Preparation of Rodent Testis Co-Cultures

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ABSTRACT

Male reproductive development is a complex process that is sensitive to disruption by a range of toxicants. There is a great need for in vitro models that can evaluate potential male reproductive toxicants. The current unit presents a protocol for preparation of a three-dimensional in vitro model of male reproductive development that reduces the number of animals required for evaluation of toxicants. A Matrigel overlay provides a three-dimensional extracellular matrix that improves cell attachment, viability, and communication, and makes the model more reflective of in vivo environments. *Curr. Protoc. Toxicol.* 55:16.10.1-16.10.7. © 2013 by John Wiley & Sons, Inc.

Keywords: male reproductive development • three-dimensional in vitro models • primary cell culture

INTRODUCTION

Given the time- and resource-intensive nature of reproductive toxicity testing in vivo, well-defined in vitro models of male reproductive development are needed to facilitate evaluation of the large number of chemicals yet to be tested for reproductive toxicity. While reductionist cell culture models cannot capture the full complexity of reproductive development, the relevance of cell culture models for in vivo systems can be enhanced by the creation of microenvironments that more closely resemble what is observed in vivo.

Here we present a method for preparation of a three-dimensional primary testicular cell co-culture from immature rat testis. The resulting co-culture contains germ cells, Sertoli cells, and Leydig cells, with serum-free culture conditions selecting against other neighboring cell types. A three-dimensional Matrigel extracellular matrix overlay provides an in vivo-like environment that enhances cell viability and cell communication in the culture (Yu et al., 2005) improving the relevance of the model for in vivo development.

This testicular co-culture offers a model of male reproductive development for use in evaluation of male reproductive toxicants as well as in basic research. We have previously used this model to measure cytotoxic and gene expression responses to a range of male reproductive toxicants (Yu et al., 2005, 2009).

PREPARATION OF THREE-DIMENSIONAL TESTICULAR CELL CO-CULTURE

BASIC PROTOCOL

This protocol describes a method for isolation of testicular cells from immature rats for generation of a three-dimensional primary testicular cell co-culture containing germ cells, Sertoli cells, and Leydig cells. In brief, this is accomplished by isolating testes from male rat pups and using microdissection tools and a dissecting scope, detunicating individual testes, and isolating seminiferous tubules. Tissue is digested and dispersed into a single-cell suspension through a series of enzyme digestions and wash steps. Finally, cells are plated on Primeria-coated dishes and mixed with a Matrigel extracellular matrix overlay to facilitate the creation of in vivo–like niches that enhance the viability of the culture.

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Materials

Enzyme digestion cocktail A (5 ml) and cocktail B (10 ml) (see recipes)
Complete cell culture medium (see recipe)
Matrigel (BD Biosciences; Matrigel should have protein concentration ranging from 9.2 to 10 μg/ml and endotoxin <1.5 EU/ml)
Ice and dry ice
Minimum essential medium (MEM), phenol-free
Male rat pups
Trypsin inhibitor solution (see recipe)
0.05% Trypsin and EDTA

37°C water bath
Mid-sized (e.g., 60 mm × 15–mm) cell culture dishes for testis dissection
Dissecting tools (two pairs of fine forceps per person dissecting)
Dissecting microscope and light
Waste beaker
CO_2 chamber
Surgical instruments including:
- Large scissors
- Small scissors
- Fine forceps

15-ml plastic conical tubes
3-ml plastic transfer pipets
Pulled glass pipets
Nylon mesh cell strainer (100-μm)
50-ml conical tubes
Hemacytometer
Primeria-coated 35 mm ×10–mm dishes *or* 96-well plates for plating cells (Falcon)
Pipet and pipet tips for plating cell suspension
Repeat pipets and pipet tips (any size appropriate for 30-μl aliquots)

Set up

1. Prepare enzyme digestion cocktails and complete cell culture medium and warm these in a 37°C water bath. Make sure Matrigel is thawed but kept cold on ice until needed.

2. Set up the dissecting microscope and light, dissecting tools, waste beaker, and several mid-sized cell culture dishes with clean MEM for washing and dissecting testes.

   Ideally, dissecting scopes should be set up in cell culture hoods to allow testis dissection to be performed inside the hood.

Perform testes isolation and dissection

3. Euthanize the rats by CO_2 asphyxiation, followed by decapitation of the pups and cardiac puncture of the dams.

   Note that pups are less sensitive to CO_2 and will not be fully euthanized by CO_2 asphyxiation. It is therefore crucial to perform a secondary method of euthanasia prior to removing testes.

4. Remove the testes from the male pups. To do this, apply pressure on each side of the lower abdomen to force testicular descent, and then make a small lateral abdominal incision with small sharp scissors (e.g., fine iris scissors) to remove the revealed testes. Transfer the removed testes to a mid-sized cell culture dish containing enough fresh MEM to submerge testes (e.g., 10 ml in a 60-cm dish).

5. Wash the isolated testes by transferring them into clean dishes of fresh MEM twice.

6. Under the dissecting microscope, isolate seminiferous tubules by detunicating the testes. To accomplish this, use fine-tipped forceps (we use micro-blunted, atraumatic-tipped forceps) to make a tear in the epithelial lining at one end of the testis and, holding the epithelial tissue at the other end, squeeze the testis through this tear. Once isolated, transfer the testes into a clean dish of MEM.

   Testes should be kept in culture dishes of clean MEM during dissection. Isolated seminiferous tubules will be a tightly packaged tubule that will "unravel" if pulled. The tubule should be free of smooth epithelial layers.

7. Wash the isolated tubules once by transferring into a clean dish of fresh MEM.

8. Under the dissecting microscope, use forceps to tear each detunicated testis into four to six pieces in order to give enzymes greater access to tissue in subsequent steps.

9. Transfer the tubules and MEM into a clean 15-ml conical tube.

   Use a 3-ml plastic transfer pipet. Make sure the mouth of the pipet is wide enough to prevent tissue from getting stuck in the pipet tip (≥2 mm).

10. Allow the testicular tissue to settle. Then, using a plastic transfer pipet, remove excess MEM and wash the tissue with clean MEM. Place on ice and allow the tissue to settle at the bottom of the tube.

    This is an important wash step that prevents blood components from interfering with subsequent enzyme digestions.

Enzyme digest testicular tissue to generate a single-cell suspension

11. Using a 3-ml transfer pipet, remove MEM and add 5 ml cocktail solution A. Tap the tube to ensure enzyme has access to settled tissue and incubate for 20 min in a 37°C water bath. After the first 10 min of this incubation, gently tap the tube again to ensure that the enzyme cocktail continues to have access to all tissue.

    If testes were dissected inside a cell culture hood, this is a good time to remove the dissecting microscopes and sterilize the hood. All future steps should be performed in a sterile hood.

12. Tissue will have settled during the incubation. Remove the enzyme supernatant with a transfer pipet. Wash the tissue with 10 ml MEM, tap the tube to break up settled tissue, and place on ice for 10 min.

13. Remove the MEM wash and add the first 5 ml of enzyme cocktail B. Tap the tube to disturb settled tissue and incubate for 20 min in a 37°C water bath.

14. Remove the enzyme supernatant with a transfer pipet. Wash the tissue with 10 ml MEM, tap the tube to break up settled tissue, and place on ice for 10 min.

15. Remove the MEM wash and add the second 5 ml of enzyme cocktail B. Tap the tube to break up settled tissue and incubate for 20 min in 37°C water bath.

    Following this final enzyme digestion, the pellet will be smaller and large chunks will have been broken down. With each progressive wash step, remaining supernatant will become more clear. Pellet will become smaller and finer as chunks of tissue are digested.

16. Remove the enzyme supernatant with a transfer pipet. Wash the tissue with 10 ml MEM, tap the tube to break up settled tissue, and place on ice for 10 min.

    During these final wash steps, prepare 30 ml trypsin inhibitor solution for steps 18 to 21 and prepare materials for cell plating and Matrigel addition.

17. Remove MEM and add 0.05% trypsin and EDTA (roughly 5 to 6× the amount of testicular tissue). Incubate for 3 min in the 37°C water bath.

18. Double the volume with trypsin inhibitor solution and centrifuge for 5 min at 150 × *g*, room temperature.

19. Remove the supernatant and resuspend the pellet in 5 ml trypsin inhibitor solution.
20. Centrifuge for 5 min at 150 × *g*, room temperature.
21. Remove the supernatant and resuspend the pellet in 5 ml trypsin inhibitor solution.
22. Disperse the cell pellet with a pulled glass pipet and filter through nylon mesh cell strainer into a 50-ml conical tube.

Count and plate the cells

23. Dilute the filtered cell suspension in warm complete medium for cell counting (40 ml is appropriate for a preparation from 60 pups).
24. Determine the cell concentration using a hemacytometer.
25. Add additional warm complete medium to bring the final cell density to 0.8×10^6 cells/ml.
26. Plate the cells at the appropriate volume (e.g., 2 ml/35-mm dish).
27. Using a repeat pipet, add cold Matrigel to the center of each dish for a final concentration of 150 μg/ml (e.g., 30 μl of Matrigel/35-mm dish). Immediately after addition, gently swirl the plates to ensure dispersal of Matrigel.

    Keep thawed Matrigel on ice and use pipet tips chilled on dry ice to add Matrigel to each dish. Warm Matrigel will polymerize, making it difficult to add to plates and disperse evenly.

28. Cells should attach within several hours of plating. Allow the cells to acclimate for 48 hr prior to chemical treatment. Maintain co-cultures in complete medium, feeding every 2 to 3 days.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete medium

For 500 ml medium combine:
480 ml Eagle's minimum essential medium (without phenol red, with calcium and magnesium)
150 μl of 0.01 mg/ml epidermal growth factor (final concentration 0.003 μg/ml)
5 ml ITS+ (insulin, human transferrin and selenous acid; Universal Culture Supplement Premix; Becton Dickinson, cat. no. 354352) (final concentration 1%)
5 ml of 0.1 mM nonessential amino acids (final concentration 0.001 mM)
5 ml penicillin-streptomycin (final concentration 1%)
5 ml of 1 mM sodium pyruvate (final concentration 0.01 mM)
127.6 μl of 3 mM sodium lactate (final concentration 0.77 μM)
Store up to 1 week at 4°C

Enzyme cocktail A

For 5 ml cocktail combine:
500 μl of 10 mg/ml collagenase (final 1 mg/ml)
50 μl of 0.1 mg/ml DNase I (final 0.001 mg/ml)
4200 μl Eagle's minimum essential medium (without phenol red, with calcium and magnesium)
250 μl of 20 mg/ml hyaluronidase (final 1 mg/ml)
Make fresh on day of use and warm in 37°C water bath

Enzyme Cocktail B

For 10 ml cocktail combine:
1000 μl of 10 mg/ml collagenase (final 1 mg/ml)
100 μl of 0.1 mg/ml DNase I (final 0.001 mg/ml)
8900 μl Eagle's minimum essential medium (without phenol red, with calcium and magnesium)
Make fresh on day of use and warm in 37°C water bath

Trypsin inhibitor solution

For 30 ml solution combine:
120 μl of 0.1 mg/ml DNase I (final 0.4 μg/ml)
30 ml Eagle's minimum essential medium (without phenol red, with calcium and magnesium)
120 μl of 0.1 mg/ml soybean trypsin inhibitor (final 0.4 μg/ml)
Make fresh on day of use

COMMENTARY

Background Information

Developmental processes occurring during male reproductive development and spermatogenesis are particularly susceptible to perturbation by a diverse range of toxicants (Benson, 2009; Rider et al., 2009; Bonde, 2010; Ema et al., 2010). However, animal models for evaluation of male reproductive toxicity are not well suited for evaluation of the large number of untested chemicals, as they are animal, resource, and time intensive. Validation of robust in vitro models is therefore of particular importance for reproductive and developmental toxicology (Hartung et al., 2011).

Co-cultures of multiple cell types from fetal and neonatal rodent testis offer a promising model of male reproductive development for toxicity testing. Several two-dimensional testicular co-cultures have been established, including Sertoli-germ cell co-cultures from both 10- and 28-day-old rats (Hadley et al., 1985; Gray, 1986) and Sertoli-germ co-cultures from 18-day-old rats, which were successfully enriched for Sertoli cells (Chapin et al., 1988). Leydig-Sertoli cell co-cultures were also developed using tissue from mice (Bilinska, 1989; Bilinska et al., 1989) and from humans (Lejeune et al., 1998). Unfortunately, early incarnations of these co-cultures were poorly reproducible and difficult to maintain (Gregotti et al., 1992; Li et al., 1998). Precoating cell culture dishes with extracellular matrix material, such as Matrigel, was shown to enhance cell attachment in some of these two-dimensional testicular cell cultures (Hadley et al., 1985; Orth et al., 2000). Furthermore, Sidhu and colleagues demonstrated that addition of a Matrigel overlay to hepatocyte cultures facilitates maintenance of enzyme activity (Sidhu et al., 1993). Subsequent work showed that addition of a three-dimensional extracellular matrix overlay improved the relevance of in vitro cell cultures for modeling in vivo environments (Cukierman et al., 2001).

In response to these findings, our laboratory developed a neonatal rat testicular cell co-culture using a Matrigel overlay to provide a three-dimensional extracellular matrix. At day 5, male rat pups have testes large enough to make this procedure possible but immature enough to produce a culture that is representative of an immature testis with potential for development and differentiation. The addition of a three-dimensional extracellular matrix overlay dramatically enhanced the viability and consistency of the culture, increasing Sertoli cell attachment and significantly increasing cell communication. The Matrigel overlay also decreased stress signaling and improved cell viability (Yu et al., 2005). We have used this three-dimensional testis co-culture model to successfully distinguish between reproductively toxic and nontoxic phthalate esters (Yu et al., 2009) and establish a dose-response curve for cadmium (Yu et al., 2005). The proceeding paper from the Testicular Toxicity In Vitro Methods workshop held in Baltimore in 2011 provides an excellent review of in vitro systems for male reproductive systems (Saldutti et al., 2013).

Critical Parameters and Troubleshooting

Note that all volumes are designed for a preparation from 60 rat pups to generate ~240 to 300 ml of cell suspension. Larger volumes of

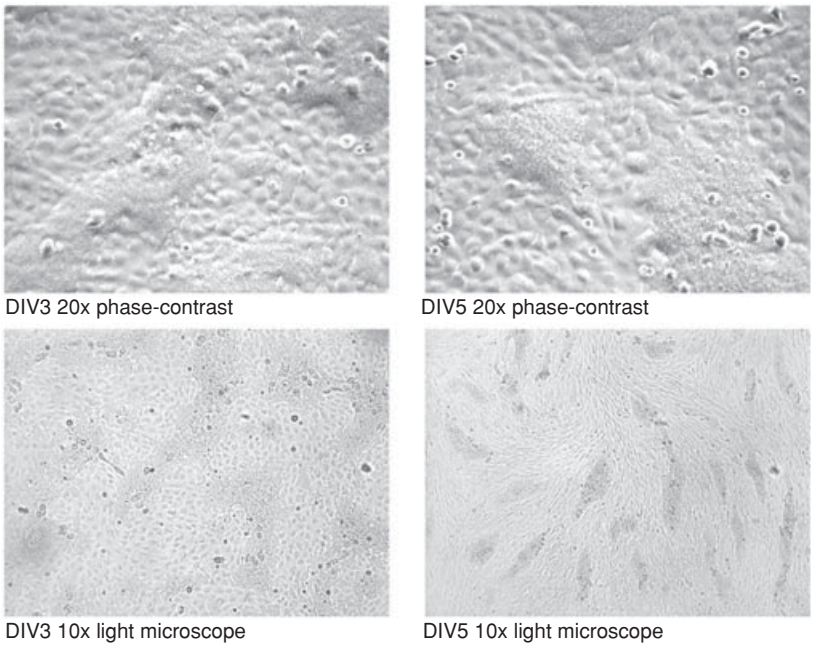

Figure 16.10.1 Typical microscope images of three-dimensional testicular cell co-cultures**.** Testicular cell co-cultures were prepared as described in the Basic Protocol. Representative light microscope (10×) and phase-contrast images (20×) were captured 3 (DIV3) and 5 (DIV5) days after plating.

tissue will require larger volumes of enzyme digestion cocktails, trypsin, and MEM wash steps. Significantly larger volumes of tissue should be split into two conical tubes to maximize access of enzymes to tissue.

The wash steps during testes isolation and dissection are important in removing blood and interstitial fluid, which can interfere with enzyme digestions.

If cells have died during enzyme digestions, DNA released from dead cells can create a stringy gel that interferes with dispersal of cells into a single-cell suspension. If this occurs, an additional DNase wash and centrifugation step may rescue the remaining live cells by removing free DNA, though cell yield will likely be compromised.

Primeria-coated culture plates are essential for the proper attachment and growth of this culture. Ensure that you have the proper cell culture plates before starting the preparation.

Matrigel must be kept cold prior to addition to cell cultures to prevent premature polymerization. Therefore, Matrigel should be thawed on ice. When adding Matrigel to plates, it is important to use cold repeat pipet tips, move quickly, and gently swirl culture dishes immediately after adding Matrigel to ensure an effective and consistent coating.

Anticipated Results

Cells typically attach within 2 hr of plating and can be maintained upwards of several weeks in culture. Co-cultures should contain germ cells, Sertoli cells, and Leydig cells. Once the culture is established, the Matrigel facilitates three-dimensional cell growth (Fig. 16.10.1).

Time Considerations

Time required for dissection of tissue will depend on the number of animals used and the number of people involved. Typically, a preparation from 60 rat pups will require 2 hr for sacrificing and dissection of testes if there are at least two people (one to euthanize the animals and remove the testes while the other dissects the testes to isolate tubules).

Enzyme digestion steps and washes will take ~2 hr regardless of volume and can easily be performed by one person.

Cell counting, plating, and Matrigel addition will also depend on volume, but will generally take under an hour. Ideally, at least two people will be involved in this step so that one can begin adding Matrigel immediately after cells are plated while the other finishes plating the remaining cells.

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Literature Cited

Benson, R. 2009. Hazard to the developing male reproductive system from cumulative exposure to phthalate esters–dibutyl phthalate, diisobutyl phthalate, butylbenzyl phthalate, diethylhexyl phthalate, dipentyl phthalate, and diisononyl phthalate. *Reg. Toxicol. Pharmacol.* 53:90-101.

Bilinska, B. 1989. Interaction between Leydig and Sertoli cells in vitro. *Cytobios* 60:115-126.

Bilinska, B., Viklicky, V., Draber, P., and Wojtusiak, A. 1989. Luteinizing hormone-induced modifications of the cytoskeleton in cultured mouse Leydig cells. *Cytobios* 58:25-34.

Bonde, J.P. 2010. Male reproductive organs are at risk from environmental hazards. *Asian J. Androl.* 12:152-156.

Chapin, R.E., Gray, T.J., Phelps, J.L., and Dutton, S.L. 1988. The effects of mono-(2-ethylhexyl)-phthalate on rat Sertoli cell-enriched primary cultures. *Toxicol. Appl. Pharmacol.* 92:467-479.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. 2001. Taking cell-matrix adhesions to the third dimension. *Science* 294:1708-1712.

Ema, M., Kobayashi, N., Naya, M., Hanai, S., and Nakanishi, J. 2010. Reproductive and developmental toxicity studies of manufactured nanomaterials. *Reprod. Toxicol.* 30:343-352.

Gray, T.J. 1986. Testicular toxicity in vitro: Sertoli-germ cell co-cultures as a model system. *Food Chem. Toxicol.* 24:601-605.

Gregotti, C., Di Nucci, A., Costa, L.G., Manzo, L., Scelsi, R., Berte, F., and Faustman, E.M. 1992. Effects of thallium on primary cultures of testicular cells. *J. Toxicol. Environ. Health* 36:59-69.

Hadley, M.A., Byers, S.W., Suarez-Quian, C.A., Kleinman, H.K., and Dym, M. 1985. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. *J. Cell Biol.* 101:1511-1522.

Hartung, T., Blaauboer, B.J., Bosgra, S., Carney, E., Coenen, J., Conolly, R.B., Corsini, E., Green, S., Faustman, E.M., Gaspari, A., Hayashi, M., Wallace Hayes, A., Hengstler, J.G., Knudsen, L.E., Knudsen, T.B., McKim, J.M., Pfaller, W., and Roggen, E.L. 2011. An expert consortium review of the EC-commissioned report "alternative (Non-Animal) methods for cosmetics testing: Current status and future prospects - 2010". *Altex* 28:183-209.

Lejeune, H., Sanchez, P., and Saez, J.M. 1998. Enhancement of long-term testosterone secretion and steroidogenic enzyme expression in human Leydig cells by co-culture with human Sertoli cell-enriched preparations. *Int. J. Androl.* 21:129-140.

Li, L.H., Jester, W.F. Jr., and Orth, J.M. 1998. Expression of 140-kDa neural cell adhesion molecule in developing testes in vivo and in long-term Sertoli cell-gonocyte cocultures. *J. Androl.* 19:365-373.

Orth, J.M., Jester, W.F., Li, L.H., and Laslett, A.L. 2000. Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. *Curr. Top. Dev. Biol.* 50:103-124.

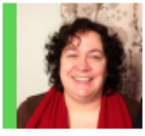
Rider, C.V., Wilson, V.S., Howdeshell, K.L., Hotchkiss, A.K., Furr, J.R., Lambright, C.R., and Gray, L.E. Jr. 2009. Cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development. *Toxicol. Pathol.* 37:100-113.

Saldutti, L.P., Beyer, B., Breslin, W., Brown, T., Chapin, R.E., Campion, S., Enright, B., Faustman, E.M., Foster, P., Kelce, W., Kim, J.H., Loboa, E.G., Piersma, A., Sasaki, J.C., Seyler, D., Turner, K., Yu, H., and Yu, X. 2013. Proceedings Paper from Testicular Toxicity In Vitro Models Workshop: Opportunities for Advancement via Biomedical Engineering Techniques.

Sidhu, J.S., Farin, F.M., and Omiecinski, C.J. 1993. Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. *Arch. Biochem. Biophys.* 301:103-113.

Yu, X., Sidhu, J.S., Hong, S., and Faustman, E.M. 2005. Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat Sertoli cell/gonocyte co-cultures: An improved in vitro model for assessment of male reproductive toxicity. *Toxicol. Sci.* 84:378-393.

Yu, X., Hong, S., Moreira, E.G., and Faustman, E.M. 2009. Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: Lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. *Toxicol. Appl. Pharmacol.* 239:325-336.



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