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Isolation of Primary Leydig Cells from Murine Testis

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[Abstract] In males, Leydig cells are the primary source of testosterone, which is necessary for testis development, masculinization, and spermatogenesis. Leydig cells are a valuable cellular model for basic research; thus, it is important to develop an improved method for isolation and purification of Leydig cells from testes. The available methods for Leydig cell isolation have some drawbacks, including the need for sophisticated instruments, high cost, tediousness, and time consumption. Here, we describe an improved protocol for isolation of primary Leydig cells from testicular tissue by digestion with collagenase IV.

Keywords: Primary Leydig cell, Testis, Testosterone, Collagenase IV, Late-onset Hypogonadism

[Background] Leydig cells, located in the interstitial tissue of testis, are steroidogenic cells that contribute to testosterone synthesis and secretion. Leydig cell steroidogenesis is strictly regulated by luteinizing hormone (LH) secreted by the pituitary gland. LH binds luteinizing hormone receptor (LHCGR) located on the surface of Leydig cells to activate adenylyl cyclase and increase the production of cyclic adenosine monophosphate (cAMP). The cAMP signaling cascade stimulates the expression of steroidogenic acute regulatory protein (StAR), which transports cholesterol from cytoplasm into the inner membrane of mitochondria. Cholesterol is converted to pregnenolone by a P450 side-chain cleavage enzyme (CYP11A1) and translocated to the endoplasmic reticulum. Finally, pregnenolone is converted to testosterone by a series of lyase reactions. Male hypogonadism is characterized by testosterone deficiency and affects approximately 6% of males, with an increasing incidence and prevalence in recent years. However, the mechanisms of testosterone deficiency are far from clear. Immortalized cell lines provide useful tools to investigate the molecular mechanisms underlying testosterone deficiency. Characterized Leydig cell lines, such as MA-10, BLTK1, and TM3 (Engeli *et al.*, 2018), are unable to produce testosterone due to a lack or low expression of testosterone synthase genes. In contrast, the expression level of these genes in primary Leydig cells is closer to the cells *in vivo*. Furthermore, primary Leydig cells feature a complete signaling pathway of testosterone synthesis and are widely used to investigate late-onset hypogonadism (LOH) and diseases of the reproductive system. Additionally, Leydig cell and stem Leydig cell transplantation have been developed as alternative therapies for the treatment of testicular damage, LOH, and sexual dysfunction, achieving good curative effects (Artyukhin

et al., 2007; Sun *et al.*, 2009; Jiang *et al.*, 2014). Therefore, procedures for isolation and enrichment of Leydig cells from testes are vital for establishing the role of Leydig cells in hypogonadism. In recent years, various approaches have been used to isolate Leydig cells, but gradient centrifugation is the most frequently used (Conn *et al.*, 1977; Gale *et al.*, 1982). Therefore, these methods are complicated, costly, time-consuming, and provide low yield. Here, we describe a cost-effective and time-saving protocol for isolating Leydig cells via digestion with collagenase VI.

Materials and Reagents

1. Sterile 50 ml centrifuge tubes (Corning, catalog number: 430829)
2. 40 μm cell strainers (Falcon, catalog number: 352340)
3. Sterile 100 × 20 mm cell culture dish (Corning, catalog number: 430167)
4. 0.22 μm filters (Millipo, catalog number: GNWP02500)
5. 8-week-old healthy Kunming (KM) mice
6. DMEM basic (1×) (Gibco, catalog number: C11995500BT)
7. Fetal bovine serum (FBS) (ExCellBio, catalog number: FSD500)
8. Collagenase IV (Biosharp, catalog number: C-5138)
9. NaCl (Guangzhou Chemical Reagent Factory, catalog number:7647-14-5)
10. KCl (Guangzhou Chemical Reagent Factory, catalog number: 7447-40-7)
11. $Na_2HPO_4·12H_2O$ (Guangzhou Chemical Reagent Factory, catalog number: 7558-79-4)
12. KH_2PO_4 (Guangzhou Chemical Reagent Factory, catalog number: 7778-77-0)
13. Penicillin G Potassium Salt (MDBio, catalog number: W627099)
14. Streptomycin Sulfate (Diamond, catalog number: G821BA0034)
15. 100% ethyl alcohol (Sinopharm, catalog number: 64-17-5)
16. Nitrotetrazolium Blue chloride (NTB) (Sigma-Aldrich, catalog number: N6876-50G)
17. DHEA (Sigma-Aldrich, catalog number: 252805-10MG)
18. DMSO(Sigma-Aldrich, catalog number: D2650-100ML)
19. β-NAD (Sigma-Aldrich, catalog number: N1511-250MG)
20. I^{125}-testosterone Coat-A-Count RIA kits (Beijing North Institute of Biological Technology, catalog number: 201202)
21. Luteinizing Hormone (Sigma-Aldrich, catalog number: 869003)
22. HSD3B antibody (Santa Cruz Biotechnology, catalog number: sc-515120)
23. StAR antibody (CST, catalog number: D1Z2A)
24. SF-1 antibody (CST, catalog number: D10H12)
25. Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam, catalog number: ab150077)
26. Goat Anti-Mouse IgG H&L (Alexa Fluor® 647) (Abcam, catalog number: ab150115)
27. Primary & secondary antibody diluent for immunostaining (Yeasen, catalog number: 36323ES60)
28. Paraformaldehyde (Shanghai Lingfeng, catalog number: 30525-89-4)
29. 1× PBS (see Recipes)

30. D-PBS (see Recipes)
31. Penicillin/streptomycin solution (see Recipes)
32. 0.75 mM KCl hypotonic buffer (see Recipes)
33. 75% ethyl alcohol (see Recipes)
34. 10 mg/ml Collagenase IV solution (see Recipes)
35. HSD3B staining solution (see Recipes)
36. Complete medium (see Recipes)
37. 4% paraformaldehyde solution (see Recipes)
38. StAR antibody dilution solution (see Recipes)
39. HSD3B antibody dilution solution (see Recipes)
40. SF-1 antibody dilution solution (see Recipes)

Equipment

1. Surgical Instruments:
   Scissors (Yuyan instruments, catalog number: Y15103)
   4× forceps (Yuyan instruments, catalog number: Y15201)
   Pointed forceps (Fine Science Tools, catalog number: 11252-00)
2. Low-speed centrifuge (Zonkia, model: SC-3610)
3. Biological safety cabinet (Heal Force, model: HFsafe-I200LC)
4. Water bath kettle (Jing Hong, model: XMTD-8222)
5. Cell incubator (Thermo Fisher, model: 371)
6. Fully automatic autoclave sterilizer (STIK, model: MJ-78A)
7. Inverted microscope (Nikon, model: TS100)
8. Electronic analytical balance (Sartorius, model: BSA124S-CW)
9. Gamma radioimmunoassay counter (Anhui ustc zonkia scientific, model:GC -1200γ)
10. Hemacytometer (Marienfeld, catalog number: 0650030)

Procedure

The protocol described in this manuscript is summarized in Figure 1.

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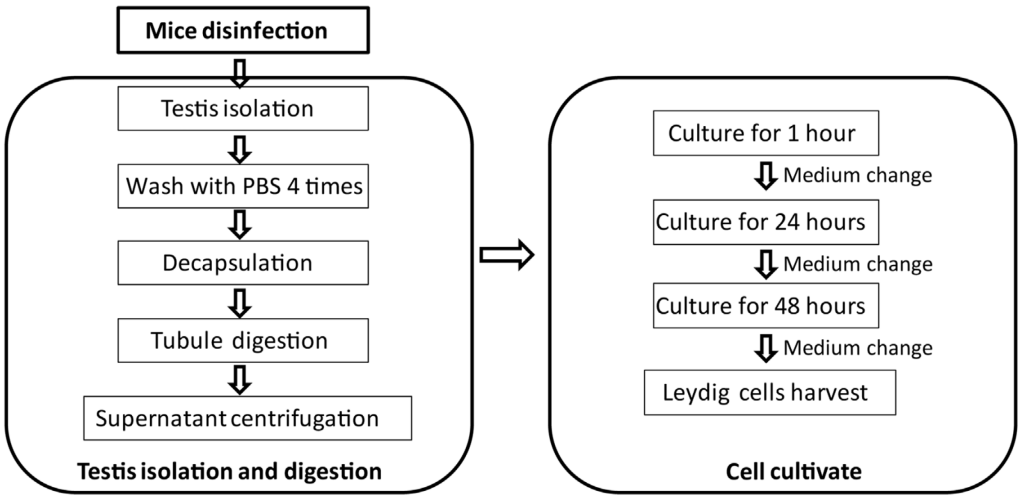

Figure 1. Protocol work flow.

Schematic summarizing the major steps of the protocol.

See Video 1 for procedure of Leydig cell isolation.

Video 1. Testis isolation, wash, and decapsulation.

1. Sacrifice ten 8-week-old healthy Kunming (KM) mice in a desiccator using CO_2 (Leary, 2013). Wait until breathing stops, soak the mice in 75% alcohol for 10 min to sterilize them, and place them on the operating table (Figure 2A).
2. Pull up the skin in the lower abdomen region, cut it horizontally with sterile surgical scissors, locate and remove the testes with sterile forceps, and transfer them into a 50 ml tube containing 30 ml of precooled PBS.
3. Move the testes to the biosafety cabinet, pour testes and PBS into an empty clean cell dish, add 20 ml of PBS (with 1× penicillin/streptomycin solution) to each of the four dishes in advance, and wash the testes four times (Figure 2B).
4. Clamp the testes one by one to an empty cell dish using forceps and puncture the testis with

another pointed forceps. Squeeze out the content and place it into a new cell dish containing 20 ml of PBS (Figure 2C). Finally, collect all into a 50 ml tube with 10 ml of collagenase IV (1 mg/ml) solution.

5. Put the tube in a shaking water bath to digest for 7 min at 37°C.
6. Add 20 ml of complete medium to the tube to terminate the digestion at room tempreture (figure 2D).
7. Let the contents settle for 5 min until the seminiferous cords sink to the bottom of the centrifuge tube.
8. Transfer the supernatant to a new 50 ml centrifuge tube, filter it on a 40 μm cell strainer, and centrifuge at 250 × *g* for 10 min.
9. After centrifugation, discard the supernatant and add 2 ml of complete medium to resuspend the cells in a tube.
10. Seed the cell suspension evenly into two 100 mm culture dishes containing 5 ml of complete medium.
11. Incubate the dishes in 5% CO_2 incubator at 37°C for 1 h (Figure 2E).
12. One hour later, remove the non-adherent cells, and add 6 ml of fresh complete medium to the dish to continue culturing for 24 h (Figure 2F).
13. Twenty-four hours later, discard the original medium, treat the cells with 4 ml of KCl hypotonic solution for 5 min to further remove the myoid cells, and wash with PBS three times.
14. Add 6 ml of fresh complete medium per dish and continue culturing for 48 h (Figure 2G).
15. After 48 h of culture, harvest the Leydig cells for further experiments.
16. Stain for HSD3B enzyme to identify the purity of Leydig cells (figure 2H). Perform total cell counts using a hemacytometer. Approximately 2 million cells can be obtained from ten mice. Harvest approximately 20,000 cells, resuspend them in PBS, smear on the slides, and leave to dry. Add 50-100 μl HSD3B enzyme staining solution to cover the cells and incubate for 30 min at 37°C away from light. Discard the staining solution and wash the slide twice with PBS. Use a TS100 Inverted microscope to capture the images, and perform analysis with ImageJ.
17. Add complete medium (with 1ng/ml LH) to the Leydig cells, and continue to culture for 48 h, following on from step 15. Then collect the supernatants to measure testosterone with I^{125}-testosterone Coat-A-Count RIA kits by radioimmunoassay (RIA) (Figure 2I) according to the manufacturer's protocol.
18. Perform immunostaining for Leydig cell markers StAR, HSD3B, and SF-1 (Figures 2J-2L). Fix the cells in 4% paraformaldehyde solution for 10 min, wash the cells three times with PBS, permeabilize cells in 0.5% Triton-X for 10 min, and then block nonspecific adhesion sites using 5% BSA for 1 h at room temperature. Dilute the primary and second antibodies in the antibody diluton solution. Incubate cells with primary antibody solution (such as StAR, HSD3B, and SF-1) overnight at 4°C. Wash with PBS three times and then incubate cells with secondary antibody for 1 h at room temperature. Wash with PBS three times and stain nuclei with DAPI. Use a LSM710 confocal microscope (Zeiss) to capture the images.

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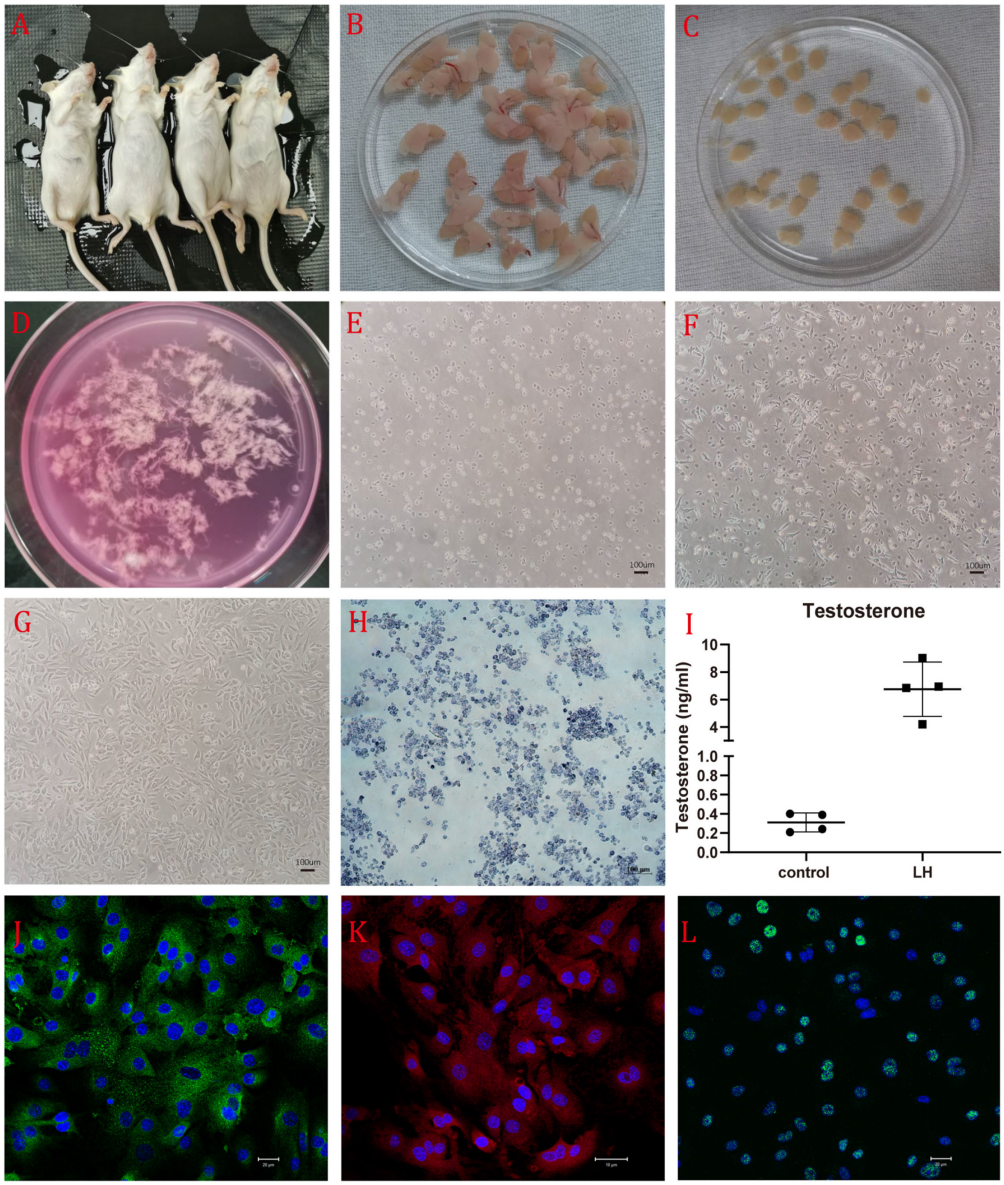

Figure 2. Isolation and identification of primary Leydig cell from mouse.

A. The KM mice are sacrificed and disinfected in 75% alcohol. B. Testis are taken from the sacrificed mice. C. Testis with tunica albuginea removed. D. Testis after digestion by collagenase IV. E. Cells adhere for 1 h (Scale bar: 100 μm). F. Cells adhere for 24 h (Scale bar: 100 μm). G. Cells adhere for 72 h (Scale bar: 100 μm). H. The isolated cells stained for HSD3B enzyme (purple) (Scale bar: 100 μm). I. Testosterone levels of cell supernatants contein 1 ng/ml LH (LH) and without LH (control). J. Immunostaining of Leydig cell marker StAR (Scale bar: 20 μm). K. Immunostaining of Leydig cell marker HSD3B (Scale bar: 10 μm). L. Immunostaining of Leydig cell marker SF-1 (Scale bar: 20 μm).

Data analysis

1. Five random microscopic fields were examined per slide. The positive rate of HSD3B enzyme staining is calculated with ImageJ using the Analyze Particles function. Set 1,000 as the minimum particle size.
2. Only the isolated cells with more than 85% positive rate are used in further experiments.

Recipes

1. 1× PBS (pH = 7.4)
   8 g NaCl
   0.2 g KCl
   3.63 g $Na_2HPO_4·12H_2O$
   0.24 g KH_2PO_4
   Add 1 L ddH_2O
   Sterilize it at 121°C for 30 min.
2. D-PBS (pH = 7.4)
   8 g NaCl
   0.2 g KCl
   1.15 g $Na_2HPO_4·12H_2O$
   0.2 g KH_2PO_4
   Add 1 L ddH_2O
3. Penicillin/streptomycin solution (10×)
   3.1 g Penicillin G Potassium Salt
   5 g Streptomycin Sulfate
   50 ml 1× PBS (pH = 7.4)
   Perform filter sterilization through 0.22 μm filters.
4. 0.75 mM KCl hypotonic buffer
   14 g KCl
   Add 250 ml ddH_2O
5. 75% alcohol
   750 ml 100% ethyl alcohol
   250 ml ddH_2O
6. 10 mg/ml Collagenase IV solution
   100 mg Collagenase IV
   10 ml 1× PBS (pH = 7.4)
7. HSD3B staining solution
   a. Solution A:
      1 mg NTB

0.6 mg DHEA

0.6 ml DMSO

b. Solution B:

10 mg β-NAD

9.5 ml D-PBS

Add 0.30 ml of solution A to 4.75 ml of solution B and mix thoroughly.

8. Complete medium

10% FBS

90% DMEM basic (1×)

9. 4% paraformaldehyde solution

4.0 g paraformaldehyde

Add 100 ml 1×PBS

10. StAR antibody dilution solution

5 μl StAR antibody

Add 5 ml Primary & secondary antibody diluent for immunostaining

11. HSD3B antibody dilution solution

5 μl HSD3B antibody

Add 5 ml Primary & secondary antibody diluent for immunostaining

12. SF-1 antibody dilution solution

5 μl SF-1 antibody

Add 5 ml Primary & secondary antibody diluent for immunostaining

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Competing interests

The authors declare that they have no competing interests.

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