Optimal culture conditions are critical for efficient expansion of human testicular somatic and germ cells in vitro

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Objective: To optimize culture conditions for human testicular somatic cells (TSCs) and spermatogonial stem cells.
Design: Basic science study.
Setting: Urology clinic and stem cell research laboratory.
Patient(s): Eight human testicular samples.
Interventions(s): Testicular tissues were processed by mechanical and enzymatic digestion. Cell suspensions were subjected to differential plating (DP) after which floating cells (representing germ cells) were removed and attached cells (representing TSCs) were cultured for 2 passages (P0–P1) in StemPro-34– or DMEM-F12–based medium. Germ cell cultures were established in both media for 12 days.
Main Outcome Measure(s): TSC cultures: proliferation doubling time (PDT), fluorescence-activated cell sorting for CD90, next-generation sequencing for 89 RNA transcripts, immunocytochemistry for TSC and germ cell markers, and conditioned media analysis; germ cell cultures: number of aggregates.
Result(s): TSCs had significantly prolonged PDT in DMEM-F12 versus StemPro-34 (319.6 ± 275.8 h and 110.5 ± 68.3 h, respectively). The proportion of CD90-positive cells increased after P1 in StemPro-34 and DMEM-F12 (90.1 ± 10.8% and 76.5 ± 17.4%, respectively) versus after DP (66.3 ± 7%). Samples from both media after P1 clustered closely in the principle components analysis map whereas those after DP did not. After P1 in either medium, CD90-positive cells expressed TSC markers only, and fibroblast growth factor 2 and bone morphogenetic protein 4 were detected in conditioned medium. A higher number of germ cell aggregates formed in DMEM-F12 (59 ± 39 vs. 28 ± 17, respectively).
Conclusion(s): Use of DMEM-F12 reduces TSC proliferation while preserving their unique characteristics, leading to improved germ cell aggregates formation compared with StemPro-34, the standard basal medium used in the majority of previous reports. (Fertil Steril® 2017;107:595–605. ©2017 by American Society for Reproductive Medicine.)
Key Words: Human testicular somatic cells, germ cells, spermatogonial stem cells, male fertility preservation

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Cancer treatment outcomes among children have improved tremendously in recent years, allowing the majority of them to survive (1). Consequently, the prevalence of adults who were previously treated with the use of chemotherapy and/or radiotherapy has increased significantly (2). One of the unfortunate complications of cancer treatment is infertility (3). Unlike adolescents (4) and adults, semen cryopreservation before gonadotoxic exposure is not possible for boys, because spermatogenesis does not commence until puberty (5). This has led to worldwide initiatives to develop spermatogonial stem cell (SSC)–based strategies for fertility preservation.

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SSC homeostasis requires a balance between self-replication to maintain the stem cell pool and meiotic differentiation to fuel spermatogenesis (6). This equilibrium is tightly regulated by the testicular microenvironment (7). Testicular somatic cells (TSCs), including Sertoli and peritubular cells, play essential roles during spermatogenesis by providing structural and nutritional support for differentiating germ cells (8). The frequency of SSCs is estimated to be low in mammalian testes; it is thought to represent 0.03% of testicular cells in mice (9). Therefore, propagation of SSCs in vitro would likely be required before attempting fertility restoration by either autotransplantation to the testis (5) or in vitro spermatogenesis (10). Unfortunately, early reports of long-term human SSC expansion have been controversial (1, 11, 12).

Human testicular cell suspension cultures are characterized by two main populations: fibroblastic-like somatic cells which tend to attach to culture dishes, and small round cells which are presumed to be germ cells and tend to adhere to the attached cells. The latter may divide and form colonies which disappear in early cell passages because the adherent cells quickly grow and become confluent (13). In keeping with this, there is an observed loss of SSC-associated marker expression (UTF1, FGFR3, and DAZL) during early culture stages, together with a concomitant increase in the expression of TSC markers (VIM, ACTA2 and GATA4) (12). TSCs are crucial for SSC expansion in vitro, but their overgrowth remains a major challenge (13). Therefore, TSC overgrowth in vitro is one of the major hurdles to overcome when attempting to achieve efficient SSC expansion. Previous studies tried to characterize the different testicular cell subpopulations according to specific markers (14, 15). CD90 has been suggested to be exclusively expressed in human somatic cells compared with germ cells, especially in the postnatal period (16).

Media composition is a critical aspect of cell culture. Medium formulations used for ex vivo human SSC propagation by the majority of researchers (1, 12, 13, 17, 18) have not changed significantly from the StemPro-34–based medium used in early mouse studies, where efficient long-term expansion of SSCs was reported (19). A recent study found that human SSCs display limited proliferation in vitro under murine SSC StemPro-34–based medium culture conditions (20). In contrast, the use of DMEM-F12–based medium has rarely been reported (21). To our knowledge, despite the crucial role that media composition plays in such systems, there are no publications directly comparing these two types of media for human testicular cell culture. Because TSC overgrowth remains a crucial obstacle for human SSC expansion in vitro, we decided to compare the effects of these two types of media on testicular somatic and germ cell growth. Specifically, our aim was to compare the proliferation rates and phenotypes of human TSCs expanded in culture with the use of growth factor–supplemented StemPro-34 or DMEM-F12 medium. In addition, we contrasted the effect of these two types of media on germ cell survival.

METHODS

Tissue Collection and Cell Dissociation

Human testicular samples were obtained from seven patients who underwent orchiectomy (four owing to testicular malignancy and three because of testicular pain) and one patient that underwent microscopic testicular sperm extraction (microTESE) for nonobstructive azoospermia (NOA; Supplemental Table 1, available online at www.fertstert.org). All patients provided written informed consents to participate in this study (University of Toronto Research Ethics Board [REB] no. 30252 and Mount Sinai Hospital REB no. 14-0032-E). After pathologic analysis and/or reproductive use (microTESE), the remaining testicular tissue was transferred to the research lab in isolation medium composed of Dulbecco Modified Eagle Medium, Nutrient Mix F-12 (DMEM-F12; Life Technologies) with 7.5% sodium bicarbonate, nonessential amino acids (Invitrogen), 1% penicillin/streptomycin, 1 μl/mL gentamicin (G1397-10MI; Sigma-Aldrich), and 10 mg/mL DNAse (cat. no. d-5025 150KU; Sigma-Aldrich). Two of the orchiectomy tissue samples were immediately cryopreserved and used subsequently for these studies. Samples were washed three times with the use of phosphate-buffered saline solution (PBS) followed by chopping with scissors in Modified Eagle Medium (MEM) + 20% fetal bovine serum (FBS) to get 1–2-mm pieces; 10–20 pieces were transferred to 1 mL freezing medium consisted of MEM + 20% FBS + 5% dimethylsulfoxide. Vials were thawed in water at 37°C for 3 minutes. The thawed tissue was washed in isolation medium and then subjected to mechanical and enzymatic digestion as performed in the four fresh samples. Briefly, mechanical digestion was conducted with the use of fine scissors and needles to disrupt the tissue and to separate the seminiferous tubules as much as possible. Tissue pieces were transferred to a 50-mL tube containing isolation medium and washed repetitively until the supernate became clear. Five mL Enzyme Mix I containing 2 mg/mL collagenase type I (cat. no. LS004196; Warthington), 2 mg/mL hyaluronidase (cat. no. H2126; Sigma-Aldrich), 2 mg/mL trypsin (cat. no. T1005; Sigma-Aldrich), and 4 μL DNAse (cat. no. d-5025 150KU; Sigma-Aldrich) was added to the tube containing 5 mL isolation medium and testicular tissue (total volume of 10 mL) and incubated in a shaker-incubator at 32°C and 120 rpm for 15 minutes. The cell suspension was centrifuged for 5 minutes at 400 rpm without brake, and the supernate was removed. Tubules were washed in isolation medium, followed by centrifugation for 5 minutes at 400 rpm without brake. The supernate was removed and 5 mL Enzyme Mix II containing 2 mg/mL collagenase type I, 2 mg/mL hyaluronidase, and 4 μL DNAse in 5 mL isolation medium (total 10 mL) was added to the tissue pellet, following by incubation at 32°C on a shaker at 120 rpm. Microscopic observation was performed to assess the thinning and perforation of the peritubular cell layer. Accordingly, up to 10 additional minutes of enzymatic incubation time was included. Undigested tissue was removed by subjecting the cell suspension to sequential filtration with 70-μm and 40-μm nylon filters, followed by centrifugation for 5 minutes at 1,800 rpm without brake. To lyse red blood cells, testicular cells suspensions were incubated with a 1:4 isolation medium to ammonium chloride ratio on ice for 10 minutes and washed twice with isolation medium with 10% FBS (lot no. AWK24007; Hyclone) and without DNAse.