Published in final edited form as:

Methods Mol Biol. 2016 ; 1502: 111–118. doi:10.1007/7651_2016_334.

Use of Stirred Suspension Bioreactors for Male Germ Cell Enrichment

Sadman Sakib,

Department of Comparative Biology and Experimental, Medicine, Faculty of Veterinary Medicine, Cumming School, of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1, Department of Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1

Camila Dores,

Department of Comparative Biology and Experimental, Medicine, Faculty of Veterinary Medicine, Cumming School, of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1

Derrick Rancourt, and

Department of Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1, Department of Oncology and Medical Genetics, Cumming, School of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1

Ina Dobrinski

Department of Comparative Biology and Experimental, Medicine, Faculty of Veterinary Medicine, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1, Department of Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, HMRB 404, Calgary, AB, CanadaT2N 4N1

Abstract

Spermatogenesis is a stem cell based system. Both therapeutic and biomedical research applications of spermatogonial stem cells require a large number of cells. However, there are only few germ line stem cells in the testis, contained in the fraction of undifferentiated spermatogonia. The lack of specific markers makes it difficult to isolate these cells. The long term maintenance and proliferation of nonrodent germ cells in culture has so far been met with limited success, partially due to the lack of highly enriched starting populations. Differential plating, which depends on the differential adhesion properties of testicular somatic and germ cells to tissue culture dishes, has been the method of choice for germ cell enrichment, especially for nonrodent germ cells. However, for large animals, this process becomes labor intensive and increases variability due to the need for extensive handling. Here, we describe the use of stirred suspension bioreactors, as a novel system for enriching undifferentiated germ cells from 1-week-old pigs. This

method capitalizes on the adherent properties of somatic cells within a controlled environment, thus promoting the enrichment of progenitor cells with minimal handling and variability.

Keywords

Germ cells; Spermatogonial stem cell; Testes; Stirred suspension bioreactor; Spinner flasks

1 Introduction

Male germ line stem cells, located at the basement membrane of seminiferous tubules, form the foundation of spermatogenesis. These cells have widespread potential applications: for treating male infertility, preserving endangered species, regenerative medicine, and generating transgenic animals [1–4]. Germ line stem cells, once isolated from the testis and placed into a conducive environment, have the ability to attain pluripotency and differentiate into tissues of all three germ lineages [5–11]. However, a significant number of cells are required for these applications.

Expansion of germ cell number is a challenge due to the lack of efficient enrichment protocols, especially for nonrodent model species such as pig. The current enrichment method being employed is differential plating, which is well established for rodent germ cells [12]. The differential plating technique depends on the differential adhesion of germ and somatic cells to tissue culture surfaces. It involves the sequential plating of testicular cell suspensions, where Sertoli cells readily adhere to the plastic surfaces of tissue culture plates while germ cells remain suspended in the supernatant or attach slightly to the somatic cells. In addition to collecting germ cells from the supernatant, those in the slightly attached cell fraction can be obtained by treatment with 1:20 and 1:5 dilution of trypsin–EDTA 0.25 % .

Although this technique has been employed with satisfactory results [13, 14], when dealing with large animal models, this technique becomes too labor intensive due to the large number of cells involved. It also increases the risk of introducing variability due to extensive cell handling. Therefore, a protocol that allows efficient enrichment with minimum handling within a controlled environment is needed.

Stirred suspension bioreactors provide an efficient system for the expansion of cells in a controlled environment, reducing sources of variability. Bioreactors have been employed in the culture and long term maintenance of a number of different cell types [15–17]. Here, we report two protocols for enriching prepubertal (1 week old) porcine germ cells by exploiting the adhesive of Sertoli cells in bioreactors [18]. One protocol involves using the bioreactor solely. The starting cell suspension is stirred at 100 rpm for 66 h allowing Sertoli cells to form aggregates. At 18, 44, and 66 h, cell suspensions are filtered through a 40 µm mesh to remove the aggregates, resulting in a seven to eightfold enrichment of germ cells. The second protocol involves two rounds of bioreactor filtration as above (44 h) followed by differential plating. Cell suspensions were stirred at 100 rpm for 44 h. At 18 and 44 h the cells were filtered to remove the Sertoli cell aggregates. After the second round of filtration, the remaining cells were trypsinized and plated for 22 h. The supernatant and the slightly adhered fraction of germ cells were collected at 66 h. This approach consistently resulted in

a 9-fold enrichment of germ cells [18]. A schematic of the experimental design is shown in Fig. 1.

2 Materials

All solutions must be prepared under sterile conditions and reagents tested for cell culture must be used.

2.1 Testes Tissue Enzymatic Digestion Reagents

1. DPBS (w/o Ca^{++} and Mg^{++}): Dulbecco's phosphate-buffered saline (w/o Ca^{++} and Mg^{++}), 1× penicillin/streptomycin (p/s).
2. HDMEM 1× p/s: High glucose Dulbecco's modified Eagle medium (HDMEM), 1× p/s.
3. HDMEM: High glucose Dulbecco's Modified Eagle medium (HDMEM) without additives.
4. Collagenase IV: HDMEM, 2 mg/mL collagenase IV (*see* ^{Note 1}).
5. Hyaluronidase: HDMEM, 2.5 µg/mL hyaluronidase (*see* ^{Note 2}).
6. DNase I: HDMEM, 7 mg/mL DNase I (*see* ^{Note 3}).
7. 0.25 % trypsin–EDTA.
8. Culture dish: 100 mm tissue culture dishes.
9. Sterile forceps, scissors.

2.2 Stirred Suspension Bioreactor Culture

1. Bioreactor vessel: 125 mL Stirred Suspension Bioreactors (Corning Style Spinner Flask; NDS Technologies Inc. Vineland, NJ, USA) with magnetic impellers.
2. Magnetic stir plate: Variable speed magnetic stir plates that can maintain a speed of 100 rpm and can be housed within a CO₂ cell culture apparatus.
3. Bioreactor culture medium (HDMEM): High-glucose Dulbecco's modified Eagle medium (DMEM), 50 U/mL penicillin, 50 U/mL streptomycin, 5 % fetal bovine serum (FBS).
4. DPBS (w/o Ca^{++} and Mg^{++}): Dulbecco's phosphate-buffered saline DPBS (w/o Ca^{++} and Mg^{++}).
5. 0.25 % trypsin–EDTA.

¹Dissolve 80 mg of Collagenase IV powder in 40 mL H-DMEM w/o additives in a 50 mL centrifuge tube and filter through a 0.2 µm vacuum filter.

²Dissolve 0.05 mg Hyaluronidase in 20 mL of HDMEM w/o additives and filter through a 0.2 µm vacuum filter.

³Resuspend 56 mg of DNase I in 8 mL H-DMEM in a 15 mL centrifuge tube and filter through a 0.2 µm syringe filter.

2.3 Stirred Suspension Bioreactor Followed by Differential Plating

1. Bioreactor culture medium (HDMEM): High-glucose Dulbecco's modified Eagle medium (DMEM), 50 U/mL penicillin, 50 U/mL streptomycin, 5 % fetal bovine serum (FBS).
2. 0.25 % trypsin-EDTA.
3. 0.25 % trypsin-EDTA (1:10): 36 mL DPBS, 4 mL 0.25 % trypsin-EDTA.
4. Culture dish: 100 mm tissue culture dishes.
5. DPBS (w/o Ca^{++} and Mg^{++}): Dulbecco's phosphate-buffered saline DPBS (w/o Ca^{++} and Mg^{++}).

3 Methods

3.1 Testes Tissue Enzymatic Digestion

1. The testes are obtained by castration of 1 week old pigs (*see* ^{Note 4}). The isolated porcine testes are washed in 3 changes of DPBS 1× p/s at room temperature (*see* ^{Note 5}).
2. Using sterile forceps the testis the tunica vaginalis and epididymis are cut and the testis tissue is placed in a new clean dish.
3. The dish is closed and tissue is weighed together (*see* ^{Note 6}).
4. Using sterile forceps and scissors, the testis capsule is peeled from the parenchyma and the capsule is placed in a clean dish.
5. The capsule and the dish are weighed together and subtracted from the initial weight to obtain the actual weight of the testes tissue being digested.
6. The tissue is cut in half and then in half again lengthwise and the mediastinum is removed (*see* ^{Note 7}).
7. The remaining tissue is minced with sterile scissors into ~1–2 mm size pieces and placed into 40 mL of collagenase IV (2 mg/mL) using a spatula (*see* ^{Note 8}).
8. The tubes are then placed in 37 °C water bath and inverted every 5 min until the tissue appears more uniform and the fluid becomes cloudy (~30 min).
9. The tissue is allowed to sediment and the supernatant is discarded.
10. Then, 20 mL collagenase IV (2 mg/mL), 20 mL hyaluronidase (2.5 µg/mL) and 1 mL DNase I (7 mg/mL) are added to the tissue.

⁴This protocol was established for germ cells from prepubertal (1 week old) testes. Germ cells from older animals may result in lower enrichment.

⁵DPBS 1× P/S is only required for these initial three washes.

⁶If the weighing apparatus is outside of the biosafety cabinet, take care to keep the dish closed to avoid contaminating the tissue.

⁷1–2 cm pieces of tissue may be cut and fixed for subsequent histology.

⁸8 mL (less than 10 mL) of tissue per tube should be added.

11. The tubes are placed back in 37 °C water bath and inverted every 5 min for ~30 min.
12. When almost no tissue fragments remain and seminiferous cords are floating, the tissue is centrifuged at $75 \times g$ for 5 min.
13. The supernatant is removed and the tubules are resuspended in DPBS and transferred to a new 50 mL tube leaving the chunks behind.
14. The tubules are then centrifuged at $75 \times g$ for 2 min and washed with DPBS thrice.
15. The supernatant from the last wash is removed and 5 mL of H-DMEM, $1 \times p/s$ is added.
16. 10 mL of 0.25 % trypsin–EDTA is added and mixed by inverting and placed in the 37 °C water bath.
17. After 5 min the tube is inverted and observed for the presence of cloudy white DNA. If DNA is present, 500–1000 μ L of DNase I is added to the tube.
18. The tubes are continuously shaken and observed every 5 min; if necessary trypsin and DNase may be added (*see* ^{Note 9}).
19. Upon satisfactory digestion, 10 % FBS is added to neutralize the trypsin.
20. The cell suspension is then filtered through 100, 70 and then 40 μ m cell strainers.
21. The tubes are then centrifuged at $500 \times g$ for 5 min.
22. The supernatant is discarded and the cells are resuspended in 30–40 mL fresh H-DMEM, which is considered here as the starting cell suspension.

3.2 Stirred Suspension Bioreactor Culture

1. An appropriate volume of starting cell suspension containing 500×10^6 cells is introduced into the bioreactor.
2. Pre-warmed high-glucose Dulbecco's modified Eagle medium (HDMEM) supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, and 5 % fetal bovine serum (FBS) is added to make a volume of 100 mL.
3. The bioreactor is then placed on magnetic stirrer in the cell culture incubator and the cells are stirred at 100 rpm in 37 °C and 5 % CO₂ (*see* ^{Notes 10 and 11}).
4. After 18 and 44 h, all the media and the cells are removed from the bioreactor; filtered through a 40 μ m mesh to remove large cell aggregates and placed into two sterile 50 mL centrifuge tubes (*see* ^{Note 12}).

⁹The amount of cord digestion may be visualized using a microscope. If digestion is going slowly, mechanical digestion by pipetting up and down may be considered.

¹⁰It is recommended to count the rotation of the magnetic bar per minute rather than depending solely on the display of the magnetic stir plate.

¹¹Higher rpm result in smaller aggregate size, whereas lower rpm, e.g., 80 rpm will result in aggregates of larger size. Different cell types may require different rotation speeds for satisfactory enrichment.

5. The two sterile 50 mL centrifuge tubes are then centrifuged at $500 \times g$ for 5 min and the supernatant is discarded.
6. The sedimented cells are then resuspended in fresh pre-warmed HDMEM.
7. The suspension is again placed in the bioreactor and cultured as described in **step 3** of this section.
8. After 66 h from the initial loading, the cells are again filtered through a $40 \mu\text{m}$ filter and concentrated as cell pellets after 5 min centrifugation at $500 \times g$.
9. The cell pellet is then resuspended in 0.25 % trypsin–EDTA for 5 min to obtain a single cell suspension from the remaining cells by breaking up aggregates smaller than $40 \mu\text{m}$ (*see* Note 13).
10. The trypsin is neutralized by addition of 10 % FBS and the single cell suspension is centrifuged at $500 \times g$ for 5 min.
11. The supernatant is then discarded and the cells are washed twice in PBS and resuspended in HDMEM.

3.3 Stirred Suspension Bioreactor Followed by Differential Plating

1. Testicular cells (5×10^6 cells/mL) will be subjected to enrichment by SSB as described above in Sect. 3.1 for 44 h. The recovered cells are centrifuged at $500 \times g$ for 5 min and concentrated as pellets.
2. The pellet is then resuspended in 0.25 % trypsin–EDTA (5 min) until a single cell suspension is observed. The trypsin is neutralized by the addition of 10 % FBS, the cells are washed with PBS twice and resuspended in high-glucose DMEM with 5 % FBS (*see* Note 13).
3. The cells are seeded at 5×10^6 cells/mL concentration on 100 mm tissue culture dishes in 10 mL of high-glucose Dulbecco's modified Eagle medium (HDMEM) and incubated for 22 h at 37°C in 5 % CO_2 in air.
4. After 22 h (66 h since the initial loading), the cells in suspension and the slightly attached fractions are collected by trypsinization for 30 s with 1:10 dilution of 0.25 % trypsin–EDTA.
5. The trypsin is then neutralized by adding 10 % FBS and the cells are centrifuged at $500 \times g$ for 5 min.
6. The supernatant is removed and the pellet is washed twice with DPBS.
7. The cells are then resuspended in high-glucose Dulbecco's modified Eagle medium (HDMEM).

¹²The aggregates may be preserved for fixing and staining later to determine the ratio of somatic and germ cells in the aggregates, as some germ cells will be adhered to the somatic cell clumps.

¹³In some instances, trypsinization can result in cell degradation and release of the cell DNA. If the presence of DNA (appearance of cloud like structures) is observed, some DNase can be added.

References

1. Sehgal L, Usmani A, Dalal SN, Majumdar SS. Generation of transgenic mice by exploiting spermatogonial stem cells in vivo. *Methods Mol Biol.* 2014; 1194:327–337. DOI: 10.1007/978-1-4939-1215-5_18 [PubMed: 25064112]
2. Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D, Tang W, Liao J, Zhou C, Liu W, Zhu P, Guo H, Pan H, Wu C, Shi H, Wu L, Tang F, Li J. Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Res.* 2015; 25(1):67–79. DOI: 10.1038/cr.2014.160 [PubMed: 25475058]
3. Sato T, Sakuma T, Yokonishi T, Katagiri K, Kamimura S, Ogonuki N, Ogura A, Yamamoto T, Ogawa T. Genome editing in mouse spermatogonial stem cell lines using TALEN and double-nicking CRISPR/Cas9. *Stem Cell Rep.* 2015; 5(1):75–82. DOI: 10.1016/j.stemcr.2015.05.011
4. Aponte PM. Spermatogonial stem cells: current biotechnological advances in reproduction and regenerative medicine. *World J Stem Cells.* 2015; 7(4):669–680. DOI: 10.4252/wjsc.v7.i4.669 [PubMed: 26029339]
5. Kanatsu-Shinohara M, Lee J, Inoue K, Ogonuki N, Miki H, Toyokuni S, Ikawa M, Nakamura T, Ogura A, Shinohara T. Pluripotency of a single spermatogonial stem cell in mice. *Biol Reprod.* 2008; 78(4):681–687. DOI: 10.1095/biolreprod.107.066068 [PubMed: 18199882]
6. Simon L, Ekman GC, Kostereva N, Zhang Z, Hess RA, Hofmann MC, Cooke PS. Direct transdifferentiation of stem/progenitor spermatogonia into reproductive and nonreproductive tissues of all germ layers. *Stem Cells.* 2009; 27(7):1666–1675. DOI: 10.1002/stem.93 [PubMed: 19544441]
7. Ning L, Goossens E, Geens M, Van Saen D, Van Riet I, He D, Tournaye H. Mouse spermatogonial stem cells obtain morphologic and functional characteristics of hematopoietic cells in vivo. *Hum Reprod.* 2010; 25(12):3101–3109. DOI: 10.1093/humrep/deq269 [PubMed: 20940138]
8. Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. *Stem Cells.* 2009; 27(1):138–149. DOI: 10.1634/stemcells.2008-0439 [PubMed: 18927477]
9. Dym M, He Z, Jiang J, Pant D, Kokkinaki M. Spermatogonial stem cells: unlimited potential. *Reprod Fertil Dev.* 2009; 21(1):15–21. [PubMed: 19152741]
10. Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI, Dym M. Pluripotent stem cells derived from adult human testes. *Stem Cells Dev.* 2009; 18(8):1115–1126. DOI: 10.1089/scd.2008.0347 [PubMed: 19281326]
11. Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K, Yuen C, Greilach S, Zhao HH, Chow M, Chow YC, Rao J, Barritt J, Bar-Chama N, Copperman A. Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. *Hum Reprod.* 2011; 26(6):1296–1306. DOI: 10.1093/humrep/der026 [PubMed: 21349855]
12. Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL. Production of transgenic rats by lentiviral transduction of male germ-line stem cells. *Proc Natl Acad Sci U S A.* 2002; 99(23):14931–14936. DOI: 10.1073/pnas.222561399 [PubMed: 12391306]
13. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol.* 2005; 279(1):114–124. DOI: 10.1016/j.ydbio.2004.12.006 [PubMed: 15708562]
14. Izadyar F, Spierenberg GT, Creemers LB, den Ouden K, de Rooij DG. Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction.* 2002; 124(1):85–94. [PubMed: 12090922]
15. Shafa M, Sjonnesen K, Yamashita A, Liu S, Michalak M, Kallos MS, Rancourt DE. Expansion and long-term maintenance of induced pluripotent stem cells in stirred suspension bioreactors. *J Tissue Eng Regen Med.* 2012; 6(6):462–472. DOI: 10.1002/term.450 [PubMed: 21761573]
16. Krawetz R, Taiani JT, Liu S, Meng G, Li X, Kallos MS, Rancourt DE. Large-scale expansion of pluripotent human embryonic stem cells in stirred-suspension bioreactors. *Tissue Eng Part C Methods.* 2010; 16(4):573–582. DOI: 10.1089/ten.TEC.2009.0228 [PubMed: 19737071]
17. Fluri DA, Tonge PD, Song H, Baptista RP, Shakiba N, Shukla S, Clarke G, Nagy A, Zandstra PW. Derivation, expansion and differentiation of induced pluripotent stem cells in continuous suspension cultures. *Nat Methods.* 2012; 9(5):509–516. DOI: 10.1038/nmeth.1939 [PubMed: 22447133]

18. Dore C, Rancourt D, Dobrinski I. Stirred suspension bioreactors as a novel method to enrich germ cells from pre-pubertal pig testis. *Andrology*. 2015; 3(3):590–597. DOI: 10.1111/andr.12031 [PubMed: 25877677]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Experimental design. 1. 1-week-old pig testes are enzymatically digested into a single cell suspension. 2. Cell suspensions were split into two groups and assigned to enrichment method A and B. 2A. Cell suspensions were placed in stirred suspension culture, after 18, 44, and 66 h cell suspensions were filtered through a 40 µm strainer to remove larger cell aggregates and poured back into culture until the next time point. 2B. Cell suspensions were placed in stirred suspension culture for 44 h, then subjected to one round of differential plating. 3. Enriched germ cell suspension is obtained. Figure is modified from [18] and reproduced with permission