. Immunohistological analysis revealed that cytochrome P450, family 11, subfamily A, polypeptide 1 **(**CYP11A1) specifically mark the Leydig cells in prepubertal buffalo testis. Using enzymatic digestion and Percoll density gradient centrifugation, a cell population that consisted of approximately 95% pure Leydig cells was obtained as indicated by CYP11A1 staining. Purified Leydig cells were cultured in DMEM/F12 supplemented with 10% foetal bovine serum (FBS) for 72 h. The cultured Leydig cells proliferated, expressed Leydig-cell specific transcripts (*STAR*, *HSD3B1*, *HSD3B6,* and *CYP17A1*) and proteins (CYP11A1, HSD3B and LHCGR), and secreted testosterone. It was concluded from the present study that buffalo Leydig cells can be maintained in culture for 72 h. The primary culture of buffalo Leydig cells can be used for studying acute responses, biochemical properties and other factors regulating testicular steroidogenesis, independent of other testicular cell types.

Keywords: Male buffalo, testis, Leydig cells, cell culture, CYP11A1, testosterone.

1. INTRODUCTION

Water buffalo (*Bubalus bubalis*) has a wide geographical distribution in the Indian subcontinent, Middle East, Eastern Europe and several other Asian countries [1]. India emerged as the world's leading milk producing country, accounting for 15.6% of the estimated 671.3 million tones of the world milk production, in the year 2007 [2]. There are 105 million buffaloes in India and approximately 140 million tons of buffalo milk is produced annually, thereby, making buffalo an economically important livestock species here. Buffaloes are better at converting poor quality forage into meat and milk. They are reported to have a 5% higher digestibility of crude fiber than high yielding cows and 4-5% higher efficiency of metabolic energy for milk production [3]. However, buffalo has been regarded as a sluggish breeder with low reproductive efficiency, characterized by delayed puberty, seasonality, anoestrus, low conception rate and long calving intervals [4]. Similarly, delayed sexual maturity is a serious problem in male buffalo. Establishment of spermatogenesis in buffalo testis is delayed in comparison to cattle [5, 6]. This delay in sexual maturation can be attributed to lower plasma testosterone level in male buffalo as compared to cattle [7, 8]. Interestingly, serum luteinizing hormone (LH) level in male buffaloes is comparable to that of cattle [9, 10]. Therefore, it is likely that other factor(s) may be necessary to stimulate testosterone synthesis in buffalo Leydig cells. However, these factors remain elusive.

In most species, testosterone is a critical regulator of male sexual maturation. The mammalian testis produces testosterone, which is secreted by the Leydig cells. The secreted testosterone promotes sexual differentiation in the foetus; secondary sexual maturation at the time of puberty and is essential for normal spermatogenesis in the adult [11]. Testosterone biosynthesis, which takes place in Leydig cells, is a complex process involving series of enzymes. Steroidogenic acute regulatory (STAR) is required for the transfer of cholesterol from the outer to the inner mitochondrial membrane, the first and rate-limiting step in steroid hormone biosynthesis [12]. Cholesterol in the inner mitochondrial membrane is converted into pregnenolone by the Cyp11a1 enzyme [13]. Pregnenolone diffuses across the mitochondrial membranes and is further metabolized by hydroxy-delta-5-steroid dehydrogenase, 3 beta (HSD3B) enzyme to progesterone in the smooth endoplasmic reticulum. Progesterone is a substrate for the cytochrome P450 17a (CYP17A) which catalyzes two distinct reactions, the hydroxylation of progesterone at

*Address correspondence to this author at the Testis Biology Group, Laboratory for the Conservation of Endangered Species, Centre for Cellular and Molecular Biology, Council for Scientific and Industrial Research, Uppal Road, Hyderabad, 500 007, India; Tel: +91-40-24006437;
Fax: +91-40-24006441; E-mail: sandeep@ccmb.res.in
[†]These authors contributed equally to this study.

ISSN: 1927-5196 / E-ISSN: 1927-520X/13

C_{17} followed by the cleavage of the two-carbon side-chain to yield the C_{19} steroid, androstenedione, the immediate precursor of testosterone. The final reaction is the reduction of the 17-ketone of androstenedione by hydroxysteroid (17-beta) dehydrogenase (HSD17B) to yield testosterone [14].

Reproductive performance of male buffaloes can be improved by stimulation of endogenous Leydig cells to produce testosterone. Manipulation of Leydig cells, to optimize testosterone secretion, requires their pure population in culture [15]. The characterization of buffalo Leydig cells in the culture and the assessment of their functional activity would thus, aid in studying the intracellular biochemical events in testosterone secretion. Leydig cells in monolayer culture, which maintains morphological and functional integrity, can be an excellent method to investigate the factors which regulate testicular steroidogenesis. Primary culture for buffalo Leydig cells, if established, could be used for studying acute response and biochemical properties of these cells, independent of other testicular cell types. Further, this would also be a valuable tool to examine the temporally regulated events in Leydig cell function.

The objective of the present study was to identify a specific marker for buffalo Leydig cells. Using the identified marker, an almost pure population of Leydig cell was obtained from prepubertal buffalo testis, which was cultured *in-vitro* and characterized.

2. MATERIAL AND METHODS

2.1. Ethics Statement

All animal experiments were approved by the Institutional Animal Ethics Committee of the Centre for Cellular and Molecular Biology, Hyderabad, India.

2.2. Collection of Testis

All chemicals for cell culture were from Sigma (www. sigmaaldrich.com) unless mentioned otherwise. The testes from prepubertal (aged 4-6 months; n=5) and adult (aged 2-3 years; n=3) water buffaloes (*Bubalus bubalis*) of Murrah breed were collected from Municipal Slaughterhouse, Hyderabad, India. Permission was obtained from the slaughterhouse to use these animal parts. A small piece of testis tissue from adult buffalo testes was submerged immediately after collection in RNALater® (Ambion, Inc; www.ambion.com) following manufacturer's instructions and stored at -20°C until isolation of RNA. Testes tissues for histochemical analysis were fixed immediately in 4% paraformaldehyde solution after collection. For Leydig cell isolation and culture, the testes from prepubertal buffaloes (n=5) were transported in DMEM/F12 containing 15 mM HEPES (DMEM/F12-HEPES; Invitrogen, www.invitrogen.com), antibiotics and antimycotic on ice within 1-2 hour to the laboratory.

2.3. Histochemistry

Fixed testicular tissues were dehydrated, embedded in paraffin and sectioned (6µm thick). Dilutions of primary and secondary antibodies were done in PBS with 1% BSA (Sigma, www.sigmaaldrich.com). Sections were dewaxed, rehydrated and stained with Leydig cell specific maker CYP11A1. Briefly, sections were blocked with 10% foetal bovine serum (Gibco, www.invitrogen.com) and 3% (w/v) BSA (Sigma, www. sigmaaldrich.com) in PBS for 30 min, incubated with rabbit anti-Cyp11a1 antibody overnight at 4ºC, washed several times with PBS, incubated with 3% (v/v) H_2O_2 for 10 min, washed three times with PBS, incubated with anti-rabbit HRP-conjugated secondary antibody (Calbiochem, www.calbiochem.com; 1: 200) for 30 min at 37°C, rinsed three times with PBS, incubated for 3-5 min in DAB substrate kit (Vector Laboratories, www.vectorlabs.com) according to the manufacture's instruction, rinsed thoroughly in distilled water, dehydrated and mounted in Vectamount (Vector Laboratories, www.vectorlabs.com) and observed under a Zeiss Axioplan 2 microscope. In negative controls, primary antibody was omitted and instead the section was incubated with 1% (w/v) BSA in PBS.

2.4. Isolation of Leydig Cells

All chemicals for cell culture were from Sigma (www.sigmaaldrich.com*)* unless mentioned otherwise. On arrival, the testes were washed several times with distilled water and phosphate buffer saline (PBS) and processed immediately for cell isolation. Testes were treated as described previously [16] with some modifications. Briefly, tunica and other visible connective tissues were removed. The testes were minced with scissors and incubated in DMEM/F12 medium supplemented with 15 mM HEPES (Invitrogen), 100IU/mL-50µg/mL penicillin-streptomycin, 40-mg/mL gentamycin, 1.5-mg/mL collagenase type-IV and 5 µg/mL DNase at 37ºC for 15 min in a shaking water bath operated at 100 cycles/min. The dispersed cells suspension was filtered through 40-µm cell strainer (BD Falcon, www.bdbiosciences.com). Collected cells were

subjected to Percoll Density Gradient for enrichment of Leydig cell population.

Buffalo Leydig cells were further purified using discontinuous Percoll gradients by a modification of the method described by Moundipa *et al*. [17]. Briefly, an iso-osmotic Percoll suspension (Pharmacia Biotech, www.gehealthcare.com) was prepared by the addition of 9 parts of Percoll to 1 part of 1.5M NaCl (Sigma). A discontinuous density gradient was made by diluting the iso-osmotic Percoll suspension with HEPES buffered DMEM/F12. The gradient consisted of 30 and 60% Percoll phases. The filtered cell suspension was placed over the gradient and centrifuged at 800 ×g for 20 min at 18º C. The band (cells situated between the 30% and the 60% Percoll phases) was collected, washed with DMEM/Ham F12 medium. The cells were counted using a hemocytometer and analyzed for Leydig cells by CYP11A1 staining.

2.5. Leydig Cells in Isolated Cells

Cells collected following isolation and after Percoll separation were fixed in 4% paraformaldehyde solution for 10 min, washed twice with PBS and attached to poly-l-lysine (Sigma)-coated slides (Fisher Scientific, www.fishersci.com). Cells were stained with anti-CYP11A1 antibody for identifying Leydig cells. Briefly, after permeablizing with 0.1% Triton X 100 (Sigma), cells were washed with PBS several times, incubated in 10% FBS in PBS. Cells were incubated with rabbit anti-CYP11A1 (1:200) at 4ºC overnight, washed several times with PBS, incubated with goat anti-rabbit-Cy3 (Molecular probes, www.invitrogen.com; 1:200) for 1 h at 37ºC, rinsed three times with PBS, stained with 1µg/mL Hoechst 33342 (Sigma) for 10 min, mounted in slowFade (Molecular Probes) and observed under an Axioplan 2 microscope fitted with an epifluorescent lamp. In negative controls, primary antibody was omitted and instead the cells were incubated with 1% BSA in PBS. To evaluate the average number of CYP11A1-positive cells, 10 random fields from each trail were counted. Approximately 1×10^4 cells (n = 5) were counted to evaluate CYP11A1-positive cells.

2.6. Culture and Characterization of Leydig Cells

Viability of Leydig cells collected after Percoll density gradient centrifugation was determined by trypan blue dye exclusion and cultured for six days *in vitro*. Cells were cultured in 24-well culture dishes (TPP; www.tpp.ch) with a micro cover glass (Fisher Scientific) at a density of 2×10^5 cells/cm^2 for staining with Leydig cell specific markers. Cover glasses were coated with 0.1% gelatin (Sigma) overnight (12-17 h) before seeding cells onto them. The culture medium used was DMEM/F12 supplemented with 10 µg/mL insulin, 10 µg/mL apo-transferrin, 100 IU/mL penicillin, 50 µg/mL streptomycin, 40 µg/mL gentamycin sulfate, single strength non-essential amino acid solution (Gibco, www.invitrogen.com), 1 mM pyruvate and 10% FBS (Gibco). Cells were cultured at 37ºC in a water-saturated atmosphere with 95% air and 5% CO_2. Cells were analyzed histochemically after 72 h of culture. The spent medium was collected and stored at -20° C for testosterone assay.

The cells cultured on cover glasses were stained with Leydig cell-specific markers such as CYP11A1, HSD3B (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase) and LHCGR (luteinizing hormone/choriogonadotropin receptor, previously known as LHR). Briefly, after fixing in 4% paraformaldehyde solution and permeablizing with 0.1% Triton X 100 (Sigma), cells were washed with PBS several times, incubated in 10% FBS (rabbit serum for anti-3β-HSD antibody) in PBS. Cells were incubated separately with one of the antibody, i.e., rabbit anti-CYP11A1 (1:200), goat anti-HSD3B1 (previously known as HSD3B; Santa Cruz Biotechnology; www.scbt.com; 1:30) and rabbit anti-LHCGR (previously known as LHR; Santa Cruz Biotechnology; www.scbt.com; 1:50) at 4ºC overnight, washed several times with PBS, incubated with corresponding secondary antibody, i.e., goat anti-rabbit-Cy3 or rabbit anti-goat-Alexa fluor 488 (both from Molecular probes; 1:200) for 1 h at 37ºC, rinsed three times with PBS, stained with 1µg/mL Hoechst 33342 (Sigma) for 10 min, mounted in slowFade (Molecular Probes, Eugene, USA) and observed under an Axioplan 2 microscope fitted with an epifluorescent lamp. In negative controls, primary antibody was omitted and instead the cells were incubated with 1% BSA in PBS.

2.7. Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

The cultured Leydig cells were characterized for the expression of Leydig cell-specific transcripts. Total RNA was prepared from testes of adult buffaloes (as positive control), freshly purified Leydig cells and from cells of primary cultures collected at 72 h. The stored adult testes tissue pieces were removed from RNALater® and processed for RNA isolation. The cells and testes tissue pieces were processed for RNA

isolation using TRIZOL reagent (Invitrogen; www.invitrogen.com) according to the manufacturer's instructions. Extracted RNAs were diluted with DEPC-water and incubated with 10 units of RNase free-DNase (Roche, www.roche.com) for 30 min at room temperature. Incubating the samples at 70°C for 15 min inhibited DNase activity and samples were stored on ice. Random Primers and RNase OUT (both from Invitrogen) were added to the RNA solution, incubated for 5 min at 65°C and set on ice. For reverse transcription, MMLV high performance reverse transcriptase (Epicentre biotechnologies, www.epibio.com) was added to the RNA solution and incubated for 10 min at 25°C, for 60 min at 37°C and for 5 min at 90°C (RT +). At the same time, the reactions without the addition of reverse transcriptase enzyme were done to check genomic DNA contamination (RT -). PCR amplification was carried out on 1 µL of the cDNA per 19 µL of PCR reaction mixture containing 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 × PCR buffer, 5 pmol of each primers and 1 U of *Taq* DNA polymerase (AmpliTaq Gold™, Applied Biosystems, www.appliedbiosystems.com). The primer sequences used for the amplification of specific genes are given in Table **1**. The PCR products were separated and visualized by 2% agarose gel electrophoresis containing 0.5 µg/ mL ethidium bromide. All PCR products were sequenced to confirm identity.

2.8. Measurement of Leydig Cell Proliferation

The proliferation of Leydig cells was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) rapid colorimetric assay. The purified Leydig cells were counted in haemocytometer, and the viability was determined by trypan blue dye exclusion. The cells were cultured in 96-well flat-bottom tissue culture dishes (TPP) at a density of 2×10^3 cells/well. After every 24 h for the following 72 h, the proliferation state of the Leydig cells was analyzed by MTT. The reagent of 20 µL MTT (Sigma; 5 mg/mL) was added to each well and incubated for 4 h at 37°C. After the incubation, the medium was then carefully removed and 200 µL of DMSO was added in each well to dissolve the formazan crystals. The absorbance was then measured at 470 nm using microplate reader. Each experiment was repeated 3 times, and all values represent data of a minimum of 9 wells.

2.9. Testosterone Assay

The spent media collected at 72 h of culture were analyzed for testosterone concentration using a commercial kit (CAN-TE-250; Diagnostics Biochem Canada Inc., www.dbc-labs.com). Media collected from the cultures described above were stored at -20° C before assaying and no steroid extraction procedure was employed. The hormone kit was used according to manufacturer instructions although modified by replacing the standards with fresh standard curves prepared in medium from the same batch as the medium used for the Leydig cell cultures. The assays were validated for use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve, and by recovery of the unlabeled ligand. No further modifications of the standard procedures were needed. Samples were measured in duplicate. Each experiment was repeated 3 times.

Table 1: RT-PCR Primer Sequences Used for Amplification of Specific Genes

Gene (ID)	Primers	GeneBank accession no.	Product size (bp)	Annealing Temperature (°C)
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*)	CCCAAACATCCAAGGACAGT CACGCTGTTGGAAAGAGTCA	NM_174343.2	241	56
Steroidogenic acute regulatory protein (*STAR*)	AAGCTGTGTGCTGGAAGCTCCTA ATAGAGTCTGTCCATGGGCTG	BC110213.1	387	58
Cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*)	CAGAGAAGTGCTCCGAATCC CTGCTCCAAAGGGCAAGTAG	NM_174304.1	242	56
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6 (*HSD3B6*)	ATGTGGCCTGGGCACATATTCT TTCTGCTTGGCTTCCTCCCAG	NM_017265.4	375	58
Glyceraldehyde-3-phoshate dehydrogenase (*GAPDH*)	GCGCCAAGAGGGTCATCATCT AAGGCCATGCCAGTGAGCTT	NM_001034034.1	355	58

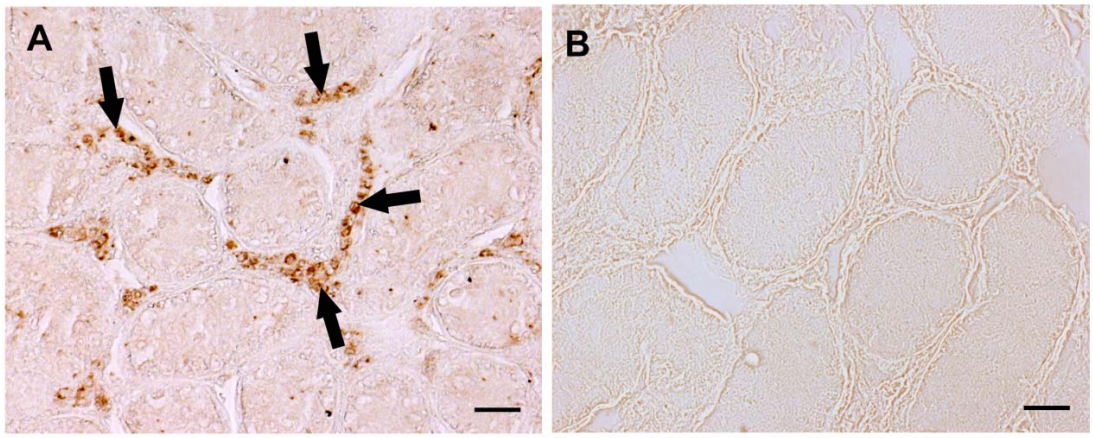

Figure 1: Expression of CYP11A1 in prepubertal testis. (**A**) Testis section from 5-month-old buffalo. Arrows indicate CYP11A1-positive cells. Note that CYP11A1 staining is seen only in the interstitial Leydig cells and other cells in testis are not stained. (**B**) In a negative control where primary antibody was omitted, no positive cells are present. Scale bar = 20 µm.

2.10. Data Analyses

The results are presented as mean ± SEM. The statistical analysis was conducted using analysis of variance (ANOVA). Differences were determined by analyzing the data with Fisher's PSLD test for significance between the means. The level of significance was set at $P < 0.05$.

3. RESULTS

CYP11A1 staining was specifically observed in the Leydig cells of prepubertal buffalo testis, and other cell-types such as Sertoli, peritubular myoid and germ cells were unstained (Figure **1A**). CYP11A1 protein expression in stained Leydig cells was present in cytoplasm. In negative control, where primary antibody was omitted, CYP11A1 staining was not observed in any cells (Figure **1B**).

Having found a specific marker, we next attempted to isolate a pure population of Leydig cells from the prepubertal buffalo testis. The abundant interstitial tissue in buffalo testis yielded large number of Leydig cells. The isolated cells after collagenase-IV digestion contained 82.6 ± 9.6% CYP11A1-positive. A total of $2.1 \pm 0.5 \times 10^7$ Leydig cells/gm of testis could be isolated. The viability of cells as determined by trypan blue dye exclusion was 72–74%. To further purify the Leydig cell population, we subjected the isolated cells to discontinuous density Percoll centrifugation between 30% and 60% density gradients. The cell population collected between the Percoll layers was rich in Leydig cells and consisted of 95.4 ± 3.3% CYP11A1-positive cells (Figure **2**). The number of Leydig cells collected was $1.2 \pm 0.3 \times 10^6$/gm of testis tissue and the viability of the purified cells were greater than 75%, as determined by trypan blue exclusion.

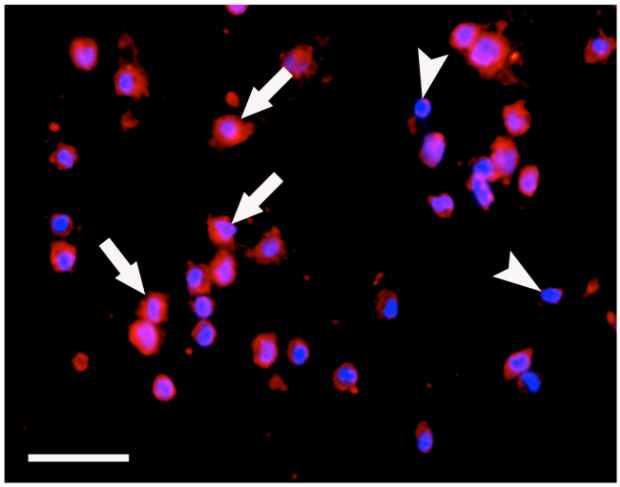

Figure 2: Fluorescence staining of purified Leydig cells after Percoll purification from prepubertal buffalo testis. CYP11A1-positive cells (arrows) appear red and negative cells (arrowheads) do not stain. The nuclei (blue) are stained with Hoechst 33342. Scale bar = 50 µm.

The enriched Leydig cell population collected after Percoll density gradient was cultivated *in-vitro*. The isolated Leydig cells were cultured for 72 h in DMEM/F12 medium containing 10% FBS without any growth factors and hormone supplements. Within few hours of seeding, most of the live cells attached onto the surface of culture dish and showed epithelial morphology. Cultured cells formed flat focal colonies within 72 h of culture (Figure **3A**). The Leydig cell colonies could be identified as a patch of epithelial cells in culture and grew in size, reaching an average diameter of 123 ± 4.5 µm. Some of the colonies reached 160 ± 5.6 µm in diameter by 72 h of culture. When viewed at higher magnification, the cultured cells

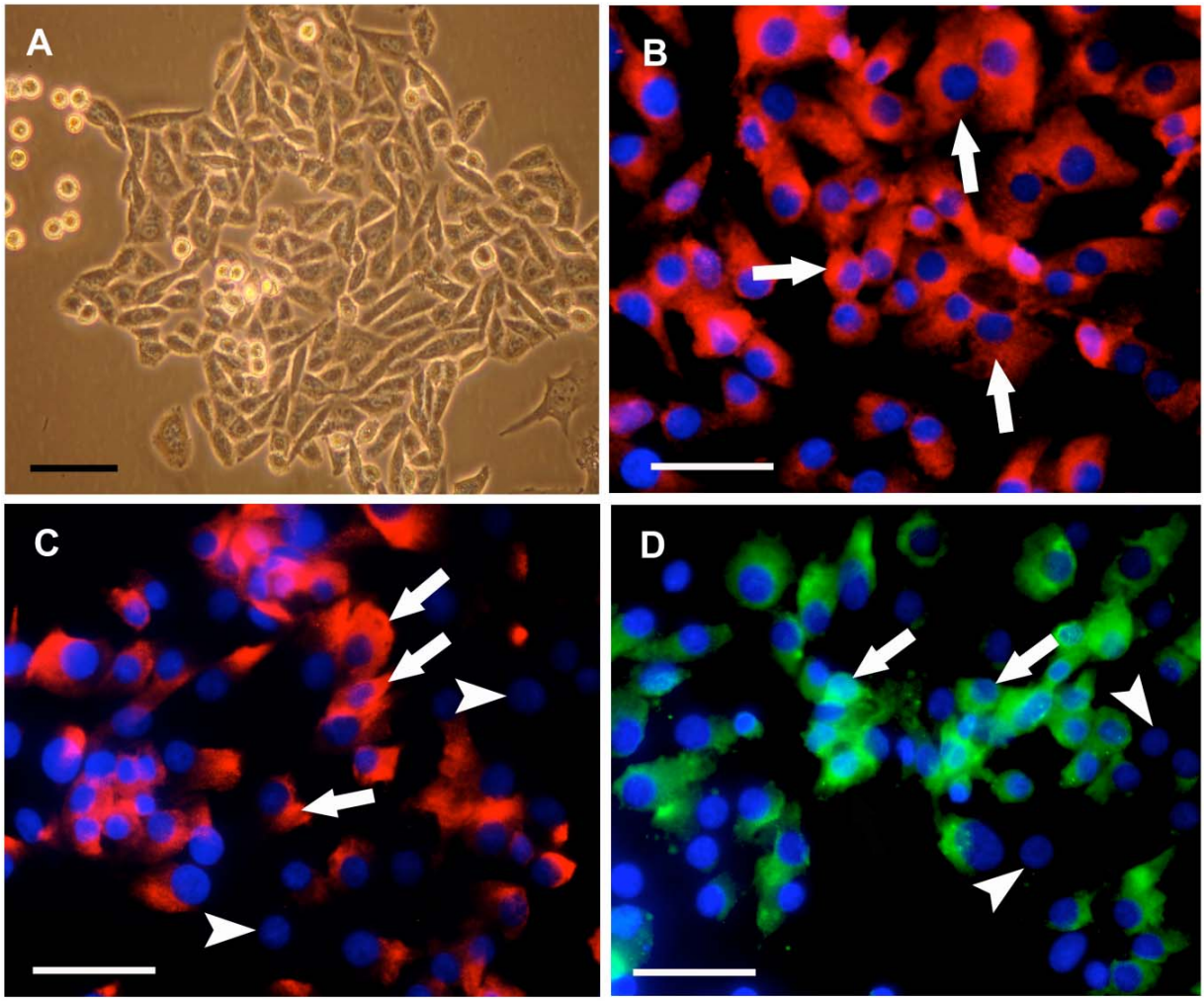

Figure 3: Buffalo Leydig cells in culture. (**A**) Leydig cells grow as focal colonies of adherent cells with epithelial morphology. Cultured Leydig cell colonies express (**B**) CYP11A1, (**C**) LHCGR and (**D**) HSD3B. Positively stained cells are marked by arrows and unstained cells by arrowheads. The nuclei are stained with Hoechst 33342. Scale bar = 50 µm.

appeared granular and contained numerous cytoplasmic lipid droplets (data not shown).

At 72 h, the cultured cells were characterized for the expression of Leydig cell-specific proteins. The CYP11A1 (Figure **3B**), LHCGR (Figure **3C**) and HSD3B (Figure **3D**) staining was seen in most cells in focal colonies. However few cells remained unstained. All proteins showed cytoplasmic localization and the staining intensity was variable in the stained cells.

As shown in Figure **4**, RT-PCR analysis of freshly purified Leydig cells showed either weak or no expression of steroidogenic enzyme-specific transcripts such as *STAR*, *HSD3B1*, *HSD3B6* and *CYP17A1*. However, after 72 h culture period, expression of all transcripts was clearly up-regulated.

The MTT colorimetric assay was employed to assess the growth and proliferation of Leydig cells in culture. As shown is Figure **5**, Leydig cells proliferation showed exponential increase until 72 h in culture.

The concentration of testosterone in the spent media collected at 72 h of culture was measured using a commercial ELISA kit. The mean testosterone concentration after 72 h of culture was 2.1 ± 0.7 ng/mL in the spent medium.

4. DISCUSSION

The present study is the first successful attempt to isolate and culture Leydig cells from buffalo testis. We established that CYP11A1 specifically marks the Leydig cells in prepubertal buffalo testis. CYP11A1 is a haem-containing protein, which mediates the conversion of cholesterol into pregnenolone [13, 18]. Immunoreaction of CYP11A1 is reported in Leydig cells of rat [19], pig [20-22], mouse [23], goat [24], and horse [25]. Therefore, it is indicative that CYP11A1

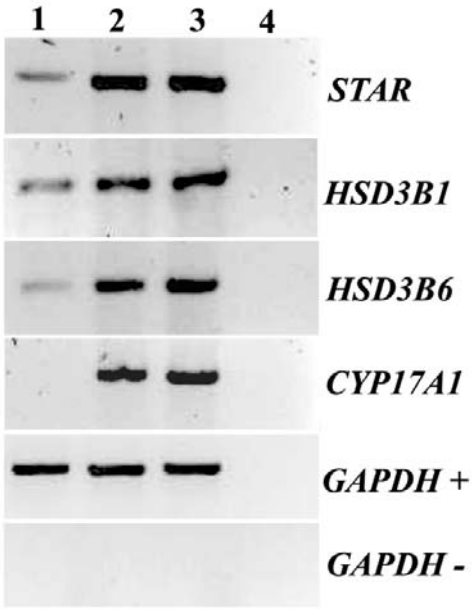

Figure 4: Reverse transcription–polymerase chain reaction (RT-PCR) analysis of purified (lane 1), and cultured cells at 72 h (lane 2) for Leydig cell-specific gene expression. The steroidogenic enzyme-specific transcripts are markedly up-regulated in cells of the primary cultures. *GAPDH* is used for normalizing RNA samples. The expression of genes in adult buffalo testis is shown as a positive control (lane 3). *GAPDH-*, isolated RNA without reverse transcription to check genomic DNA contamination. Lane 4, negative control, where cDNA was omitted.

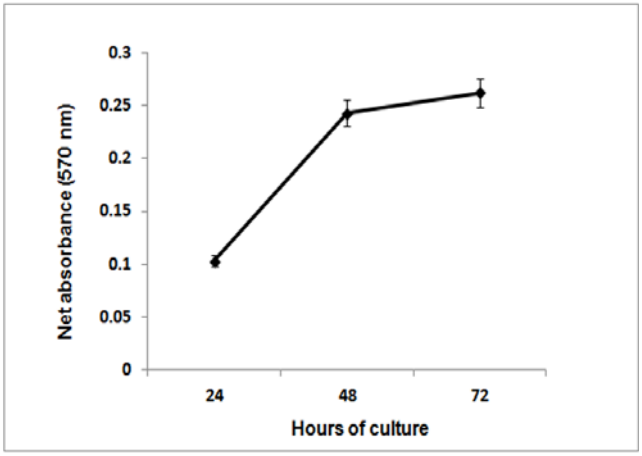

Figure 5: Evaluation of proliferation of buffalo Leydig cell in primary cultures by MTT assay. Leydig cells show exponential proliferation until 72 h in culture. Data are presented as mean ± SEM.

specifically marks Leydig cells in a variety of species, including buffalo.

Purified Leydig cell population from buffalo testis was obtained in the present study by using Percoll discontinuous density gradient centrifugation. Purification of Leydig cells using similar technique is reported in pig [15, 26, 27] rabbit [28], bull [17], rat [17, 29, 30], monkey [31], mouse [32] and horse [33-35]. In the present study, interface between 30% and 60% (corresponding to 1.067-1.075 g/mL buoyant densities) Percoll fraction had enriched Leydig cell population (95% of the cells were CYP11A1-positive). These data are in agreement with previous reports in bull where, Leydig cell enrichment was observed in similar Percoll layers [17].

The purified buffalo Leydig cells were cultured in DMEM/F-12 that contained 10% FBS and other supplements but without any supplemental growth factors or hormones. Due to the high serum content of the culture medium, Leydig cell proliferation was significant. Attached Leydig cells formed colonies that showed epithelial morphology during the culture. The flat colonies grew in size, suggesting that they were composed of actively proliferating cells and not merely aggregations of Leydig cells. A similar observation was made for mice Leydig cells cultured *in-vitro* [32]. Abundant cytoplasmic granules were observed in cultured Leydig cells in the present report. These cytoplasmic granules point towards the lipid droplets, which are rich in cholesterol [19].

Regulation of the steroidogenic machinery in gonadal cells involves a complex interaction of a diversity of hormones and numerous signalling pathways [36, 37]. In this report, expression of transcripts (*STAR*, *HSD3B1*, *HSD3B6,* and *CYP17A1*) and proteins (CYP11A1 and HSD3B) essential in testosterone biosynthesis were examined. Sun *et al*. [32] reported expression of Cyp11a1 and Hsd3b proteins in mouse Leydig cells cultured *in-vitro*. The freshly isolated Leydig cells showed a weak or absence of expression of transcripts essential for testosterone biosynthesis. However, the expressions of steroidogenic enzymes-specific transcripts were up-regulated in cultured Leydig cells (Figure **4**). Similar up-regulation of steroidogenic enzyme-specific transcripts in unstimulated Leydig cells isolated from neonatal pig testis is reported [15]. The up-regulation of steroidogenic enzyme specific-transcripts in cultured cells corroborates with elevated testosterone level in spent medium in the present study.

Expression of LHCGR protein was also observed in cultured Leydig cells in the present study. Localization of LHCGR in cultured rat Leydig cell is also reported [38]. The biosynthesis of testosterone is dependent on stimulation of Leydig cells by luteinizing hormone (LH),

which specifically binds to LHCGR receptor present on the surface of Leydig cells [39]. Presence of LHCGR expression is suggestive of a robust *in-vitro* culture of buffalo Leydig cells.

Buffalo Leydig cells in culture showed progressive proliferation until 72 h of culture, as assessed by the MTT assay. Proliferation of Leydig cells in culture has been assessed using the MTT assay in pig [40] and mouse [41, 42]. It can be concluded from the present study that buffalo Leydig cells survive and proliferate in primary cultures until 72 h.

Leydig cells from prepubertal buffalo testis secreted testosterone in culture medium. The Leydig cell culture media was assayed for testosterone using an ELISA kit. Testosterone in culture media using ELISA kit has been assayed previously [43, 44]. Buffalo naturally have low plasma testosterone level [7, 8] (an average of 0.6 ± 0.2 ng/mL in sexually mature males [45]). Therefore, presence of a considerable level of testosterone in the spent media is an interesting finding as culture medium was not supplemented with any specific factor to stimulate the Leydig cells for testosterone secretion. Elevated testosterone in spent media of unstimulated pig Leydig cells culture is reported [15], at levels which are higher than concentrations measured in the present study. This discrepancy could be due to difference of species in two studies. The elevated level of testosterone in spent media in the present study can also be attributed to a high number of purified Leydig cells in culture. The possibility that FBS may have stimulated the Leydig cell to secrete testosterone cannot be ruled out.

In conclusion, CYP11A1 specifically marks Leydig cells in buffalo testis. An almost pure population of Leydig cells can be isolated from prepubertal buffalo testis using enzymatic digestion and Percoll density gradient centrifugation. The purified Leydig cells can survive, proliferate, express Leydig cell-specific transcripts and proteins, and secrete testosterone. These cells can provide an excellent tool to investigate the factors which regulate testicular steroidogenesis in buffalo.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. B Prabhu for assistance in collecting testes. This work was supported by grants from Council for Scientific and Industrial Research (CSIR), Government of India.

REFERENCES

[1] Nagarajan M, Kumar N, Nishanth G, Haribaskar R, Paranthaman K, Gupta J, *et al*. Microsatellite markers in buffalo. BMC Genet 2009; 10: 68. http://dx.doi.org/10.1186/1471-2156-10-68

[2] Srivastava AK. Dairy scenario, In: Mallik M. Ed. National Dairy Research Institute Vision 2030, 2011; p. 1-7. Available from: URL http://www.ndri.res.in/ndri/Design/Vision.pdf.

[3] Mudgal VO. Comparative efficiency for milk production of buffaloes and cattle in the tropics. Proceedings of II World Buffalo Congress. New Delhi, India 1988; Vol II, Part II, pp. 454-462.

[4] Madan ML, Prakash BS. Reproductive endocrinology. Soc Reprod Fertil Suppl 2007; 64: 261-81.

[5] Ahmad N, Umair S, Shahab M, Arslan M. Testicular development. Theriogenology 2010; 73: 20-25. http://dx.doi.org/10.1016/j.theriogenology.2009.06.034

[6] da Luz PA, Andrighetto C, Santos PR, Jorge A, Constantino MV, Pereira FT, *et al*. Daily sperm production. Spermatogenesis 2012; 2: 88-93. http://dx.doi.org/10.4161/spmg.20220

[7] Sharma IJ, Agarwal SP, Agarwal VK, Dwaraknath PK. Changes in profiles of serum sex steroids. Theriogenology 1984; 22: 175-86. http://dx.doi.org/10.1016/0093-691X(84)90430-8

[8] Gulia S, Sarkar M, Kumar V, Meyer HH, Prakash BS. Divergent development of testosterone secretion. Trop Anim Health Prod 2010; 42: 1143-48. http://dx.doi.org/10.1007/s11250-010-9538-x

[9] Hemeida NA, el-Baghdady YR, el-Fadaly MA. Serum profiles of androstenedione, testosterone and LH. J Reprod Fertil 1985; 74: 311-16. http://dx.doi.org/10.1530/jrf.0.0740311

[10] Dixit VD, Singh B, Singh P, Georgie GC, Galhotra MM, Dixit VP. Circadian and pulsatile variations. Theriogenology 1998; 50: 283-92. http://dx.doi.org/10.1016/S0093-691X(98)00136-8

[11] Aumuller G, Seitz J. Protein secretion. Int Rev Cytol 1990; 121: 127-31.

[12] Stocco DM. The role of the StAR protein. J Endocrinol 2000; 164: 247-53. http://dx.doi.org/10.1677/joe.0.1640247

[13] Payne AH, Hales DB. Overview of steroidogenic enzymes. Endocr Rev 2004; 25: 947-70. http://dx.doi.org/10.1210/er.2003-0030

[14] Payne AH, Youngblood GL. Regulation of expression. Biol Reprod 1995; 52: 217-25. http://dx.doi.org/10.1095/biolreprod52.2.217

[15] Lervik S, von Krogh K, Karlsson C, Olsaker I, Andresen O, Dahl E, *et al*. Steroidogenesis in primary cultures. Theriogenology 2011; 76: 1058-69. http://dx.doi.org/10.1016/j.theriogenology.2011.05.011

[16] Goel S, Reddy N, Mandal S, Fujihara M, Kim SM, Imai H. Spermatogonia-specific proteins. Theriogenology 2010; 74: 1221-32. http://dx.doi.org/10.1016/j.theriogenology.2010.05.025

[17] Moundipa PF, Beboy NS, Zelefack F, Ngouela S, Tsamo E, Schill WB, *et al*. Effects of Basella alba. Asian J Androl 2005; 7: 411-17. http://dx.doi.org/10.1111/j.1745-7262.2005.00056.x

[18] Lin D, Gitelman SE, Saenger P, Miller WL. Normal genes. J Clin Invest 1991; 88: 1955-62. http://dx.doi.org/10.1172/JCI115520

[19] Sun XR, Risbridger GP. Site of macrophage. Biol Reprod 1994; 50: 363-67. http://dx.doi.org/10.1095/biolreprod50.2.363

[20] Moran FM, Ford JJ, Corbin CJ, Mapes SM, Njar VC, Brodie AM, *et al*. Regulation of microsomal. Endocrinology 2002; 143: 3361-69. http://dx.doi.org/10.1210/en.2002-220329

[21] Weng Q, Medan MS, Watanabe G, Tsubota T, Tanioka Y, Taya K. Immunolocalisation of steroidogenic enzymes. J Reprod Dev 2005; 51: 299-304. http://dx.doi.org/10.1262/jrd.16077

[22] Choi I, Kim JY, Lee FJ, Kim YY, Chung CS, Chang J, *et al*. Ontogeny of expression. J Androl 2009; 30: 57-74. http://dx.doi.org/10.2164/jandrol.107.004796

[23] Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF, Dieter M. Progenitor cells. J Cell Biol 2004; 167: 935-44. http://dx.doi.org/10.1083/jcb.200409107

[24] Weng Q, Medan MS, Ren L, Watanabe G, Tsubota T, Taya K. Immunolocalisation of steroidogenic enzymes. Exp Anim 2005; 54: 451-4. http://dx.doi.org/10.1538/expanim.54.451

[25] Almeida J, Conley AJ, Mathewson L, Ball BA. Expression of steroidogenic enzymes. Reproduction 2011; 141: 841-48. http://dx.doi.org/10.1530/REP-10-0499

[26] Bernier M, Gibb W, Haour F, Collu R, Saez JM, Ducharme JR. Studies with purified immature porcine Leydig cells in primary culture. Biol Reprod 1983; 29: 1172-78. http://dx.doi.org/10.1095/biolreprod29.5.1172

[27] Geiger TL, Khan M, Whisnant CS, Prien SD, Khan SA. Regulation of DNA synthesis. Domest Anim Endocrinol 1999; 17: 65-75. http://dx.doi.org/10.1016/S0739-7240(99)00024-7

[28] El-Sherbiny AM, Amin SO, Hernandez C, Carreau S. The immature rabbit testis: presence of two distinct populations of Leydig cells. World Rabbit Sci 1994; 2: 141-46.

[29] Hedger MP, Risbridger GP, de Kretser DM. Autoradiographical localization. Aust J Biol Sci 1985; 38: 435-44.

[30] Machluf M, Orsola A, Boorjian S, Kershen R, Atala A. Microencapsulation of Leydig Cells. Endocrinology 2003; 144: 4975-79. http://dx.doi.org/10.1210/en.2003-0411

[31] Anbalagan M, Sriraman V, Jagannadha Rao A. Isolation and characterization. J Endocrinol 2003; 179: 175-82. http://dx.doi.org/10.1677/joe.0.1790175

[32] Sun J, Zhong L, Zhu Y, Liu G. Research on isolation. J Reprod Dev 2011; 57: 433-6. http://dx.doi.org/10.1262/jrd.10-123N

[33] Hess MF, Roser JF. A comparison of the effects. Anim Reprod Sci 2005; 89: 7-19. http://dx.doi.org/10.1016/j.anireprosci.2005.06.014

[34] Yoon MJ, Roser JF. Insulin-like growth factor-I. Anim Reprod Sci 2010; 122: 353-8. http://dx.doi.org/10.1016/j.anireprosci.2010.10.001

[35] Yoon MJ, Roser JF. A synergistic effect. Anim Reprod Sci 2011; 126: 195-99. http://dx.doi.org/10.1016/j.anireprosci.2011.05.008

[36] Ascoli M, Fanelli F, Segaloff DL. The lutropin/ choriogonadotropin receptor, a 2002 perspective. Endocr Rev 2002; 23: 141-74. http://dx.doi.org/10.1210/er.23.2.141

[37] Stocco DM, Wang X, Jo Y, Manna PR. Multiple signaling pathways. Mol Endocrinol 2005; 19: 2647-59. http://dx.doi.org/10.1210/me.2004-0532

[38] Teerds KJ, Rijntjes E, Veldhuizen-Tsoerkan MB, Rommerts FF, de Boer-Brouwer M. The development of rat Leydig cell. J Endocrinol 2007; 194: 579-93. http://dx.doi.org/10.1677/JOE-06-0084

[39] Dufau ML. Endocrine regulation. Annu Rev Physiol 1988; 50: 483-508. http://dx.doi.org/10.1146/annurev.ph.50.030188.002411

[40] Brun HP, Leonard JF, Moronvalle V, Caillaud JM, Melcion C, Cordier A. Pig Leydig cell culture. Toxicol Appl Pharmacol 1991; 108: 307-20. http://dx.doi.org/10.1016/0041-008X(91)90120-4

[41] Krockova JZ, Massanyi P, Sirotkin AV, Pivko J, Makarevich AV, Lukac N, *et al*. Nickel induced structural. J Trace Elem Med Biol 2011; 25: 14-18.

[42] Wu L, Zhang A, Sun Y, Zhu X, Fan W, Lu X, *et al*. Sirt1 exerts anti-inflammatory effects. Fertil Steril 2012; 98: 194-9. http://dx.doi.org/10.1016/j.fertnstert.2012.04.008

[43] Lai KP, Wong MH, Wong CK. Inhibition of CYP450scc expression. J Endocrinol 2005; 185: 519-27. http://dx.doi.org/10.1677/joe.1.06054

[44] Mondillo C, Falus A, Pignataro O, Pap E. Prolonged histamine deficiency. J Androl 2007; 28: 86-91. http://dx.doi.org/10.2164/jandrol.106.000257

[45] Gunarajasingam D, Rajamahendran R, Downey BR, Lague PC. Testosterone secretion. Theriogenology 1985; 24: 185-95. http://dx.doi.org/10.1016/0093-691X(85)90182-7

Received on 20-11-2012 Accepted on 10-01-2013 Published on 22-03-2013

DOI: http://dx.doi.org/10.6000/1927-520X.2013.02.01.5