Isolation and Culture of Leydig Cells from Adult Rats

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INTRODUCTION

Bouin and Ancel (1), based on a number of observations, provided compelling evidence for an endocrine role for Leydig cells. Subsequently, Christensen and Mason (2) developed techniques for the separation of Leydig cells from the seminiferous tubules and demonstrated the ability of the Leydig cells to transform radiolabelled progesterone to androgens. Later, Hall *et al.* (3) showed that the conversion of cholesterol to androgens occurred only in the interstitial tissue.

It is now well established that androgens play a vital role in spermatogenesis, sperm maturation and maintenance of accessory sex organs and sexual behaviour. The synthesis of androgens by the Leydig cells is under the influence of LH. Exposure to contraceptive agents, environmental toxicants etc. adversely affect Leydig cell production of testosterone. Hence, it is important to be able to assess the steroidogenic capacity of Leydig cells which in turn requires a highly purified sample of Leydig cells.

The method described in this chapter for isolation of Leydig cells, is a multi-step method with 98% purity and maintains Leydig cells in primary culture.

ANIMALS, REAGENTS AND THEIR SUPPLIERS

Animals

Adult male (Sprague Dawley) rats (90-120) days old weighing 350-450 g were routinely used for the isolation of Leydig cells.

Reagents

- a) Medium 199, Hanks balanced salts solution (without calcium and magnesium salts),

Dulbecco's phosphate - buffered saline solution, sodium bicarbonate, HEPES, gentamicin sulfate and fetal bovine serum were purchased from GIBCO, Grand Island, New York.

- b) Dulbecco's modified Eagle's medium/Ham's F12 phenol red-free culture medium containing 15 mM HEPES, protease-free bovine serum albumin, soybean trypsin inhibitor, heparin, EDTA, bovine lipoprotein, nitroblue tetrazolium salt, etiocholan - 3 β -01-17-one and β -nicotinamide adenine dinucleotide were purchased from Sigma (St. Louis. M. O.)

The other reagents used were: Collagenase from Worthington Biochemical (Freehold, NJ, USA), bovine serum albumin (BSA) and ovine luteinizing hormone (LH) from Schwarz/Mann Biotech, Division of ICN Biochemicals, Cleveland, OH.

Buffers and preparation of media

a) Dissociation Buffer (ph 7.1-7.2)

Dissolve one packet of medium 199 in 800 ml of double distilled de-ionized water (DDDW) and add sodium bicarbonate (8.5 mm), and soybean trypsin inhibitor (25 g/ml). The volume was adjusted to 1 lit. by DDD water.

b) Elutriation Buffer (pH 7.1-7.2)

Dissolve one packet of medium 199 in 800 ml of DDD water and add sodium bicarbonate (8.5 mM), HEPES (9 mM), BSA (0.5%), heparin (282 g/ml), EDTA (336 g/ml) and soybean trypsin Inhibitor (25 ug/ml). Adjust the volume to 1 lit with DDD water.

c) Percoll Buffer (pH 7.1-7.2)

One liter buffer was prepared by adding 15 mM HEPES, 4mm sodium bicarbonate, 0.25% BSA and 25 mg/ml soybean trypsin inhibitor; the pH was adjusted between 7.1 to 7.2.

d) Leydig Cell Culture Medium (LC Medium; pH 7.1-7.2)

One liter of LC medium was buffered by adding HEPES (15 mM), sodium bicarbonate (26 mM), PF-BSA (0.1%), and gentamycin sulfate (12 g/ml) in DDD water; the pH was adjusted to 7.1-7.2.

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These buffers should be filtered through suction sterilized filters under the culture hood into sterilized bottles and stored at 4°C.

e) Internal Collagenase (IC)

Internal collagenase is prepared by adding 12 mg of collagenase and 1 ml of dispase to 12 ml of dissociation buffer in a 50 ml Falcon tube; mix well before use.

f) External Collagenase (EC)

External collagenase was prepared by adding collagenase (16 mg), DNase (0.25 ml), and dispase (1 ml) to 32 ml of dissociation buffer in a 50 ml Falcon tube; mix well and stored at 4°C.

IC and EC should be prepared at the time of Leydig cell separation.

g) Percoll Tubes

To 4 ml of 10x Hank's or HBSS solution in a 50 ml Falcon tube, 44 ml of Percoll (Pharmacia, Piscataway, USA) was added and mixed well; 19 ml of above solution was taken in two sterilized Oakridge centrifuge tubes. To one of these tubes, add 16 ml Percoll buffer and 10 μ l of each density marker beads i.e. green, red and blue and mix well. In another tube, add 15.5 ml Percoll buffer and 0.25 ml DNase. These Percoll tubes should be prepared in culture room under hood and under strict sterilized conditions.

PROCEDURE

The multi-step method for isolation of adult rat Leydig cells is almost similar to the method described by Kleinfelter *et al.* (4,5). The method described here resulted in a preparation of 18×10^6 highly purified Leydig cells when 12 adult rat testes were utilized. Fig. 1 is a schematic representation of this multi-step method. The method is described under the following steps.

1. Perfusion of testis

After CO₂ asphyxiation of the rat on the day of Leydig cell isolation, the testes from the rats were quickly removed and placed in ice-cold dissociation buffer (DB). With the help of a fine scalpel blade, a small longitudinal incision was made through the outer wall of the testicular artery and the vasculature was perfused with freshly prepared internal collagenase (IC) solution (0.3-0.5 ml) through a very fine cannula until the vasculature is devoid of red blood cells (Fig. 2). Each perfused testis is transferred to a beaker containing fresh chilled dissociation buffer.

2. Enzymatic Dispersion of Interstitial Cells

After perfusion, each testis was decapsulated by making a fine nick on the tunica with a sterilized

iridectomy scissors and the seminiferous tubules were squeezed into a 50 ml centrifuge tube (Falcon) containing 5 ml DB. If this has been done correctly, the parenchyma will be suspended by the excised testicular artery still attached to the capsule. By pushing down on the parenchyma, the artery remaining in the parenchyma was stripped out, and the parenchyma was allowed to fall into the tube. Two decapsulated testes were placed in each centrifuge tube, and 5 ml of external collagenase (EC) solution was added to all tubes containing testes. The centrifuge tubes were tightly capped and incubated horizontally with shaking (70 cycles/min) at 34°C for approximately 10 minutes. The duration of the incubation varied, depending on the final appearance of the dissociated parenchyma. The incubation was terminated when the final appearance of the dissociated parenchyma appeared loose, but retained the original ovoid architecture of the original mass. This was critical because prolonged dissociation of the parenchymal wall would liberate increasing number of contaminating germ cells, thereby decreasing the efficiency of Leydig cell isolation. Moreover, extended dissociation would result in extensive damage to collagenase-sensitive LH receptors on the Leydig cells.

Incubation was terminated by filling each centrifuge tube with fresh collagenase-free DB. The contents of each tube were mixed by inversion several times and poured through double 100 μ m nylon mesh (Spectrum Medical Industries, LA, USA) into an empty Falcon centrifuge tube. The volume of the cell suspension in each of the receiving tube was adjusted to 50 ml and the dissociated cells were pelleted by centrifugation at 1000 rpm at 4°C for 10 min. The supernatant was decanted and the pellet was resuspended immediately in 52 ml of DB, without making any bubbles.

3. Centrifugal Elutriation

Before killing the animals for isolation of Leydig cells, the elutriation system is assembled as shown in Fig. 3. After assembling the elutriator, the elutriation rotor (JE-6B) was fixed into the Beckman centrifuge machine and attached with peristaltic pump (Cole Parmer, Chicago, USA) and bubble trap as shown in Fig. 3. The required flow rate (in present isolation procedure, at 16 ml/min.) was set up; 70% ethanol was run through the whole system and the elutriator was run for 5 min at 2000 rpm at 10 to 15°C. During collagenase incubation, the ethanol was flushed out and replaced with elutriation buffer (EB). Bubble trap was filled to the top of the inflow needle with EB (around 100 ml) and the rest of the elutriation system i.e. the inflow tubing, elutriation rotor, and outflow tubing was filled with EB. While EB was pumped through the system at a rate of 16 ml/min, the elutriation rotor was accelerated to 2000 rpm and while

Fig. 1. Flow chart of different steps involved in the elutriation method and culture of isolated leydig cells

* indicate % purification of Leydig cells.

Fig. 2. Testicular perfusion by pushing the medium through testicular artery

Fig. 3. Illustration of elutriationn system

buffer flow rate was 16 ml/min, the rotor was decelerated to 0 rpm to remove air bubbles trapped in the system. After the rotor had decelerated, the rotor must be spun and stopped abruptly several times to dislodge residual air bubbles. Air bubbles left in the system could severely compromise the success of cell separation by increasing back pressure on the system, which, in turn, would alter the buffer flow velocities in the elutriation chamber. After the system was determined to be bubble free, the peristaltic pump was turned down to provide minimal buffer flow through the system while the dissociated testicular cells were harvested. The 52 ml cell suspension was aspirated into a 60 ml sterilized syringe. The filled syringe was connected to an elutriation loading tube (Fig. 3) fashioned with silastic tubing (0.4 cm inside diameter, 1.0 cm outside diameter, 285 cm long) and three-way valves. The cell suspension was injected slowly into the loading tube without pressure. The peristaltic pump of elutriation system was set to pull EB at a rate of 16 ml/min. and the elutriation was accelerated to 2000 rpm. The loading tube was taken to the elutriator and attached to three-way valves in line to the loading chamber/double trap. The dissociated testicular cells were pumped at a rate of 16ml/min through the loading tube, into loading chamber/bubble trap. The cells were mixed and diluted with EB in the loading chamber/bubble trap and were subsequently transported at 16 ml/min flow rate into the separation chamber within the elutriation rotor.

To elutriate effectively and discard smaller contaminating cells and sperm, the area between adjacent cells in the separation chamber must be maximized. Therefore, it was critical to monitor the concentration of the cells ($< 5 \times 10^6$ ml) during loading, the volume in which these cells become diluted in the loading chamber (> 100 ml) and the loading flow rate (16 ml/min). These factors exert a combined influence on the efficiency of cell separation. During the process of elutriation, cell separation occurs largely as a

function of cell size, with larger cells partitioning towards the centrifugal end of the separation chamber, while intermediate and smaller cells partition towards the centripetal end of the chamber. Under the centrifugal force exerted by the elutriation rotor operating at 2000 rpm and the centripetal force exerted by the 16 ml/min counter flow of buffer, a 300 to 500 ml fraction of contaminating sperm, condensed spermatids, round spermatids, endothelial cells and other small germ cells were collected. After 20 to 25 min, all dissociated cells have exited the loading chamber and have partitioned within the separation chamber. The fraction of cells that is retained in the separation chamber is comprised of Leydig cells, macrophages, pachytene spermatocytes and multinucleated germ cells. These cells were collected in two 50 ml centrifuge tubes by decelerating the rotor to 0 rpm while the pump runs at the same flow rate.

Step 4: Density Gradient Percoll Centrifugation

The cells collected from the elutriator were pelleted by centrifugation as described above (250x g for 10 min. at 4°C). While the cells are pelleting, isotonic Percoll solution was prepared by diluting 4.0 ml of 10xHBSS with 44 ml of unreconstituted stock Percoll under strict sterile condition (as described before). 0.25 ml DNase was added to the tube receiving cells. The contents of both tubes were properly mixed, balanced and the tubes were placed in a JA-20 rotor for centrifugation at 14500 rpm at 4°C for 45 to 60 min. In this type of separation, cell types were partitioned as a function of their buoyant densities, as a continuous linear density gradient was generated. After centrifugation, the gradient was divided into a fraction lighter than 1.068 gm/ml which contained germ cells, macrophages and damaged Leydig cells and a fraction heavier than 1.068 gm/ml containing intact, steroidogenically active Leydig cells (Fig. 4). The fraction between green and red beads contained germ cells and fraction below red beads contained mostly

Fig. 4. The density profile of different cell types obtained following density gradient percoll centrifugation (a) without and (b) and (c) with separation by centrifugal elutriation

macrophages; the fraction from mid-point between red and blue beads to the bottom of blue beads contained highly purified Leydig cells. The upper fraction was removed by gentle aspiration with a pipette and the Leydig cell fraction was transferred to a 50 ml Falcon centrifuge tube, diluted to 50 ml with Percoll buffer (PB) and pelleted by centrifugation (250x g for 10 min) as described before. The supernatant was discarded and the pellet was dissolved with the required volume of LC medium. This tube, containing highly purified Leydig cells was kept in ice and the number of Leydig cells/ml was counted using a haemocytometer and used as per requirement.

CULTURE OF PURIFIED ADULT RAT LEYDIG CELLS

Before collecting the Leydig cells, the cytodex III beads (Cat. No 17-0485.01) were prepared for culture. Cytodex III beads were hydrated by soaking 10 gm of beads in 50 ml of 1x HBSS buffer, the beads allowed to settle, and the process repeated twice. To sterilize, the beads were immersed in 70% ethanol overnight. Finally, the beads were prepared for culture by equilibrating the beads in LC culture medium overnight. The culture medium was decanted and 5 ml of fetal bovine serum was added, diluted to 20 ml with LC medium and incubated at 34°C for about 2 hours. The beads were reequilibrated for each culture. Approximately 10×10^6 highly purified Leydig cells were resuspended in 2.0 ml LC medium. The volume should be adjusted if yields vary so that 1×10^6 Leydig cells can be plated in 0.2 ml volume. Leydig cells were added to 35 mm culture dishes (Fischer Scientific) containing 0.2 ml of cytodex III beads. Bovine lipoprotein was added to provide a final concentration of 0.5 mg/ml. This volume of lipoprotein runs between 0.1 and 0.2 ml, depending on the protein concentration of the particular preparation. Finally, LC medium was added to bring the final culture volume to 2.0 ml (0.5×10^6 Leydig cells/ml); the final Leydig cell concentration is critical for optimal testosterone production and should be determined within each laboratory prior to initiating experiments. Testosterone production from Leydig cells was stimulated with 100 ng/ml LH (10 μ l of a 20 mg/ml stock) and these cultures were maintained at 34°C in 5% CO₂ in air (21% O₂), overnight. To maintain steroidogenic activity in culture for a period of several days, Leydig cells were stimulated with only 0.1 ng/ml of LH. These cultures were maintained at reduced oxygen tension (5% CO₂, 5% O₂, 90% air) (6). Medium was changed daily. To change medium, the entire contents of the culture dish were transferred to a 12 x 75 mm culture tube and the cytodex beads with attached Leydig cells were allowed to settle to the bottom. The supernatant, containing unattached Leydig cells and spent medium was centrifuged and frozen for assay of testosterone. Leydig cells attached to the beads were resuspended

in fresh culture medium and returned quickly to the culture dishes and placed in their reduced oxygen environment. After termination of culture, attached Leydig cells may be recovered for morphological and biochemical evaluation.

Recovery of Leydig cells

Leydig cells attached to beads were incubated in a 35 mm culture dish with LC medium containing 2.0 mg/ml collagenase while shaking at 34°C for 30 min. This was followed by gentle pipetting to complete the Leydig cell detachment. The contents of the dish were transferred to a 50ml centrifuge tube, diluted to 50ml with LC medium and filtered through 100/ μ m nylon mesh. Leydig cells were recovered from filtrate after centrifugation (250x g for 10 min).

Staining for 3 β - hydroxysteroid dehydrogenase activity (3 β HSD)

To stain Leydig cells for the presence of 3 β -HSD activity, the pelleted Leydig cells were resuspended in fresh culture medium; 0.015 ml aliquot (150,000 cells) was placed on the surface of a clean glass slide and allowed to dry. While the cells were drying 3 β -HSD solution was prepared by dissolving 2mg nitroterazolium salt in 0.6 ml of 1 mg/ml etiocholanolone solution mixed with 9.5ml of warm Dulbecco's Phosphate Buffer Saline (DPBS) containing nicotinamide adenine dinucleotide (10 mg). The dried smear was covered with the staining solution and left undisturbed until staining was complete (1-2 h). The stained cells were fixed with 10% formalin in DPBS containing 5% sucrose for 5 min. The slide was blotted dry and a drop of glycerol: DPBS (1:1, v/v) was added, a cover slip placed and sealed with nail polish.

Morphological evaluation

For morphological evolution, $1-2 \times 10^6$ cells were gently pelleted by centrifugation (250x g for 10 min.) and fixed on ice with 5% glutaraldehyde in 0.05 M collidine buffer containing 0.1 M sucrose, pH 7.4. After 1 hr, the cells were rinsed twice with buffer, centrifuged and then post-fixed on ice with 1% aqueous osmium tetroxide and 2.5% potassium ferrocyanide for an additional 1 hr. After this, standard dehydration and embedding procedures were applied.

PRECAUTIONS AND TROUBLE SHOOTING

1. Perfusion of the testicular vasculature with collagenase-supplemented medium is a very important step. This perfusion removes majority of the red blood cells from the testis. The remaining red blood cells are eliminated during the elutriation step, which increases the purity of the final Leydig cell preparation. Perfusion increases both yield and the steroidogenic response of the Leydig cells in the final cell

preparation.

2. Check the flow rate of elutriation buffer at each time of isolation.
3. Cellular attachment to a porous substrate is important in the successful culture of Leydig cells. Even in the presence of bovine lipoprotein, Leydig cells cultured on tissue culture plastic or glass coverslip failed to respond to LH as effectively as Leydig cells attached to the porous substratum provided by cytodex III beads. The beads may provide a three dimensional architecture that closely mimics the testicular architecture.
4. Reduced oxygen tension and sub-maximal LH stimulation are also very important for optimal maintenance of the steroidogenic capacity of the purified Leydig cells in culture.
5. All glassware, tubings and other required instruments should be autoclaved.

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